

# The Microbiological Synthesis of Ergosterol

## I. Assay Procedure

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The determination of ergosterol in microbial products has been described using a wide variety of methods. Generally, the extraction procedures utilized a saponification of either the wet or dry cell material followed by a solvent extraction. Bills *et al.* (1930) reported the use of a direct ethanol-benzene extraction of dried yeast while Wenck *et al.* (1935) utilized a direct ethanol extraction followed by a saponification. Following saponification and extraction, Castille and Ruppel (1933) utilized the digitonin precipitation to isolate the sterol fraction. The actual measurements have generally utilized the Liebermann-Burchard reaction (Wenck *et al.*, 1935) or the ultraviolet absorption properties of ergosterol (Bills *et al.*, 1930 and Castille and Ruppel, 1933).

This study was initiated as part of a program which required substantial numbers of ergosterol determinations using relatively small samples of materials. In addition, sufficient versatility was required so as to permit application to a wide variety of microbial products. The basis of the procedure is dependent on the fact that ergosterol, in the main, is present in cells as free ergosterol or as simple esters of fatty acids. In these forms, ergosterol is readily extractable by a wide range of organic solvents. The ability to carry out determinations directly on the crude extracts has also contributed to the simplicity of the procedure. Comparative isolation data presented here is typical and lends support to the validity of the procedure.

### EXPERIMENTAL METHODS

Determinations are generally made on the solids obtained from 50 to 100 ml of fermentation broth. The broths are routinely centrifuged, and the solids washed and centrifuged two times with distilled water. This washing treatment is essential in removing interfering materials, particularly in the case of complex media. The solids are spread evenly on a 5 cm filter paper and air dried for 20 hours at 65–70 C. Vacuum drying at reduced temperatures yields comparable results.

The dried cell pads are micropulverized with a semi-micro model of a Wiley mill. The importance of particle size was established as described below and rou-

tinely a particle size capable of passing an 80 mesh screen with agitation is used. Samples of 200 to 500 mg are extracted in Soxhlet extractors for 6 hours with ether. The relative merits of a wide range of solvents were tested and the results are discussed below.

The ether extracts are taken to dryness at room temperature. By using a series of three appropriation dilutions with chloroform, a final volume of 5 ml containing approximately 0.1 to 0.4 mg ergosterol is prepared in calibrated colorimeter tubes. Exactly 5 ml of the Liebermann-Burchard reagent (Hoffman, W. S., 1940) is added with shaking. Relatively constant temperatures are maintained during the reaction period with 25 C being used in this work.

The absorption maxima in the visible region for the Liebermann-Burchard reaction on ergosterol are 420  $m\mu$  and 670  $m\mu$ . Both are relatively broad maxima with the maximum at 670  $m\mu$  being attained in less than 2 minutes at 25 C while maximum at 420  $m\mu$  appears after approximately 25 minutes. The times selected for reading are slightly off the maxima but were selected for the convenience of setting up approximately 20 samples in a series.

After exactly a 10 minute interval, the density determination is made on a Lumetron colorimeter using a 660  $m\mu$  filter of the narrow band width type. After exactly a 20 minute interval, the measurement is repeated at 420  $m\mu$ . The blank correction consists of 5 ml of the reagent, and 5 ml of the chloroform used in the sample dilutions. The observed densities are used to calculate ergosterol concentrations from a prepared standard curve.

In the event of poor correlation between the determinations made at 660  $m\mu$  and 420  $m\mu$ , the lower determination is retained as the more probable. An ultraviolet absorption curve on the original chloroform solution using a Carey spectrophotometer is used to further check the assay. It is felt that the most reliable determinations are based on the 285  $m\mu$  and 296  $m\mu$  maxima, using the lowest assay obtained by the use of either maximum or by the difference of the maxima.

The direct isolations were carried out following saponification of micropulverized dried preparations with 8 per cent potassium hydroxide in 95 per cent

ethanol. The reaction was maintained at reflux for 6 hours after which the insolubles were filtered off and washed with hot ethanol. The clear filtrate was set in the refrigerator overnight for crystallization. The precipitate was filtered and washed with water. The filtrate was diluted with two volumes of water and set aside at refrigerator temperatures for further precipitation. The precipitates were combined, recrystallized from ethanol, and ultraviolet spectra were determined from appropriate chloroform dilutions. Calculations were based on the difference between the 285 and 296  $m\mu$  maxima.

#### EXPERIMENTAL RESULTS

The close correlation of ergosterol recoveries which are obtained by direct solvent extraction as compared to the saponification procedure is illustrated in table 1 which compares the analysis of five samples of a relatively wide range of ergosterol content.

The importance of particle size in direct solvent extraction is illustrated by the extraction of a series of particle size ranges of a yeast sample in table 2.

The selection of the most suitable solvent was based upon the following major requirements: 1) relatively rapid efficient extraction of the ergosterol and 2) the production of crude extracts suitable for direct determinations by the Liebermann-Burchard reaction and ultraviolet absorption.

Acetone, ethanol, methanol, and dichloroethylene are equally efficient in the extraction of ergosterol. However, these solvents produce extensive ultraviolet background as well as interfering background for the colorimetric determination. Hexane, benzene, cyclo-

TABLE 1. Comparison of direct ether extraction and isolation methods for the determination of ergosterol

Sample	Per cent Ergosterol* by Direct Ether Extraction	Per cent Ergosterol by Isolation
No. 1 yeast.....	0.75	0.63
No. 2 yeast.....	0.29	0.22
No. 3 yeast.....	0.12	0.11
No. 4 yeast.....	2.60	2.50
No. 5 yeast.....	0.75	0.78

\* Per cent ergosterol is calculated on a dry weight basis.

TABLE 2. Relationship of particle size to ergosterol extractability

Particle Size*	Per cent Ergosterol
10 mesh to 20 mesh.....	0.53
20 mesh to 40 mesh.....	0.70
40 mesh to 60 mesh.....	0.86
60 mesh to 80 mesh.....	0.85
80 mesh to 100 mesh.....	0.97
Less than 100 mesh.....	0.95

\* The lower mesh number was passed with agitation while being retained by the higher mesh number.

TABLE 3. Comparative measurements of ergosterol by the Liebermann-Burchard reaction and ultraviolet absorption

Sample	Per cent Ergosterol by Ultraviolet Absorption	Per cent Ergosterol by Colorimeter Determination
No. 1 mold.....	0.43	0.40
No. 2 mold.....	0.58	0.50
No. 3 yeast.....	0.71	0.64
No. 4 yeast.....	0.95	0.91

hexane, isopropyl ether, and acrylonitrile are somewhat less efficient in extraction of the sterol although interfering background materials are somewhat less than in the first group. Diethyl ether is relatively efficient in the extraction of the sterols and interfering background is at a minimum both for colorimetric determinations and direct ultraviolet measurements.

Four hours, using a reflux producing 5 to 6 passes per hour, is adequate to effect complete extraction. The ergosterol stability in ether at reflux was tested with no appreciable loss up to 6 hours. Twenty-four hour reflux results in approximately 15 to 30 per cent loss as determined by ultraviolet absorption.

Relatively close correlation is generally observed in results obtained by ultraviolet absorption and the colorimetric determination as illustrated by typical data in table 3.

#### DISCUSSION

Assay precision was estimated from 27 sets of duplicate colorimetric determinations made during normal runs. The coefficient of variation was calculated to be 9.6 per cent. However, there is some indication that the standard deviation does not vary directly with the amount of ergosterol in the sample.

The evidence presented above indicates rather clearly that saponification procedures do not liberate substantial quantities of ergosterol bound by the particulate portion of the cells. This implies, excluding any conjugates not subject to the rather vigorous alkaline hydrolysis used, that ergosterol is present in the main as the free sterol or readily extractable esters. It is felt that saponification procedures which are in general use serve the purpose of digesting and breaking up cells rather than liberating ergosterol by the hydrolysis of insoluble conjugates.

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#### SUMMARY

A relatively simple and precise direct solvent extraction procedure for the determination of ergosterol is

presented. The method has been found applicable to a wide range of microbiological products using relatively small samples for determinations.

## REFERENCES

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