2. A method for the quantitative estimation of the vitamins D has been developed. The determination has an overall accuracy of $\pm 5\%$.

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Studies on the Analysis of Vitamins D

2. THE ANALYTICAL PURIFICATION OF VITAMIN D BY DIFFERENTIAL SOLUBILITY, PRECIPITATION REACTIONS, AND CHROMATOGRAPHY

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Attempts to analyse synthetic irradiation products and fish-liver oils for calciferol and vitamin D_3 are fraught with unusual difficulties. These arise, in the main, from the very high biological potency of vitamin D, the wide range of potency in materials to be assayed and the low specificity of known chemical reactions. The difficulties have not been generally appreciated, and the literature shows that, with few exceptions, published methods are empirical. There have thus been few attempts to deal fundamentally with the analytical separation of the vitamins D from known interfering substances.

In fish-liver oils, the chief interfering substance is vitamin A, the removal of which is the main problem to be solved. Vitamin A may be present, weight for weight, in at least a hundred times the concentration of vitamin D, which it masks in all known reactions. Milas, Heggie & Reynolds (1941) attempted to remove vitamin A and carotenoids by preliminary treatment of concentrates with maleic anhydride in dioxan. Ewing, Kingsley, Brown & Emmett (1943) developed chromatographic methods for the elimination of vitamin A and claimed good results. Müller (1947) also described a complicated chromatographic procedure.

The analysis of vitamins D in irradiation products appears at first sight to be easier and advances have been made, particularly by Ewing, Powell, Brown & Emmett (1948). It is often assumed that most of the sterols in an irradiation product of ergosterol or

7-dehydrocholesterol do not interfere with the analysis of vitamin D by the best known colorimetric methods. Experience has shown, however, that without the best attainable purification of vitamin D by removal of sterols, such methods do not give results agreeing with biological assays, when applied to widely varying products. This is so for the method of Ewing *et al.* (1948).

In the present work, two reactions have been used to study the analytical behaviour of calciferol and vitamin D_3 . Since the two substances can differ in behaviour, they have been studied independently, and conclusions drawn from study of one have not been, without test, accepted for the other. The two reactions are the antimony trichloride-acetyl chloride reaction of Nield, Russell & Zimmerli (1940) and the iodine trichloride reaction described by Green (1951).

The antimony trichloride reaction is the more specific for vitamins D, but is interfered with, to varying degrees, by the presence of cholesterol, 7-dehydrocholesterol, tachysterol (and possibly protachysterol), vitamin A and carotenoids. A disadvantage in its use is the lack of reproducibility of the reagent, which makes it difficult to obtain precise results even with fairly pure vitamin D_3 . The iodine trichloride reagent is less specific, but gives a high degree of precision, and is additionally useful in analytical studies of the sterols produced by irradiation which do not react with the antimony trichloride reagent.

EXPERIMENTAL

Materials and apparatus

(1) *Sterols* (for specification, see Part 1 (Green, 1951)). (2) *Vitamin A alcohol*. Isolated by alkaline hydrolysis from pure synthetic vitamin A acetate. (3) *Light petroleum*. A.R. grade, b.p. 40–60°. (4) *Ethanol*. Pure absolute. All aqueous ethanol solutions used are recorded as v/v concentrations. (5) *Carbon tetrachloride*. Pure, redistilled from glass apparatus. (6) *Ether*. Anaesthetic. (7) *Digitonin*. Purest commercial. British Drug Houses Ltd. (8) *Floridin earth*. Prepared for chromatography by British Drug Houses Ltd. Sieved to remove particles passing 160 mesh.

All the solvents used in the spectroscopic and chromatographic studies were stringently purified and suitable for spectroscopic purposes.

The Spekker micro-absorptiometer was used throughout for colorimetric measurements. Spectrum green 604 filters were used for the ICl_3 titrations. Spectrum blue-green 603 filters were used for the SbCl_3 determinations in conjunction with a standard curve obtained from determinations made on pure vitamin D_3 and calciferol. Spectroscopic determinations, including some vitamin D estimations by the method of Nield *et al.* (1940) were carried out on the Uvispek spectrophotometer.

The quantitative separation of provitamins from vitamins D

The separation of the provitamins D is advisable as a preliminary step, especially with crude irradiation products of 7-dehydrocholesterol. It is also essential to remove cholesterol. Suggestions have been made, for example by Gudlet (1941), for freezing out interfering sterols from solution in methanol. Digitonin precipitation has been recommended by Raoul & Meunier (1939) and other workers, but experimental conditions for quantitative removal from vitamin D have not previously been stated.

Primary separation by differential solubility. Although ergosterol, 7-dehydrocholesterol and the vitamins D are appreciably soluble in most concentrations of aqueous ethanol above 50 %, it has been found that, at a concentration of 72 % (v/v) ethanol, differences in solubility are large enough to permit a primary separation of the provitamins from the vitamins D.

Solubilities of the sterols examined were determined by two methods. (a) By shaking an excess of the sterol with 10 ml. of 72 % ethanol to give a saturated solution. (b) By dissolving an excess of the sterol in a known amount of pure ethanol and then bringing the concentration to 72 % by the dropwise addition of water and centrifuging off precipitated material.

Amounts of dissolved sterol were measured by direct evaporation of the solution *in vacuo*, dissolving the dry residue in CCl_4 , and titration on the micro-absorptiometer with ICl_3 . Table 1 shows the solubilities for a number of sterols.

Table 1. *Solubilities of sterols in 72 % ethanol*

	Solubility (mg./100 ml.)
Vitamin D_3	80
Calciferol	>100
Ergosterol	6.5
7-Dehydrocholesterol	<2.0
Cholesterol	80
Lumisterol	>100
Tachysterol	>100
Suprasterols	>100

7-Dehydrocholesterol is so insoluble in 72 % ethanol that only traces could be detected with the ICl_3 reagent. Ergosterol solubility is very low, being less than a tenth of that of calciferol. Cholesterol, however, cannot be adequately separated from the vitamins D by solubility difference.

Quantitative separation by digitonin. Digitonin precipitation of sterols has been widely used for many years, but, although its use in the determination of vitamin D has been mentioned by some authors, information of a quantitative nature concerning the best procedure has been lacking. Solvents used in digitonin precipitation have been, variously, pure ethanol, 95 % ethanol, acetone-ethanol, and methanol. In many methods, precipitation takes several hours to complete, which is unsatisfactory when the purpose is vitamin D analysis. Thaysen (1914) showed that precipitation in 95 % ethanol is variable and often incomplete, and this was supported by Dam (1928).

Many experiments have now shown that 72 % ethanol is an admirable medium for the quantitative precipitation of ergosterol, cholesterol, and 7-dehydrocholesterol digitonides, precipitation being maximal within 1 hr., and the removal of these sterols more complete than from other solvents. It is not known if the vitamins D form soluble compounds with digitonin in ethanolic solution. However, after precipitation of the provitamins from a mixture containing vitamins D, the latter substances are easily and quantitatively extracted from the filtrate by simple extraction with CCl_4 . The latter appears to be a better solvent than light petroleum, which has been customarily used. If an ethanolic solution of a vitamin D, to which digitonin has been added, is evaporated to dryness, the vitamin D may also be easily extracted from the residue by washing with several small quantities of CCl_4 . If, therefore, a soluble vitamin D-digitonin complex exists, it is easily split to yield unchanged the original vitamin D by simple solvent treatment.

The method which has been developed for the quantitative separation of vitamin D from ergosterol, 7-dehydrocholesterol, and cholesterol is, therefore, as follows. The sample of mixed sterols (20–50 mg.) is dissolved in 20 ml. of ethanol and 8–6 ml. of water added slowly, with stirring. The

resulting 72 % ethanol solution is allowed to settle for 5 min. and any precipitate is removed by centrifuging: the precipitate is washed with a little of the same solvent. Alternatively, the sample may be directly extracted by shaking for 10 min. with 30 ml. of 72 % ethanol in a flask fitted with a ground-glass stopper, and the solution filtered. To the filtrate obtained by either method is then added a slight excess of digitonin (about 100–150 mg. in 5 ml. 72 % ethanol) and the mixture stirred and allowed to stand for 1 hr. Precipitated material is removed by centrifuging, the clear supernatant extracted twice with 10 ml. CCl₄, and the extracts filtered and evaporated to dryness *in vacuo*. The residue is dissolved in a standard volume of CCl₄ for titration. Alternatively, the aqueous ethanol solution may be evaporated directly to dryness *in vacuo*, and the residue extracted four or five times with CCl₄ and made up to volume for titration: small losses of vitamin D, however, may occur in this latter method.

*Chromatographic behaviour of vitamin D
and other sterols*

Vitamins D have been separated from irradiation products and fish-liver oils by the use of alumina columns. Thus Brockmann (1936) used columns of this type to separate pure vitamin D₃ from tunny liver oil by processes involving many supplementary methods of separation, and used marker dyes as vitamin D indicators. It does not appear that with alumina columns quantitative adsorption and elution have been achieved.

Three chromatographic methods have been suggested for use in vitamin D analysis. Ewing *et al.* (1943) used 'Superfiltral' columns, but the procedure is complicated by many stages and vitamin D is determined by a difference method. Later, Ewing *et al.* (1948) employed a more satisfactory chromatographic separation of sterols and vitamin A on 'Superfiltral'. De Witt & Sullivan (1946) claimed separation of vitamin A and sterols from vitamin D

Table 2. Separation of vitamins D from sterols precipitable with digitonin

Interfering sterol in mixture (mg.)			Vitamin D in mixture (mg.)	Vitamin D recovered by analysis (mg.)
Cholesterol	7-DHC*	Ergosterol		
—	—	2.002	—	0.005
—	15.000	—	5.002 D ₃	4.820
—	—	20.000	2.010 D ₂	2.002
—	10.020	9.982	0.505 D ₃	0.498
20.050	10.010	—	0.525 D ₃	0.504
—	22.000	10.010	0.625 D ₃	0.648

* 7-Dehydrocholesterol.

Table 2 gives a summary of a great number of experiments. Vitamin D was determined, in synthetic mixtures containing interfering sterols, by ICl₃ titration, which reveals the presence of other sterols (except cholesterol): frequent checks, using the SbCl₃-acetyl chloride reagent were made and in all cases showed very good agreement with the titration method.

The method gives an absolute separation of the vitamins D from the provitamins D and cholesterol in a wide range of admixture. Vitamins D are recovered quantitatively and unchanged.

Effect of other substances on solubility separation. Other sterols do not interfere with the solubility or digitonin procedures. Small amounts of fat have little effect. Larger amounts of fat (over 50 mg.) interfere with quantitative extraction and must be removed by saponification. The solubility of β-carotene in 72 % ethanol is very low (3.8 μg./10 ml.), but may be slightly increased by the presence of other substances. Vitamin A alcohol and esters are fairly soluble in 72 % ethanol.

by zoning on a column prepared from a mixture of magnesia and diatomaceous earth.

Adsorption of vitamins D and other sterols on floridin earth. In their method for the analysis of vitamin E, Emmerie & Engels (1939) used floridin earth as an adsorbent. It has now been found that this earth is suitable for separating vitamins D from tachysterol, vitamins A, and some other interfering substances. In the present work, the chromatographic tubes used were 13 mm. diameter and about 12.5 cm. long, above the constriction. The portion of the tube below the constriction was of narrow bore to induce slow passage. The earth was prepared by boiling a thin slurry of about 5 g. with 15–20 ml. of conc. HCl for 1 min. and rapidly pouring the stirred mixture, without using suction, into the tube until a column about 7.5 cm. high was obtained. After passage of the acid, the column was washed successively with 15–20 ml. of ethanol and 25 ml. of light petroleum in small portions. During the ethanol washing, a small filter paper was placed on the top surface of the earth, and the whole

Table 3. *Adsorption and elution of sterols on floridin earth*

Ethanol concentration used for prewashing columns (%)	Sterol adsorbed (μ g.)	Solvent for elution	Sterol recovered (μ g.)
100	2,560 calciferol	50 ml. EtOH/ether	2,540
	95,400 calciferol	50 ml. EtOH/ether	94,800
	82,600 calciferol	100 ml. CCl ₄	79,200
90	5,100 calciferol	50 ml. EtOH/ether	5,120
	4,950 calciferol	100 ml. CCl ₄	4,890
	2,520 D ₃	100 ml. CCl ₄	2,510
	500 D ₃	100 ml. CCl ₄	485
	84 D ₃	100 ml. CCl ₄	86.5
	21 D ₃	100 ml. CCl ₄	20.5
	1,080 D ₃	100 ml. cyclohexane	260
	1,080 D ₃	100 ml. benzene	1,100
	1,520 tachysterol	100 ml. CCl ₄	210
	1,680 tachysterol	100 ml. CCl ₄	120
85	510 D ₃	100 ml. CCl ₄	395

column pressed down about 1 cm. with a glass plunger.

Vitamin D is dissolved in light petroleum for the adsorption process, which is preferably carried out without suction. Quantitative adsorption always occurs, and even protracted washing with light petroleum does not result in any elution. Ergosterol, 7-dehydrocholesterol, lumisterol, tachysterol and the suprasterols are also quantitatively adsorbed on floridin. The capacity of the column is variable, as described later, but quantities of calciferol up to 100 mg. have been adsorbed on columns prewashed with absolute ethanol. In general, the capacity for mixed sterols appears very high.

Adsorption on moist columns. The adsorptive capacity of floridin is affected by its moisture content. For experimental study, columns were prepared at a standard moisture content by prewashing with aqueous ethanol, the columns then being washed with 25 ml. of light petroleum, as previously described. Columns prewashed with 90–100% ethanol still retained a high adsorptive capacity and were capable of adsorbing up to 50 mg. of calciferol. Prewashing with 85% ethanol severely reduced the capacity of the column, not more than about 1 mg. of calciferol or vitamin D₃ being adsorbed. Columns prewashed with 85% ethanol are not suitable for quantitative adsorption and elution.

Adsorption in the presence of fat. Adsorption on floridin is not affected to a marked degree by the presence of small quantities of fat. Even when 0.5 g. of arachis oil is added to a light petroleum solution of vitamin D₃, none of the latter is eluted in the first 25 ml. of filtrate.

Elution of sterols. Columns prewashed with all strengths of ethanol may be completely eluted with 50 ml. of a 3% (v/v) solution of ethanol in ether. The most useful eluting solvent, however, is CCl₄, 100 ml. of which elutes all adsorbed vitamins D from

columns prewashed with 90% ethanol and over 90% of the adsorbed material from columns prewashed with absolute ethanol. From columns prewashed with 90% ethanol, quantitative elution occurs with many other solvents, such as benzene and ethylene dichloride. Even as weak an eluting solvent as cyclohexane slowly removes adsorbed vitamin D, on prolonged washing.

All the irradiation sterols behave similarly to the vitamins D, with the single and important exception of tachysterol. Tachysterol, adsorbed on floridin prewashed with 90% ethanol, is eluted with CCl₄ to an extent of less than 15%. This phenomenon does not occur when columns prewashed with absolute ethanol are used for the adsorption of tachysterol, and is probably caused by the specific destructive properties of certain types of floridin earth column towards certain structures, which are described in more detail in the section dealing with vitamin A. This difference in behaviour between the vitamins D and tachysterol, however, provides a method for their analytical separation.

Table 4. *Separation of tachysterol-calciferol mixtures on floridin earth*

Calciferol in mixture (μ g.)	Tachysterol in mixture (μ g.)	Calciferol recovered by elution (μ g.)
890	2400	910
720	1420	760
1500	1620	1450
490	1620	475

Table 3 presents some experimental results obtained from a large number of trials of the adsorption and elution of vitamins D and other sterols. Table 4 shows the experimental separation of simple mixtures of calciferol and tachysterol. In these experiments, the mixture was adsorbed on a standard floridin earth column, prewashed with 90% ethanol,

from light petroleum solution, the columns washed with 25 ml. of the same solvent, and then eluted with 100 ml. of CCl_4 . The eluates were analysed for calciferol, after evaporation and making up in the requisite solvent.

Variations in adsorptive capacity of floridin. Different batches of floridin vary in adsorptive behaviour. Some batches have low adsorptive capacities, others adsorb sterols strongly and elution with the weaker solvents may become non-quantitative. The main differences are due to variations in particle size. It has been found advisable to remove particles which pass a 160-mesh sieve. The fraction not passing the sieve is about 50–70 % of the total and is satisfactory for chromatography. Each batch of earth, however, should be tested for (a) total adsorptive capacity, (b) non-elution of vitamins D with light petroleum, and (c) quantitative elution with carbon tetrachloride.

Chromatographic behaviour of vitamin A on floridin

Although Emmerie & Engels (1939) stated that floridin earth columns, 5 cm. long and 1.3 cm. in diameter, would adsorb up to 320 i.u. of vitamin A, and Awapara, Mattson, Mehl & Deuel (1946) used the adsorption of vitamin A on floridin in a method for the analysis of margarine, no study appears to have been made of the very complex behaviour of vitamin A on this adsorbent. The purpose of the present study was to devise a simple chromatographic procedure for the quantitative separation of vitamins A and D on floridin.

Effect of moisture on adsorptive capacity. The adsorption of vitamin A on floridin earth is greatly affected by the presence of moisture.

A preliminary study of this effect was carried out in the following manner. Three samples of earth were taken, one dried at 105° until moisture-free, the second (containing 3 % water) used direct from the stock bottle, and the third mixed with an added 5 % by weight of water. Each sample (1 g.) was placed in a test tube and to each was added a measured volume of a light petroleum solution (1000 i.u./ml.) of a high potency vitamin A oil. The tubes were

stoppered and shaken vigorously for 1 min. Adsorption of vitamin A was indicated by the earth assuming a light-blue colour. The solutions, after filtration, were tested for unadsorbed vitamin A. Increasing quantities of vitamin A solution were added until, after adsorption, the filtrate was found to contain vitamin A. As the results in Table 5 show, the adsorptive capacity is large and is markedly reduced by the presence of water.

Table 5. *Adsorption of vitamin A on floridin earth*

Moisture content of earth (%)	Max. vitamin A adsorbed/g. of earth (i.u.)
<0.1	10,000
3	7,000
8	3,000

Vitamin A on floridin columns. The vitamin A source was a high potency fish-liver oil (140,000 i.u./g.), dissolved in light petroleum for adsorption. The prepared columns (7.5×1.3 cm.) after acid treatment, were prewashed with various strengths of ethanol (15–20 ml. used), followed by 25 ml. light petroleum.

Known amounts of vitamin A were adsorbed on the columns, which were then washed with 25–50 ml. light petroleum. After collection of the filtrates, elution was carried out with 3 % ethanolic ether or CCl_4 . The residual vitamin A and its decomposition products were determined in the evaporated filtrates and eluates by the SbCl_3 reaction, and expressed as 'apparent vitamin A' recovered. Table 6 shows the recoveries and illustrates the enormous extent of vitamin A destruction which occurs under varying conditions.

The absorption of more concentrated sources of vitamin A, such as non-saponifiable extracts of fish-liver oils, molecularly distilled concentrates or pure vitamin A alcohol was investigated. In general, it was found that the more concentrated the source, the greater the yield of SbCl_3 -reactive substance obtained in the eluates from the columns.

When a solution of vitamin A is passed through floridin earth, an intense blue colour develops at the top of the column. Depth of colour and degree of

Table 6. *Destruction of vitamin A on acid-washed floridin*

Ethanol concn. for prewash (%)	Vitamin A adsorbed (i.u.)	Apparent vitamin A in filtrate (i.u.)	Elution solvent	Apparent vitamin A in eluate (i.u.)
100	1,500	0	50 ml. EtOH/ether	100
100	3,000	0	50 ml. EtOH/ether	120
100	31,000	300	50 ml. EtOH/ether	650
100	70,000	Not determined	50 ml. EtOH/ether	1700
90	30,000	550	50 ml. EtOH/ether	280
90	70,000	650	50 ml. EtOH/ether	450
90	140,000	Not determined	50 ml. EtOH/ether	100–2000
85	70,000	1250	50 ml. EtOH/ether	90
90	3,000	Not determined	100 ml. CCl_4	80
90	10,000	Not determined	100 ml. CCl_4	150

spreading depends on three factors: (a) concentration of ethanol used for prewashing, (b) amount of vitamin A adsorbed, and (c) nature of vitamin A source, i.e. the presence of other saponifiable or non-saponifiable factors. On washing with light petroleum, the blue band weakens in intensity and spreads slightly, but no vitamin A is removable even on prolonged washing, except where very large amounts are put on the column, when it appears in the first 25 ml. of filtrate.

Vitamin A destruction products on floridin

Since vitamin D can be quantitatively adsorbed on and eluted from floridin earth whereas vitamin A may be quantitatively destroyed by the same procedure, the method is most valuable as a means of separating the two substances. A more detailed study of the vitamin A destruction process was made, with particular regard to obtaining eluted fractions giving the minimal interference with the estimation of vitamin D.

For these experiments, pure vitamin A alcohol, freshly prepared by alkaline hydrolysis from synthetic vitamin A acetate, was employed.

The destruction of vitamin A by acid-treated floridin results in a mixture which separates, on chromatographic analysis, into a number of products. In most experiments, the standard 7.5 cm. column was used and the amount of vitamin A used for adsorption was 7–8 mg. or about 25,000 i.u. In order to obtain additional data, columns measuring 12.5 × 2.5 cm. were occasionally used, with up to 100 mg. of vitamin A alcohol. The solvents used were all purified until a blank chromatographic procedure showed no preferential adsorption or elution of ultraviolet-absorbing impurities. The absorption data on the products of destruction were obtained by evaporation of fractions and solution of eluted material in pure ethanol, measurements being made on the Uvispek spectrophotometer, using 1 cm. cells throughout.

Columns prewashed in pure ethanol. Adsorption of 7–8 mg. of vitamin A alcohol in light petroleum on columns prewashed with pure ethanol, followed by washing with light petroleum, yields as eluate about 2% of the original material. 100 ml. of solvent are necessary for complete elution, and the eluate, by careful fractionation, can be separated into three main substances.

The first 25 ml. of light petroleum contains a substance which shows absorption bands at 230 m μ . and 280–290 m μ ., the latter peak being variable in position. In addition, the first fraction sometimes contains a small quantity of anhydro-vitamin A, with peaks at 350, 370 and 390 m μ . (the 350 m μ . band often being obscured). The second 25 ml. of light petroleum sometimes contains a little unchanged vitamin A which is adsorbed slightly more

strongly than anhydro-vitamin A. Not more than 0.1% of the original vitamin A can ever be recovered unchanged. The last fraction of the light petroleum eluate contains only a small amount of material with adsorption peaks at 240 and 290 m μ . The smallness of the total light petroleum fraction and the changes in its composition which are brought about by minute variations of chromatographic conditions combine to render the total composition of this solution non-reproducible. Anhydro-vitamin A and vitamin A, for example, are often absent. The ratio between the intensities of the 230 and 290 m μ . peaks varies considerably, perhaps indicative of the fact that two separate substances are involved. The $E_{1\%}^{1\text{cm}}$ values at 230 and 290 m μ . appear to be between 150 and 300. The absorption curves of the fractions are shown in Fig. 1.

Fig. 1. Absorption curves of light petroleum filtrates produced by chromatographic destruction of 10 mg. vitamin A alcohol. All fractions in 5 ml. ethanol.

Light petroleum was usually followed by CCl_4 as eluting solvent. This solvent increases the intensity of the blue adsorption zone and diffuses it downwards to a small degree. The CCl_4 eluate is colourless and contains substances which adsorb over the whole ultraviolet range with two variable peaks, the main one at 280–290 m μ . and a subsidiary peak at 230–250 m μ . The total weight of the eluted fraction comprises about 10–15% of the original vitamin A, and 100 ml. of CCl_4 suffices for complete elution. The $E_{1\%}^{1\text{cm}}$ value of the total fraction at 290 m μ . is about 150–200. As with the light petroleum washings, careful fractionation results in a partial separation of the two absorption peaks. The substances in the CCl_4 eluate, although qualitatively similar to those found in the light petroleum washings, cannot be eluted by prolonged washing with light petroleum.

If the solvents used in the chromatographic analysis are not spectroscopically pure, selective adsorption and elution of the impurities occur on the column, and the second 25 ml. of CCl_4 may slowly elute a purple band which yields a material having a strong absorption peak at $280\text{ m}\mu$. This is not found to occur with pure solvents, and the substance is probably not a vitamin A decomposition product (Fig. 2).

Fig. 2. Absorption curve of total CCl_4 eluate by chromatography on florisil of 10 mg. vitamin A alcohol; dissolved in 25 ml. ethanol. Absorption curve of 'purple band' material shown.

Carbon tetrachloride was followed in succession by ether, 3% ethanolic ether, and ethanol as eluting solvents. These solvents decrease the intensity of the blue band and impart to it a greenish hue. Each of the three solvents elutes a bright yellow fraction, depth of colour increasing with each fraction. The ethanol fraction is bright orange. 100–150 ml. of each solvent is sufficient to elute its own fraction. On evaporation, these coloured vitamin A decomposition products are obtained as yellow or orange solids. The ether, 3% ethanolic ether, and ethanol fractions comprise, respectively 15–20, 15–20 and 25–30% the original weight of vitamin A.

On examination of the spectroscopic curves of the three fractions, dissolved in ethanol, the substances are found to absorb over the whole ultraviolet range and up to about $470\text{ m}\mu$. in the visible region. All three contain three peaks, at 220–230, 280–290 and at $335\text{ m}\mu$. The ratio of the extinctions of the three bands in each fraction is variable, but the extinctions are of the same order. The 280–290 $\text{m}\mu$. band is generally the strongest in all fractions, but tends to

decrease in relative intensity from the ether to the ethanol fractions. However, the $E_{1\text{ cm}}^{1\%}$ values at the peaks decrease markedly in the fractions from ether to ethanol. The total fractions from the ether, ethanolic ether, and ethanol eluates have $E_{1\text{ cm}}^{1\%}$ values at $290\text{ m}\mu$. of approximately 800–1000, 200–350 and 70–100, respectively (Fig. 3).

Elution of the column with a more polar solvent only yields small amounts of a pale-yellow material.

Fig. 3. Absorption curves of coloured ether, EtOH-ether, and EtOH eluates by chromatography of 10 mg. vitamin A alcohol on column washed with 100% ethanol, showing marked decrease in E values. All fractions in 100 ml. ethanol.

Columns prewashed with aqueous ethanol. Pre-treatment of the florisil earth column with aqueous ethanol (in concentrations above 85%) changes the character of the vitamin A destruction process as well as decreasing the capacity of the column. In general, as the concentration of ethanol is decreased to 85%, the intensity of the blue adsorption band decreases and becomes more diffuse, while the proportion of decomposition products eluted into the light petroleum washings increases, and the proportion of the yellow and orange products eluted into the ether and ethanol fractions decreases. The substances obtained from the light petroleum fractions are, in general, similar to those obtained from ethanol-washed columns, with a great preponderance of the $230\text{ m}\mu$. band substance. Only traces of unchanged vitamin A are recovered. However, washing with 85% ethanol is peculiar in producing, as the main substance in the light petroleum fraction, a large amount of anhydrovitamin A, easily determined by its characteristic three peaks at 351, 371 and $392\text{ m}\mu$. (Fig. 4).

The CCl_4 fractions are closely similar to those obtained from ethanol-washed columns, showing mainly the 290 $\text{m}\mu$. band. The products obtained in the ether, ethanolic ether and ethanol eluates are also similar to those obtained from ethanol-washed columns, but do not appear to be identical. In the

Fig. 4. Absorption curve of first light petroleum filtrate, by chromatography of 10 mg. vitamin A alcohol on column washed with 85% ethanol. Fraction dissolved in 50 ml. ethanol. Anhydro-vitamin A is the main constituent.

latter three fractions, the main band at 280–290 $\text{m}\mu$. is present, but may be displaced to shorter wavelengths. In the ethanol eluates, this band may be displaced to as low as 260 $\text{m}\mu$. The 230 $\text{m}\mu$. band is variable, and the 335 $\text{m}\mu$. band is not found in products from these columns.

The effect of floridin columns containing free hydrochloric acid. All the types of columns so far described have contained no appreciable amounts of free unadsorbed hydrochloric acid. Experiments carried out to investigate the effect of columns containing free acid show that, under such conditions, the character of the vitamin A destruction process is again changed.

If the vitamin A solution is made up in light petroleum containing dry HCl gas and then chromatographed on floridin, no blue band appears on the column, and the vitamin A is quantitatively destroyed to yield a new type of product which passes completely through the column in light petroleum. It is a pale-yellow waxy solid, which is almost insoluble in ethanol, very soluble in light petroleum, and has an absorption spectrum in cyclohexane showing three bands at 240–250, 280–290 and 335 $\text{m}\mu$., all with $E_{1\text{cm}}^{1\%}$ values of the order of 200 (Fig. 5).

Increasing the free HCl content still further by prewashing with ethanol containing dry HCl, preparatory to adsorbing the vitamin A, yields a substance which is slowly eluted by light petroleum as a vivid rose-pink band. However, the substance appears to be closely similar to, if not identical with, the yellow waxy solid, the absorption curve of which is shown in Fig. 5.

Fig. 5. Absorption curve of light petroleum filtrate containing total destruction products from vitamin A alcohol, formed on column containing free HCl.

The presence of the pronounced peak at 335 $\text{m}\mu$. is interesting, since this peak is only found in the normal type of column eluate in the brightly coloured ether and ethanol fractions.

Vitamin D undergoes considerable destruction on free acid-containing columns, and this type of column cannot be adapted for use in the separation of vitamins A and D.

Interference of vitamin A products after chromatography in vitamin D analysis

As has already been shown, chromatography on floridin earth, under widely varying conditions, suffices to destroy quantitatively very large amounts of vitamin A. Using columns prewashed with absolute ethanol or 90% ethanol, vitamin D may be quantitatively adsorbed and eluted with either CCl_4 or 3% ethanolic ether. It is therefore possible to measure the residual interference by known amounts of vitamin A in methods of estimating vitamin D. 10 mg. (approx. 30,000 i.u.) quantities of pure vitamin A alcohol were chromatographed on floridin earth columns and residual apparent vitamin D in eluted fractions determined by ICl_3 titration and SbCl_5 colorimetric analysis.

Most eluted fractions containing vitamin A destruction products react to a small degree with the

SbCl₃ reagent. However, the method of determining vitamin D recommended by Nield *et al.* (1940) eliminates even the small residual interference due to vitamin A decomposition products in the eluates from 90 % ethanol prewashed columns. By this method, which takes advantage of the fact that the absorption of the vitamin D-SbCl₃ colour reaction is zero at 550 m μ ., the peak extinction at 500 m μ . is measured against an 'internal blank' extinction reading at 550 m μ .

Measurements on the residual interference due to vitamin A after chromatography are recorded in Table 7.

Table 7. *Residual interference after chromatography of 10,000 μ g. of vitamin A*

Column prewash (% ethanol)	Type of eluate	ICl ₃ reaction	'Apparent vitamin D' (μ g.)*	'Apparent vitamin D' (μ g.)†
100	3 % ethanolic ether CCl ₄	Strongly positive Negligible	— 11.0	— 6.0
90	3 % ethanolic ether CCl ₄	Strongly positive Negligible	— 20.0	— 0.5
85	CCl ₄	Negligible	14.0	2.5

* Maximum 'apparent D' by measuring at 500 m μ . against external blank.

† 'Apparent D' measured against internal blank at 550 m μ .

The orange-coloured decomposition products of vitamin A eluted with ether react strongly with ICl₃, behaving to this reagent like carotenoid pigments in giving a delayed reaction, accompanied by transient blue colour formation. The CCl₄ eluates react to a negligible extent with ICl₃. As measured by the SbCl₃-acetyl chloride reagent, using the internal blank method, the interference due to 'apparent vitamin D' is very small in all cases. The 90 % ethanol-washed column is, from every point of view, the most suitable for the separation of vitamins A and D. The CCl₄ eluates from vitamin A destruction on this type of column react with the SbCl₃ reagent to give a product whose absorption curve over the range 500–550 m μ . is almost linear and of equal intensity: the internal blank method of measurement therefore eliminates interference almost entirely.

Table 8. *Separation of vitamin A and vitamin D₃*

Vitamin A alcohol in mixture (mg.)	Vitamin D ₃ in mixture (μ g.)	Recovery of vitamin D ₃ (μ g.)	
		ICl ₃ assay	SbCl ₃ assay
10,000	120	Not determined	112
25,000	300	280	292
19,000	505	520	485
10,000	50	Not determined	43

Table 8 shows the experimental separation of mixtures of pure vitamin A alcohol and pure vitamin D₃ by chromatographic analysis on floridin columns, prewashed with 90 % ethanol and eluted with CCl₄.

DISCUSSION

The vitamins D can be quantitatively separated from their provitamins and other precipitable sterols by differential solubility in 72 % ethanol followed by digitonin precipitation. The precipitation in 72 % ethanol is very rapid and is characterized by the completeness of its removal of precipitable substances. This step is particularly necessary in the analysis of vitamin D₃ concentrates obtained by irradiation of crude sterol mixtures.

Chromatography on floridin earth, under the conditions described, places on a firm basis the

efficient separation of tachysterol and vitamin A from the vitamins D, being particularly valuable in that the former two substances are the main causes of gross interference encountered in vitamin D analysis. The behaviour of acid-washed floridin earth columns towards tachysterol, vitamin A, and vitamin D appears to be complex. Provided that the columns contain no free hydrochloric acid, vitamin D can always be recovered quantitatively. The capacity of the columns for vitamin D is very large and decreases as more dilute alcohol is used for pre-washing, there being, however, a fairly sharp threshold between adsorption and non-adsorption at about 80 % ethanol. There are no other qualitative effects.

Tachysterol is destroyed to a large extent by single passage through a column washed with 90 % ethanol, but not if the column is washed with pure ethanol. This effect of aqueous ethanol in changing the character of adsorption is more marked with vitamin A. The complex destruction of vitamin A is conditioned by two main factors, acidity and moisture content of the column. When the vitamin A decomposition products are examined spectroscopically, a remarkable feature observed is the persistence of the position of the three main absorption bands (one of which may be missing) in all the fractions tested. This fact indicates that the destruction is probably due to a process of polymerization. A series of substances is formed, the general spectroscopic absorption of which is extended into longer wavelengths with increased

molecular weight. The basic molecular structure of the substances is probably similar.

The consistent appearance of the 280–290 m μ . band of low intensity is of some interest, since a similar low intensity band is characteristic of kitol, a dimeric polymer of vitamin A, described by Embree & Shantz (1943*a*) and was also observed in the sub-vitamin A isolated from shark-liver oil by Embree & Shantz (1943*b*). A structural relationship between these substances is indicated.

The influence of prewashing with 85 % ethanol in producing large amounts of anhydro-vitamin A from the column is unexpected, since 'cyclization' of vitamin A is usually produced by the presence of acid in anhydrous solvents. After 85 % ethanol washing, however, the columns have a high moisture content, and it is evident that there is a water-acid relationship which profoundly affects the decomposition and which, under certain conditions, leads to 'cyclization' of vitamin A.

For analytical purposes, the 90 % ethanol washed column is the most useful. By its use, with one liquid chromatogram, vitamin D can be separated from tachysterol and vitamin A. β -Carotene is destroyed on the column and does not interfere.

SUMMARY

1. Methods for the quantitative separation of vitamins D from interfering sterols and vitamin A have been developed.

2. The methods involve removal of provitamins and precipitable sterols by differential solubility and precipitation with digitonin under controlled conditions and a single chromatographic analysis on floridin earth.

3. The destruction of vitamin A on floridin earth has been studied.

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Studies on the Analysis of Vitamins D

3. EXPERIMENTS ON THE SEPARATION OF VITAMIN D FROM VITAMIN A AND OTHER SUBSTANCES BY CONDENSATION TECHNIQUES AND SELECTIVE IRRADIATION

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Proposals for the analytical separation of vitamins D from interfering substances include the method of selective condensation with maleic or citraconic anhydrides and that of removing interference by selective destruction with filtered ultraviolet radiation. The first method has been recommended for the removal of vitamin A in fish-liver oils and also for the removal of tachysterol in synthetic irradiation

products. The second method has been recommended for the destruction of vitamin A.

Vitamin A, carotenoid substances in general, the vitamins D, tachysterol and some other sterols condense, under suitable conditions, with maleic and citraconic anhydrides to give inactive substances capable of easy elimination. Windaus & Lüttringhaus (1931) stated that vitamin D reacted very