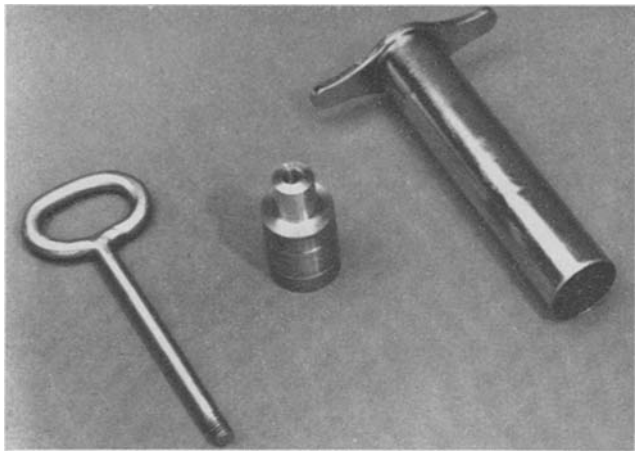

FIG. 5. Disassembled sampler for use with plastic fat sample holder.

to cut down heat radiation to or from the sample, which becomes quite a problem at times with metal holders. The above holder is also easily disassembled for cleaning.

The sample may be introduced into the sample holder in a number of ways. One such way is illustrated in Figure 5. The sampler was made from a cork borer of the appropriate size fitted with a piston and plunger. The piston has to be inserted from the cutting end because of the manner in which the cylinder is attached to the handle of the borer.

In using the above described sampler, the piston comes in contact with the top of the fat surface being sampled. This is satisfactory for samples where the condition of the original surface is not a prerequisite.

For persons desirous of obtaining a sample without disturbing the original surface the following procedure is suggested. The handle of the cork borer should be removed. This cylinder is then inserted into the fat, and the cylinder and fat core withdrawn and inverted. Insert the piston on the cutting side of the cylinder and push out the fat core. The first surface emerging will then be undisturbed and in its original condition.

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Adsorption Analysis of Lipids. IV. Fractionation of Cholesterol and Ergosterol[1]

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PREVIOUS work (1, 2, 3, 4) has indicated that cholesterol and ergosterol can be separated by chromatographic adsorption methods. In no instance however have data of purity and recovery been sufficiently complete to assess the efficiency of the adsorption analysis technique as a practical tool for the isolation of sterols. Before the complex problem of the analysis of soybean sterols could be undertaken, information concerning the efficiency of adsorption analysis was required. The cholesterol-ergosterol system was chosen as a model system primarily because of availability of materials and ease of analysis of mixtures by physical methods.

This paper describes the fractionation of cholesterol, ergosterol, and mixtures of the two upon columns of alumina by use of the "flowing chromatogram" technique. The efficiency of fractionation was evaluated by determinations of weights, melting points, optical rotations, and spectral adsorption measurements on eluted fractions.

Materials

Cholesterol used was of commercial grade: m.p. 146-148°C.; $[\alpha]_D^{25°C.} = -39.76°$. The constants reported in the literature for pure cholesterol are as follows: m.p. 150-151°C.; $[\alpha]_D = -39.5°$ (5). The cholesterol purified by the procedure of Anderson (6) was also used: m.p. 148.5-149.5°C.; $[\alpha]_D^{25} = -39.87°$.

Commercial ergosterol (m.p. 147-151°C.; $[\alpha]_D^{25} = -119.80°$) was purified by recrystallizing twice from acetone, according to the method of Lamb *et al.* (7). Its spectral absorption coefficients (a = gms./liter[1] cms.[1]) were: 31.95 at 2,710 Å; 33.23 at 2,810 Å; 19.48 at 2,930 Å. Its melting point was 149-156°C., and $[\alpha]_D^{25°C.} = -119.53°$. Values reported in the literature are: 28.38 at 2,710 Å; 29.16 at 2,810 Å; 16.79 at 2,930 Å (8); m.p. 163°C.; $[\alpha]_D = -132°$ (5).

Aluminum oxide (Harshaw's Al-2 powder[3]) was used for adsorbent and was selected for its characteristics of filtration, fractioning power, and high recovery of solutes. It is in Class V of the Brockman Scale of adsorbent strengths. Petroleum ether (B.P. 50-60°C.) was purified by distillation and passage through a silica column to remove ultra-violet absorbing impurities (9).

Experimental Procedure

Considerable exploratory work was necessary before the conditions of adsorption were determined. This survey work was, of course, complicated by the colorless characteristics of the sterols. Choice of the adsorbent was a compromise between the fractionating power of strong adsorbents and the high recoveries of weaker adsorbents. Choice of solvents was

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[2] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Report of a study made under the Research and Marketing Act of 1946.

[3] The mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

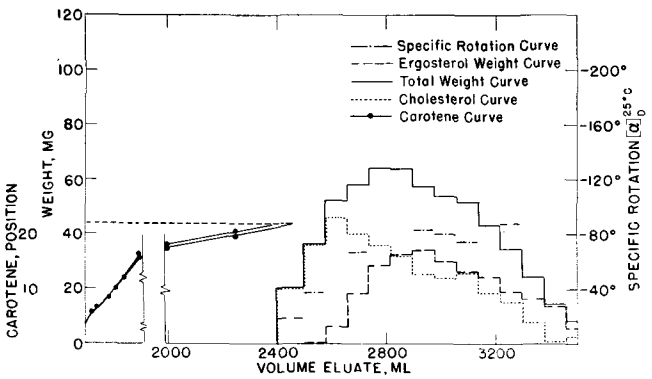

FIG. 1. Adsorption analysis of cholesterol and ergosterol (commercial preparations): Alumina, 263 gm. wet pack; cholesterol, 0.333 gm.; ergosterol, 0.333 gm.; solvent for sample, 50 ml. of 4% ethanol in petroleum ether; solvent for retardation, 300 ml. of petroleum ether; developing agent, 0.5% ethanol in petroleum ether; recovery, 92%.

limited by their solutility for ergosterol and their ultra-violet transparency as well as by their efficacy as developers. The technique of chromatography described and experimental results obtained represent "best results" of a large number of trials.

Alumina (285 gm.) was poured as a slurry in petroleum ether into a glass column 4.5 cm. in diameter and 26 cm. long and was packed with the aid of a few centimeters of negative pressure at the base of the column. Samples dissolved in a mixture of ethanol in petroleum ether were added to the column when the solvent level approached the top of the column of adsorbent. After the entry of the sample solution into the column, 300 ml. of petroleum ether were passed into the column to retard the movement of bands and prevent rapid elution. Development of the chromatogram was then effected with 0.5% purified ethanol in petroleum ether. Solvents were introduced by means of a glass reservoir which permitted the continuous application of pressure (0.5 p.s.i.) by prepurified nitrogen (0.002% oxygen) at the top of the column.

The rate of solvent flow under these conditions was about 1 liter per hour. Progress of the chromatogram was followed by adding approximately 0.2 mg. of carotene (90% α and 10% β) to each sample. The carotene served as a visible indicator of evenness of packing. Observation of fractionation of the carotene into α and β bands further attested to the satisfactory operation of the column.

The carotene pigments were apparently displaced by the following sterols and were eluted first. The plots of the progress of this band on the column given in the figures illustrate the retardation by petroleum ether and the development in 0.5% ethanol. In the experiments 40-, 50-, or 80-ml. fractions of percolate were collected in tared flasks. The weight of sterols in each fraction was then determined after the removal of solvent *in vacuo* at room temperature.

In those solutions which contained ergosterol and could be analyzed spectrophotometrically, optical densities were measured at wavelengths 2,710 Å, 2,810 Å, and 2,930 Å prior to evaporation. The concentration of ergosterol in the cholesterol-ergosterol system was calculated from spectrophotometric determinations. Specific rotations ($[\alpha]_D^{25°C.}$) and melting points were also measured in some instances.

Results

Results of the adsorption analysis of commercial preparations of ergosterol and cholesterol are shown in Figure 1. The total weight of the fractions (mg. per sample), the spectrophotometrically-determined ergosterol weight per fraction, the cholesterol weight per fraction (total wt. minus ergosterol wt.), and the specific rotations are plotted against the volume of eluate. Judged by the optical rotation and absence of ergosterol, only the first fraction appears as pure cholesterol. The higher specific rotations for the later fractions generally confirm spectrophotometric data and indicate increasing amounts of ergosterol. The only conclusion from this experiment is that cholesterol is less strongly adsorbed than ergosterol and that fractionations rather than separations of crude sterol materials are to be expected.

Since impurities of the commercial preparations could obscure the separations obtained, the purity of the commercial cholesterol was studied by passing it through an alumina column. The adsorption analysis of this preparation gave strong evidence of its impurity. Figure 2 shows a wide variation in specific

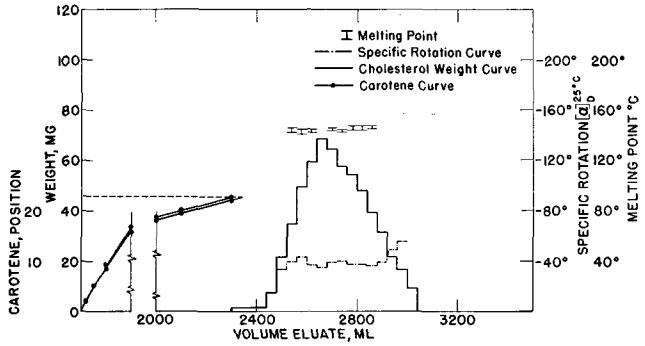

FIG. 2. Adsorption analysis of cholesterol (commercial preparation): Alumina, 276 gm. wet pack; cholesterol, 0.6665 gm.; solvent for sample, 50 ml. of 0.50% ethanol in petroleum ether; developing agent, 0.50% ethanol in petroleum ether; recovery, 91%.

rotation from —33.52° to —55.92°. The impurity may account, in part, for the poor fractionation apparent in Figure 1.

Adsorption analysis of the purified cholesterol was subsequently performed. Figure 3 shows that the seemingly pure compound as obtained by the crystallization of the debrominated cholesterol (6) still contains a small amount of impurities. This fact is evidenced by a 7° spread in specific rotation of the fractions as well as the slight variations in their melting points.

Results of the adsorption analysis upon the commercial ergosterol preparation (Figure 4) show a wide variation in specific rotation from —101.20° to —121.70°. A further evidence of impurity is shown by the departure of the spectrometrically determined weight curve from the gravimetric weight curve.

The fractionation of cholesterol from ergosterol appears greatly improved when the mixture is composed of purified components as shown in Figure 5. The first two fractions of eluate are nearly free of ergosterol as an impurity. It is interesting to note that the third fraction, which contains 4.3% ergosterol in cholesterol, seemed to be relatively pure according to

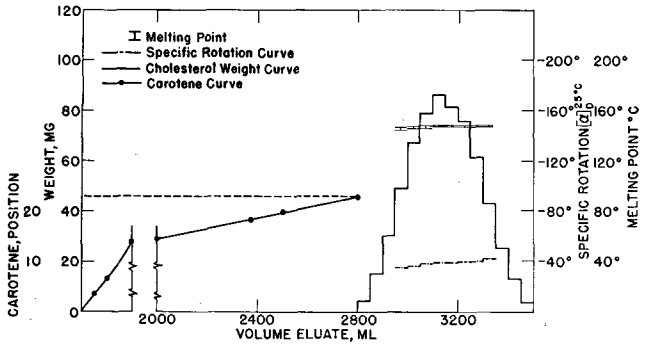

FIG. 3. Adsorption analysis of purified cholesterol: Alumina, 307 gm. dry pack; cholesterol, 0.6661 gm. (purified); solvent for sample, 30 ml. of 3.3% ethanol in petroleum ether; solvent for retardation, 300 ml. petroleum ether; developing agent, 0.50% ethanol in petroleum ether; recovery, 95%.

its specific rotation. However this amount of impurity has a decided effect upon its melting point. The narrow range of melting points and of optical rotation values found in the initial fractions suggest the presence of nearly pure cholesterol. In the first three fractions 48.4% of the original cholesterol was obtained with 98% purity. These were followed by three fractions of intermediate composition.

The wide range and spread of the melting points in the later ergosterol fractions indicate that they are not characterized by any single entity. It is apparent that in several instances the calculated ergosterol weight is greater than the gravimetrically determined weights of the respective fractions. These fractions therefore have higher specific absorption values than the value which was reported by Huber (8) ($a^{2,810\,Å}$-29.16) and which was used in the present calculations. Three fractions exhibiting this anomaly have absorption coefficients at 2,810 Å of 31.61, 32.35, and 31.27. On the basis of these data it may be postulated that vitamin D_2 and other irradiated products may be present. The "ergosterol" of the seventh and following fractions, calculated with Huber's constant, account for 64.9% of the ergosterol originally placed on the column.

Discussion

Adsorption analysis has been shown to be of value in determining the purity of sterol preparations. Although the cholesterol obtained by recrystallization of the debrominated products gives indications of a pure product by reference to its melting point and specific rotation, the fractions obtained by absorption analysis exhibit slight variation in their melting points and specific rotations. Furthermore it has been shown that the use of one physical property, such as optical rotation, is not an adequate criterion for judging purity. When ergosterol comprises a minor contaminant of cholesterol, optical density data are necessary to establish its presence. Although this work indicates the improbability of a complete separation of the two compounds, adsorption analysis has been sufficiently effective that small fractions of "pure" cholesterol and ergosterol can be obtained.

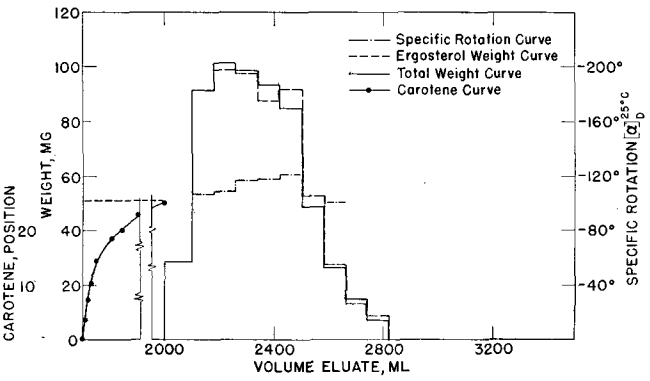

FIG. 4. Adsorption analysis of ergosterol (commercial preparation): Alumina, 290 gm. wet pack; ergosterol, 0.6295 gm.; solvent for sample, 100 ml. of 6% ethanol in petroleum ether; solvent for retardation, 460 ml. of petroleum ether; developing agent, 0.75% ethanol in petroleum ether; recovery, 95%.

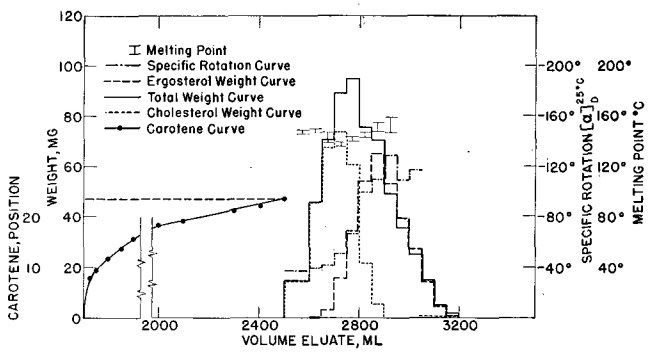

FIG. 5. Adsorption analysis of "purified" cholesterol and "purified" ergosterol: Alumina, 285 gm. wet pack; purified cholesterol, 0.3123 gm.; purified ergosterol, 0.3112 gm.; solvent for sample, 79 ml. of 6.3% ethanol in petroleum ether; solvent for retardation, 300 ml. of petroleum ether; developing agent, 0.55% ethanol in petroleum ether; recovery, 98%.

From the shape of the elution curves it appears that a displacement type of adsorption analysis (10) characterizes the fractionation of sterols. The molecules forming the cholesterol band are closely followed by ergosterol molecules during the progress of development and appear to be displaced by them. If displacement is occurring, a complete separation evidenced by two major peaks in the weight curve cannot be expected. Since adsorption analysis is more an art than a science, the possibility remains that better separations may be achieved after further investigation with various adsorbent and solvent combinations.

Although separation of sterols in naturally occurring mixtures presents a difficult problem, adsorption analysis should constitute a useful tool for fractionation, even with its apparent limitations.

Summary

The adsorption analysis of the cholesterol-ergosterol system has been studied. Since purified cholesterol and ergosterol were shown to be multi-component by adsorption analysis on alumina, calculation of the degree of separation of their mixture involves certain approximations. Of the cholesterol originally placed on the column, it was calculated that 48.4% was recovered with a purity of 98%. Ergosterol recovered in the later fractions had a calculated purity of 97% and represented 64.9% of the ergosterol originally placed on the column.

Acknowledgment

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REFERENCES

1. Winterstein, A., and Stein, G., Z. Physiol. Chem., *220*, 247 (1933).
2. Karrer, P., and Nielsen, N., Ber. ges Physiol. Exptl. Pharmakol, *86*, 529.
3. Landenburg, K., Fernholz, E., and Wallis, E. S., J. Org. Chem., *3*, 294 (1938).
4. Brockmann, H., and Volpers, F., Chem. Ber., *80*, 77 (1947).
5. Gilman, H., "Organic Chemistry," John Wiley and Sons inc., New York, (1942).
6. Anderson, R. J., J. Biol. Chem., *71*, 407 (1927).
7. Lamb, F. W., Mueller, A., and Beach, G. W., Ind. Eng. Chem. Anal. Ed. *18*, 187 (1946).
8. Huber, W., Ewing, G. W., and Kriger, J., J. Am. Chem. Soc., *67*, 609 (1945).
9. Graff, M. M., O'Connor, R. T., and Skau, E. L., Ind. Eng. Chem. Anal. Ed., *16*, 556 (1944).
10. Tiselius, A., Arkiv. Kem. Mineral Geol. 16A, *18* (1943)

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Comparison of the Whole Fruit and Component Methods of Analysis of Tung Fruit

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THE estimation of oil in tung by grinding the whole fruit was described by McKinney, Halbrook, and Agee in 1948 (1, 2). This method was adopted by the Commodity Credit Corporation as the official method of analysis in its price support for the 1947 crop. Further comparisons between the whole fruit and the so-called *component method* in which the hull- and shell-free kernels are analyzed for oil were contained in a report of the Subcommittee on Tung Fruit and Meal Analysis of the American Oil Chemists' Society in 1948 (3). The most recent report of this committee is given elsewhere in this journal. No data have been previously reported for the effects of moisture content and fineness of grinding in the whole fruit method of analysis. The present report contains the results of a comparison of the two methods which were obtained in the same laboratory, using different portions of the same samples of tung fruit. The samples were chosen to cover a wide range in the contents of moisture and oil.

In the whole fruit method the sample was first ground in a Wiley[2] mill equipped with a quarter inch screen, thoroughly mixed, and an aliquot of about a pint was reground in a Bauer laboratory mill with the plates set close enough to give a fine meal. Since moisture is lost during the fine grinding, it was necessary to determine the moisture content of the sample before and after grinding so that the results could be calculated to the original moisture basis. Moisture was determined on five-gram samples by drying them in a vacuum oven for 2½ hours at 104°C. The oil content was determined by extracting five grams of the finely ground sample with petroleum naphtha (Skelly F) in a Butt extraction tube for four hours.

Because of the extrusion of oil, kernels and seeds cannot be ground finely enough in a Bauer laboratory mill for complete extraction of the oil. Samples ground coarsely enough to avoid extrusion of oil can be analyzed after regrinding the partially extracted sample with sand in a mortar, but particles of shell in the sample interfere with the proper regrinding of the sample.

[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[2] The specification by firm name of equipment and special reagents used throughout this article is for identification purposes and implies no endorsement of the manufacturer or product mentioned.

The samples analyzed by the component method were weighed; the kernels were separated from all hulls and shells; and all hulls and shells were collected and dried to constant weight; care was taken not to lose any particles. The cleaned kernels were then weighed, ground, and analyzed for oil and moisture content as described for the whole fruit method, except that after extraction for four hours the partially extracted kernels were ground for five minutes with mortar and pestle and then extracted for two additional hours. The percentage of oil in the fruit was calculated from the total weight of sample, and the weight and percentage of oil in the kernels. The percentage of moisture was calculated from the total wet weight and the total dry weight of sample.

In both methods the bulk of the solvent in the micella was removed rapidly on a steam bath. Thirty minutes in a vacuum oven heated to 100°C. served to remove any residual solvent.

TABLE I

Percentage of Oil Extracted From Tung Fruit as a Function of Fineness of Grinding and Moisture Content. Calculated to Dry Basis

Sample No.	Moisture content, %	Distance between plates, inch			
		0.004	0.008	0.012	0.020
1	7.9	25.64	26.22	.....	.....
2	7.8	26.50	26.49	.....	.....
3	9.0	25.56	25.65	25.55	24.81
4	8.4	26.23	25.68	26.10	25.64
5	40.4	24.23	24.16	24.07	23.46
6	9.3	24.60	23.78	24.64	23.56
7	8.4	25.24	24.79	24.62	.....
8	17.0	23.99	23.67	23.56	.....

The complete extraction of oil from ground tung kernels is known to be difficult (5), and in order to be certain that substantially all of the oil is extracted when using the component method it is necessary to interrupt the extraction and thoroughly regrind the partially extracted kernels with mortar and pestle. However presence of hull and shell particles precludes effective regrinding in a mortar after partial extraction of the oil in the whole fruit method. For this reason the effect of fineness of grinding on the amount of petroleum naphtha-extractable material in the ground whole fruit is very important.

The effect of fineness of grinding on the amount of oil extracted from whole fruit is shown in Table I. Reference to this table shows that there are no dif-