ISOLATION OF THE STEROLS OF DEHYDRATED ALFALFA MEAL

by

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INTRODUCTION

The carotene in dehydrated alfalfa can be extracted and isolated from the rest of the plant materials by a process which involves radial chromatography. The Department of Chemistry is interested in preparing such carotene concentrates for use as a vitamin A supplement in feeds. To make the process economical, it probably will be necessary to isolate other materials of commercial importance which can assume part of the cost of operation. Alfalfa sterol may be such a material. α-spinasterol, the chief sterol of alfalfa (1), perhaps can be converted to vitamin D under the influence of ultraviolet light, and may be of value as a starting material for the manufacture of certain pharmaceuticals. Therefore, an investigation was initiated to devise a method of isolating it in conjunction with the process for preparing carotene concentrates.

Little work has been done on the isolation of sterols in green plants such as alfalfa because other plant pigments which are present interfere in the isolation process. Fernholz and Moore (2) isolated α-spinasterol from alfalfa, while Heyl, Wise, and Speer (3) isolated it from spinach. Morgal, Petering, and Miller (4) isolated the sterol fraction of alfalfa and irradiated it with ultraviolet light. They reported that the sterols in the fraction possessed vitamin D activity after irradiation. Hickman (5) used high-vacuum distillation to separate sterol from seed oils. Wall (6), using a falling film molecular still similar to the one used by Hickman, separated the sterols of green

plants from the other plant pigments. He found that the recovery of sterol was 90 per cent or more, but that the recovery of carotene was poor.

EXPERIMENTAL

Removal of Sterols from Adsorbent

Preliminary experiments had shown that when a Skellysolve B extract of dehydrated alfalfa meal was adsorbed on calcium phosphate in a radial chromatograph, the carotene could be eluted by washing with Skellysolve B, but that most of the sterols would remain on the adsorbent. Hence, the first objective in developing a procedure for isolating the sterols was to find a means of removing them from the adsorbent. Acetone was selected as a possible eluting agent. Fifty milliliters of a Skellysolve B extract of alfalfa containing 2.47 mg of sterol were drawn by vacuum into a 7 cm column of calcium phosphate contained in a 25 x 200 mm adsorption tube. The adsorbent was washed successively with 200 ml portions of Skellysolve B containing 0, 1, 2, 5, and 10 per cent of acetone. Each fraction was collected separately and was analyzed for sterols by the micro method of Wall and Kelley (1). The data, presented in Table 1, show that the sterols were eluted by the Skellysolve B-acetone mixtures, the major portion being eluted by the 2 per cent acetone solution.

Having determined that acetone was capable of eluting sterols from calcium phosphate in a conventional adsorption tube, it was necessary to evaluate it on a larger scale with the radial

chromatograph (7), since this apparatus was being used in the preparation of carotene concentrates. This apparatus consists of a perforated drum mounted within a housing. The cylindrical portion of the drum is lined on the inside with filter paper. The drum is packed with the calcium phosphate adsorbent and is free to rotate on its axis. The extract is fed in a continuous stream through the hollow axis and is thrown by centrifugal force into the adsorbent. After passing through the perforations of the drum, the eluate is collected in the housing, from which it is removed by means of a stopcock.

Table 1. Elution of sterols from a calcium phosphate column by successive washings of Skellysolve B-acetone mixtures (Total sterols adsorbed: 2.47 mg).

Acetone in eluting agent	Volume of wash	Sterols eluted	
per cent	ml	mg	per cent
0	200	0.17	6.9
1	"	0.20	8.2
2	"	2.10	85.0
5	"	0.00	0.00

Six kilograms of dehydrated alfalfa meal were extracted with Skellysolve B and the extract was passed through the radial chromatograph. Ten gallons of Skellysolve B were used as an eluent to remove most of the carotene. Five gallons each of 2, 5, and 50 per cent acetone in Skellysolve B then were used to wash the adsorbent. Samples of each eluate were analyzed for sterol con-

tent. The results are presented in Table 2.

Table 2. Elution of sterols from the radial chromatograph by various concentrations of acetone in Skellysolve B (Original extract contained 3,000 mg of sterol).

Acetone in eluting agent	Volume of wash	Sterols eluted	
per cent	gallons	mg	per cent
0	10	198	6.6
2	5	240	8.0
5	5	1250	41.6
50	5	720	24.0

The Skellysolve B wash removed most of the carotene from the chromatograph, but eluted very little of the sterols. Acetone in Skellysolve B eluted most of the sterols, 5 per cent acetone eluting the major portion. Fifty per cent acetone was needed to elute the other plant pigments and remaining sterols from the adsorbent.

For such a process to be feasible industrially, most of the chlorophyll must be removed from the adsorbent before it can be used again. Since high concentrations of acetone were required to effect chlorophyll removal, a search was made for a more efficient eluting agent. Additions of ethyl, isopropyl and n-butyl alcohols to Skellysolve B all appeared to be superior to acetone when tested with small adsorption tubes. Isopropyl alcohol was selected for pilot plant tests because of its low cost and the ease of removing it from the solutions after elution.

The same procedure was used as in the preceding experiment

with acetone. Table 3 shows the results obtained when mixtures of isopropyl alcohol and Skellysolve B were used as eluting agents.

Table 3. Elution of sterols from the radial chromatograph by various concentrations of isopropyl alcohol in Skellysolve B (Original extract contained 3,530 mg of sterol).

Isopropyl alcohol in eluting agent	Volume of eluting agent	Sterols eluted	
per cent	gallons	mg	per cent
0	10	200	5.6
2	5	1300	36.9
5	5	2020	57.1

It will be seen that the 2 and 5 per cent isopropyl alcohol solutions were effective as eluting agents for the sterols. The major portion of the sterols was found in the 5 per cent solution. Isopropyl alcohol was more efficient than acetone, for a 5 per cent isopropyl alcohol solution efficiently eluted the sterols and cleaned the adsorbent for re-use, while a 50 per cent acetone solution was needed to accomplish this feat. Furthermore, elution with isopropyl alcohol was accomplished with a smaller volume of eluting agent. Approximately 94 per cent of the sterols were eluted from the chromatograph by isopropyl alcohol-Skellysolve B, as compared with 73 per cent of sterols eluted by acetone-Skellysolve B.

Ninety-five per cent ethanol in Skellysolve B also was tested with the radial chromatograph, but was found to be of

little value, since the chromatograph was not cleaned sufficiently for re-use. Apparently this was due to the water in the ethanol, which adsorbed on the calcium phosphate and prevented normal functioning of the adsorbent.

Isolation of Sterols from Eluate

Although the elution procedures which were studied separated the sterols from the major portion of the carotene, they did not effect a separation from the other plant pigments. It was necessary, therefore, to search for a method of doing this. Fernholz and Moore (2) used phasic distribution to separate sterols from carotene and other plant materials. They extracted a Skellysolve B solution of the pigments with 95 per cent methanol in a separatory funnel. The sterols were more soluble in the methanol solution and passed into this phase. Most of the other plant materials remained in the Skellysolve B solution.

Two types of continuous liquid-liquid extractors were constructed to investigate the phasic separation of the pigments. One of the extractors was similar to that of Chapman and Hammett (8) and utilized compressed air to bring the two immiscible solutions into intimate contact. The other extractor operated on a continuous distillation principle (9), by which the condensate of the heavier liquid is allowed to percolate through a column of the lighter liquid.

In one method of extraction which was investigated, the eluate from the chromatograph was evaporated to remove the eluting agent and the residue was dissolved in Skellysolve B. This solu-

tion was placed in the percolation reservoir of the continuous distillation extractor. A 10 per cent solution of potassium hydroxide in 90 per cent methanol was placed in the distilling flask of the apparatus. The Skellysolve B solution was then extracted with the methanol solution for 4 hours. It was found that the condensate from the distillation flask contained 95 per cent methanol (Table 4). Hence, the phasic system of Fernholz and Moore was duplicated.

Table 4. Determination of per cent methanol in condensate from the distilling flask by comparison of its refractive index with those of known mixtures of methanol and water.

Solution	Refractive index
90 per cent methanol	1.3326
95 " " "	1.3308
100 " " "	1.3259
Condensate	1.3308

During the extraction it was noted that chlorophyll was removed from the Skellysolve B solution and was concentrated in the distilling flask, where it was saponified by the potassium hydroxide. The Skellysolve B retained a small amount of carotene which had not been removed by previous separations, and various other materials such as fats and waxes.

It still was necessary to separate the sterols from the saponification mixture obtained from the above extraction. This was accomplished by diluting the alkaline 90 per cent methanol

solution to about 80 per cent by adding water, transferring this solution to the air-lift extractor, and recycling it through Skellysolve B. The sterols were more soluble in the Skellysolve B than in the 80 per cent methanol, and after three hours of recycling were transferred from the methanol to the Skellysolve B. The Skellysolve B solution was evaporated, the residue was dissolved in 95 per cent ethanol, and the sterols were allowed to crystallize. The sterol was identified by preparing the acetate (m. p. 183-184° C.) and the benzoate (m. p. 194-195° C.). These derivatives indicate the sterol to be α-spinasterol. The data of Table 5 indicate the amount of sterols which was extracted by this procedure.

Table 5. Extraction of sterols from the eluate by a method involving saponification and partition between Skellysolve B and methanol.

Experiment	Sterols in eluate	Sterols extracted	
	mg	mg	per cent
1	500	468	94.0
2	1300	1220	94.0

A second method was studied for isolating sterols from the substances eluted from the chromatograph. In this procedure, activated magnesia was used to remove chlorophyll, thus avoiding the necessity of saponification. The eluate from the chromatograph was evaporated to dryness, and the residue was dissolved in Skellysolve B. Into this solution was stirred activated magnesia until all of the chlorophyll was adsorbed. The adsorbent was re-

moved by filtration and was washed thoroughly with 10 per cent acetone in Skellysolve B to elute sterols and xanthophylls. The acetone was removed by evaporation, and the residue was dissolved in Skellysolve B. The latter was extracted with 90 per cent methanol for four hours in the continuous distillation extractor, as previously described. The sterols thus were transferred to 90 per cent methanol, from which they were crystallized and purified as described previously. Table 6 shows the amount of sterols that was extracted by this method.

Table 6. Extraction of sterols from the eluate by a method involving adsorption and partition between Skellysolve B and methanol in a continuous distillation extractor.

Experiment	Sterols in eluate	Sterols extracted	
	mg	mg	per cent
1	688	670	97.3
2	720	640	91.5

The air-lift extractor also was used in conjunction with the adsorbent as in the experiment described above. Ninety-five per cent methanol was used as the extracting solution. The results of this experiment are shown in Table 7.

Table 7. Extraction of sterols from the eluate by a method involving adsorption and partition between Skellysolve B and methanol in an air-lift extractor.

Experiment	Time of extraction	Volume of methanol	Sterols in eluate	Sterols extracted	
	min.	liters	mg	mg	per cent
1	480	2	2420	2317	95.8
2	240	1	350	326	93.0

Each of the methods described separated the sterols from the rest of the plant pigments in the eluate from the chromatograph. The method involving saponification was the most difficult to use because of emulsification which occurred in the air-lift extractor due to the presence of chlorophyll degradation products. This difficulty was not encountered in the method utilizing adsorption.

Efficiency of Extractors

As previously shown, the single extraction required in the adsorption method can be accomplished with either of the extractors used. To determine their relative efficiencies, a Skellysolve B solution containing a known amount of sterols was divided into two equal portions. One of the portions was extracted in the air-lift and the other in the continuous distillation extractor. At various time intervals, 2 ml samples of the Skellysolve B phase were withdrawn from each extractor and were analyzed for sterols. The data, presented in Fig. 1, show that the initial rate of sterol extraction by the air-lift extractor was consider-

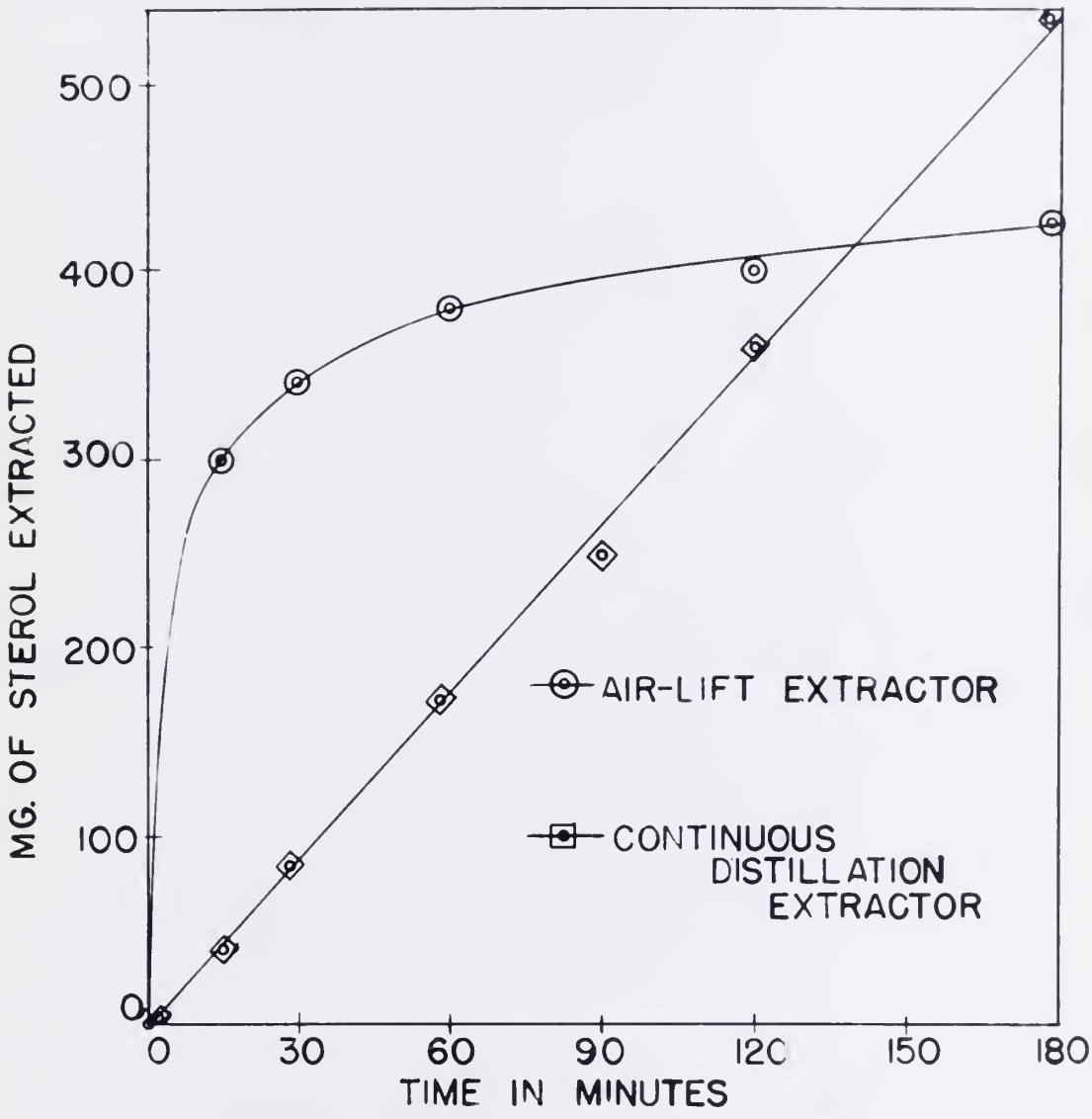

FIG. I. EXTRACTION OF STEROLS BY CONTINUOUS DISTILLATION AND BY AIR-LIFT EXTRACTORS

ably greater than by the continuous distillation extractor. However, extraction by the continuous distillation extractor eventually was greater. This was true because, with the air-lift extractor, sterols were partitioned between the two solvents until an equilibrium was reached (10). Such an equilibrium could not result with the distillation extractor because the sterols were not vaporized during the distillation. Hence, the sterols were partitioned continuously between the Skellysolve B solution and fresh methanol.

Ultraviolet Absorption Spectrum of α-spinasterol

The absorption spectrum of α-spinasterol has not been reported in the literature. Since a quantity of this sterol was isolated from alfalfa meal during the course of this investigation, its ultraviolet absorption spectrum was determined. The α-spinasterol was recrystallized from methanol, ethanol, and benzene until the melting point remained unchanged on further recrystallization. The method used by Hogness, Sidwell, and Zscheile (11) for the determination of the spectral curve of ergosterol was used. The absorption curve (Fig. 2) obtained in the region 2385-3135 angstroms was similar to that of ergosterol. This is to be expected, since the proposed structural formula (12) of α-spinasterol is similar to that of ergosterol. In the region 2335-2385 angstroms, the α-spinasterol curve differed from that of ergosterol in that α-spinasterol exhibited greater absorption. Whether this increased absorption was due to a structure not present in ergosterol or to an impurity has not been

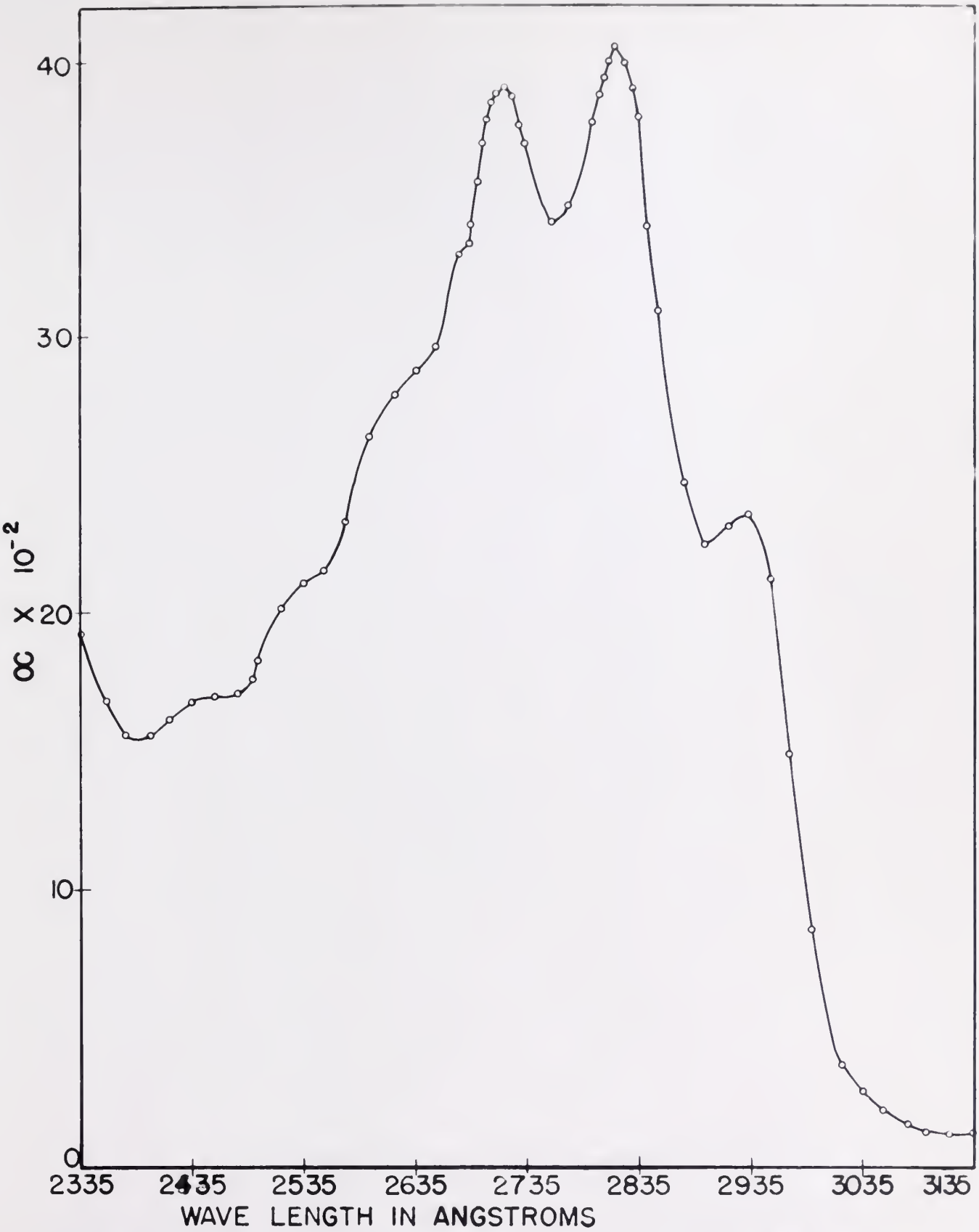

FIG. 2. ULTRAVIOLET ABSORPTION SPECTRUM OF
α — SPINASTEROL

ascertained.

DISCUSSION

Although methods for isolating sterols from alfalfa meal were developed during this investigation, the economic feasibility of such an isolation is still in doubt. Distillation Products, Inc., Rochester, N. Y., recently announced a price on soybean sterols of a dollar a pound. It is difficult to visualize the production of alfalfa sterols at this price by the methods studied in this investigation. If, however, a specific use for α-spinasterol should be found, for which other sterols could not be used, perhaps it would be profitable to isolate alfalfa sterols in conjunction with the isolation of other alfalfa constituents. Meanwhile, a further study of the methods discussed herein may lead to a more economical procedure for isolation of alfalfa sterols.

SUMMARY

Methods were investigated for eluting sterols from a calcium phosphate adsorbent in a radial chromatograph. Both acetone and isopropyl alcohol, mixed in various proportions with Skellysolve B, were capable of eluting sterols. Isopropyl alcohol was the more efficient of the two, requiring lower concentrations and smaller volumes to effect sufficient cleansing of the adsorbent to permit its re-use. Five per cent isopropyl alcohol was adequate to accomplish this.

Sterols in the isopropyl alcohol eluate were isolated by

methods involving either saponification or adsorption to remove chlorophyll, followed by phasic distribution between Skellysolve B and methanol. Two phasic distributions were necessary if chlorophyll was removed by saponification, while one was sufficient when chlorophyll was removed by adsorption on magnesia.

Two types of phasic extractors were used in the final separation. One type was a continuous distillation extractor and the other was an air-lift extractor. Both extractors utilized 95 per cent methanol percolating through a Skellysolve B solution of plant pigments to extract the sterols. The relative efficiencies of these extractors were investigated. The initial rate of extraction was greater with the air-lift extractor. However, it did not extract all of the sterols because of the formation of a partition equilibrium. Complete extraction was accomplished with the continuous distillation extractor because a partition equilibrium did not occur.

The ultraviolet absorption spectrum of α-spinasterol was determined. The spectrum was found to be similar to the ultraviolet absorption spectrum of ergosterol.

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