THE CHROMATOGRAPHIC SEPARATION OF ERGOSTEROL AND CALCIFEROL

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Laura Jean Bullard

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This is to certify that the

thesis entitled

The Chromatographic Separation
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J. T. Ewing

Major professor

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THE CHROMATOGRAPHIC SEPARATION OF

ERGOSTEROL AND CHOLESTEROL

by

Laura Jean Bellard

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The Determination of Vitamin D in Fortified and Unfortified Oils

Many attempts have been made to separate vitamin D from other compounds of similar structure by physical and chemical methods. One of the most recent attempts is the method involving the separation of ergosterol from calciferol by a chromatographic procedure. This method depends upon the removal of ergosterol, from a solution containing ergosterol and calciferol, by its adsorption on Superfiltrol from a benzene-ethyl-alcohol solution.

Another method, which is still being used and improved, for the separation of vitamin D from other compounds, is the colorimetric method involving the formation of an orange-yellow color when the vitamin D and treated with a solution of antimony trichloride. At 500 mμ this color intensity reaches an absorption maximum whose extinction ($1\%_{1cm.}$) is directly proportional to the amount of vitamin D present. However, since other compounds which usually accompany the vitamin D in natural oils also give a color and an absorption value at 500 mμ, it was found necessary to modify this method.

Tompkins' (1) work can be summarized by the following: saponification of the oil, ether and treating with digitonin to remove sterols, chromatographing to separate vitamin A from vitamin D, and determining vitamin D by measuring the absorption at 500 mμ produced when the sample was added to a solution of antimony trichloride in chloroform. This method was used on natural oils only. The vitamin A was removed by chromatographing the saponified oil as was then accomplished by extracting partially dry fatty acids with a benzene-ether solution.

Tomkins was the first user of the benzene-ethyl alcohol mixture for adsorbing vitamin A or to activated bentonite clay. He also worked with activated clay from oxide, calcium hydroxide, diatomaceous earth, fuller's earth, bentonite, oil refining, benzene, carbon tetrachloride, and benzene. Of the adsorbents, zinc carbonate, bentonite clay, and activated bentonite clay gave positive results; the latter being found to give the best.

Kingsley (2), as did Tomkins, worked only with natural oils. The procedure followed by Kingsley was somewhat of a modification of Tomkins' method, and his procedure resulted in: saponifying the oil, extracting the non-saponifiable fraction with ether, and removing of the vitamin A, sterols, and pigments by chromatographic adsorption. The vitamins D and sterols were then determined colorimetrically with antimony trichloride. A second chromatographic procedure was used to adsorb the vitamin D onto activated bentonite clay from a Skellysolve-benzene solution. The sterols which were the only components remaining in the solution, were determined by the antimony trichloride method, and the difference in the two log I_o/I measurements was used to calculate the vitamin D potency.

Kingsley also worked with adsorbents, but he made quite an extensive study of the use and properties of Skellysolve-ether-alcohol as a solvent for adsorbing both vitamin D_2 and sterols. Consequently, he found a method for applying vitamins D plus sterols, analyzing sterols alone, and obtaining the vitamin D concentration by difference.

Young (3) worked with natural oils and obtained results comparable to those of Kingsley by using his method. However, he was predominately interested in using Kingsley's method for fortified organoleptic determinations. The fortified organoleptic was contained in various oils, the principle one being corn oil. He was only able to obtain satisfactory

results by Kingsley's method to within 10%.

Hage (4) modified Kingsley's method by increasing the adsorption surface for the second chromatograph by grinding the sample with the activated bentonite clay. This modification gave very satisfactory results when applied to natural oils, but when applied to samples of irradiated ergosterol, it was not very successful.

With the use of irradiated ergosterol in vegetable oil, Hage noticed the formation of an orange band when using Skellysolve-ether-alcohol as a solvent. It seemed that this orange band varied with the ether content, because if the ether content was highly increased, the band was not formed, and if the ether content was decreased, the band moved down the column much more slowly. Since he found pure calciferol solutions to form only a faint band, he suspected the band formed to be residual ergosterol or one of the intermediate irradiation products. Upon the study of this band, he found it gave a pink color reaction characteristic of ergosterol.

Baker (5) made a study of adsorbents and concluded that different samples of Superfiltrol acted alike and that magnesia showed a few positive possibilities for future use. He also did some work verifying the Kingsley (with Hage modification) method. Since Carlson (6) had noted that when certain solvents are passed through an adsorption column containing Superfiltrol, the filtrate contained a substance, apparently eluted from the Superfiltrol, which had an appreciable absorption in the ultra-violet region, Baker decided to verify this and study this eluted substance from the Superfiltrol. He made only a few determinations, but he concluded that something was eluted from the Superfiltrol and that a constant correction for the substance could be obtained by running a

"blank" along with each sample and applying this correction to the results, which were standardized on the Beckman spectrophotometer. He was the first to make this type of estimation determination on the [illegible]. He obtained this correction by a combination of the first and second chromatographic using the [illegible] Lindley's method.

Leber worked with natural oils and irradiated ergosterol and came to much the same conclusions as his predecessors.

[illegible] did a little work with alcoholic solutions of pure ergosterol and pure calciferol and their combination, and he concluded that using benzene-ethanol-alcohol as a solvent, ergosterol was adsorbed by activated bentonite and calciferol proceeded on through the tube. However, he did no quantitative or any conclusive qualitative work along this line.

Therefore, in view of what has been done in the field of separating vitamins D from their pro-vitamins and in view of what is yet to be done, it is the writer's purpose to present a method, using only pure stock alcoholic solutions of ergosterol and calciferol respectively, for:

1. Presenting and verifying the calculated and experimental proofs for the additivity of the two compounds with respect to their concentrations.
2. Quantitatively and qualitatively separating various dilute concentrations of the two compounds by chromatographic method which makes use of selective adsorption.
3. Quantitatively and qualitatively separating various strong concentrations of ergosterol and calciferol by the above mentioned method.

and to make a further study of heating, which appears during the development of the chromatogram, and of solvents.

Material and apparatus.—The three solvents used were hexane, ether, and ethyl alcohol.

Commercial hexane (from petroleum) was used without further purification as a filler for giving polarity to the reagents used for the developing of the chromatogram. This hexane, in order to be useable for the given purpose, must transmit from 210 mμ using a wide open slit on the Beckman quartz spectrophotometer (7).

Commercial anhydrous ethyl ether (U. S. P. filled every 6 days, and meeting A. C. S. specifications) was used without further purification and must be reusable here (8). This ether must transmit down to about 215.5 mμ using a wide open slit on the Beckman quartz spectrophotometer.

The absolute ethyl alcohol is a high grade chemically pure product, but it must be further purified. Purification was carried out allowing the ethyl alcohol to stand for 36 hours over potassium hydroxide and silver nitrate crystals. This solution may be shaken frequently and the precipitate allowed to settle out. It was then distilled, and the alcohol fraction was collected and allowed to stand for approximately one week over activated aluminum amalgam (9). The alcohol was distilled from the aluminum amalgam activated solution, and this alcohol fraction must transmit down to 215 mμ using a wide open slit on the Beckman quartz spectrophotometer.

All reagents must be free from any traces of benzene because of its high absorption value in the ultra-violet range in which we are interested. It also must be purified to be classified by Spectrol-

Supercel, the adsorbent used, is finely divided activated bentonite clay, and it was obtained from the Filtrol Corp., 315 West 5th Street, Los Angeles, California.

Mount Mesa ergosterol, which was obtained from Merke, Lewis, and Company, Detroit, Michigan, was used. This sterol is of a very high degree of purity. A stock alcoholic solution of the ergosterol, which was purchased in the form of crystals, was made up by weighing out a known amount of the crystals and making a solution of the crystals up to a known volume with purified ethyl alcohol (prepared as previously described).

The calciferol used, which was obtained from the Winthrop Chemical Company, New York City, New York, was made up into a stock alcoholic solution exactly as described above for the ergosterol. The white calciferol crystals were contained in a capsule. They are also of a very high degree of purity.

The suction apparatus was designed so that the pressure was controlled by the aid of an open-tube mercury manometer which was attached to the suction flask and had been calibrated so as to give a pressure differential of 6 cm. and 10 cm. of mercury. It was also an apparatus containing six banks so that six adsorption columns could be developed simultaneously.

Procedure.--The tubes used for the chromatographic determinations are made of pyrex and are about 15 cm. long and about 1.7 cm. in diameter. One end of each tube is constricted to a diameter of about 0.4 cm. (3). These tubes were thoroughly cleaned before filling by letting stand over night in sulfuric-dichromic acid solution. They

were then washed free of the clearing solution with distilled water and alcohol and were dried in a drying oven.

The adsorption columns were prepared by placing a small wad of cotton as a support for the adsorbent in the unconstricted portion of the tube. The tube was fitted to a suction flask as illustrated in Fig. 4 and packed with Superfiltrol. The adsorption columns must be carefully and uniformly prepared in order to obtain reproducible results. A suction of 6 cm. was applied. The quantity of Superfiltrol needed was measured by filling a 15 cm. long and 1.5 cm. in diameter test tube to a height of 6 cm. This amount of Superfiltrol was packed very firmly into the adsorption tube, using a large glass rod flattened on one end to a diameter just slightly less than that of the adsorption tube, under the above pressure. The pressure was applied so as to eliminate any air pockets in the column, because they would introduce irregularly shaped adsorption bands. This gave a packed column of 3.5 cm. A second and equal portion of the adsorbent was then added, the above process of packing repeated, and the column obtained measured 7 cm. in height. The column was now ready for use (Fig 4).

The various concentrations of ergosterol and cholesterol samples used were made up as desired by mathematical calculation from respective original stock solutions (prepared as previously described). The stock solution of ergosterol contained 0.0260 g./100 ml. of absolute ethyl alcohol. The stock solution of cholesterol contained 0.0276 g./100 ml. absolute ethyl alcohol.

The methods of preparation for the solutions to be chromatographed were varied. These methods were:

(a) Each desired percentage concentration of ergosterol

and calciferol was prepared so that the final concentration would give a corrected $A_{1\%,\ 1\ cm.}$ value of 0.700 at 272 mμ. This was done to prove that the curves are additive and the desired concentration of each constituent could be determined mathematically. Consequently, it was proven that the experimental and calculated values of the mixtures of different percentages of ergosterol and calciferol were identical. This has been illustrated in Table I and Fig. 1.

A known volume of solution prepared in such a manner was chromatographed, the filtrate taken to dryness, the residue dissolved in the original known volume of alcohol, any necessary dilutions made, and the absorption curve of this solution determined on the machine. As will later be described, it was found that this method required a solvent correction factor. With the use of this correction factor, this method was a very satisfactory way to determine very small amounts of calciferol present in a mixture of the two compounds. The application and use of this correction factor is shown in Table II and Fig. 2.

(b) A known amount of calciferol was used, and a correspondingly large amount of ergosterol, so as to give the desired relative percentage concentration of each. Then after chromatographic analysis, the calciferol residue, after the solvents were removed by evaporation to dryness, required a dilution of over 1:100 in order to determine its absorption curve. Consequently, the necessary correction factor in the above method was eliminated by dilution.

This method was advantageous in that it did not require accurate volumetric measurements and also showed that the percentage recovery of calciferol from chromatographic analysis did not depend upon the original concentration of calciferol. An example of this has been illustrated in the results shown in Table III and Fig. 3.

From the alcoholic samples which were prepared as described above, a measured aliquot was taken from each and placed in an Erlenmeyer flask. Regardless of the method of preparation used for the sample, the chromatographic procedure was the same. The aliquots were evaporated to dryness from their alcoholic solutions in the vinyl evaporation apparatus. Under the reduced pressure, the evaporation was hastened by placing the Erlenmeyer flask containing the sample in a beaker of hot water.

While the sample was evaporating, a solution of mixed solvents, known as "developer" or "50-10-1", was prepared. The developer consisted of 50 parts of hexane, 10 parts of anhydrous ethyl ether, and 1 part of absolute ethyl alcohol by volume. (These were the previously mentioned purified solvents.)

The dry residue from this evaporation was taken up in 5 ml. of the special mixture of solvents.

The previously prepared 7 cm. adsorption column was wetted and washed down with developer until no absorption was observed when the developer that had gone through the column was run against a portion of the original solution of developer in the beaker. This condition existed after the addition of about 30-35 ml. of this special mixture of solvents. At this point a yellowish-brown band developed in the Superfiltrol about one third of the way down the column (Fig. 2_1). This band seemed to be

important and should be left in the tube, because without it and the proper amount of developer present when it went through the tube, the superfiltrol was found to show no selective adsorption properties [illegible].

The sample was added, followed by 5 ml. of the solvent for rinsing the flask and 30-35 ml. of the same solvent for developing the adsorption bands. A separatory funnel was used as a container for the 35 ml. of developer and acted as a dripping funnel (Fig. 1) so that the top of the adsorption column did not become dry, otherwise it would shrink away from the glass walls and development of the column would be irregular. A pressure differential of 6 cm. of mercury was maintained while the sample and the 5 ml. of each level were being added, and then this differential was increased to 10 cm. while the 35 ml. of the developer was being added. Further increase of the suction would result in cracking in the adsorption column and packing of the adsorbent to such a degree that the flow of the developing solution would be nearly stopped.

It was noticed that upon the addition of the 35 ml. of developer, the afore-mentioned yellowish-brown band acquired a definite uniform bluish-green color (Fig. C_2). And, when the 35 ml. of developer had passed through the column, the band had progressed down the tube to within about ½ cm. above the cotton support and was restored to its original yellowish-brown color (Fig. C_3).

When the last of the developer had passed through the column, the adsorbent was dried by drawing air through the column for 5 to 10 minutes. Upon letting the adsorbent stand for one half hour or more and becoming more completely dry, the yellowish-brown band at the bottom

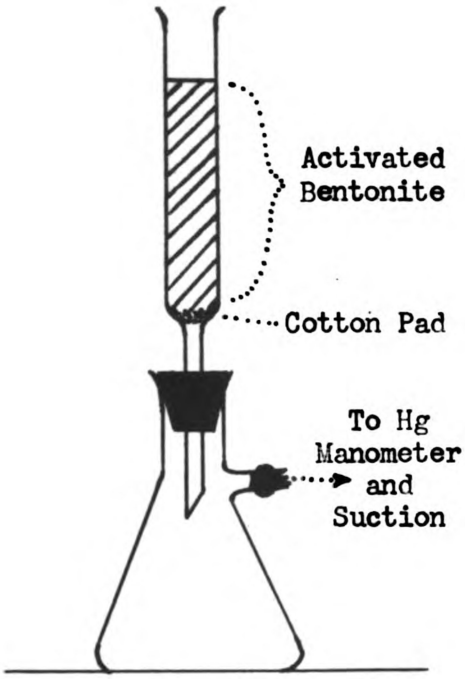

Fig. A

Packing the Adsorption Tube

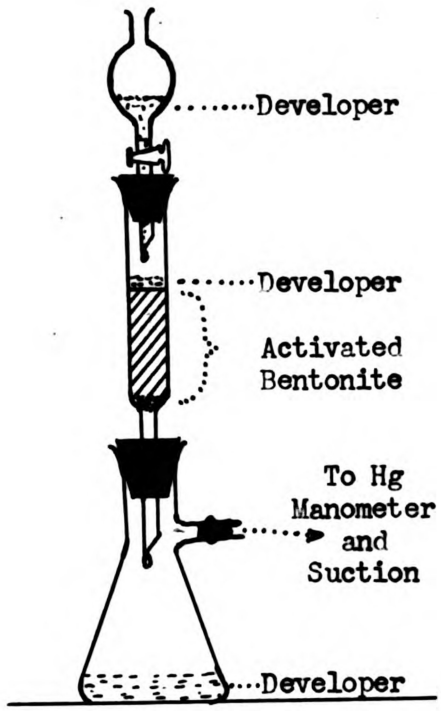

Fig. B

Developing the Chromatogram

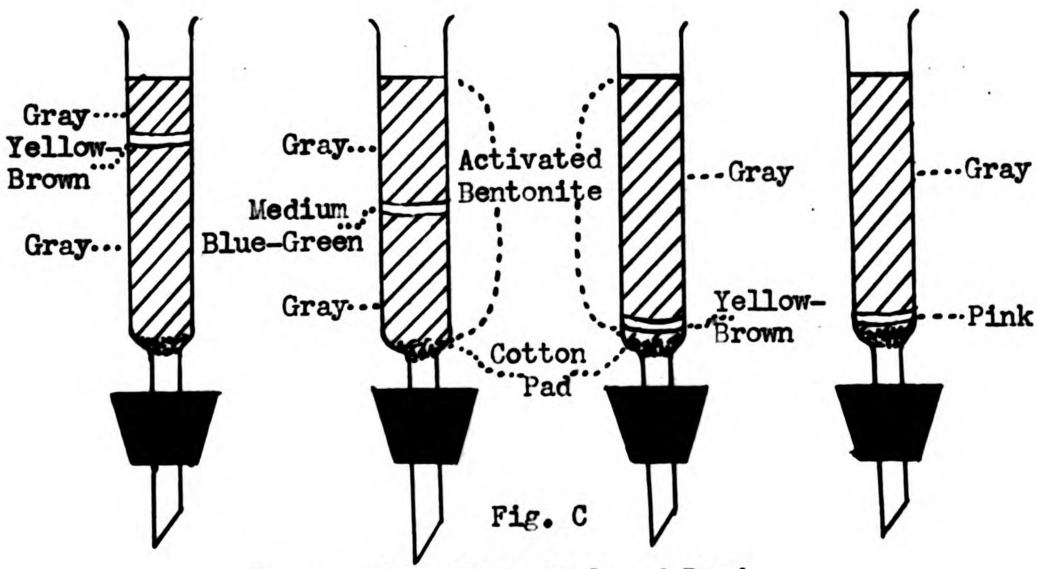

Fig. C

Progression of the Colored Band

(1) Before addition of the sample

(2) Addition of the sample

(3) After addition of the sample

(4) After drying ½ hr. or more

of the column changed to a very pink color (Fig. 8) when very concentrated solutions of ergosterol and calciferol had previously been passed through the tube.

This filtrate was evaporated to dryness under reduced pressure and with a beaker of hot water surrounding the flask to hasten evaporation.

The filtrate residue was taken up in 10 ml. of absolute ethyl alcohol, and the correct dilutions were determined (when necessary) and made so as to give an absorption value for log I_0/I between 0.500 and 0.800 at the critical wave length desired which shows maximum absorption. Since this residue had alcohol as its solvent, its absorption curve was determined on the Beckman using the absolute ethyl alcohol as the reference, or blank.

Results can be calculated from the standpoint of the determination of: the additivity of the ergosterol and calciferol curves, the necessary use of the solvent correction factor, and the elimination of the solvent correction factor to determine the final composition of the filtrate.

I. The additivity of ergosterol and calciferol curves.

A. To obtain a complete curve of any percentage mixture of ergosterol and calciferol by mathematical calculations.

1. Determine the curve of each of the pure compounds to be used.

2. Apply the fundamental equation, which is:

$$E\ (1\%,\ 1\ cm.) = \frac{\log I_0/I}{(l)(c)}$$

where E (1%, 1 cm.) is the extinction of a solution that has a concentration of one gram of solid per one hundred milliliters of solution

and the absorption is measured through a thickness of one centimeter of solution; I_o is the incident intensity; I is the transmitted intensity; l is the thickness of the solution in centimeters; c is the concentration of the solution in grams per one hundred milliliters.

3. For each wave length desired, substitute the proper values in the above equation and determine the required $E^{1\%}_{1cm.}$ (1%, 1 cm.) value.

For example, let the desired wave length for a mixture of 20% ergosterol and 80% calciferol be 260 mμ. Then:

$$E\ (1\%,\ 1\ cm.) = E^{1\%}_{1cm.}\ (\text{for erg. at } 260\ m\mu) \times 0.20 + E^{1\%}_{1cm.}\ (\text{for calc. at } 260\ m\mu) \times 0.80$$

(desired total)

Values taken from graph of pure samples

$$= 172 \times 0.20 + 423 \times 0.80$$

$$= 338 + 36$$

$$= 374$$

B. To obtain a complete curve of any percentage mixture of ergosterol and calciferol by experimental results:

1. Calculate mathematically the final concentration of the mixture which will give a constant E (1%, 1 cm.) value of 0.700 at 272 mμ.

Using alcoholic stock solutions of ergosterol (0.0260 g./100 ml.) and calciferol (0.03276 g./100 ml.) and the fundamental equation as a reference, the following equation are employed:

$$\log I_o/I = E^{1\%}_{1cm.}\ (\text{of ergosterol at a given } \lambda) \times \text{Concentration} + E^{1\%}_{1cm.}\ (\text{of calciferol at a given } \lambda) \times \text{Concentration}$$

(total)

Letting the desired wavelength be 272 mμ, then:

0.700 = 260 x 0.20 x "C" + 408 x 0.80 x "C"

where 260 and 408 are obtained from a graph of the pure compounds; 0.20 and 0.80 are the desired concentration concentrations; "C" is a certain total concentration.

Solving for "C":

"C" = 0.01890 g./100 ml. = total number of solids that needs to be present in 100 ml. to give this log I_o/I value..

2. Determine the exact number of milliliters of orcinol and salicylol which should be taken from the stock solution by:

$$\frac{0.20 \times 0.01890}{0.0260} = 0.0143 \text{ ml. of stock solution for every ml. of total solution desired}$$

and

$$\frac{0.80 \times 0.01890}{0.02376} = 0.6362 \text{ ml. of stock solution for every ml. of total solution desired.}$$

where, for orcinol and salicylol respectively, 0.20 and 0.80 are the desired final concentrations of each with respect to the total concentration of the solution; 0.01890 is the calculated number of grams/100 milliliters necessary for the solution; 0.0260 and 0.02376 are the concentrations of the stock solutions.

Therefore, in order to have a large enough volume to be able to do accurate work volumetrically, multiply each of the above results by one hundred giving the values 1.43 ml. and 63.62 ml. respectively. Take these portions, add them together, and add enough absolute ethyl alcohol to the mixture to bring the total volume up to 10 ml. This solution is

now by three to stock, or light to 1:10, and you have the concentration desired.

With this solution, determine its absorption curve on the spectrograph using the prism designed. As an example, it was found that the log I_0/I value for this solution was 0.700 at 272 mμ and the Δ (1/λ. Imp.) was 37². The 0.700 value is the exact figure previously calculated for this solution. In order to obtain any other exposure value, calculate the desired concentration or curve, as set up in values previously described, and determine the absorption curve. The results of such calculated and exposed strip are graphically tabulated in Table I, Table IV, and Fig. 1.

II. The use of the solvent correction factor.

When such small amounts of concentrated (as described above) were absorption-tested, it was found necessary to determine a solvent correction factor, because the solvent had appreciable absorption in wave length in which we were interested.

A definite absorption curve was determined by carefully and volumetrically determining the absorption for 10 ml. of developer. This was done by accurately taking 10 ml. of unchromatographed developer, evaporating this to dryness, and taking this residue up to 10 ml. in absolute ethyl alcohol. The absorption curve of this solution was determined and is recorded in Table II and Fig. 2.

Therefore, knowing the exact absorption for a definite amount of developer, chromatographing all samples volumetrically, and making all necessary dilutions with great accuracy, it is easy, by mathematical calculation, to determine the percent of original solvent correction that

TABLE I

Absorption Curve Values for Pure Ergosterol and Pure Calciferol and a Percentage Mixture of Both

Wave Length mu.	Pure Ergosterol g./100 ml. 0.0026		Pure Calciferol g./100 ml. 0.00166		20% Ergosterol, 80% Calciferol g./100 ml. 0.0018[illegible]		
	log I_0/I	$E^{1\%}_{1cm.}$	log I_0/I	$E^{1\%}_{1cm.}$	Calculated $E^{1\%}_{1cm.}$	Experimental log I_0/I	$E^{1\%}_{1cm.}$
230	.130	50	.382	230	194	.362	196
232	.130	50	.396	238	200	.376	203
234	.130	50	.418	250	210	.392	212
236	.131	50	.435	263	220	.407	220
238	.134	52	.453	273	228	.425	230
240	.144	55	.472	284	238	.444	240
242	.151	58	.498	300	252	.468	253
244	.165	64	.522	314	264	.490	265
246	.181	70	.544	328	276	.514	278
248	.207	80	.568	342	290	.536	290
250	.234	90	.590	356	303	.562	304
252	.284	109	.610	368	316	.583	315
254	.305	117	.646	389	334	.619	335
256	.340	131	.665	401	347	.646	350
258	.404	155	.680	410	362	.670	362
260	.466	179	.704	423	374	.694	375
262	.481	185	.715	431	382	.707	382
264	.483	186	.725	437	386	.716	387
266	.516	198	.725	437	389	.721	390

Table I
(Page 2)

Wave Length mμ.	[illegible] g./100 ml. 0.00[illegible]6		[illegible] g./100 ml. 0.00[illegible]		[illegible] g./100 ml. 0.00[illegible]		
	log I_0/I	$E^{1\%}_{1cm.}$	log I_0/I	$E^{1\%}_{1cm.}$	Calculated $E^{1\%}_{1cm.}$	Experimental log I_0/I	$E^{1\%}_{1cm.}$
268	.683	22[illegible]	.718	[illegible]	39[illegible]	.71[illegible]	39[illegible]
270	.656	252	.703	[illegible] (corrected: 23)	[illegible]	.72[illegible]	[illegible]
272	.675	260	.666	[illegible]	378	.700	378
274	.62[illegible]	[illegible]	.645	388	359	.665	360
276	.598	[illegible]	.608	367	339	.628	[illegible]
278	.610	[illegible]	.573	345	323	.601	325
280	.675	260	.522	31[illegible]	303	.558	[illegible]
282	.704	270	.475	286	283	.506	[illegible]
284	.635	[illegible]	.426	256	25[illegible]	.[illegible]72	[illegible]
286	.500	19[illegible]	.378	228	220	.[illegible]1	[illegible]
288	.[illegible]	[illegible]	.328	197	180	.351	[illegible]
290	.38[illegible]	1[illegible]7	.283	171	166	.307	[illegible]
292	.405	156	.245	[illegible]7	1[illegible]8	.277	150
294	.[illegible]8	160	.210	126	13[illegible]	.2[illegible]	135
296	.354	136	.174	105	111	.203	110
298	.223	86	.140	8[illegible]	8[illegible]	.157	85
300	.116	45	.113	68	6[illegible]	.118	[illegible]

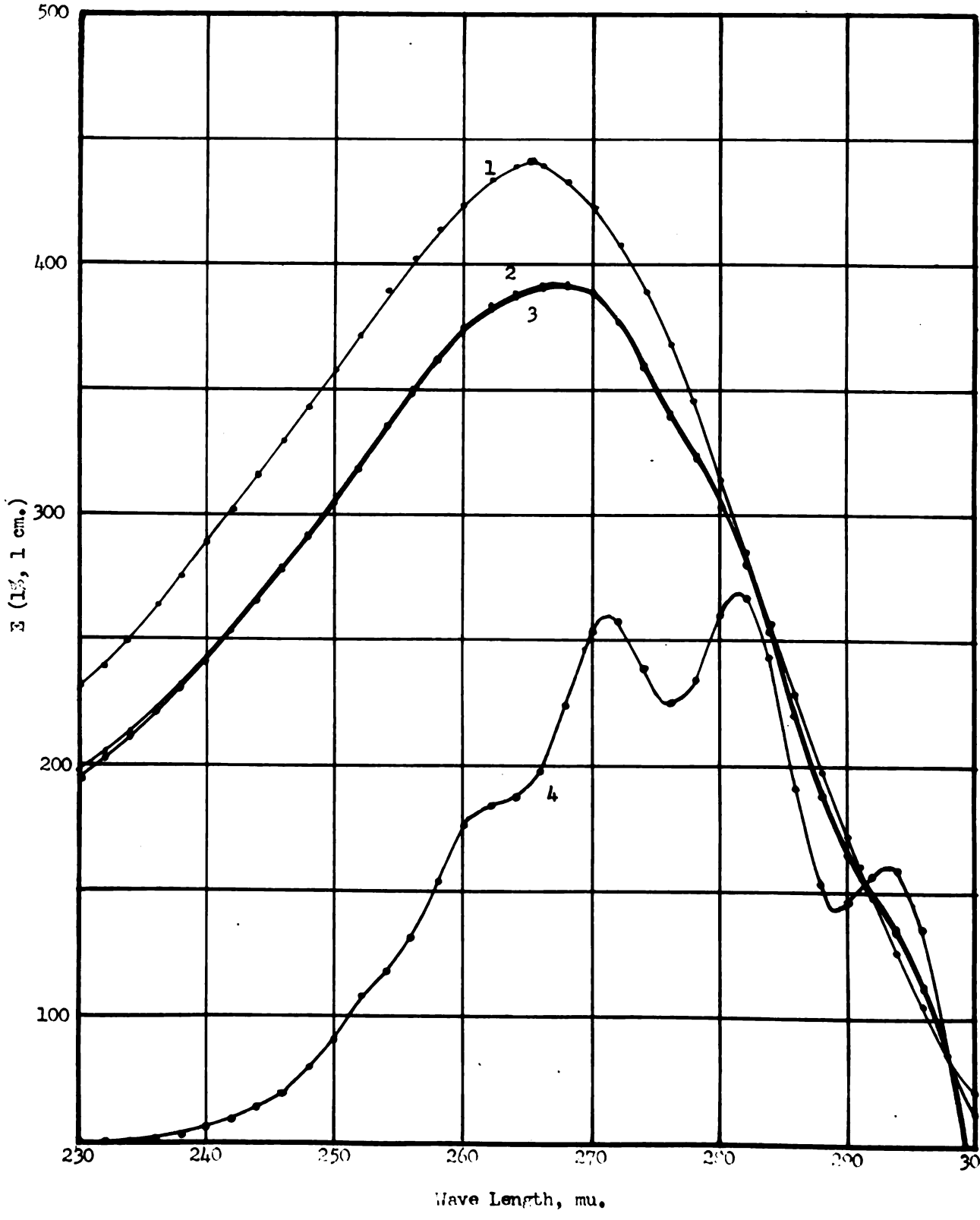

Fig. 1.— Absorption curves of ergosterol and calciferol in absolute ethyl alcohol: 1, pure calciferol; 2, 20% ergosterol - 80% calciferol (experimental); 3, 20% ergosterol - 80% calciferol (calculated); 4, pure ergosterol.

Table II

Absorption Curve Values for a Solution of 20% Ergosterol and 80% Cholesterol with the Application of the Solvent Correction Factor

Wave Length mμ.	ES-10-1 Orig. Vol. of 10 ml. Final Vol. of 10 ml. Dilution; none	20% Ergosterol - 80% Cholesterol Orig. Vol. of 10 ml. Final Vol. of 10 ml. Dilution: 1:2 0.00158 g./100 ml.		$E^{1\%}_{1cm.}$
	log I_0/I	Original log I_0/I	Corrected log I_0/I	
230	.304	.179	.175	237
232	.294	.174	.180	245
234	.279	.168	.189	255
236	.263	.163	.200	270
238	.245	.156	.211	285
240	.232	.154	.222	300
242	.221	.151	.233	315
244	.216	.158	.244	330
246	.211	.164	.253	342
248	.210	.177	.267	361
250	.210	.188	.278	376
252	.210	.197	.287	389
254	.212	.509	.297	401
256	.212	.516	.304	412
258	.212	.527	.315	425
260	.211	.533	.323	435
262	.206	.533	.327	442
264	.199	.530	.331	448
266	.194	.526	.332	449
268	.189	.518	.329	445
270	.187	.508	.321	434

Table II

Wave Length	50-10-1 Orig. Vol. of 2 ml. Final Vol. of 10 ml. Dilution: None	20% Isopropanol - 80% Dilutent Orig. Vol. of 2 ml. Final Vol. of 10 ml. Dilution: 1:2 0.001% E./100 ml.		
mμ.	log I_o/I	Original log I_o/I	Corrected log I_o/I	E1%
272	.184	.495	.311	421
274	.180	.480	.300	405
276	.174	.455	.281	380
278	.163	.429	.267	361
280	.156	.406	.250	338
282	.151	.380	.229	310
284	.144	.347	.203	275
286	.143	.325	.183	247
288	.139	.301	.163	220
290	.136	.277	.141	190
292	.132	.254	.122	165
294	.129	.229	.100	135
296	.123	.207	.084	114
298	.123	.193	.070	95
300	.119	.174	.055	74

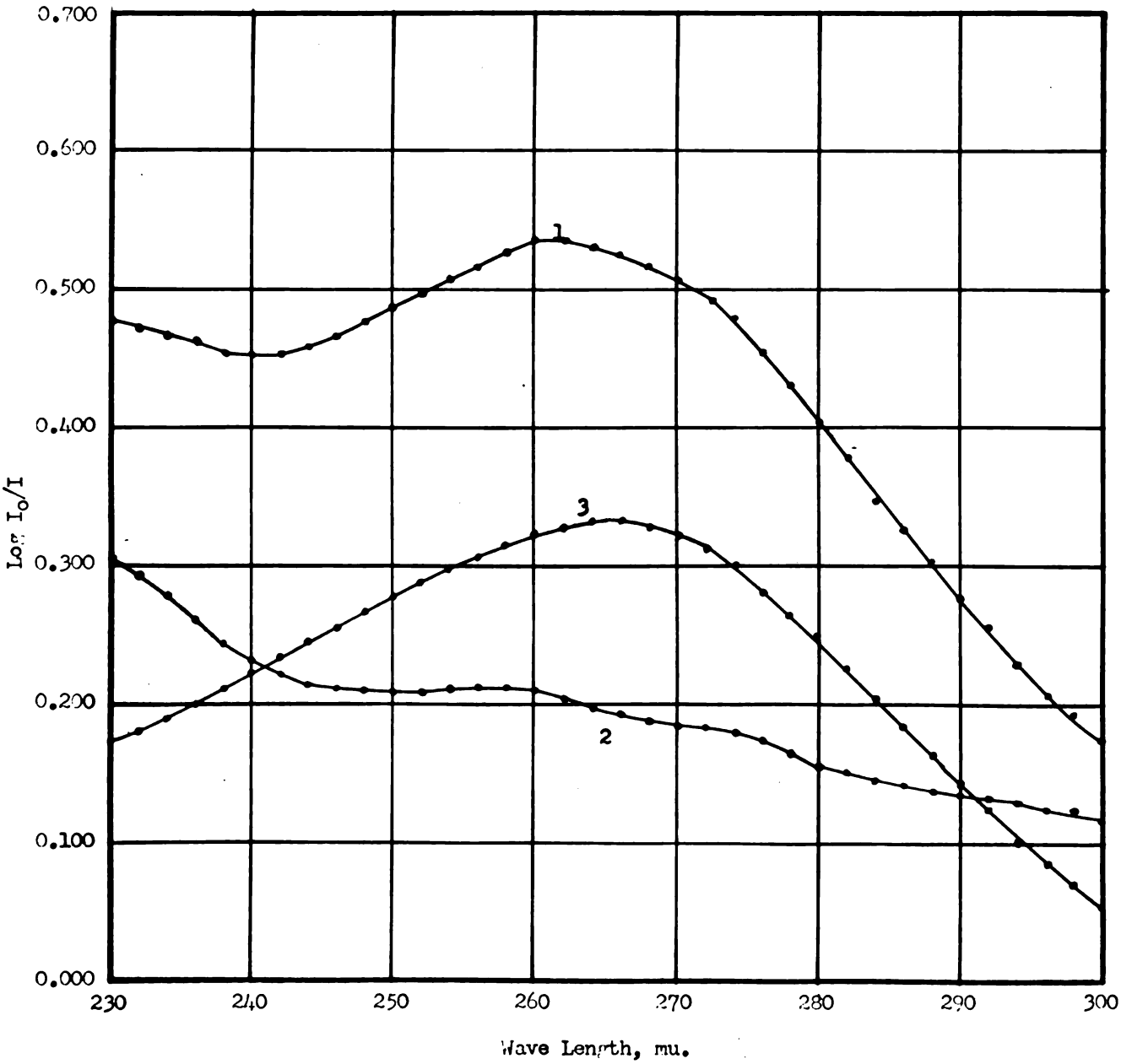

Fig. 2.— Absorption curves of calciferol and solvent (equal final volumes and same total dilutions): 1, recovered calciferol and solvent correction (original filtrate); 2, solvent correction; 3, corrected recovered calciferol (pure).

ready to be applied in each case.

The form of the fundamental equation that is used is:

$$E\ (1\%,\ cm.) = \frac{\log I_o/I\ (\text{of solution}) - \log I_o/I\ (\text{of developer})}{l \times c}$$

This equation has been used to calculate the results shown in Table II, and Fig. 2.

III. The elimination of the solvent correction factor.

It has been found if a solution containing a high amount of solids of calciferol is used, that by a certain progression of dilutions in order to obtain the absorption curve, the solvent correction factor can be almost entirely diluted out.

For example: An extraction of 0.1% at 266 mμ for 40 ml. of developer which had been taken to dryness and brought up to 10 ml. of alcohol was observed. Then this pure volume was taken to dryness and brought up into a total dilution of 1:100, an absorption of only 0.00085 would exist. However, this absorption affects the Beckman readings only five in the third decimal place, or only five in a thousand. This is beyond the accuracy of the instrument in the range of 0.500 to 0.800, because it is only accurate to plus or minus five in the thousandths place.

A typical chromatographic run and calciferol recovery with results is presented in Table III and in Fig. 3. Since 40 ml. of solvent have been involved in the chromatographing of the above cited solution, and the dilutions are 1:10; 1:9; 1:4; at 266 mμ, the correction factor will be:

$$0.1\% \times \frac{1}{36} = 0.005$$

which is within the limitation of the instrument, and it illustrates how

Table III

Absorption Curve Values of a Preserved Solution from 2 ml. of Unpreserved Solution in Erlenmeyer and Solution without the Application of the Solvent Dilution Factor

Wave Length mμ.	30% Alcohol - 70% Chloroform $\log I_0/I$ .1 % 1 cm. .1750 g./100 ml. Dilutions: 1:10; 1:9; 1:8	
230	.352	246
232	.358	271
234	.368	279
236	.379	287
238	.392	299
240	.412	312
242	.428	324
244	.441	334
246	.459	347
248	.476	361
250	.492	373
252	.505	383
254	.521	395
256	.535	405
258	.546	414
260	.557	422
262	.567	430
264	.572	434
265	.575	436
266	.572	434
268	.565	428

Table III
(Page 2)

Wave Length mμ.	30% [illegible] 1 - 70% [illegible] 1 log I_0/I	$E^{1\%}_{1\,cm.}$ 0.1750 g./100 cc. Dilutions: 1:10; 1:9; 1:4
270	.553	419
272	.538	407
274	.518	392
276	.490	371
278	.461	349
280	.429	325
282	.398	301
284	.362	274
286	.329	249
288	.296	224
290	.261	198
292	.227	172
294	.200	151
296	.174	132
298	.152	115
300	.132	100

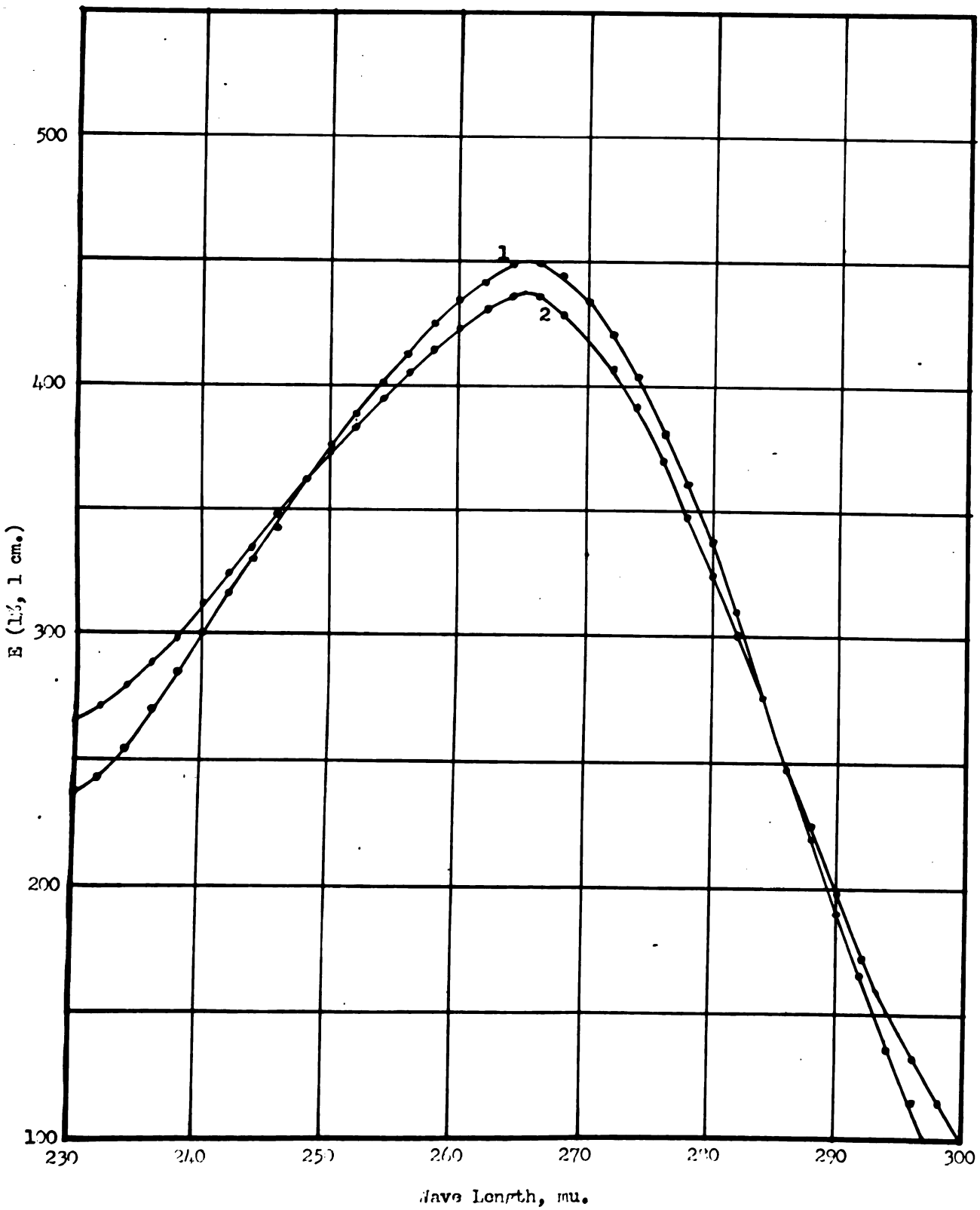

Fig. 3.— Absorption curves of pure recovered calciferol in absolute ethyl alcohol: 1, calciferol recovered from a 20% ergosterol - 80% calciferol solution; 2, calciferol recovered from a 30% ergosterol - 70% calciferol solution.

Table IV

Comparison of Calculated and Experimental E (1%, 1 cm.) Values for Various Concentrations of Ergosterol and Calciferol at the Wave Length During Maximum Extinction

Composition		Total Solids Present g./100 ml.	Wave Length mμ.	E (1%, 1 cm.)	
Erg.	Cal.			Calculated	Experimental
100	0	0.0026	282	268*	270
90	10		272	275	
50	50	0.00200	270	338	[illegible]
40	60	0.002[illegible]	270	355	358
30	70	0.001935	269	371	374
20	80	0.001[illegible]8	268	390	391
10	90	0.0178	266	413	415
0	100	0.00166	265	460*	461

*Literature value (11)

the solvent power factor may be slightly by dilution.

Variation of solvents for developing.— A study was made to see if anything could be substituted for the list of solvents used as a developer and also to see if all of the properties of this mixture were essential.

In working with various combinations of the developer which were used throughout the study, it was found that a solution of ether-ethyl alcohol gave results identical to those of a solution of benzene-ethyl alcohol. However, during the development of the chromatograms, no visible bands were formed in the chromatogram, so it was not possible to know where or when to stop the developing. When ether alone was used as a developer, it also gave good positive results, but again no bands were noted. Ether is very volatile and impractical to use for any type of volumetric work. Also, it has a decided residue which would interfere with absorption results. Ethyl alcohol, when used as a developer, permitted both unretarded and retarded to pass through the chromatrol, but it was very large and seemed to stop up the column. Solutions of benzene-ether and benzene-ethyl alcohol permitted neither unretarded nor retarded to go through the chromatrol, but the chromatic yellowish-brown band was formed in each case.

Other mixtures of solvents investigated were: benzene-isoamyl alcohol benzene-acetone, ether-isopropyl alcohol, and ether-acetone. Since the composition of the mobile phase were kept constant, all of the four systems involved here, upon determining their absorption curves, gave exactly the same curve. The curve then disclosed that one of the original solution

had been held back and corresponded exactly to a 25% crystoral - 75% colloformol curve (which was the composition used for the determinations). Therefore, the solvents do not afford a way to separate crystoral from colloformol on even both of the brick.

The above observations have been compiled and presented in Table V.

Discussion.— It was not possible to find any previous work that had been done on the additivity of alcoholic solutions of pure crystoral and pure colloformol and their resultant absorption curves. Therefore, it was decided to try to formulate a mathematical way to add the respective $E\,(1\%,\ 1\ cm.)$ values of the two pure substances and determine an "ideal" or "hypothetic I" (until further proven) solution curve. The next step was, by titration, to find a method for ascertaining the correct concentration of each compound and combining them into decimal percentages. A solution prepared in this manner had its absorption curve determined on the Lovibond, and this brought about the verification of the above mathematical calculations. This was very important, and very good results were obtained as was shown in Table IV. There is no obvious reason from the experimental results being consistently higher than the calculated results by about 2 or 3 units. The reason for the curves of the 100% solutions not agreeing exactly with the $E\,(1\%,\ 1\ cm.)$ value given in the literature is that the compounds used were only of a very high degree of purity, and not of 100% purity.

Using a solution prepared in the above way, it was then desired to separate the two compounds by adsorption of the crystoral upon Super-filtrol from a benzene-alcohol solution. This separation proved very satisfactory, but the small quantities of the compounds used introduced a necessary correction for solvents in order to obtain correct results.

Table V

Summary of Specificity of Solvents

Solvents part/part	Ethyl Alc. 1	Ether 10	Hexane 50	Isopropyl Alc. 10	Acetone 10
Ethyl Alc. 1	..	[illegible]	C	x	x
Ether 10	[illegible]	[illegible]	C	A	A
Hexane 50	C	C	C	..	A
Isopropyl Alc.	x	A	A	A	x
Acetone 10	x	A	A	x	x

A, both ergosterol and cholesterol in the filtrate; B, cholesterol only found in the filtrate; C, neither ergosterol nor cholesterol in the filtrate; x, not tried due to other preceding results.

Baker (5) thought the necessary correction was due to [illegible] eluted from the Superfiltrol. However, it has been shown here that this correction can be entirely eliminated by washing the column along with developer until no absorption is shown when this solution is run on the Beckman against some of the original developer. The compounds that need a correction applied for their absorption are the residues of the constituents of the developer. These always appear, especially when dilute concentrations of the compounds are used, and they can not be eliminated except by continuous dilution.

The bands, as shown in Fig. 1, which appeared in every single irradiated mixture of ergosterol and calciferol, have previously been observed by Page ( ). It was his opinion that the band was associated with the relative concentrations of ether in the developer. His work and these results both indicated this same conclusion. However, he believed this yellowish-brown band to be associated with ergosterol or some other intermediate product of irradiation. In this work the band was run on out of the column with additional developer, evaporated to dryness, taken up in ethyl alcohol, and its absorption curve determined. All results, which seemed to vary slightly but gave relatively the same curve, indicated no ergosterol present in this band.

Several attempts were made by using various solvents to recover the ergosterol from the Superfiltrol, but no satisfactory results were obtained.

It was shown to be helpful if concentrated solutions of ergosterol and calciferol could be used, because the large number of dilutions shortened the time required for calculating the results by "diluting out" the

solvent connection. This also helps to improve the accuracy of the results, since [illegible] this [illegible] required for the amount of solvent used for developing. It [illegible] only in the absence are that such concentrated solutions as these would be used.

A further check on the results obtained by this chromatographic method of analysis for vitamin D_2 may be made by the antimony trichloride method as shown by Ewell (10). He also has shown that for natural oils not only can ergosterol be separated from calciferol by this chromatographic method of analysis, but that a complete separation of all of the remaining irradiation products can be separated from calciferol.

Table VI and Fig. 4 illustrate very clearly how the percentage recovery of calciferol varies inversely with the concentration of ergosterol present and how the points of the percentages vs. concentration when placed on a graph seem to fall along a straight line.

It was first thought that the developer could be varied or completely changed by using other solvents, but their combinations were found not to be selective in their ability to promote the adsorption of either ergosterol or calciferol upon activated bentonite clay.

From the results obtained by the work done on the various solvents for developing, it appeared that hexane was present merely as a "filler", because it either retained or released the compounds that were dissolved in it depending on what it was used with. The combination of ethyl alcohol and ether seemed to be the necessary combination. The alcohol evidently acted as a polar compound for the selective adsorption of the ergosterol upon the Superfiltrol, but with it present in any great quantity, it lost this property and clogged up the column. Ether seemed to be the most effective solvent,

Table VI

Compilation of Calciferol Recovery Results from Twelve Solutions Containing Various Percentages of Ergosterol and Calciferol with an Absorption Maximum at 265 mμ.

Composition % Erg.	Composition % Calc.	Observed E(1%,1 cm.)* at 265 mμ.	Literature E(1%,1 cm.) at 265 mμ.	Calciferol Recovery %
100	0	4	460	.9
60	40	436	460	94.8
50	50	430	460	93.6
50	50	433	460	94.1
40	60	428	460	93
40	60	447	460	97.5
30	70	432	460	94
30	70	436	460	94.8
25	75	440	460	95.8
20	80	449	460	97.6
10	90	452	460	98.2
0	100	455	460	99

* Value given by chromatographed solution (filtrate) containing only pure Calciferol.

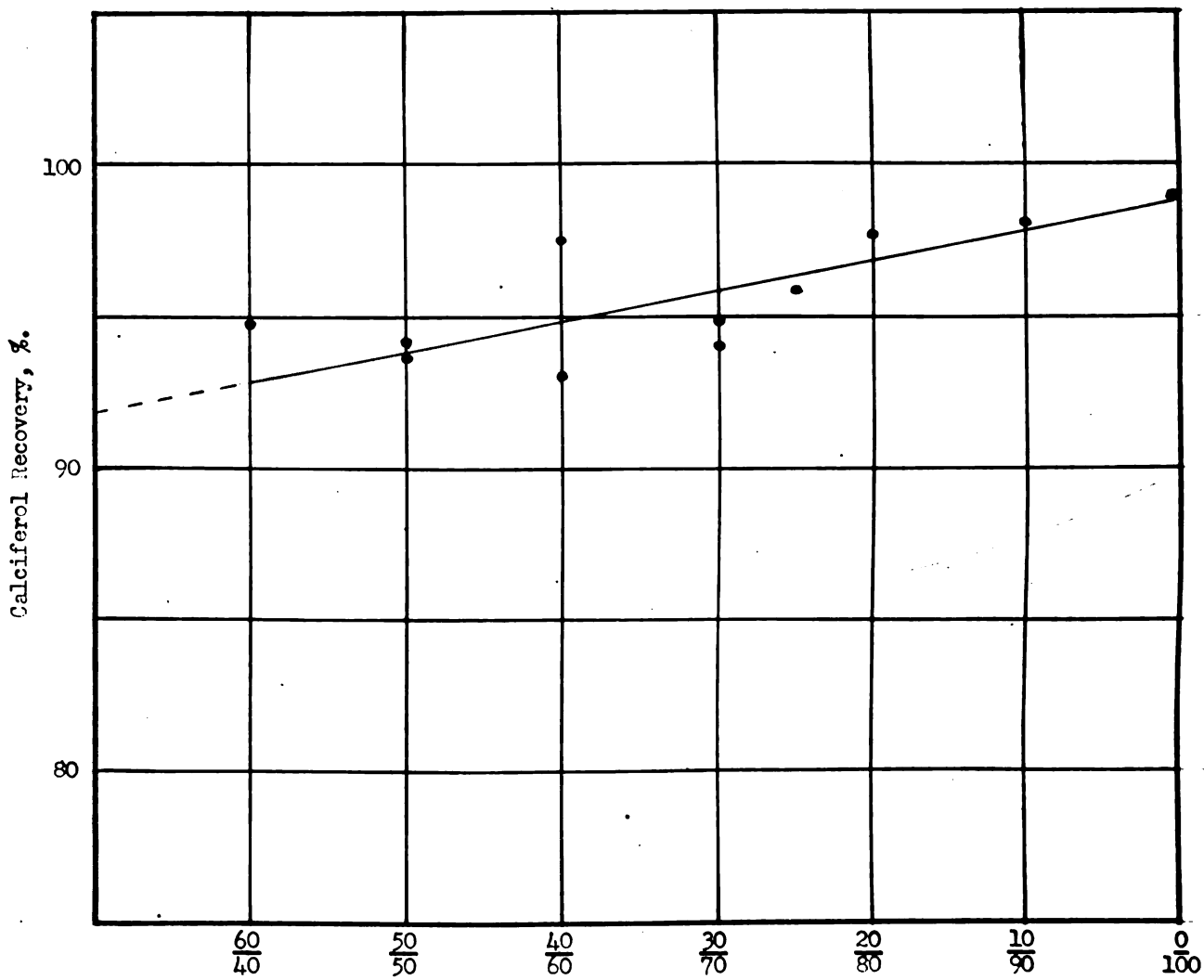

Fig. 4.— Percentage recoveries of calciferol from chromatographed solutions containing ergosterol and calciferol.

but as mentioned before, it has several disadvantages.

Summary:—

1. The pure curves of the respective solutions of ergosterol and calciferol were additive.

2. A complete separation of ergosterol and calciferol was obtained by this procedure.

3. All determinations were done quantitatively and qualitatively.

4. A calciferol recovery and separation from ergosterol between 93% - 95% was obtained by chromatographing a solution of ergosterol and calciferol. There seemed to be a strong indication that this percentage recovery varied inversely as the concentration of ergosterol was increased.

5. Hexane-ether-alcohol developer was found to be the only solution which would permit ergosterol to be adsorbed onto Superfiltrol and let calciferol proceed on through the column.

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