# ERGOSTEROL AS AN INDICATOR OF FUNGAL INVASION IN GRAINS[1,2]

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**ABSTRACT**

The extent of preharvest fungal invasion in grain sorghum, wheat, and corn samples measured by an ergosterol assay was compared to results from plating surface-disinfected seeds. Ergosterol content and percentage of kernels with fungi increased in sorghum which was harvested at successively later dates. Assays of wheat samples showed a close relation between ergosterol level and invasion by field fungi and/or with weather conditions known to favor fungal invasion. The ergosterol assay gave a quantitative estimate of previous invasion by fungi even though the fungi were no longer viable. Corn kernels with obvious fungal damage had as much as 200 μg/g ergosterol, while freshly harvested sound kernels contained as little as 0.2 μg/g. Ergosterol was quantitated by high-pressure liquid chromatography, and also could be estimated from ultraviolet spectroscopy of sterols obtained by thin-layer chromatography.

Determining the extent fungi have invaded cereal grains and seeds has been the subject of considerable investigation and discussion (1–3). Microbiological methods can be used to determine the species of fungi and their relative abundance in a sample, but such methods only measure live fungi and require several days of incubation time. Furthermore, fungi that grow in and damage grain, then die, cannot be detected by microbiological methods. Fungal mycelium can sometimes be observed by direct microscopic examination of removed, stained pericarp (4,5), but quantitation is difficult and the procedure is too tedious for routine use.

Seed germination, visible discoloration, and various chemical indicators such as fat acidity are sometimes used as indirect measures of fungal activity. However, such measures may reflect damage from other causes, or may change only after extensive fungal growth.

While any of the above tests can be useful for certain purposes, none directly measures the amount of fungal activity that has occurred, or the amount of fungal biomass present. Chitin content has been proposed as a measure of fungal growth in grains (6–8). Chitin assay involves acid hydrolysis of chitin to glucosamine, which can be determined colorimetrically, gas chromatographically, or by use of an amino acid analyzer. However, the usefulness of a chitin assay is limited by long assay time (4 to 6 hr/sample), and by misleading results from samples containing insect parts.

Use of an ergosterol determination is herein described as a sensitive indicator of fungal invasion in grain. Ergosterol production by *Alternaria alternata,* which

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commonly invades small grains in the field (9), has recently been confirmed. Ergosterol has been considered the predominant sterol component of nearly all fungi (10). It has been proposed that the primary role of sterols in nature is as architectural components of membranes (11). Ultraviolet (uv) spectroscopic and high-pressure liquid chromatographic methods are discussed as ways to measure ergosterol. Ergosterol contents of sorghum, wheat, and corn samples are compared with results from mycological measures of fungal invasion and are related to weather conditions during grain maturation.

## MATERIALS AND METHODS

### Grains

The sorghum grains from Riley County, KS, were commercial and "regional selection" hybrids grown in plots managed by Kansas State University. Wheats were from performance test plots of the Kansas Agricultural Experiment Station. Yellow corn was grown in Riley County, KS.

### Mycological Method—Whole-Seed Plating

Grains were surface-disinfected with 2% hypochlorite solution. Fifty or 100 kernels were placed on malt agar containing 4% NaCl and 200 ppm Tergitol NPX (Union Carbide Co., New York, NY). Petri plates were incubated 5 days at 25° C; then fungi growing from kernels were identified.

### Extraction and Cleanup

Fifty grams of grain was blended with 100 ml of methanol for 2 min. The blended mixture, plus an additional 50-ml portion of methanol used to rinse the blender jar, was poured into a 200-ml glass centrifuge bottle and centrifuged 5–7 min at 1000–1200 rpm. The supernatant was poured off. The residue was resuspended in 50 ml of methanol, shaken for about 30 sec, and centrifuged. The two supernatants were combined, mixed with 20 g KOH and 50 ml of ethanol, and refluxed for 30 min. The cooled saponified mixture was diluted with 50 ml water and extracted with 100- then 50-ml portions of petroleum ether, bp 60°–70° C. Petroleum ether extracts were combined and evaporated to about 10 ml over a steam bath with aid of a gentle flow of nitrogen.

### uv Spectroscopy

Early in this study, uv spectroscopy was used to verify the presence of ergosterol and to compare concentrations of ergosterol in sterol fractions from sorghum grains. The petroleum ether extract was evaporated to complete dryness and the residue dissolved in 0.5 ml benzene-acetonitrile (98:2, v/v). A precoated, 20 × 20-cm thin-layer chromatography plate (Brinkman MN Silica Gel G-HR, 0.25 mm soft layer, activated 1 hr at 110° C) was streaked with 350 $\mu$l of the sample. The plate was developed in benzene-acetone (90:10, v/v) to about 10 cm and dried at room temperature. The sterol band between $R_f$ 0.37 and 0.50 was scaped into a small fritted-glass filter funnel. Several 0.5-ml portions of ethanol were used to extract the silica. Final volume was adjusted to 3.0 ml. A Perkin-Elmer Model 350 spectrophotometer was used to record uv spectra.

**High-Pressure Liquid Chromatograph (HPLC)**

For further extract cleanup prior to HPLC a small glass column (300 × 10 mm o.d.) was used, packed successively with a small wad of glass wool, 10 mm anhydrous sodium sulfate, 40 mm silica gel (0.063–0.200 mm, EM Reagents), and 15 mm anhydrous sodium sulfate. The silica was adjusted to 1% water content.

The evaporated petroleum ether extract was added to the column and eluent collected. The column was washed with two 10-ml portions of methylene chloride-isopropanol (99:1, v/v) or with three or four 10-ml portions of 100% methylene chloride. The washes were combined with the sample eluent, evaporated (over a steam bath with a gentle flow of nitrogen) to near dryness, transferred to a 2-dram vial, and then evaporated to complete dryness. The residue was dissolved in 0.5 ml of methylene chloride-isopropanol (99:1, v/v).

Usually, 10 or 25 μl of the final extract was injected into the HPLC. When the ergosterol level of the sample was high, the final extract was diluted so the amount of ergosterol injected would be within the calibrated range (0.10 to 22 μg) of the detector.

The HPLC equipment consisted of a Varian Associates 4200 solvent delivery system, a μ-Porasil (300 × 4.0 mm i.d., 6.35 mm o.d.) column from Waters Associates, and a Schoeffel Model SF770 variable wavelength detector set at 282

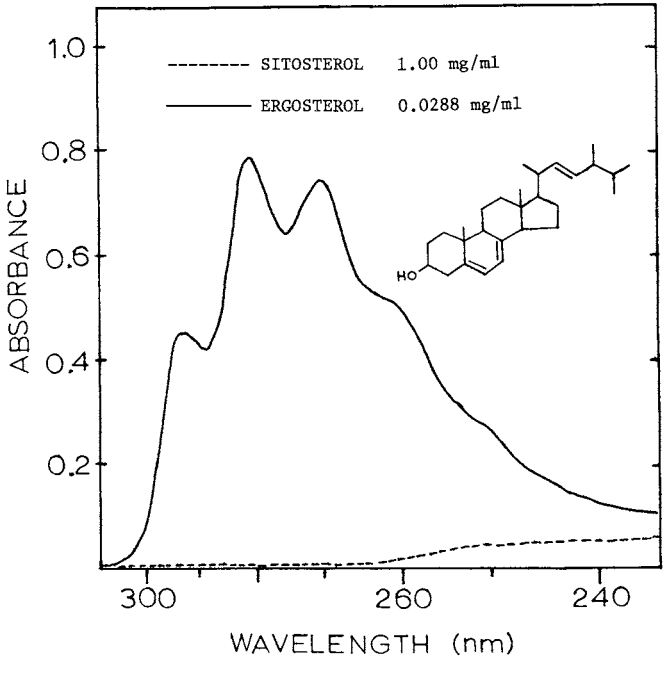


Fig. 1. Ultraviolet (uv) spectra of ergosterol and sitosterol in absolute ethanol, and structure of ergosterol.

nm and 0.1 absorption units full scale. The mobile phase was methylene chloride-isopropanol (99:1, v/v) at 1.67 ml/min (550 psi). A guard column (50 × 2.0 mm i.d., 3.2 mm o.d. packed with Corasil II) was placed between the injector (Valco rotary valve) and the μ-Porasil column. Peak areas were measured by a computing integrator (minigrator) from Spectra Physics Inc. Reagent-grade methylene chloride was washed twice with distilled water, dried over anhydrous calcium chloride, and then distilled.

**Ergosterol Standard**

Ergosterol from Eastman Kodak Company was recrystallized twice from absolute ethanol and dried under vacuum at room temperature for at least 3 hr.

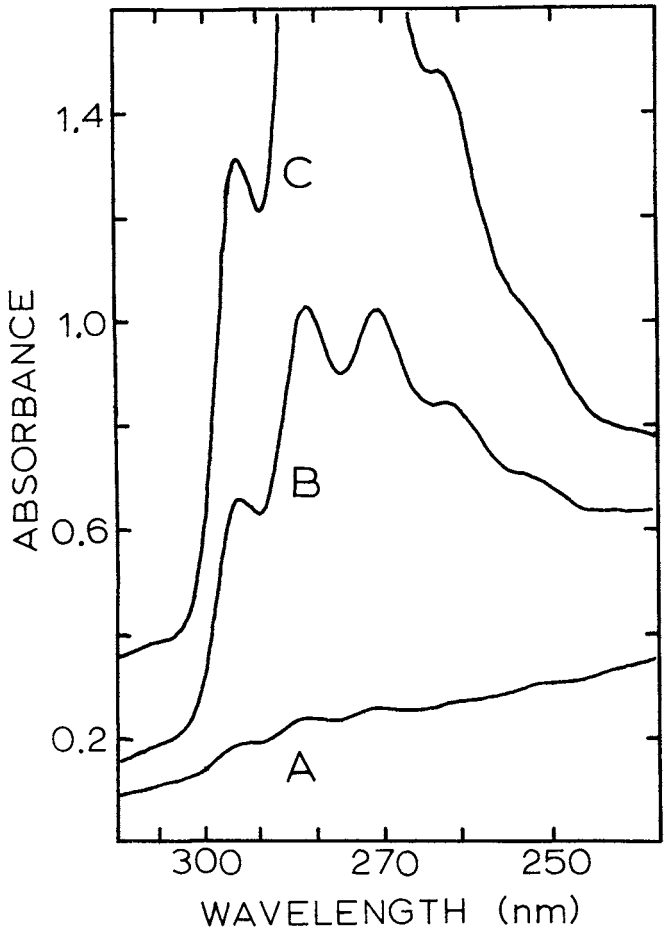


Fig. 2. Ultraviolet (uv) spectra of sterol fractions, in absolute ethanol, of hand-picked samples from a single lot of sorghum grain: A) bright kernels, B) grain as harvested, and C) discolored kernels.

## RESULTS AND DISCUSSION

### Methodology

Ergosterol's characteristic uv absorption, which differs significantly from those of the higher plant sterols, *i.e.*, sitosterol, campesterol, stigmasterol, and smaller amounts of brassicasterol and cholesterol, was used for detection of ergosterol in grains. Because ergosterol has a conjugated pair of double bonds at carbons 5–6 and 7–8 (Fig. 1), it absorbs uv light strongly between 300 and 240 nm with a characteristic pattern. The plant sterols, of which sitosterol is used as an example in Fig. 1, lack the 7–8 unsaturation and, therefore, absorb very little uv light at wavelengths greater than 240 nm.

Because ergosterol in grain may be both free and bound, the percentage of ergosterol recovered from the grain is not easily determined. For example, the ergosterol in one sorghum sample determined by our blender-methanol extraction and by a 12-hr Soxhlet extraction of ground grain with petroleum ether gave values of 13.5 and 9.0 μg ergosterol/g grain, respectively. The

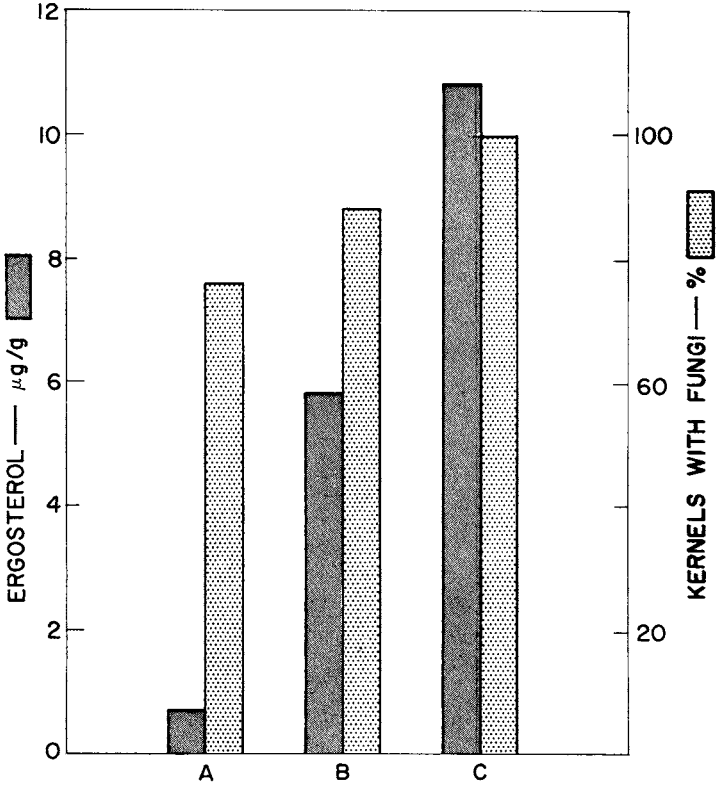


Fig. 3. Ergosterol contents and percentages of kernels with internal fungi in sorghum samples identified in Fig. 2.

difference may be partially accounted for by extraction of more bound ergosterol by methanol than by petroleum ether. Deleting the saponification step lowered detectable ergosterol by about 38%, which suggests that about 62% of the ergosterol is in the "free" form. After studying saponification conditions, it was concluded that 30 min of reflux is adequate and nitrogen atmosphere is needed if the reflux period is 3 hr or more. Our method recovered 93 ± 5% of ergosterol which had been added to fungus-free, milled rice (9).

An early experiment was designed to test the hypothesis that a uv spectrum of isolated sterol fraction would indicate the presence and approximate concentration of ergosterol. A small lot of hand-harvested grain sorghum was separated into three parts: A) bright kernels, B) kernels as harvested, and C)

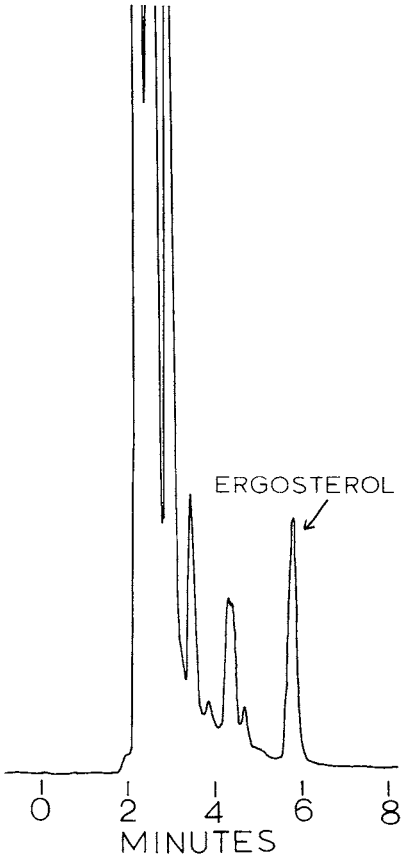


Fig. 4. High-pressure liquid chromatogram (HPLC) of an extract from 1973 crop wheat containing 0.66 μg/g ergosterol. Volume injected was 10 μl. Detailed chromatographic conditions are described in **Materials and Methods**; the detector was set at 282 nm and 0.1 absorption units, full scale.

discolored kernels. uv Spectra (Fig. 2) of sterol fractions from tlc showed the presence of ergosterol (compare with Fig. 1), and an increase in concentration as discoloration increased. Ergosterol contents, estimated from the absorbance difference between 310 and 293 nm, corresponded with whole-seed plating data on extent of preharvest fungal invasion (Fig. 3). Although 75% of the bright kernels had viable fungi, the extent of invasion in each kernel apparently was slight, as reflected by the low ergosterol level.

In subsequent analyses HPLC was a sensitive, convenient, reliable method for determining ergosterol in grains. Saponified extracts from sorghum, wheat, and corn gave similar chromatograms. A typical chromatogram of an extract from wheat is shown in Fig. 4. Other sterols were eluted along with ergosterol at 5.8 min; however, only ergosterol contributed significantly to absorption of 282 nm light (refer to Fig. 1). Sensitivity is more than adequate since levels as low as about 0.05 $\mu$g/g were detected and no interferences were encountered. Detector output was calibrated from about 0.1 to 22 $\mu$g ergosterol, although it appeared that linearity extended up to at least 55 $\mu$g. Not shown in Fig. 4 are two uncharacterized peaks at 12.7 and 14.6 min which are minor with sorghum and wheat but fairly large with corn. The sorghum, wheat, and corn samples next discussed were analyzed by the HPLC method.

**Sorghum**

From a plot in Riley County, KS, sorghum hybrids RS 671, RS 702, RS 610, TAM 680, and Acco 1019R were hand-harvested September 14 and 24 and October 4, 1976. Each sample was plated and analyzed for ergosterol. The data, summarized in Fig. 5, show that ergosterol levels correlated with degree of fungal

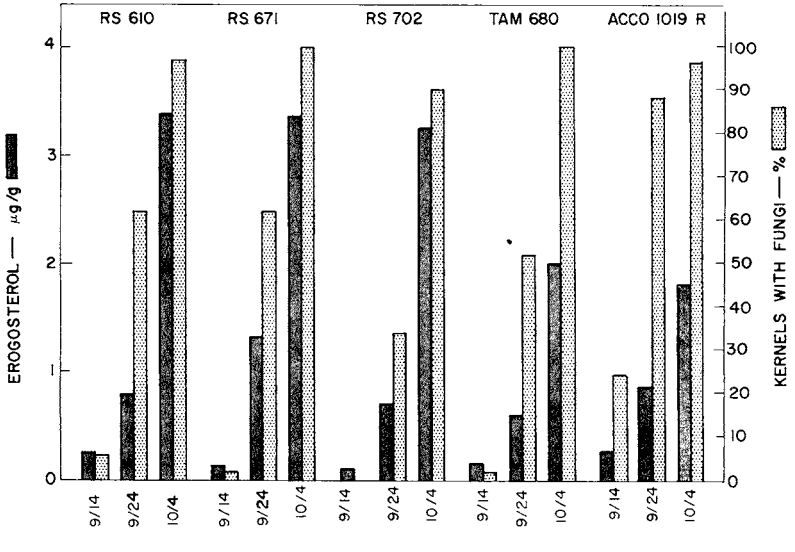


Fig. 5. Ergosterol content and fungal invasion percentages of five sorghum hybrids, each harvested on three dates in 1976: September 14, September 24, and October 4.

invasion as measured by whole-seed plating. Ergosterol levels were lowest in September 14 samples, in which a very low percentage of kernels was invaded, and highest in October 4 samples, which had *Alternaria* in all, or nearly all, of the kernels. Of samples harvested October 4, lower ergosterol levels in TAM 680 and Acco 1019R suggested that the average amount of fungal growth per kernel in those two samples was less than in samples of RS 671, RS 610, and RS 702. About two-thirds of the samples were analyzed for ergosterol two to four times; coefficients of variations ranged from 5 to 20% and averaged about 10%.

The data in Fig. 5 indicate that fungal invasion increased in each hybrid from the first to the third harvest. A similar trend was apparent in whole-seed plating data collected in 1969–1971 grain from 15 sorghum hybrids harvested at intervals beginning when seeds were formed until normal harvest time (Sauer and Burroughs, unpublished). From 1000-kernel weight data and appearance of the 1976 grain, we believe the September 14 harvest was shortly after physiological maturity. The grain was quite light colored September 14 and only slightly darker October 4. In 1969–1971, 1975, and 1976, *Alternaria* was the predominant fungus, with a much smaller percentage of kernels invaded by *Fusarium* and *Cladosporium*. Other fungi sometimes present included *Nigrospora, Epicoccum, Curvularia, Phoma,* and *Helminthosporium*.

Although the 1975 and 1976 sorghum samples showed little evidence of extensive fungal growth and discoloration, they did show differences in quantities of ergosterol. Secondary metabolites, such as alternariols appear to be less sensitive indicators of fungal growth. For example, 1975 sorghum with ergosterol at 5 to 8 μg/g had barely detectable (about 0.1 μg/g) alternariols. A

**TABLE I**
**Ergosterol Contents of Eagle Wheat Representing Three Crop Years from Three Kansas Locations**

| Location | Year | Ergosterol[a] | Rainfall[b] | |
|---|---|---|---|---|
| | | μg/g | No. days[c] | Total in. |
| Hutchinson | | | | |
| South Central | 1973 | 0.67 | 0 | 0 |
| | 1974 | 1.68 | 7 | 1.92 |
| | 1975 | 3.92 | 10 | 8.67 |
| Minneola | | | | |
| Southwest | 1973 | 0.73 | 3 | 0.38 |
| | 1974 | 1.78 | 6 | 2.53 |
| | 1975 | 3.54 | 8 | 2.50 |
| Colby | | | | |
| Northwest | 1973 | 0.78 | 4 | 0.75 |
| | 1974 | 1.20 | 5 | 4.78 |
| | 1975 | 1.49 | 12 | 8.83 |

[a]Average of 2 to 4 analyses per sample; average coefficient of variations was 17%.
[b]From 5 days before estimated date of physiological maturity to harvest, approximately 21, 23, and 28 days in 1973, 1974, and 1975, respectively.
[c]Receiving at least 0.01 in.

group of 1973 sorghum samples with low levels of invasion had nondetectable levels of alternariols (12).

Information regarding the distribution of ergosterol in the sorghum kernel is not available. The ergosterol content of sorghum containing 13.5 μg/g ergosterol was not lowered when the grain was washed with hot benzene to remove the outer wax layer (13). Furthermore, uv spectra of the wax dissolved in warm ethanol did not indicate ergosterol.

**Wheat**

Eagle, Trison, and Centurk wheats were analyzed for 3 crop years from Hutchinson, Minneola, and Colby Experiment Stations in Kansas. Ergosterol content of Eagle wheat was lowest in 1973 and highest in 1975 at each location (Table I). The same ergosterol-year relation also was observed with Trison and Centurk varieties from Hutchinson and Minneola, respectively. The greater degree of fungal invasion in 1975 than in 1973 and 1974, as indicated by ergosterol contents, may be explained by weather conditions from a few days before physiological maturity to harvest. Number of rainy days and total rainfall showed that 1973 was the driest and 1975 was the wettest year. Also, relative humidity at each location was generally lowest in 1973 and highest in 1975. Minneola and Hutchinson had similar average daily temperatures, a few degrees warmer than at Colby (14).

Our results agree with Hyde's observation (4) that degree of invasion depends on atmospheric humidity while grain ripens. From worldwide surveys in 1947–49, Hyde found that amount of subepidermal fungi in wheat tended to be high in samples from localities where higher relative humidities prevailed.

In the 1975 Eagle wheat samples (Table I), about 90% of the kernels were invaded with *Alternaria,* 30% with *Fusarium,* and 4% with other fungi; none of the kernels were free of fungi. The 1974 and 1973 samples of Eagle wheats scored, respectively, 16 and 9% kernels invaded with *Alternaria,* 7 and 5% with *Fusarium,* and 80 and 88% with no fungi; these samples had been kept at room temperature for many months, during which time most of the original microflora probably died. The 1975 samples were kept in cold storage, which should have

**TABLE II**
**Ergosterol and Whole-Seed Plating Data from Corn Samples Representing a Composite of Five Freshly Harvested Ears Shelled in Thirds as Tip, Midsection, and Butt Portions**

| Sample | Kernels Invaded by | | | Kernels Not Invaded | Ergosterol |
|---|---|---|---|---|---|
| | Fusarium % | Cephalosporium % | Other fungi % | % | μg/g |
| Tip | 17 | 15 | 6 | 64 | 2.10 |
| Midsection | 17 | 30 | 5 | 51 | 0.28 |
| Butt | 5 | 37 | 4 | 57 | 0.23 |

kept the original microflora alive. Because of their different storage histories, the 1973–1975 wheat samples cannot be used to compare ergosterol levels with fungal invasion percentages.

### Corn

Ergosterol in corn samples ranged from 0.15 to about 200 μg/g. The lower level was observed in freshly harvested, hand-shelled ears which had no visible fungal damage; 88 to 98% of surface-disinfected kernels were free of internal fungi. The upper level was measured in several samples of hand-selected, rotted, discolored kernels, invaded primarily by *Fusarium*.

We plated seeds and determined ergosterol in a set of three samples representing a composite of five freshly picked ears. The ears were hand-shelled in thirds into tip, midsection, and butt portions. Although half the kernels from the midsection and butt portions had internal fungi, the low ergosterol level suggests the extent of invasion was very slight (Table II). The data also indicate that, even though fewer kernels were invaded in the tip portion, invasion in those kernels was more extensive than in kernels from the other two portions.

Ergosterol assays seem to have a greater sampling variability in corn than in wheat or sorghum. Besides having more kernels per gram of sample, the small grain kernels tend to be more uniformly invaded. Corn at harvest may have many fungus-free or lightly invaded kernels, along with a few that are heavily invaded or completely rotted. The heavily damaged kernels produce great sampling variability.

## SUMMARY AND CONCLUSIONS

An ergosterol determination is a useful measure of fungal invasion in grains because ergosterol is 1) a constituent of nearly all fungi, 2) not a native constituent of grains, and 3) reliably and rapidly determined by the HPLC method. For convenience and quantitation, the HPLC method is superior to uv spectroscopy of a sterol fraction from tlc. The latter method was useful to confirm the presence of ergosterol in the grains and could be used to estimate ergosterol levels if HPLC equipment were not available.

Adequate comparisons of ergosterol and chitin as measures of fungal growth are not available, and the tests are being compared in our laboratory. The assay for ergosterol requires about 1 hr and that for chitin 4–6 hr. Whether or not there is fungal growth in grains that goes undetected by ergosterol assay remains to be determined. Our data indicate, however, that growth of common field fungi, *i.e.*, *Alternaria, Fusarium,* etc., are adequately detected by the ergosterol assay.

Viable and nonviable fungal biomass is measured by an ergosterol assay, whereas whole-seed plating detects only viable fungi. Ergosterol levels would be an important aid in evaluating the extent of fungal invasion in grains in which fungi may have died from unfavorable storage conditions. Ergosterol values are expected to correlate best with whole-seed plating when freshly harvested, slightly invaded grain is used for the comparison, such as the 1976 sorghums (Fig. 5). Even here, samples with the same percentage of kernels invaded may have different ergosterol contents, depending on the extent of fungal invasion in each kernel. When 100% of the kernels are invaded, whole-seed plating has essentially reached its limit as a measure of fungal invasion; that is not the case with an

ergosterol assay. The assay, however, does not identify fungal species and so does not distinguish between preharvest invasion and invasion by storage fungi.

The ergosterol assay may be useful in selecting grain sorghum hybrids for resistance to weathering and discoloration caused primarily by *Alternaria alternata*. Data from the 1976 sorghums (Fig. 5) strongly suggest that such a selection process should not rely on a single date of harvest. Each hybrid should be harvested at several intervals to take into account increases in invasion after physiological maturity. Secondary metabolites, such as alternariols, are useful as relative measures of invasion only when the sorghum grain is considerably discolored from extensive invasion by *Alternaria*.

**Literature Cited**

1. CHRISTENSEN, C. M., and KAUFMANN, H. H. Grain storage: the role of fungi in quality loss. University of Minnesota Press, Minneapolis (1969).
2. POMERANZ, Y. Biochemical, functional, and nutritive changes during storage. In: Storage of cereal grains and their products (2nd ed.), ed. by C. M. Christensen, Chap. 2. Amer. Ass. Cereal Chem.: St. Paul, Minn. (1974).
3. CHRISTENSEN, C. M., and KAUFMANN, H. H. Microflora. In: Storage of cereal grains and their products (2nd ed.), ed. by C. M. Christensen, Chap. 4. Amer. Ass. Cereal Chem.: St. Paul, Minn. (1974).
4. HYDE, M. B. The subepidermal fungi of cereal grains. I. A survey of the world distribution of fungal mycelium in wheat. Ann. Appl. Biol. 37: 179 (1950).
5. HYDE, M. B., and GALLEYMORE, H. B. The subepidermal fungi of cereal grains. II. The nature, identity and origin of the mycelium in wheat. Ann. Appl. Biol. 38: 348 (1951).
6. GOLUBCHUK, M., CUENDET, L. S., and GEDDES, W. F. Grain storage studies. XXX. Chitin content of wheat as an index of mold contamination and wheat deterioration. Cereal Chem. 37: 405 (1960).
7. COCHRAN, T. W. Hexosamine biosynthesis by fungi as a reliable index of their growth in liquid and solid media. Thesis, Virginia Polytechnic Institute and State University (1975).
8. DONALD, W. W., and MIROCHA, C. J. Chitin as a measure of fungal growth in stored corn and soybean seed. Cereal Chem. 54: 466 (1977).
9. SEITZ, L. M., and PAUKSTELIS, J. V. Metabolites of *Alternaria alternata*: ergosterol and ergosta-4,6,8(14),22-tetraen-3-one. J. Agr. Food Chem. 25: 838 (1977).
10. WEETE, J. D. Fungal lipid biochemistry: distribution and metabolism. Plenum Press: New York, NY, p. 160 (1974).
11. NES, W. R. Role of sterols in membranes. Lipids 9: 596 (1974).
12. SEITZ, L. M., SAUER, D. B., MOHR, H. E., and BURROUGHS, R. Weathered grain sorghum: natural occurrence of alternariols and storability of the grain. Phytopathology 65: 1259 (1975).
13. HUBBARD, J. E., HALL, H. H., and EARLE, F. R. Composition of the component parts of the sorghum kernel. Cereal Chem. 27: 415 (1950).
14. DEPARTMENT OF COMMERCE. National Climatic Center, Asheville, NC. Climatological data, Kansas, Vol. 87-89 (1973-75).

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