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YEAST ANALYSIS

Spectrophotometric Semimicrodetermination of Ergosterol in Yeast

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In a method for spectrophotometric determination of ergosterol in yeast samples containing 5 to 100 mg. of yeast solids, the yeast is digested with alcoholic alkali and ergosterol is extracted in a single extraction with a measured volume of pure *n*-heptane. Absorbances at 281.5 and 230 mμ are determined on an aliquot of the heptane diluted in absolute ethyl alcohol. Amount of "ergosterols" are determined from the 281.5-mμ measurement, and amount of 24(28)-dehydroergosterol is determined from the 230-mμ measurement. The difference in these values gives the ergosterol content.

A RAPID METHOD OF ERGOSTEROL DETERMINATION, applicable to small samples, was needed in a study of the formation of ergosterol by yeast cultures under a variety of propagational conditions. It was desirable that the method give results corresponding to the amount of purified ergosterol that could be isolated from the yeast.

Methods for the determination of ergosterol in yeast (*1, 2, 5–10, 12, 17*) usually employ saponification of the yeast with strong aqueous or alcoholic alkali, extraction of the unsaponifiable matter with an immiscible solvent, and measurement of ergosterol by colorimetric or spectrophotometric means, either with or without digitonin precipitation.

In this laboratory the method of Castille and Ruppol (*5*), except for use of stronger alkali in the saponification and omission of the digitonin precipitation, was used as a reference method. It required 2 to 5 grams of compressed yeast, and an operator could analyze 6 to 12 samples per day. In attempts to simplify the method, it was found that *n*-heptane, which could be obtained in a spectroscopically pure grade, extracted the yeast sterols from the yeast digested with alcoholic alkali cleanly and quantitatively in a single extraction. Dilution of the *n*-heptane with absolute ethyl alcohol and determination of absorbances at 281.5 and 230 mμ gave values closely agreeing with the reference method. A determination could be made on as little as 5 mg. of yeast solids, and an operator could carry out 24 or more analyses per day.

Ergosterol is generally considered to be the primary sterol of yeast and is by far the most readily isolated. A number of other yeast sterols which have been reported are zymosterol (*11*), "α-dihydroergosterol" (*4*), since shown to be 5-dihydroergosterol (*13*), ascosterol (*15*), fecosterol (*15*), episterol (*14*), hyposterol (*14*), and 24(28)-dehydroergosterol (*3*). Under the usual conditions of yeast growth, such as used in baker's yeast manufacture, the sterol produced is predominantly ergosterol (about 80%). Except for 24(28)-dehydroergosterol, the other sterols can be considered to be minor constituents. It has been found in this laboratory that, although there are variations with yeast strains, any of a wide variety of yeast strains is capable of making as much 24(28)-dehydroergosterol as ergosterol.

Because of the complexity of the yeast sterols, colorimetric methods of measurement cannot be expected to be satisfactory for determination of ergosterol content. Work in this laboratory on the fractionation of yeast unsaponifiable matter showed nearly all the spectral absorption in the 220- to 300-mμ region to be due to ergosterol and 24(28)-dehydroergosterol. As the latter had an E (1%, 1 cm.) value at 281.5 mμ almost equal to that of ergosterol, and showed an intense spectral absorption band at 230 mμ where that of ergosterol was low, it was possible to calculate percentage of "ergosterols" from the 281.5-mμ measurement, and

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Table I. Spectral Absorption Maxima and Minima in Absolute Ethyl Alcohol

	Wave Length, Mμ	$E^{1\%}_{1cm.}$ Ergosterol	$E^{1\%}_{1cm.}$ 24(28)-Dehydroergosterol
Maxima	293	162.5	167
	281.5	290	308
	271	275	295
	230	...	518
Minima	289	146	153
	276	227	240
	248	...	146
	230	37.5	...

24(28)-dehydroergosterol from the 230-mμ measurement. The difference between the two percentage values gave ergosterol content. Values for ergosterol content determined in this way were found to be a good index of the amount of purified ergosterol that could be isolated from various yeasts.

Physical Properties of Ergosterol and 24(28)-Dehydroergosterol

Crystalline ergosterol for use as a reference material was prepared by the following procedure.

Ten grams of a good grade of commercial ergosterol was recrystallized twice from 2 to 1 alcohol-benzene made by mixing 133 ml. of USP 95% ethyl alcohol or S.D. 3A ethyl alcohol (95% ethyl alcohol denatured with 5% methanol), 66 ml. of C.P. benzene, and 2 ml. of water, and clarifying the hot solution by filtration in a hot funnel prior to the final crystallization. The crystals obtained upon slow cooling to 10° C., were filtered, and air-dried overnight; they consisted of ergosterol monohydrate, $C_{28}H_{44}O.H_2O$, molecular weight 414.64, melting point 165–166° C., $(\alpha)^{29}_D$ — 128 (1% in chloroform), and gave an absorption spectrum in absolute ethyl alcohol having the maxima and minima shown in Table I. The spectral absorption curve is shown in Figure 1.

The isolation of 24(28)-dehydroergosterol has been described (*3*). The sterol monohydrate, melting point 118–120° C., $(\alpha)^{29}_D$ — 78 (1% in chloroform), gave maxima and minima in absolute ethyl alcohol as shown in Table I, with the spectral absorption curve shown in Figure 1. As isolation of this material is difficult, crystalline ergosterol will serve as reference material to which the spectral absorption characteristics of 24(28)-dehydroergosterol can be related.

Reagents

Absolute Alcohol. U.S.P. absolute ethyl alcohol from U. S. Industrial Chemicals Co., was used as received.

Alcoholic Potassium Hydroxide. A 25% solution in water–ethyl alcohol was made as required by dissolving 250 grams of C.P. potassium hydroxide pellets in 400 ml. of water and diluting to 1 liter with U.S.P. absolute ethyl alcohol. The solution should be freshly prepared daily.

***n*-Heptane.** Pure grade *n*-heptane obtained from Phillips Petroleum Co., Bartlesville, Okla., was used as received.

Crystalline Ergosterol. Ergosterol manufactured by Standard Brands, Inc., was purified as described above for use as reference material.

Carborundum Boiling Chips. Silicon carbide grain, Grit. No. 12, from The Carborundum Co., Niagara Falls, N. Y., was used.

Saponification Tubes. These were made by sealing a test tube end on the outer member of a 19/38 standard joint. These joints are ordinarily manufactured on a 115-cm. length of tubing 17 mm. in outside diameter.

Reflux Air Condensers. These were made from the inner member of a 19/38 joint having a tubing extension 9 mm. in outside diameter. The lower extension was cut off to a drip tip and the upper extension made 12 inches long.

Stoppers. Standard taper 19/38 glass stoppers were used.

Water Bath. An electric water bath which could be held at 85° to 90° C. was used.

Spectrophotometers. Absorption spectra and absorbance readings were taken on a Model DU Beckman quartz spectrophotometer using slit width settings varying from 0.4 mm. at 281.5 mμ to 1.2 mm. at 230 mμ.

Procedure

Alcoholic Alkali Digestion. Accurately weigh about 100 mg. of compressed yeast or, about 25 mg. of dry yeast into a saponification tube. Add 4 ml. of alcoholic potassium hydroxide and a Carborundum boiling chip. For liquid yeast creams, pipet 2 ml. of the yeast cream into the saponification tube, add 1 gram of C.P. potassium hydroxide pellets (eight normal sized pellets = 1 gram), swirl until potassium hydroxide is completely dissolved, and add 2 ml. of U.S.P. absolute ethyl alcohol and a Carborundum chip. Apply a few drops of glycerol to the joint surface of a reflux air condenser, insert into the saponification tube, and place in a water bath at 85° to 90° C. After digesting for 1.5 hours, add 1 ml. of absolute alcohol through the top of the reflux condenser to wash down the walls of the saponification tube. Continue digestion an additional 1.5 hours, for a total digestion time of 3 hours.

Extraction. At the end of the digestion, remove the tube from the bath, remove the reflux condenser, and cool.

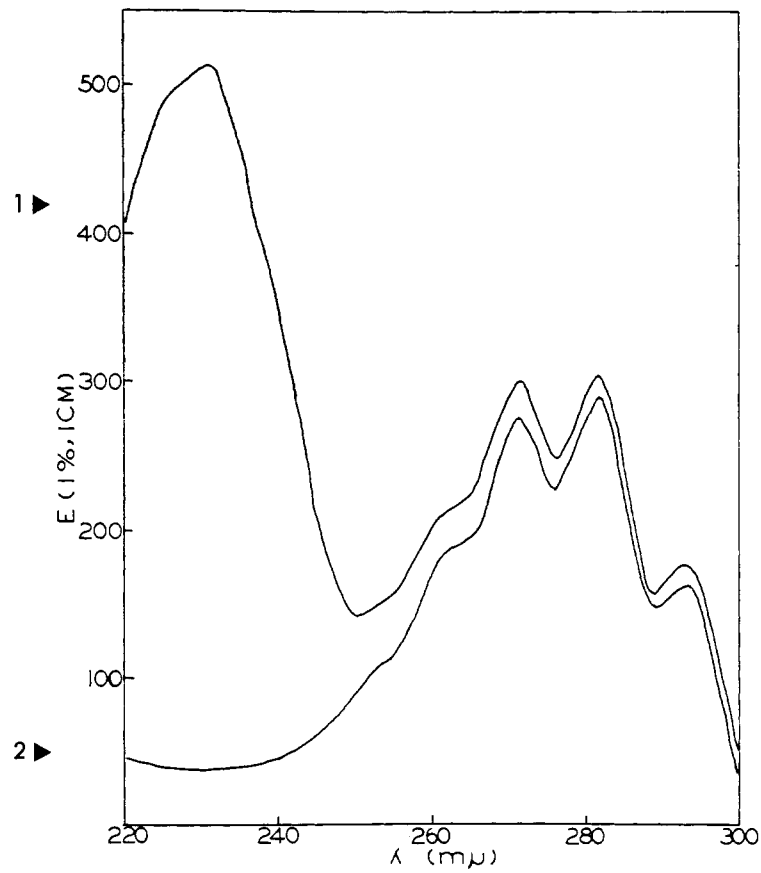

Figure 1. Ultraviolet absorption spectra

1. Ergosterol
2. 24(28)-Dehydroergosterol

Table II. Recoveries of Ergosterol

Ergosterol Introduced, Mg.	Ergosterol Found, Mg.	% Recovery
0.270	0.264	98.0
0.270	0.263	98.0
0.535	0.515	96.3
0.535	0.521	97.5
0.540	0.531	98.5
0.540	0.518	96.0
0.810	0.803	99.3
0.810	0.775	95.3
1.070	1.020	95.5
1.070	1.030	96.2
1.600	1.550	96.6
1.600	1.520	95.0

Table III. Comparative Determination of Ergosterols

Yeast Strain	% Ergosterols, Yeast Solids Basis: Reference procedure	Semi-micro-procedure
Saccharomyces cerevisiae Hansen		
Strain fulliensis	4.23	4 12
Var. ellipsoideus, strain Delft II	1.70	1.78
Strain batatae	2.46	2.44
Unidentified	2.78	2.80
Wine yeast	1.17	1.14
Tokay yeast	2.63	2.52
Champagne yeast	2.70	2.58
Burgundy yeast	1.80	1.76
Wine yeast	2.50	2.50
Distiller's yeast	2.92	2.80
Baker's yeast, Gebrüder Mayer	1.70	1.74
Saccharomyces chevalieri (Guilliermond)	4.41	4.64
Saccharomyces carlsbergensis		
Chubut	2.33	2.12
Böhmische Mucor Hefe	2.06	2.12
Obergärige Hefe A	2.14	2.16

To each tube add 2 ml. of distilled water and exactly 5 ml. of *n*-heptane. Stopper the tube with a glass stopper and shake vigorously about 30 times. With each set of determinations, prepare a blank by shaking 5 ml. of *n*-heptane with a mixture of 4 ml. of the alcoholic potassium hydroxide reagent, 1 ml. of absolute ethyl alcohol, and 2 ml. of water. Allow the tubes to stand for 30 minutes to allow the *n*-heptane layer to clarify.

Determination. Prepare a dilute solution for spectrophotometric determination by diluting 1 ml. of the supernatant *n*-heptane layer with 10 ml. of absolute ethyl alcohol in a 16 × 150 mm. test tube. Stopper the test tube with a thumb and shake. Determine absorbances of the dilute solution at 281.5 and 230 mμ, reading against a similar dilution of the blank. If absorbance readings are very low, dilute 2 ml. of the blank with less than 10 ml. of absolute ethyl alcohol and read against a suitable blank.

Calculation. Since ergosterol and 24(28)-dehydroergosterol give nearly the same absorbance reading at 281.5 mμ, their sum, or "ergosterols" content may be calculated as follows using the $E(^{1\%}_{1\text{ cm.}})$ value of 290, determined for crystalline ergosterol:

$$\%\text{ ergosterols} = \frac{\text{absorbance at 281.5 m}\mu}{290} \times F \quad (1)$$

where F is the factor for dilutions, sample size, and percentage. The 24(28)-dehydroergosterol content is calculated from the absorbance at 230 mμ, assigning the $E(^{1\%}_{1\text{ cm.}})$ value of 518 to 24-(28) dehydroergosterol:

$$\%\text{ 24(28)-dehydroergosterol} = \frac{\text{absorbance at 230 m}\mu}{518} \times F \quad (2)$$

It has been found convenient to calculate the ratio of absorbance at 230 mμ to that at 281.5 mμ and use the ratio in determining 24(28)-dehydroergosterol content. Dividing Equations 1 and 2 above gives

$$\%\text{ 24(28)-dehydroergosterol} = \%\text{ ergosterols} \times 230/281.5\text{ ratio} \times 0.56$$

and

$$\%\text{ ergosterol} = \%\text{ ergosterols} - \%\text{ 24(28)-dehydroergosterol}$$

Results

Analytical Procedure. Table II shows recoveries (in the *n*-heptane layer) when known quantities of ergosterol were added to alcoholic alkali and put through the analytical procedure. The procedure used was that applied to liquid yeast creams, using 2 ml. of water instead of yeast cream, eight pellets of potassium hydroxide, and 2 ml. of an absolute alcohol solution of the ergosterol at the beginning of the digestion. Table III compares results obtained on a variety of yeasts using the micromethod with those obtained by the reference method.

Isolation Experiments. To determine the extent to which results obtained for ergosterol content, using the correction for 24(28)-dehydroergosterol content, agreed with amounts that could be isolated, experiments were carried out in which purified ergosterol was isolated in as quantitative as possible a manner from 100-gram samples of compressed yeast. The procedure used for isolation of unsaponifiable matter was that of Windaus and Groskopf (*16*).

The unsaponifiable residue was then weighed, and taken up in alcohol-benzene 2 to 1 (66 ml. of 95% S.D. 3A ethyl alcohol, 33 ml. of C.P. benzene, and 2 ml. of water). For each gram of unsaponifiable matter, 15 ml. of alcohol-benzene were used. The residue was dissolved by heating on a water bath, allowed to cool slowly to room temperature, and placed in a refrigerator for several hours. The crystals were then filtered, weighed, and analyzed. The material was recrystallized, if necessary, to give a purity of 80% or more as determined by spectral absorption and optical rotation measurements.

Table IV. Comparison of Ergosterol Content Shown by Analysis with Amount of Purified Ergosterol Isolated from Yeast

Yeast Culture	Analysis: % ergosterols[a]	Analysis: 230 mμ/281.5 mμ ratio	Analysis: % ergosterol[a]	Isolation: % purified ergosterol isolated[a]	Isolation: % purity
Saccharomyces chevalieri (Guilliermond)	1.50	0.50	1.08	0.99	90
	1.80	0.64	1.16	1.08	94
	3.05	0.76	1.75	1.81	96
	4.80	1.19	1.60	1.00	88
	6.28	1.32	1.64	0.79	94
Saccharomyces cerevisiae Hansen, strain fulliensis	3.71	0.54	2.59	2.01	90
	4.63	0.52	3.28	2.90	
	9.64	0.76	5.54	4.01	90
Sacch. cerev. Hansen, wine yeast	2.70	0.41	2.08	2.10	90
	8.56	0.68	5.30	3.83	96
Sacch. carlsbergensis					
Böhmische Mucor Hefe	1.71	0.37	1.36	1.10	90
Obergärige Hefe A	2.14	0.81	1.17	0.69	90
Sacch. cerev. Hansen					
Tokay yeast	2.63	0.72	1.57	1.32	88
Wine yeast	2.50	0.74	1.46	1.28	84
Burgundy yeast	1.80	0.51	1.29	0.87	96
Unidentified	2.78	0.99	1.24	0.91	82
Str. batatae	2.46	0.92	1.19	1.05	80
Var. ellipsoideus, strain Delft II	1.70	0.87	0.87	0.67	80
Guilliermond	2.69	0.58	1.82	1.63	95
Var. ellipsoideus, var. Major Castelli	3.43	0.66	2.16	1.99	90

[a] Yeast solids basis.

Table IV shows results obtained for a variety of yeast cultures. Closest agreement between ergosterol determined by analysis and purified ergosterol isolated occurs when the 230 mμ–281.5 mμ ratio is low. A low ratio indicates that the relative proportion of 24(28)-dehydroergosterol is low and fewer recrystallizations are required to give purified ergosterol.

Discussion

The method described is applicable to samples containing 5 to 100 mg. of yeast solids, and 0.05 to 2.0 mg. of ergosterols. Because of its simplicity, it is useful in obtaining reliable results on large numbers of yeast samples. By making use of the correction for 24(28) - dehydroergosterol, values for ergosterol content are indicative of the amount of ergosterol that can be isolated.

In developing the method, the use of strong alkali in digestion of the yeast was found desirable, as at alkali concentrations of 10% or less low values were obtained. Optimal range of alcohol concentration during the yeast digestion was from 20 to 80%. In partitioning the alcoholic alkali digestion mixture with *n*-heptane, the alcohol concentration was found to be more critical, and it was necessary to adhere closely to the relative amounts of alcohol and water prescribed.

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RUMEN MICROBIOLOGY

Characteristics of Free Rumen Cellulases

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By using the viscometric assay for cellulase with a carboxymethylcellulose substrate, the presence of soluble cellulases in rumen ingesta has been demonstrated. At least three enzymes appear to be involved. These enzymes are relatively thermostable and insensitive to oxygen, and have a pH optimum of about 6.5. Some samples of rumen ingesta contain a cellulase inhibitor. In other samples, no evidence of inhibitor is seen, but an activator is present.

THE ENZYMATIC HYDROLYSIS OF CELLULOSE by enzymes from aerobic fungi has been investigated actively in recent years, largely as a result of economic pressures. In the paper, wood, and textile industries, methods of preventing or controlling the process have been the ultimate goal. In the microbiological disposal of cellulosic wastes, and in ruminant nutrition, enhanced degradation of cellulose has been the long-range objective. In the balance of organic matter in soil, cellulose hydrolysis is an integral part of the humification process. A considerable volume of literature has accumulated from study of the cellulases of aerobic fungi, but aside from the recent reports of Kitts and Underkofler (*6*) and Cason and Thomas (*2*) on rumen "cellulase" and of Conchie (*3*) on rumen β-glucosidases, detailed reports on rumen enzymes are not available.

Current understanding of the cellulose-decomposing enzymes of aerobic microorganisms has been critically reviewed by Reese (*11*). From the viewpoint of comparative biochemistry, the process of cellulose hydrolysis in the rumen would be expected to resemble the process in aerobic cultures in principle, but with differences in detail. On the basis of this assumption, the cellulase activity of the rumen would probably result from a mixture of several predominantly extracellular enzymes (*4, 8*) which are relatively thermostable (*7*) and capable of attacking carboxymethylcellulose derivatives of degrees of substitution less than 1.0 (*12*).

Whether the sites of attack on the cellulose chain are random or not appears to depend on the selection of organisms. The major cellulase of *Myrothecium verrucaria* appears to cleave cellulose at random glycosidic linkages (*11*). In contrast, Nisizawa and Kobayashi (*10*) have described the cellulase of *Irpex lacteus* as yielding only cellobiose as a hydrolytic product. Such data suggest that the cleavage here is reminiscent of that seen with β-amylases, which degrade amylose from the nonreducing end yielding only maltose. Whether both