

Determination of Ergosterol Using Thin-Layer Chromatography and Ultraviolet Spectroscopy¹

M. NAEWBANIJ,² P. A. SEIB,² R. BURROUGHS,² L. M. SEITZ,³ and D. S. CHUNG⁴

ABSTRACT

Cereal Chem. 61(5): 385-388

Ergosterol in grains was determined through the use of thin-layer chromatography to purify the sterol fraction of the nonsaponifiable lipids and ultraviolet spectroscopy to quantitate at 282 nm in corn and rough rice or at 293 nm in wheat. Recovery of ergosterol from silica gel was 98% when eluted in oxygen-free ethanol within 2 hr of plate development, and recovery from spiked grain samples was 84-98% for rough rice and

91-102% for corn. Repeatability of the thin-layer chromatography-ultraviolet method was good, and ergosterol values were in close agreement with quantitation by high-performance liquid chromatography (HPLC). Sensitivity was 1 $\mu\text{g/g}$. Although not as sensitive as HPLC, the technique should be useful in grain deterioration studies when HPLC is not available.

In 1977, Seitz et al indicated that ergosterol is a sensitive measure of fungal invasion in grain. Ergosterol is the principal sterol in most fungi, but it does not occur in significant amounts in higher plants. Furthermore, ergosterol is easily quantitated because of its ultraviolet (UV) absorption peaks at 282 and 293 nm and its UV spectrum, which differs from those of other plant sterols. The conjugated double bonds in ergosterol at carbons 5-6 and 7-8 cause ergosterol to absorb UV light strongly between 240 and 300 nm, whereas other plant sterols absorb weakly at wavelengths greater than 240 nm. The assay developed by Seitz and co-workers quantified ergosterol in the unsaponified lipid fraction of grains by high-performance liquid chromatography (HPLC). At the same time, they suggested that preparative thin-layer chromatography (TLC) and spectrophotometry could be used to estimate ergosterol if HPLC equipment was not available. The objective of this study was to develop a TLC method to measure the amount of ergosterol in grain, which would be useful in monitoring fungal invasion in storage experiments.

MATERIALS AND METHODS

Standard Curve

Ergosterol from Sigma Chemical Co., St. Louis, MO, was purified by crystallizing twice from absolute ethanol. The ethanol used in the crystallization was purged 1 hr with nitrogen (prepurified grade, Matheson Gas Products, Joliet, IL). The platelike crystals were vacuum-dried and had a melting point of 164°C. Lamb et al (1946) reported that the melting point was 162-167°C.

A stock solution of ergosterol (1.5 mg/ml) was diluted with ethanol to obtain concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml. Absorbances were read at 282 and 293 nm, using a Beckman DB-G spectrophotometer (Beckman Instruments, Fullerton, CA).

Recovery of Ergosterol After TLC

Ergosterol (40-200 μg) was applied in a band to precoated TLC plates (20 \times 20 cm, coating 250 μm thick, Brinkmann G-25 HR, Brinkmann Instruments, Waterburg, NY). A second plate (2.5 \times 20 cm) was spotted to determine the migrating distance of ergosterol. The two plates were developed together in benzene-acetonitrile (9:1, v/v) until the solvent front reached the top of the plate. The smaller plate was sprayed with 50% H_2SO_4 and heated on a hot plate; ergosterol gave a gray-brown color with an R_f value of approximately 0.3. Immediately after development, the band of silica gel (approximately 2.5 cm wide) containing ergosterol on the large plate was scraped off, and the silica gel was extracted with 4.0 ml of oxygen-free absolute ethanol. The mixture was filtered by gentle suction through a coarse sintered-glass funnel, and the absorbance of the filtrate was read at 282 and 293 nm. A blank solution was prepared by extracting silica gel scraped from a plate that had not been streaked with ergosterol but that had been developed in benzene-acetonitrile. The absorbance of the blank solution was 0.015-0.046 at 282-310 nm when read against absolute ethanol.

In a separate recovery experiment, the TLC plates were treated as described previously, but the developed plates were allowed to stand at room conditions for up to 14 hr before scraping and extraction.

Ultraviolet Spectra of Nonsaponifiable Lipid Fraction Extracted from Rough Rice With and Without TLC

Medium-grain rough rice was harvested at 30% moisture at Beaumont, TX. The rice was sun-dried to 18% moisture and cleaned in a Carter dockage tester (Seedburo Equipment Co., Chicago, IL). Three sublots were treated as follows: Sample A was further sun-dried to 13% moisture, and the dull and damaged kernels were removed by hand; sample B was also sun-dried to 13% moisture and had no further treatment; sample C was rewetted to 20% moisture, stored at 35°C for 16 days, and then air-dried to approximately 14% moisture. Duplicate subsamples of A, B, and C, containing 50 g of dry solid, were ground for 1 min in a high-speed grinder (Mitey-Mill, model MX 228, Stur Dee Health Products, Island Park, NY). The nonsaponifiable lipids were isolated as described by Seitz et al (1977).

Preparative TLC was used to purify the dry nonsaponifiable lipid fractions from one sample set (A, B, C). Each residue (approximately 25 mg) was dissolved in 1.0 ml of benzene-acetonitrile (98:2, v/v), and an aliquot (250 μl) was streaked 50 μl at a time in a band 8 cm long on the TLC plate. The plate was developed with benzene-acetonitrile (9:1, v/v), dried 2-3 min at room temperature, and the sterol band located using a light box. The sterol band was visible as a well-resolved opaque streak at an R_f of approximately 0.3. Resolution of the sterol band was lost with sample sizes greater than 75 g of rough rice. The silica gel containing the sterol band was scraped from the plate and extracted with ethanol (4.0 ml). The UV spectrum of the solution was scanned

¹Contribution No. 83-137-J, Departments of Grain Science and Industry and Agricultural Engineering, Kansas Agricultural Experiment Station, Manhattan 66506. Cooperative investigation between ARS, USDA, and the Kansas Agricultural Experiment Station.

Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

²Respectively, graduate research assistant, professor, and research assistant, Department of Grain Science and Industry, Kansas State University, Manhattan 66506.

³Research chemist, Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, KS 66506.

⁴Professor, Department of Agricultural Engineering, Kansas State University, Manhattan 66506.

between 276 and 310 nm. The fractions in the second set (A', B', C') were dissolved in ethanol, and the UV spectra recorded.

Recovery of Ergosterol from Rice, Wheat, and Corn Spiked with Ergosterol

Rough rice (13% moisture) was harvested at 30% moisture in August, 1981, in Beaumont, TX, sun-dried, and stored for 12 months at 5°C. Wheat (11.5% moisture) was a U.S. No. 1 hard red winter variety harvested near Garden City, KS, in June of 1982, and stored for three months at 5°C. The corn (14.6% moisture) was grown in eastern Kansas in 1980. Following harvest, corn was dried with ambient air, then held at 5°C for 23 months. Each grain was ground to pass through a U.S. No. 40 screen before use.

In the recovery experiment, known quantities of ergosterol were added to duplicate samples (50 g of dry solids), using aliquots (100–400 μ l) of a concentrated solution in ethanol (800 μ g/ml). Ergosterol was added to the mixtures of ground grain and methanol in the initial step of the assay procedure of Seitz et al (1977). The nonsaponifiable residues were dissolved and chromatographed on thin-layer plates. Then the ergosterol band was extracted with ethanol (4.0 ml) as previously described. Quantitation of ergosterol in the ethanol solution was determined from absorbance at 282 nm, corrected for the absorbance of interfering substances by subtracting the absorbance at 310 nm from that at 282 nm.

Ergosterol Assays by TLC-UV and HPLC

To compare ergosterol values obtained by the TLC-UV technique with measurement by HPLC, samples of rough rice, yellow dent corn, and hard red winter wheat with two levels of fungal invasion were assayed. Long-grain rough rice was obtained from one lot dried to 13% moisture content immediately after harvest and from another that had been stored under controlled conditions (20% moisture, 35°C) for 15 days and then dried to 13% moisture. Corn samples were from a lot of U.S. No. 2 (14.5% moisture content) before and after storage at 25°C for 16 weeks with moisture adjusted to 16%. Before assay, corn was dried to 14% moisture content. Hard red winter wheat samples were obtained from U.S. No. 1 wheats (13% moisture content). In one lot, 2% of the kernels were invaded by *Fusarium roseum*, and in the other lot,

10% were invaded (determined by incubating surface-disinfected kernels on malt-salt (4% NaCl) agar).

Samples for ergosterol assay (300 g) were ground to about 60 mesh in a Udy cyclone sample mill (U-D Corporation, Boulder, CO) and were subdivided into four 50-g replicates. Two replicates of each sample were assayed, using the TLC-UV technique, and the other two were quantified by HPLC.

In the HPLC method, the dry, nonsaponifiable lipid fractions were dissolved in 2 ml of methylene chloride-isopropanol (99:1, v/v), and 10- μ l aliquots were injected. A 5- μ m C₁₈ "resolve" column (Waters Associates, Milford, MA) maintained at 50°C with 95% methanol flowing at 1 ml/min was used to separate the ergosterol fraction (Seitz et al 1979). Ergosterol was detected at 282 nm.

RESULTS AND DISCUSSION

Standard Curve and Recovery after TLC

Because ergosterol is susceptible to autoxidation and produces a cyclic hydroperoxide with low UV extinction at 282–293 nm (White et al 1973), we first examined the recovery of ergosterol subjected to TLC on silica gel. The curves in Fig. 1 show that the recovery was quantitative (98%) when the TLC-UV procedure was completed within 2 hr. However, if the developed plates were allowed to stand exposed to air, recovery decreased. In one instance, a developed plate stood 14 hr at approximately 25°C; the recovery of ergosterol was 29%.

UV Spectra of the Nonsaponifiable Lipid Fraction Extracted from Rough Rice

The three samples analyzed were expected to contain amounts of ergosterol related to the degree of fungal invasion and growth (Seitz et al 1979). Rough rice with kernels darkened by growth of field fungi contained more ergosterol than did a sample with damaged and dull kernels removed (Fig. 2). Storage at 20% moisture and 35°C for 16 days resulted in extensive molding, reflected in the high ergosterol content of sample C, which produced intense absorbance at 282 and 293 nm.

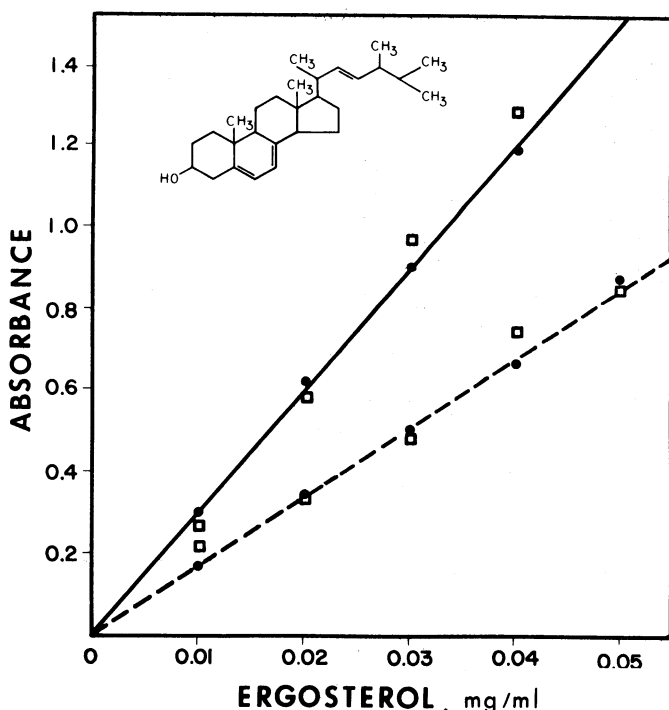


Fig. 1. Standard curves for ergosterol dissolved in oxygen-free ethanol (●) and recovered after thin-layer chromatography on silica-gel plates (□) recorded at 282 nm (—) and at 293 nm (---).

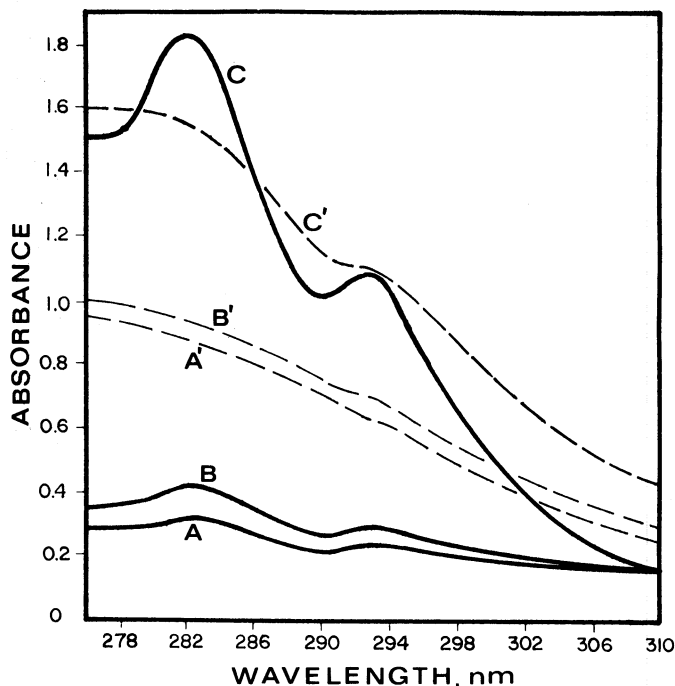


Fig. 2. Ultraviolet spectra of nonsaponifiable lipids of rough rice before (----) and after (—) purification by thin-layer chromatography. A and A' = rough rice harvested at 30% moisture and sun-dried to 13% moisture; dull kernels removed by hand. B and B' = rough rice harvested at 30% moisture and sun-dried to 13% moisture; dull kernels not removed. C and C' = rough rice adjusted to 20% moisture and stored 16 days at 35°C.

The three UV spectra provided the basis for the development of a TLC-UV method of assay for ergosterol. Ergosterol does not absorb at 310 nm (Seitz et al 1977), but the sterol fraction from rice following TLC purification absorbed somewhat at 310 nm because of interfering substances (Fig. 2). However, absorbance of the interfering substances in the sterol fraction was assumed constant between 278 and 310 nm (Fig. 2, curves A, B, and C); so subtracting the absorbance at 310 nm from absorbance of the sterol fraction at 293 or 282 nm gave a measurement of ergosterol in the samples. Curves A', B', and C' in Fig. 2 show the need for TLC to purify the steroid fraction in the nonsaponifiable lipids from rice. Without purification by TLC, the absorbance of interfering substances (curves A' and B') increased dramatically at 293 and 282 nm.

Recovery of Ergosterol after Spiking

A spiking experiment determined that ergosterol could be recovered quantitatively after a combination of the following steps: extraction of the lipids from the grain; saponification of the lipids; isolation of the nonsaponifiable lipids; and TLC purification. Recovery of ergosterol from rough rice with an initial ergosterol content of $1.3 \pm 0.1 \mu\text{g/g}$ of dry solids ranged from 84 to 98% for the four levels of added ergosterol. Figure 3 shows a linear response of recovered versus added ergosterol. When ergosterol was added to corn and wheat, recovery ranged from 91 to 102% and from 101 to 127%, respectively, and again levels in the grain samples increased linearly with added amounts (Fig. 3). The same correction for interfering substances was applied to absorbance readings for rice, corn, and wheat.

Comparison of Ergosterol Assayed by TLC-UV and HPLC

Ergosterol values obtained by TLC-UV and HPLC (Table I) show that the TLC-UV technique has good repeatability and gives values in close agreement with HPLC determinations.

In the assay of hard red winter wheat by the TLC-UV method, ergosterol content was calculated from the difference in absorbance at 293 and 310 nm. Ergosterol levels as much as $2 \mu\text{g/g}$ greater than actual values (determined by HPLC) were found in some samples when the ergosterol was calculated from 282-nm absorbance

readings. To test whether the absorbance reading obtained from the ethanol-extracted ergosterol band was attributed solely to ergosterol, the band scraped from the TLC plate was dissolved in 2 ml of methanol, and a $30\text{-}\mu\text{l}$ aliquot was injected into the HPLC. The elution profile of this extract indicated unidentified interfering substances that were eluted at 6.4, 8.0, and 10.2 min; ergosterol was eluted at 10.9 min. Spectrophotometric scans (Fig. 4) of the three interfering substances, using a photodiode ray detector (Hewlett Packard, model 1040A, Palo Alto, CA), indicated that they absorb light between 250 and 290 nm. Similar interfering substances were present in negligible amounts in rice and corn, regardless of the ergosterol level of the samples. The 101–127% recovery of ergosterol in spiked wheat samples, reported in the preceding section, resulted from absorption by the interfering compounds at 282 nm.

The TLC-UV method of determining ergosterol is not as sensitive as the HPLC method. Seitz et al (1977) reported that the HPLC method could detect as little as $0.05 \mu\text{g}$ of ergosterol per gram of grain. The method described here can detect as little as $1 \mu\text{g/g}$, which appears adequate to monitor fungal invasion in most grains during postharvest holding, drying, or storing. We found that rough rice, dried immediately after harvest to 13% moisture, contained $1.2\text{--}4.9 \mu\text{g/g}$ of ergosterol and that samples of hard

TABLE I
Ergosterol Content in Rice, Corn, and Wheat
Obtained by TLC-UV and HPLC

Sample	Ergosterol ($\mu\text{g/g}$) ^a	
	TLC-UV ^b	HPLC
Rough rice		
Newly harvested	4.59	4.90
Stored at 35°C, 20% moisture content for 15 days	10.29	10.34
for 11 days	11.91	13.71
Yellow dent corn (U.S. No. 2)		
As received	3.01	3.66
Stored at 25°C, 16% moisture content for 16 weeks	3.33	3.68
for 13 weeks	13.64	12.52
for 16 weeks	13.65	13.98
Hard red winter wheat (U.S. No. 1)		
With 2% <i>Fusarium roseum</i>	2.84 ^c	2.70
With 5% <i>F. roseum</i>	2.89 ^c	2.78
With 10% <i>F. roseum</i>	3.22 ^c	3.04
With 15% <i>F. roseum</i>	3.35 ^c	3.15

^a Two replicate subsamples analyzed by each method.

^b Values calculated from difference in absorbance at 282 and 310 nm.

^c Values calculated from difference in absorbance at 293 and 310 nm.

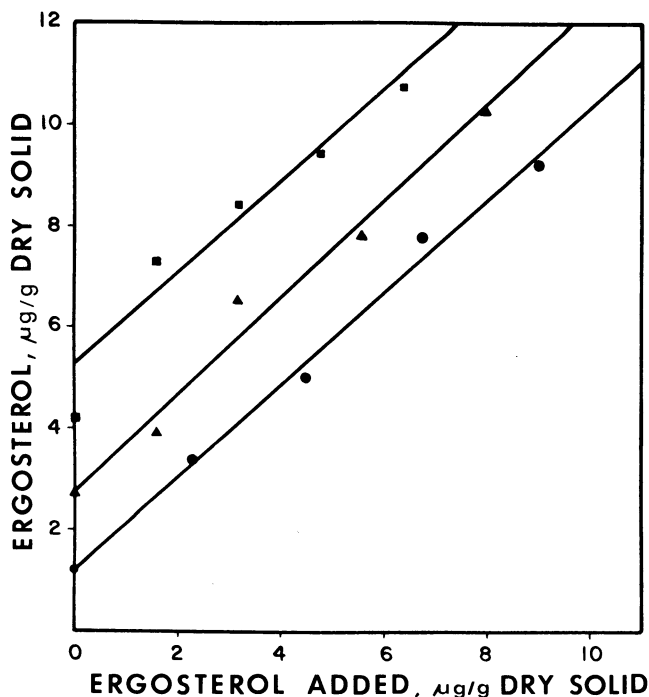


Fig. 3. Recovery of ergosterol added to rice (●), corn (▲), and wheat (■). The least-square regression lines for rice, corn, and wheat were, respectively: $\hat{Y} = 0.91X + 1.2$, $\hat{Y} = 0.99X + 2.67$, and $\hat{Y} = 0.94X + 4.95$, where \hat{Y} is the ergosterol concentration in grain ($\mu\text{g/g}$), and X is the concentration of ergosterol added ($\mu\text{g/g}$).

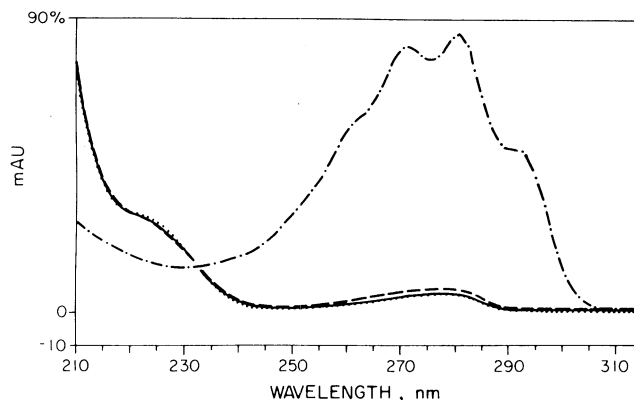


Fig. 4. Spectrophotometric scans of interfering substances (—, ·····, - - - -) and ergosterol (— · —) as eluted from the HPLC column during analysis of extracted ergosterol band from thin-layer chromatography purification of wheat containing $3.2 \mu\text{g/g}$ ergosterol. Milliabsorbance units (mAU) full-scale for the three interfering substances and ergosterol, in the order shown above, were 201, 438, 181, and 78.3.

winter wheat (U.S. No. 1) and of yellow dent corn (U.S. No. 2) contained 3.01–4.95 and 2.67–3.35 $\mu\text{g/g}$ of ergosterol, respectively. Seitz et al (1977) found 0.67–3.92 $\mu\text{g/g}$ of ergosterol in samples of freshly harvested Eagle wheat from three locations in Kansas over a three-year period.

LITERATURE CITED

LAMB, F. W., MUELLER, A., and BEACH, G. W. 1946. Quantitative determination of ergosterol, cholesterol, and 7-dehydrocholesterol.

Antimony trichloride method. *Ind. Eng. Chem.* 18:187.
SEITZ, L. M., MOHR, H. E., BURROUGHS, R., and SAUER, D. B. 1977. Ergosterol as indicator of fungal invasion in grains. *Cereal Chem.* 54:1207.
SEITZ, L. M., SAUER, D. B., BURROUGHS, R., MOHR, H. E., and HUBBARD, J. D. 1979. Ergosterol as a measure of fungal growth. *Phytopathology* 69:1202.
WHITE, J. D., PERKINS, D. W., and TAYLOR, S. I. 1973. Biosynthesis of ergosta-4,6,8,(14),22-tetraen-3-one. A novel "oxygenative pathway." *Bioorg. Chem.* 2:163.100

[Received July 18, 1983. Revision received March 12, 1984. Accepted March 13, 1984]