THE USE OF ETHER IN THE CHROMATOGRAPHIC SEPARATION OF VITAMIN D_2 AND ERGOSTEROL

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This is to certify that the

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THE USE OF ETHER IN THE CHROMATOGRAPHIC SEPARATION OF VITAMIN D_2 AND ERGOSTEROL

The development of methods for the chromatographic separation of calciferol from sterols was the result of a search for a physical-chemi- method for the estimation of the vitamin D content of both fish oils and erradiated ergosterol. Tomkins (1) employed a hexane-ether solution to separate vitamin A from the vitamin D in fish oils, using a column of "Super Filtrol," which is an activated bentonite clay. The sterols had been removed from the saponified oil previously by precipitation with digitonin. Analysis for vitamin D was made by the antimony trichloride method. Kingsley (2) found that the addition of a small amount of ethanol to the hexane-ether mixture improved the separation from vitamin A. The rate of movement of bands down the column was also increased. He found that a mixture of 50 volumes of n-hexane or Skellysolve, 10 volumes of ethyl ether and 1 volume of ethyl alcohol gave the best results. He followed the chromatographic separation from vitamin A with a separation from the sterols on short column using a hexane-benzene solution. Both of these workers used the antimony trichloride reaction which is not specific for Vitamin D.

Baker (3) used the ultraviolet absorption spectrum of calciferol in determining the potency. This permitted a more detailed study of the chromatographic process. It had the disadvantage that the solvents used dissolved some material out of the "Super Filtrol" which also had an absorption spectrum in the ultraviolet.

Meanwhile, Carlson (4) studied the adsorption characteristics of calciferol, ergosterol and cholesterol on "Super Filtrol" in various binary solvent mixtures. Results showed that when the proportion of

alcohol in a hexane-alcohol solution was increased, the adsorption of both calciferol and ergosterol fell off rapidly. With increasing proportions of ether in a hexane-ether solution, the adsorption decreased less rapidly, but ergosterol was adsorbed more than calciferol at any given per cent of ether.

Bullard (5) made a survey of the "50-10-1" method to determine its efficiency in separating synthetic mixtures of calciferol and ergosterol. She recovered amounts of calciferol ranging from 93-99%. She also investigated several binary solvent mixtures and found that pure ether or an ether-alcohol mixture also gave partial chromatographic separations. She concluded that only the "50-10-1" combination gave satisfactory results. In her work, corrections were made either by running a blank column as did Baker or by using a sufficiently large amount of calciferol and ergosterol so that the correction could be minimized by dilution. The presence of the interfering substance from "Super Filtrol" made accurate detection of small amounts of calciferol difficult.

The purpose of the work reported in this thesis is twofold. First, the effects of the various solvents and binary mixtures are examined with the intention of providing an explanation of the banding phenomena. Separations from binary mixtures are studied in order to find a more satisfactory solvent combination. Second, having found a successful mixture, a more quantitative method is developed for the chromatographic separation of calciferol from synthetic mixtures of ergosterol and calciferol. Particular attention is directed toward finding a better method of correction for the interfering substance from "Super Filtrol" and toward more successful separations of small amounts of the two compounds.

I

OBSERVATIONS ON CHROMATOGRAPHIC SEPARATIONS FROM BINARY SOLVENTS

The Action of Binary Solvents on "Super Filtrol" Columns

Before an investigation of the chromatographic behavior of ergosterol and calciferol was made, the characteristic action of the solvents on "Super Filtrol" was considered. The solvents used, singly and in binary mixtures, were n-hexane, benzene, ethyl ether, ethyl alcohol and iso-propyl alcohol.

The hexane used was either Eastman "Hexane" (from Petroleum) (Practical) or was obtained from Skellysolve B. It was purified by taking the 65-69° fraction and removing the benzene present by chromatographing on a column of silica gel activated at a temperature of 225° C. It was then redistilled, again taking the 65-69° fraction (6). The test of its purity was its ability to pass light at 230 mu. on the Beckman Spectrophotelometer and the absence of extinction maxima at 248 and 254 mu.

The benzene used was C.P. anhydrous, thiophene free.

The ether used was dried over sodium to remove both water and alcohol and was distilled from ferrous sulfate just before each operation.

The ethyl alcohol used was refluxed over silver oxide, distilled, dried over aluminum amalgam and redistilled (7).

The iso-propyl alcohol used was dried over aluminum amalgam and distilled.

The solvents may be considered in two classes, polar and non-polar. The non-polar liquids hexane and benzene behave almost identically on columns of "Super Filtrol." Both are easily desorbed by polar liquids

and both impart a dark gray shade to the column. When either is passed through a column, a darker gray area or band is formed extending from the top. The width of this band is proportional to the volume of solvent used, and its presence is correlated with the dissolving out of some factor in the "Super Filtrol" which has an absorption spectrum in the ultraviolet. When this area extends to the bottom of the column, the hexane (or benzene) is practically free of this material when it leaves the tube. The adsorption characteristics in this band are different from those of the area below it, as will be shown later.

The polar solvents, ether, ethyl alcohol and iso-proply alcohol, all dissolve a large amount of material from the "Super Filtrol." They easily displace the non-polar liquids and the alcohols will desorb ether. Ethyl ether gives a gray shade to the column between the slate-gray imparted by benzene or hexane and the nearly white shade of the clay when it is wet with the alcohols or water. The shade serves as a qualitative index of the polarity of a solvent.

All the polar liquids observed clog up the chromatograph column to such an extent that they are unfit for practical use unless they are mixed with a non-polar liquid.

When binary mixtures of ethyl or iso-propyl alcohol and benzene or hexane in low alcohol concentrations are placed on a column, an additional band is developed which is characteristic of the presence of the alcohol. This band appears as a fine line and is usually tan or brown in color. Its distance from the top of the column is proportional to the volume of solvent used and to the concentration of alcohol. If the concentration is less than two volumes per hundred of hexane or benzene this narrow band occurs within the wide gray area characteristic of the non-

polar solvents and is obscured. If the concentration is greater the alcohol line occurs in the area below. Its position in the tube for the first 3 cm. can be accurately predicted, given the volume of solvent which has passed and the concentration of the alcohol. This band plays an important part in the "50-10-1" method of separation. The volume of solvent necessary to push this line to the bottom of the column is equal to the total volume of the washing and eluting solutions used in that method.

Binary mixtures of ether and hexane or benzene show no such banding phenomenon. The chromatograph tube appears the same as when benzene or hexane alone are used, except that the shade is lighter.

It was found by optical analysis that the quality as well as quantity of material dissolved from "Super Filtrol" varied according to the solvent used. In the case of binary mixtures, the type of material coming from the tube varied through the individual run. The material in the first portion of eluant of a hexane-ether system was found to be more of the type which is found dissolved from hexane alone. In the later portions the quantity rapidly decreased but the quality was that of the substance which dissolved out of ether.

The Chromatographic Behavior of Calciferol and Ergosterol in Binary Solutions

Preliminary surveys of the chromatographic behavior of calciferol and ergosterol in binary solvents were made by Bullard (5). All solvent combinations seemed unsuitable except for the already successful ternary combination of 50 volumes of hexane, 10 volumes of ether and 1 volume of alcohol. She reported that a separation could be made from an ether-alcohol system, but that it was not complete. This choice of solvents

has been found impractical because it clogs the column as well. She also noted that a system of hexane and ether retained both compounds on the column. No quantitative work was reported. The presence of alcohol was thought essential. Tomkins had previously used a hexane-ether mixture to separate vitamin A from vitamin D (1). Kingsley (2) used alcohol in addition mainly because it gave a sharp band which could be used as a reference line. He developed his columns until this line reached the bottom of the tube. This practice became standard for chromatographing erradiated ergosterol solutions. It was decided to reinvestigate these systems, and also systems containing benzene, to determine what qualities of the solvent were necessary for separation. Attention was devoted to the mixtures hexane-ether and hexane-alcohol to find out why negative results were obtained.

From pure hexane or benzene both calciferol and ergosterol are strongly adsorbed (4). Hence, when they were chromatographed, both were removed and banded in the top of the tube. Here they remained, resisting any further elution. Combinations of benzene and hexane were found to give the same result, although they had been successfully used previously in the separation of sterols in fish oils (2). During the runs, calciferol changed from orange to purple, while ergosterol changed from pink to purple, indicating some change in the chromophoric groups during development. If a column was saturated with ergosterol, that portion which was adsorbed in the dark gray area mentioned before was purple in color while that excess which came in contact with the lower area was pink. This excess could be washed through a short column, but proved to give an entirely different absorption spectrum that that of ergosterol. The material adsorbed in the upper area could never be completely eluted.

Calciferol or ergosterol in ethyl alcohol formed no visible bands

and passed quickly through the column, considering the extreme clogging effect of these polar solvents upon the clay. This was expected since neither are adsorbed from pure ether or pure alcohol (4).

When calciferol or ergosterol were chromatographed using a hexane solution containing small amounts of ethyl alcohol (1-8 volumes/100), both banded in the fine alcohol line discussed before. Iso-propyl alcohol contains a secondary alcohol group, as do ergosterol and calciferol. It was tried next in the same concentrations, hoping that it would provide a more suitable polar component in the solvent mixture. Results were the same. When benzene was substituted for hexane, the colors of the bands developed in the alcohol line were slightly different. Yet both compounds concentrated ~~in~~ in that petition. In every case, the developing solution which passed through the column before the line reached the bottom of the tube was devoid of any material except the substance dissolved from the "Super Filtrol." The portion of eluant containing this band contained large amounts of material possessing a strong ultraviolet absorption spectrum. That portion of eluant collected after the band had completely passed through the column contained insignificant amounts of material. Sudan III, which was used previously as a marker, also banded in the alcohol line, although it is of an entirely different molecular species.

Not only was a chromatographic separation impossible, but the material which came through the tube when either calciferol or ergosterol was placed on the column had apparently decomposed. Its ultraviolet absorption spectrum bore little resemblance to either compound. However, in the case of ergosterol, slight extinction maxima were retained at 270 and 281 mμ. The bands formed were all strongly colored and the eluant

was yellow rather than clear.

In hexane-ether systems, contrary to previous results, a separation was possible. No bands were visible, but if very large amounts (greater than 0.5 mg.) of ergosterol were used, a diffuse blue area developed in the top of the tube. Calciferol came through the column in an undecomposed form, but the ergosterol could never be recovered. Since no reference band appeared, it was necessary to develop the tube by noting when a certain volume had passed. The choice of the proper volume will be discussed in Part II.

A benzene-ether system was also found successful. The column behavior was poorer than when hexane was used. The flow of eluant through the tube was prohibitively slow and the column developed fractures and air pockets.

The greatest difficulty in using benzene was encountered in optical analysis. The eluant had to be evaporated to dryness and taken up in alcohol several times in order to remove the last traces of benzene. If weights of calciferol less than 0.5 mg. were used, the interference in absorption was still too great to make a quantitative extimate of the recovered material optically. Larger amounts of calciferol permitted greater dilution and the effects of benzene could be eliminated.

Discussion

It has been stated that the type of material which comes through the chromatograph column, dissolved from the "Super Filtrol," varies with the solvent used. In the case of a binary solvent such as hexane-ether, the type of material varies during the same run. Carlson has shown (4) that ethyl alcohol is selectively adsorbed on "Super Filtrol" from hexane-alcohol mixtures when the concentration of alcohol is less than 25% by weight. Ether is positively adsorbed from hexane-ether mixtures

when the ether content is less than 4.2% by weight. These changes in the quality of material leaving the tube might therefore be expected. In making any correction for the optical absorption of this substance, the volume of wash solution used in a chromatogram must be kept constant. This not only keeps the amount of dissolved material within certain limits, but keeps the quality of the absorption spectrum uniform.

Knowing that ethyl alcohol is quite strongly adsorbed from hexane on "Super Filtrol," an explanation of the presence of the alcohol line might be offered here. During the initial part of a chromatograph using a hexane-alcohol mixture, more material would be dissolved from the top of the column than if hexane alone were used. The alcohol content should then decrease, as it is selectively adsorbed. Some of the dissolved material would then be redeposited, since its solubility would be increased. Also, any impurity present which is soluble in small alcohol concentrations but which is relatively insoluble in hexane alone will be banded in the region where the alcohol content begins to decrease.

As has been shown by observations on both hexane-alcohol and benzene-alcohol solutions of ergosterol, calciferol and Sudan III, the banding depends upon the position of the alcohol line. This means that the position of the band depends upon the volume of wash solution used before the particular compound is placed on the column and upon the alcohol concentration of the binary mixture. This type of banding is peculiar to systems containing more than one liquid in the solvent mixture. When a single solvent is used, the material to be chromatographed will initially band at the top of the column if it is adsorbed at all. In the solvent hexane-alcohol, the most important factor in banding seems to be the adsorption characteristics of the molecule

regarded as a function of changing alcohol concentration. Calciferol and ergosterol behave similarly in adsorption under varying concentrations of alcohol in hexane (4). This explains why no separation was detected.

It was observed that ergosterol chromatographed from hexane or benzene solutions was retained more strongly when it was adsorbed in the upper portion of the column. Its color was blue in the upper area, pink in the lower region. A difference in pH on the "Super Filtrol" surface might account for the variation in color. When Sudan III is adsorbed on "Super Filtrol" from hexane it is deep blue in color. If sodium carbonate and a small amount of water are added, the Sudan III is desorbed and returns to its normal red color. If the mixture is then acidified with HCl solution, the Sudan III is readsorbed and changes back to blue. Yet this oil soluble dye is not normally an indicator. The process is apparently quite reversible. This would indicate that "Super Filtrol" normally presents an acid surface. It is known that ergosterol is sensitive to the hydrogen ion, although there is only a slight change in color in the visible range. Acid causes rearrangements of the double bonds in the molecule (8).

A comparison may now be made between the "50-10-1" method and the separation from a hexane ether solution. In the "50-10-1" method, ergosterol bands in the alcohol line. Calciferol passes through the column ahead of the ergosterol because of the presence of ether. This means that the effective column length is decreased, since the alcohol line reaches the middle of the column before the compounds are added. The advantage is that the line forms a convenient reference point for stopping the development.

In a hexane-ether chromatograph, ergosterol is adsorbed at the top of the column and does not travel down the tube very fast. The full

length of the column is employed in the separation. Again, calciferol passes through because it is desorbed more effectively by the ether. Rate of flow of the eluant is slower, but this disadvantage is removed by using a shorter column. The overall running time is less. Smaller amounts of material may be separated, as will be shown in Part II.

A binary system suitable for quantitative separations must possess at least two characteristics. First, the separation must be reasonable complete on a column of practical length. Second, if analysis is to be made optically, the material appearing in the eluant must have an absorption spectrum unchanged by its contact with the column. It is pointed out that in all successful separations, ethyl ether was the essential ingredient. Systems in which ether was absent did not satisfy either of the above requirements. The results of these observations are collected in Table I.

Summary

1. When the non-polar solvents benzene or hexane are used on a column of "Super Filtrol," a dark gray area is developed in the upper portion in which the adsorption characteristics are different than those in the lower region. Ergosterol and calciferol are very strongly adsorbed and cannot be eluted.

2. When ethyl or iso-propyl alcohol are used in combination with hexane or benzene, an additional fine line is developed in the column whose length from the top is proportional to the volume of solvent passed and the concentration of alcohol. Calciferol, ergosterol and Sudan III all band in this alcohol line.

3. When ether is used with hexane or benzene, calciferol passes through the column and ergosterol is retained, making a sharp separation

TABLE I

OBSERVATIONS ON THE CHROMATOGRAPHIC

CHARACTERISTICS OF ERGOSTEROL AND CALCIFEROL

ON "SUPER FILTROL"

Solvent	Compound	Band Formation	Color	Material Recovered in Eluant	Separation
Pure Hexane	Calciferol	None -- all is adsorbed in top of column	Orange to purple	None	None
	Ergosterol	None -- all is adsorbed in top of column	Pink to Blue	None	
Hexane plus 2% ethyl or iso-propyl alcohol by volume	Calciferol	In fine band due to alcohol	Blue to Brown	Unidentified	None
	Ergosterol	In fine band due to alcohol	Blue to Green	Unidentified	
Hexane plus ethyl ether in the ratio of five vol. to one	Calciferol	Wide band	Invisible	Calciferol	Calciferol Proceeds Ergosterol
	Ergosterol	Diffuse area in top of column	Blue	Unidentified	

The table is the same if benzene is substituted for hexane with minor differences in band colors.

possible.

4. When the combination hexane-ether-alcohol is used, calciferol passes through the column due to the presence of ether, while ergosterol bands in the line due to alcohol.

5. Unless ether is present, the absorption absorption spectra of calciferol and ergosterol are altered by their contact with the column and cannot be identified. If ether is used, calciferol passes through the column unaffected.

II

A METHOD FOR THE SEPARATION OF CALCIFEROL FROM SYNTHETIC MIXTURES OF CALCIFEROL AND ERGOSTEROL USING THE BINARY SOLVENT HEXANE AND ETHER

Having decided upon a hexane-ether mixture as the most suitable binary solvent for use in the chromatographic separation of Vitamin D_2 and ergosterol, a series of separations were run on four synthetic mixtures of the two compounds. Five trials were made with each mixture, varying the weight of the sample in each case to determine the limitations of the column. The amounts of calciferol recovered from the chromatograms were determined optically on the Beckman Spectrophotelometer and are reported in Tables II-V.

Procedure

The Chromatograph Tube.-- The body of the chromatograph tube was made from glass tubing having an outside diameter of 10 mm. A slight constriction was placed between the body of the tube and the outlet in order to support a cotton plug. In addition, an adaptor was constructed to fit the tube to a 25 ml. Erlenmeyer flask and to permit partial evacuation (Fig. 1).

Packing. -- A cotton plug was placed in the tube and "Super Filtrol" was packed in it to a depth of 4 cm. while the tube and receiving flask were being evacuated. A differential pressure of - 10 mm. was used throughout. The finely divided clay was settled by gently tapping on the tube with a pencil.

The Solvent. -- A mixture of five volumes of hexane and one volume of ether was made up just before each series of separations. The solvents

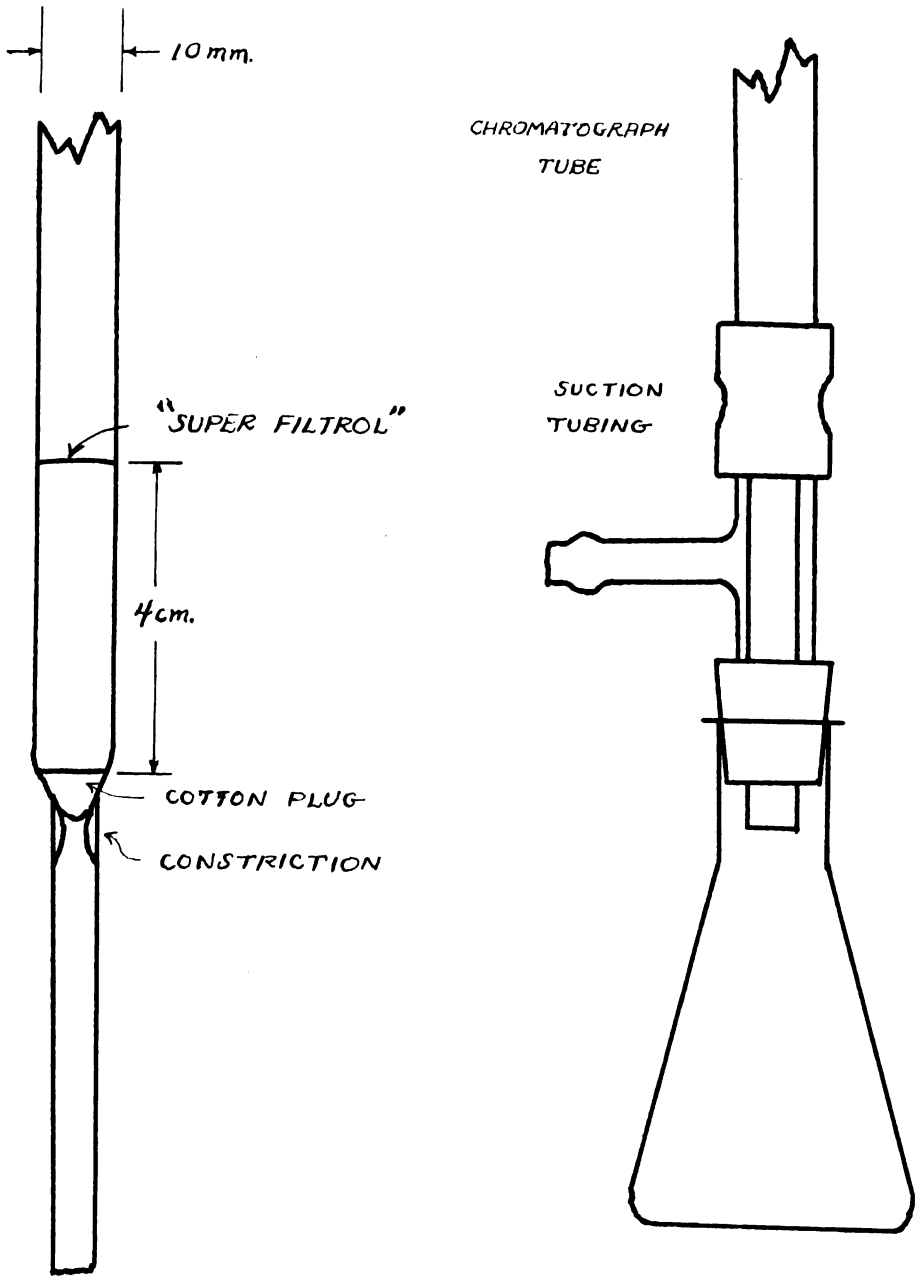

Fig. 1.-- Detail of Chromatograph Tube and the Adaptor used to Permit Evacuation of the Receiving Flask.

were purified as described in Part I. The ether had been distilled the same day. This mixture was used in washing the column, developing the chromatogram and making all dilutions of the sample.

The Sample. -- A known mixture of ergosterol and calciferol was made up from stock solutions of the compounds and evaporated to dryness, using the aspirator. The residue was dissolved in a known volume of the hexane-ether mixture. Five dilutions were made up so that the weight of calciferol to be placed on the column would be contained in one ml.

Washing and Development. -- The column was washed with 8 ml. of solvent, measured with a pipette and added to the top of the tube. This portion was then discarded and the sample mixture, in one ml. of solvent, was placed on the column. Just before the tube began to dry out, 11 ml. of solvent were added as a developer. The 12 ml. of eluant, containing the recovered calciferol and some dissolved material from the "Super Filtrol," was then prepared for optical analysis.

Optical Measurement of the Recovered Calciferol. -- The eluant was evaporated to dryness by warming the flask in a water bath and evacuating with an aspirator. The residue was immediately taken up in a known volume of absolute alcohol and a dilution was made so that the extinction would be in the range of 0.5. For weights of calciferol below 0.05 mg. the residue was dissolved in only 4 ml. of alcohol and no dilution was made. A complete extinction curve was then made from 230 to 300 mu. on the Beckman Spectrophotelometer at intervals of 2 mu. Particular care was taken in making readings at 230, 264 and 281., which were used in later calculations.

Control Runs to Determine the Characteristics of the Substance Dissolved from "Super Filtrol." -- Using the same procedure, a series

of blank chromatograms were made and the extinction values at 230 and 264 mu. were determined. These are reported in Table VI. In addition, the ratio E_{264}/E_{230} was calculated. This value is of greater use in making corrections than the individual extinction values at 264 mu.

Calculations. -- The extinction of calciferol at 264 mu. was corrected for the "Super Filtrol" impurity still present by use of the formula

$$E(\text{Calciferol at 264 mu.}) = \frac{E(\text{total at 264 mu.}) - 0.513\, E(\text{total at 230 mu.})}{0.731}$$

where 0.513 is the averaged value E_{264}/E_{230} for the material from the "Super Filtrol" and 0.731 is 1 - 0.513x0.525. The value E_{230}/E_{264} for calciferol is 0.525.

The weight of calciferol was then found by the formula

$$\text{Weight of Calciferol in mg.} = \frac{10\, ED}{E(1\%,\ 1\text{cm.})}$$

where E is the extinction of calciferol in the recovered solution and D is the dilution factor. The E(1%, 1 cm.) for the calciferol used was 468 at 264 mu.

TABLE II

Chromatographic Separations of a Mixture of 49.4% Calciferol and 50.6% Ergosterol

	Trial				
	1.	2.	3.	4.	5.
Weight of Calciferol in mg. placed on column	0.4292	0.08584	0.04292	0.02861	0.02146
Weight of Ergosterol in mg. placed on column (Both are dissolved in 1 ml. of hexane-ether mixture)	0.4390	0.0878	0.0439	0.02927	0.02295
Ml. of alcohol in which recovery from column was dissolved	40	8	4	4	4
Uncorrected extinction of recovery at 264 mu.	0.546	0.550	0.563	0.387	0.267
Uncorrected extinction of recovery at 230 mu.	0.3385	0.354	0.412	0.262	0.198
Corrected extinction of recovery at 264 mu.	0.510	0.505	0.482	0.346	0.254
Weight of Calciferol recovered, in mg.	0.436	0.0863	0.0412	0.0296	0.0217
Per cent of Calciferol recovered	101.6	100.5	95.9	103.4	101.1

Average Per Cent Recovery for Five Trials 100.50

TABLE III

Chromatographic Separations of a Mixture of 59.4% Calciferol and 40.6% Ergosterol

	Trial				
	1.	2.	3.	4.	5.
Weight of Calciferol in mg. placed on column	0.4292	0.08584	0.04292	0.02146	0.01073
Weight of Ergosterol in mg. placed on column (Both are dissolved in 1 ml. of hexane-ether mixture)	0.2927	0.05824	0.02927	0.1464	0.00732
Ml. of alcohol in which recovery from column was dissolved	40	8	4	4	4
Uncorrected extinction of recovery at 264 mu.	0.530	0.5125	0.539	0.2955	0.1725
Uncorrected extinction of recovery at 230 mu.	0.315	0.330	0.3515	0.2155	0.150
Corrected extinction of recovery at 264 mu.	0.505	0.470	0.491	0.2533	0.1307
Weight of Calciferol recovered in mg.	0.432	0.0803	0.0420	0.02166	0.0112
Per cent of Calciferol recovered	100.6	93.6	97.8	100.9	104.1

Average Per cent Recovery for Five Samples 99.40

TABLE IV

Chromatographic Separations of a Mixture of 75.1% Calciferol and 24.9% Ergosterol

	Trial				
	1.	2.	3.	4.	5.
Weight of Calciferol in mg. placed on column	0.4292	0.08584	0.04292	0.02146	0.01073
Weight of Ergosterol in mg. placed on Column (Both are dissolved in 1 ml. of hexane-ether mixture)	0.1420	0.0284	0.0142	0.00710	0.00355
Ml. of alcohol in which recovery from column was dissolved	40	8	4	4	4
Uncorrected extinction of recovery at 264 mu.	0.524	0.507	0.551	0.287	0.177
Uncorrected extinction of recovery at 230 mu.	0.301	0.307	0.336	0.201	0.158
Corrected extinction of recovery at 264 mu.	0.506	0.479	0.483	0.252	0.131
Weight of Calciferol recovered in mg.	0.433	0.0819	0.413	0.0215	0.0112
Per cent of Calciferol recovered	100.8	95.4	96.2	100.3	104.4

Average Per cent Recovery for Five Samples 99.42

TABLE V

Chromatographic Separations of a Mixture of 90.3% Calciferol and 9.7% Ergosterol

	Trial				
	1.	2.	3.	4.	5.
Weight of Calciferol in mg. placed on column	0.4292	0.0534	0.04292	0.02146	0.01073
Weight of Ergosterol in mg. placed on column (Both are dissolved in 1 ml. of hexane-ether mixture)	0.04833	0.00997	0.00483	0.00242	0.00121
Ml. of alcohol in which recovery from column was dissolved	40	8	4	4	4
Uncorrected extinction of recovery at 264 mu.	0.485	0.503	0.557	0.2885	0.1635
Uncorrected extinction of recovery at 230 mu.	0.270	0.302	0.341	0.1915	0.135
Corrected extinction of recovery at 264 mu.	0.473	0.483	0.523	0.2602	0.129
Weight of Calciferol recovered in mg.	0.404	0.0525	0.0447	0.02224	0.01102
Per cent of Calciferol recovered	94.2	96.2	104.1	103.6	102.7

Average Per cent Recovery for Five Samples 100.16

TABLE VI

Extinction Characteristics of Substance

Dissolved from "Super Filtrol"

	Trial			
	1.	2.	3.	4.
Extinction of residue* at 246 mu.	0.032	0.045	0.0315	0.0705
Extinction of residue* at 230 mu.	0.063	0.089	0.061	0.135
E_{264}/E_{230}	0.508	0.506	0.517	0.522

Average E_{264}/E_{230} 0.513

*Sample consists of residue from 12 ml. of eluant from a blank column dissolved in 4 ml. of ethyl alcohol.

TABLE VII

Absorption values for Calciferol Recovered from a Mixture of 49.4% Calciferol and 50.6% Ergosterol and Corrected Values

Wave Length in mu.	Extinction of Total Residue*	Correction	Corrected Extinction	$E_\lambda/264$	$E_\lambda/264$ for Pure Calciferol
230	0.198	-0.063	0.135	0.531	1.525
232	0.195	-0.061	0.134	0.528	0.545
234	0.196	-0.059	0.137	0.540	0.573
236	0.197	-0.055	0.142	0.560	0.605
238	0.203	-0.051	0.152	0.598	0.645
240	0.207	-0.047	0.160	0.630	0.682
242	0.213	-0.045	0.168	0.662	0.716
244	0.221	-0.044	0.177	0.697	0.750
246	0.233	-0.043	0.190	0.748	0.787
248	0.240	-0.042	0.198	0.780	0.819
250	0.248	-0.041	0.207	0.815	0.852
252	0.255	-0.038	0.217	0.855	0.885
254	0.263	-0.038	0.225	0.886	0.913
256	0.269	-0.037	0.232	0.914	0.940
258	0.275	-0.036	0.239	0.941	0.965
260	0.280	-0.035	0.245	0.965	0.983
262	0.285	-0.034	0.251	0.989	0.996
264	0.287	-0.033	0.254	1.000	1.000
266	0.285	-0.032	0.253	0.996	0.994

*The residue from an original 0.02146 mg. of calciferol was dissolved in a total of 4 ml. of ethyl alcohol.

TABLE VII (CONTINUED)

Wave Length in mu.	Extinction of Total Residue*	Correction	Corrected Extinction	E λ/264	E λ/264 for Pure Calciferol
268	0.280	-0.032	0.248	0.976	0.975
270	0.275	-0.031	0.244	0.961	0.940
272	0.265	-0.030	0.235	0.925	0.906
274	0.253	-0.029	0.224	0.875	0.855
276	0.240	-0.029	0.211	0.831	0.799
278	0.225	-0.028	0.197	0.776	0.738
280	0.208	-0.027	0.181	0.713	0.675
282	0.194	-0.027	0.167	0.658	0.603
284	0.175	-0.026	0.149	0.587	0.537
286	0.156	-0.024	0.132	0.520	0.472
288	0.140	-0.023	0.117	0.461	0.407
290	0.124	-0.022	0.102	0.402	0.346
292	0.109	-0.021	0.088	0.346	0.293
294	0.095	-0.020	0.075	0.295	0.242
296	0.082	-0.019	0.063	0.248	0.196
298	0.070	-0.017	0.053	0.208	0.158
300	0.060	-0.016	0.044	0.173	0.126

* The residue from an original 0.02146 mg. of calciferol was dissolved in a total of 4 ml. of ethyl alcohol.

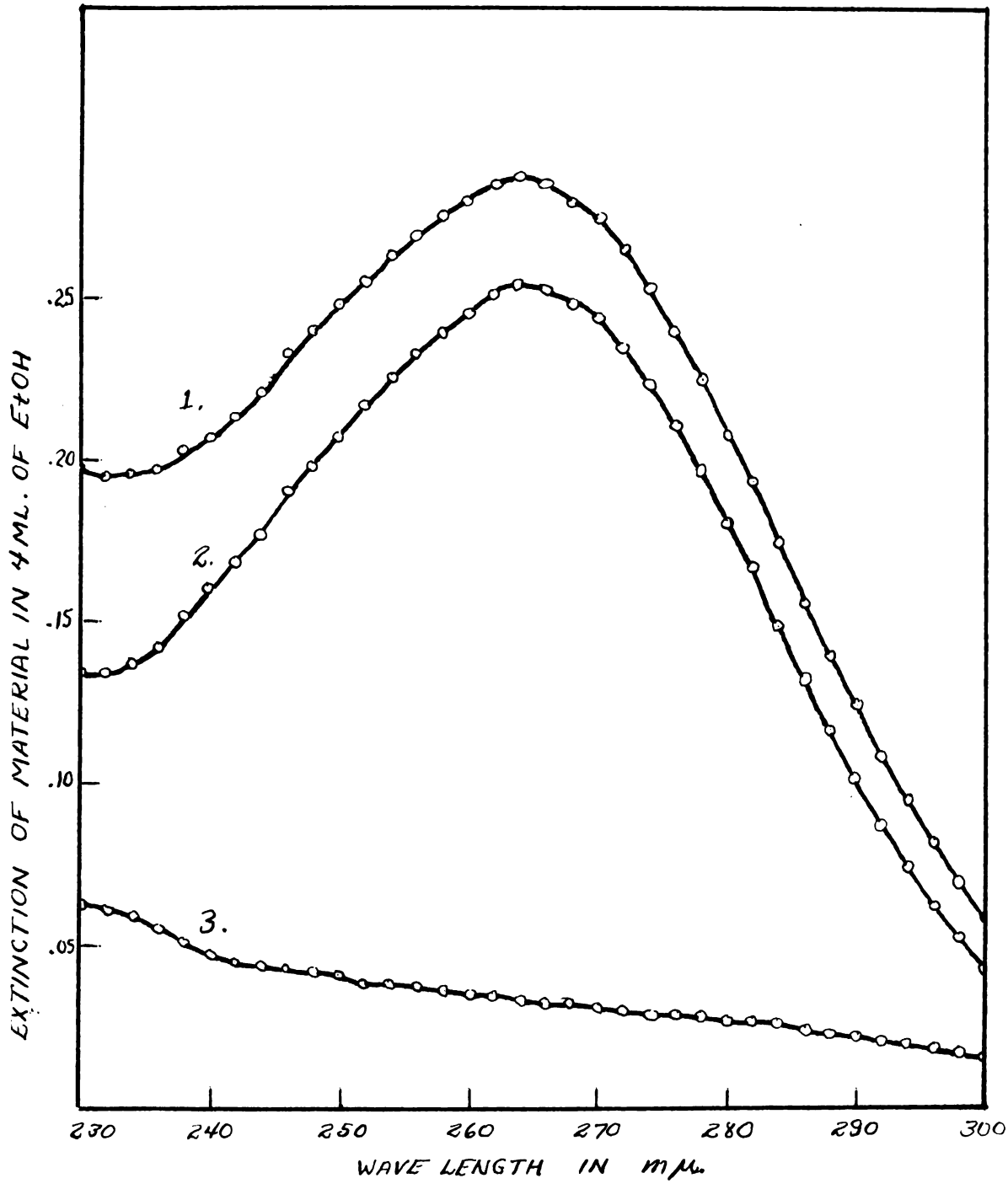

Fig. 2.-- Extinction Curves of (1) Total Residue in 12 ml. of Eluant from a Chromatographic Separation of a Mixture of 49.4% Calciferol - 50.6% Ergosterol and Containing 0.02146 mg. of Calciferol, (2) Substance Dissolved from "Super Filtrol" and (3) Corrected Calciferol Recovery Curve.

Extinction values are for the total solids dissolved in 4 ml. of ethyl alcohol.

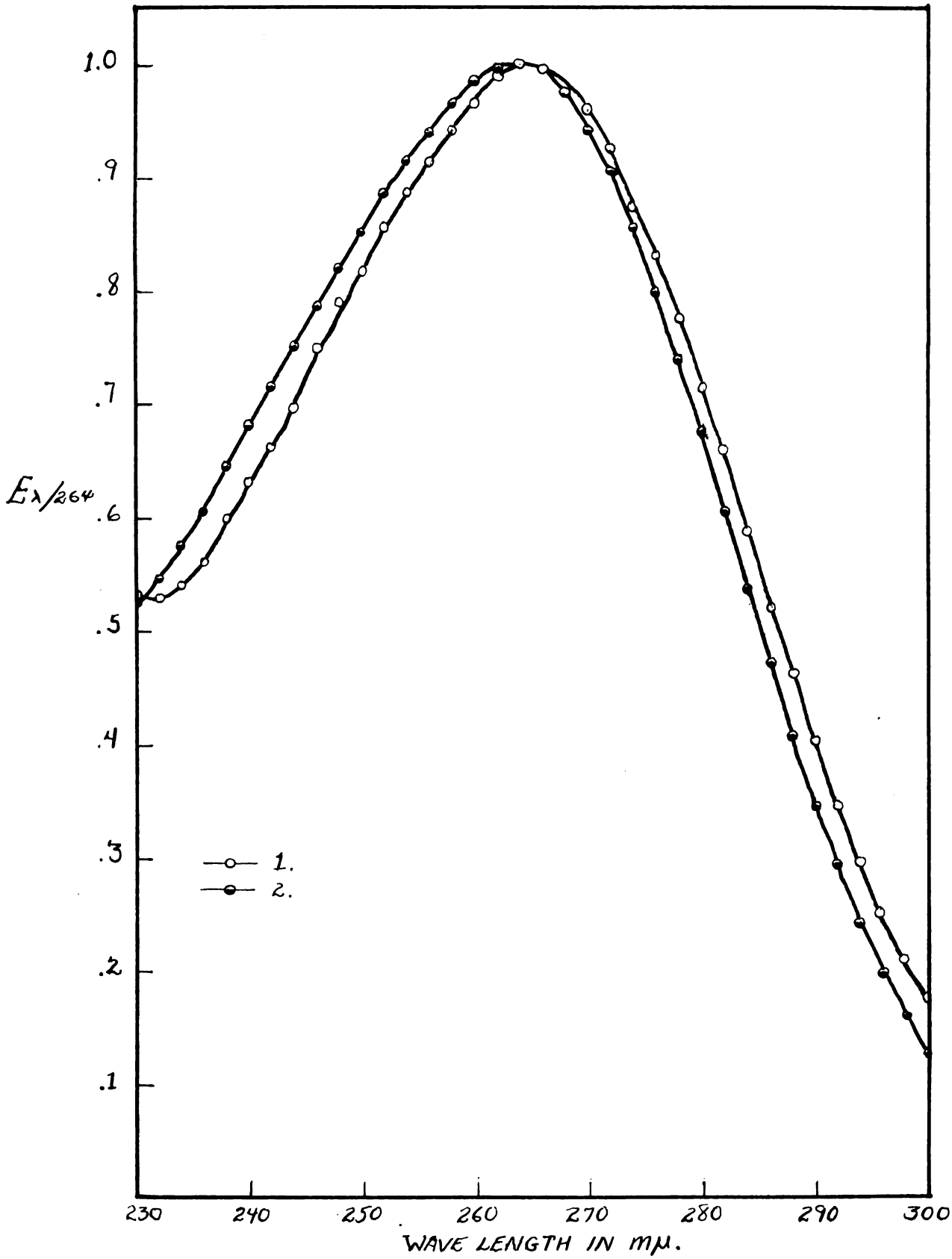

Fig. 3.-- Comparison of a corrected calciferol recovery curve (1) with the adsorption curve of pure calciferol (2). The calciferol was recovered from a 49.4% calciferol - 50.6% ergosterol mixture.

TABLE VIII

A Comparison of the Extinction Values for the Substance Dissolved from "Super Filtrol" as Calculated from the Correction Formula and the Values Expected from the Dilution

Dilution Factor		40	8	4	4	4	4
Mg. of Calciferol Placed on Column		0.4292	0.08584	0.04292	0.02861	0.02146	0.01072
		Extinction at 264 mu. of the Substance Dissolved from "Super Filtrol" from Formula					
Per cent Calciferol in Mixture	49.4	0.036	0.045	0.081	0.041	0.033	
	59.4	0.025	0.0425	0.043		0.0422	0.042
	75.1	0.013	0.023	0.034		0.035	0.046
	90.3	0.012	0.025	0.034		0.023	0.0345
		Extinction at 264 mu. of the Substance Calculated from the Averaged Blank Determinations and the Dilution					
Blank Determinations Containing No Calciferol		0.004	0.013	0.036	0.036	0.036	0.036
Dilution Factor		40	8	4	4	4	4

Discussion

The Column. -- The amount of "Super Filtrol" used was measured by the length of the column rather than by weight. A check showed that the weights of Super Filtrol in columns packed to the same length did not vary by more than 5% and that the variations in the results obtained were in no way correlated with the variations in weight. A length of 4cm. was used as a compromise. Shorter column lengths did not give complete separation, while longer lengths required not only an increase in the volumes of wash and developing solutions used but unduly increased the running time. The rate of flow through the column was considerably slower at the end of a run as clogging increased.

The Wash Solution. -- The volume of wash solution was fixed at 8 ml., which was sufficient to dissolve out most of the substance from "Super Filtrol" which interferes with optical analysis. After this volume had passed the amount of this material dropped sharply and remained almost constant. Although more washing might have removed an additional amount, the advantages to be gained would be offset by a greatly increased volume of solution and an increase in running time.

The Eluting Volume. -- It was found that after the sample in the initial ml. had been developed with 11 ml. more, all but a small fraction of the calciferol had passed into the receiving flask. After this volume, large amounts of ergosterol (or a modification) started to come through. That the choice was satisfactory is indicated by the fact that the average calciferol recovery for 20 runs was 99.87%. An idea of the purity, and hence sharpness of the separation, may be obtained from Fig. 3., which compares the absorption curve of calciferol recovered from a mixture of 49.4% calciferol and 50.6% ergosterol and the ab-

sorption curve of pure calciferol.

Analysis. -- The eluant was evaporated completely to dryness before investigation of the absorption curve because its exact volume was not known (due to evaporation losses). Further, solutions containing ether are not suitable for optical work because of their tendency to form perorides on standing. Alcohol was used at this stage because it was easier to purify and could be recovered.

Correction for the Substance from "Super Filtrol." -- The extinction effect of the material dissolved from "Super Filtrol" was too great to be minimized by dilution. This solution to the problem is only possible when the compound to be analyzed has a very strong extinction or is present in relatively large quantities. In this case, high dilution was not possible since the amount of calciferol present was very small.

Two methods of correction are available. First, the averaged extinction values at 264 mu. for the blank runs may be subtracted from the values for the total extinction at 264 mu., taking into consideration the dilution factor. Second, the extinctions of the solution at two different wave lengths may be found and the extinction of the calciferol alone calculated by solving two linear equations simultaneously. This involves the determination of certain constant ratios between extinction values at two different wave lengths for both calciferol and the interfering material (Table VI).

The latter procedure was adopted for two reasons. First, in spite of carefully duplicated runs on blank solutions, the extinction value of the residue from "Super Filtrol" could not be reproduced with any great accuracy, and no acceptable average could be found. On the other hand, the value of the ratio of extinctions at 230 and 264 mu. was fairly

constant. This was because the amounts of material dissolved from the column varied, but the kind of material was necessarily the same in each case. It can also be shown that variations in the value of this ratio as used in the formula do not cause as large variations in the result. By using this method, the limits of error were reduced.

The second reason for following this procedure in calculation was that the extinction effect of the interfering substance was correlated with the amount of calciferol passed through the column (Table VIII). The data show that for the larger amounts of calciferol (which require dilution before analysis) the correction which should be made for the interfering substance is much greater than would ordinarily be expected at the dilution used. Thus for 0.429 mg. of calciferol recovered and dissolved in 40 ml. of ethyl alcohol the correction should be about -0.004. Calculations show that the actual correction which should be made ranges from 0.012 to 0.036. It is as if the calciferol carried along with it some of this material in excess of the amount which would ordinarily come through the column in a control run.

Purity. -- It was impossible to determine to what extent ergosterol still contaminated the calciferol after its separation. The nature of the substance which came through the column after the calciferol, and which was due to ergosterol, could not be determined. However, it was observed that its absorption spectrum still had a relative maximum at 281 mu. The extent of the separation may be judged by the absorption curves for recovered calciferol and pure calciferol compared in Table VII and Fig. 3. Extinction ratios (the extinction at any particular wave length divided by the extinction at 264 mu.) are plotted rather than the actual extinction values, according to the method of Coser (9). Although

there is some slight increase in the curve between 264 and 300 mu., there is no significant rise at 281 mu. This example indicates a good separation even in the difficult case in which the original mixture contained only 49.4% calciferol. With mixtures containing smaller per cents of ergosterol, the slight distortion in the region beyond 264 mu. diminishes.

Summary

A method has been tested for the chromatographic separation of calciferol and ergosterol on "Super Filtrol" using a hexane-ether solution. The average yield of calciferol for the twenty separations tried was 99.87%. The four synthetic mixtures used contained 49.4, 59.4, 75.1 and 90.3% calciferol. Examination of the average recoveries for each of the four mixtures shows that the column gave the same quantitative results regardless of the amount of ergosterol present. The quality of the recovered product was better when the per cent ergosterol in the mixture was small. Amounts of calciferol separated by the column ranged from 0.4 to 0.01 mg. The smaller column used enabled the separation of such small quantities and also decreased the amount of material used.

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