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Rapid and Specific Method for Screening Ergosterol as an Index of Fungal Contamination in Cereal Grains

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ABSTRACT

Ergosterol is used as an effective biochemical marker for fungal contamination in food grains. A simple and rapid qualitative method for screening ergosterol in cereal grains is described. The method involves the formation of a highly fluorescent addition product of ergosterol after an iodination reaction. Iodinated ergosterol shows a characteristic greenish-blue fluorescence under longwave ultraviolet light. Further, chemical confirmatory tests are also incorporated for identifying ergosterol. The method developed may be used in the food industry, and for routine surveillance of cereal grains for fungal contamination.

INTRODUCTION

Some of the common plant foods consumed by humans, such as cereals, millets and oil seeds, are susceptible to fungal infestation. The degree of infestation varies with environmental conditions. Contamination by fungi leads to undesirable changes in colour and flavour, increase in free fatty acids and to deterioration of seed quality. Further, the elaboration of secondary metabolites such as mycotoxins, by the fungi, would lead to harmful health

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effects in humans and animals if consumed. Earlier, several attempts have been made to screen and evaluate fungal contamination in foods; namely, the Howard mould count for counting the filaments of fungi present in food, a serial dilution plate technique and also certain biochemical markers such as chitin which are used as indicators of fungal contamination (Booth, 1971; Ride and Drysdale, 1972; Donald & Mirocha, 1977; AOAC, 1984; Subrahmanyam & Rao, 1987).

Besides these, ergosterol, a structural component of most fungi (Nes, 1977) has been used as an effective index for fungal contamination based on its correlation to fungal growth (Seitz *et al.*, 1977). Methods have been developed for detection and quantitation of ergosterol. These methods need sophisticated equipment like spectrophotometers and HPLC, and thus are not useful for routine screening of fungal contamination in cereal grains.

In view of the heatlh and economic implications of mycotoxins in food grains, there is a need for rapid detection of fungal contamination. Often a large number of samples have to be routinely screened for the presence of primary or secondary metabolites of fungal origin. The objective of the present communication relates to the development of a rapid method for screening of ergosterol as a biochemical marker of fungal contamination in food grains and its chemical confirmation.

MATERIALS AND METHOD

Materials

Ergosterol standard was procured from Sigma Chemical Company (St. Louis, Missouri, USA). Silica gel-G was purchased from Glaxo Laboratories, India. Iodine crystals (extra pure) were obtained from Sarabhai Chemicals, India. All other reagents used were of analytical grade.

Six samples each of visually mouldy wheat (*Triticum vulgare*), wheat flour (whole and refined), maize (*Zea mays*) and sorghum (*Sorghum vulgare*) were analyzed by a plating technique (Booth, 1971) for fungal contamination. Similarly, undamaged grains of wheat, maize, sorghum and wheat flour (whole and refined) which served as controls were also analyzed by the same method. Simultaneously, ergosterol was extracted from the above naturally contaminated and control samples by the method of Seitz *et al.* (1977). All the samples analyzed were in triplicate. The grains were first powdered and 50 g of each were extracted with methanol and then saponified. The saponified extract was first washed with water and then ergosterol was extracted with petroleum ether, which was evaporated to dryness on a water bath and then dissolved in 1 ml of benzene:acetonitrile (98:2 v/v).

Method

Ergosterol standard (1 μg per 10 μl) was applied onto a previously coated TLC plate (20 × 20 cm) (with silica gel-G), with a thickness of 500 μm. Along with the standard, the sample extracts were also spotted. The plate was developed in a toluene:acetone (9:1 v/v) system, until the solvent front reached 15 cm height. The plate was then air-dried at room temperature and was exposed to iodine vapours in a pre-saturated glass chamber for 45 s. Development of brown spots indicated the presence and location of ergosterol. The plate was allowed to stand for 6–10 min during which time the brown coloration due to iodination totally disappears. The ergosterol spots were later visualized under longwave UV light in a dark room. Ergosterol showed an R_f value of 0·75 with a greenish-blue fluorescence. Other commonly occurring phytosterols, such as β-sitosterol, stigmasterol and animal sterols like cholesterol, were also subjected to the iodination reaction, so as to rule out any interference by other compounds.

Chemical confirmatory test

For confirming the presence of ergosterol a chemical method has also been developed. After the iodination of ergosterol and its visualization under UV, 50% sulphuric acid (v/v) and 18% hydrochloric acid (w/v) were sprayed onto the TLC plate which was dried at room temperature. The acid treatment resulted in a change to brilliant green fluorescence of ergosterol under long-wave UV light, from the original greenish-blue fluorescence. Treatment with hydrochloric acid and heating to 100°C for 5–10 min in an oven caused a reversal of fluorescence from brilliant green to the original greenish-blue fluorescence of ergosterol. A similar treatment with sulphuric acid had no effect on the brilliant green fluorescence of ergosterol.

RESULTS AND DISCUSSION

Analysis of naturally contaminated samples of wheat, wheat flour (whole and refined), maize and sorghum, indicated the presence of fungal contamination by a plating technique (Table 1). The moulds identified include species of *Aspergillus* and *Fusarium*. All the samples which were visually mouldy when screened for the presence of ergosterol by the present method were found to be positive, indicating the presence of fungi. Further, the control flour samples of wheat, positive for fungal contamination by the plating technique, were found to be negative by the present method. Perhaps the wheat flour, unlike the wheat grain sample, was not surface-sterilized

TABLE 1
Analysis of Grain/Grain Products for Fungal Contamination

Samples[a]	*Method of detection for fungal contamination*			
	Seed plating technique		*Fluorometric detection of ergosterol*	
	Control	*Naturally contaminated*	*Control*	*Naturally contaminated*
Wheat (6)	−	+	−	+
Refined wheat flour (6)	+	+	−	+
Wheat flour (whole) (6)	+	+	−	+
Maize (6)	−	+	−	+
Sorghum (6)	−	+	−	+

[a] Analysed in triplicate. Number in parentheses indicates sample size.
(−) Negative for fungal contamination.
(+) Positive for fungal contamination.

which might have resulted in the contamination by fungal spores from the environment.

The method reported makes use of the simple iodination reaction with ergosterol, resulting in the formation of a highly fluorescent addition product. The iodinated ergosterol showed a characteristic greenish-blue fluorescence under longwave UV light with an R_f of 0·75. The variation of R_f value under a given set of conditions was less than $\pm 3\%$. Figure 1 gives the spectral analysis of native ergosterol and iodinated ergosterol. The iodination reaction resulted in a change of absorption maximum of native ergosterol (λ_{max} 282 nm) to 271 nm. The iodination reaction with ergosterol is so specific that other commonly occurring phytosterols such as β-sitosterol and stigmasterol, and animal sterols like cholesterol, do not result in the formation of a fluorescent product. It is important to note that all these sterols, including ergosterol, show an R_f value of 0·75, when subjected to TLC analysis. However, only ergosterol forms the fluorescent product after iodination with iodine. The fluorescent product formed after iodination with ergosterol is a highly stable substance, unlike the native ergosterol which can undergo auto-oxidation. All the contaminated samples analyzed showed a characteristic greenish-blue fluorescence with an R_f of 0·75, which was similar in appearance to the standard ergosterol spot, indicating the presence of ergosterol. The minimum detection limit of the method developed is 500 ng/spot. Ergosterol spots were confirmed by the above-developed chemical confirmatory test. The acid treatments result in the change of fluorescence from greenish-blue to brilliant green.

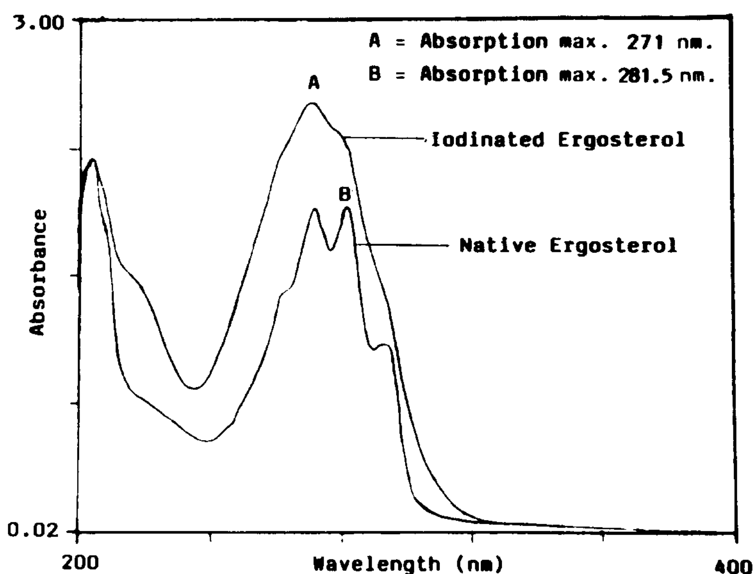

Fig. 1. Absorption spectra of (A) iodinated ergosterol ($50\,\mu g\,ml^{-1}$) and (B) Native ergosterol ($50\,\mu g\,ml^{-1}$) in absolute ethanol (oxygen-free).

Methods have been reported for the determination of the extent of fungal contamination in cereal grains. The determination of seed-borne fungi, after surface sterilization, usually takes 5–7 days to obtain results and to measure viable mycelia (Booth, 1971). However, this method does not detect the non-viable mycelia present in the grain. Chitin, which is a cell wall component of most fungi, has been used as a chemical marker for fungal contamination (Ride & Drysdale, 1972; Donald & Mirocha, 1977). The method devised for chitin estimation usually takes 4–5 h per sample analysis. However, the cereal grain samples containing insects and insect parts may lead to misleading results by this method as insects also contain chitin. Seitz *et al.* (1977) have indicated ergosterol as a sensitive measure of fungal contamination in grains. The method of ergosterol detection was based on UV absorption. Visualization was achieved in this method by spraying of sulphuric acid (50%) and heating, which resulted in a brown charred spot (Naewbanji *et al.*, 1984). The chemical confirmatory test for ergosterol reported here helps in distinguishing it from other phytosterols. The earlier methods of detection of chitin and ergosterol rely on sophisticated equipment like GLC, HPLC and spectrophotometers and are thus not useful for routine screening of fungal contamination of cereal grains.

The method reported here is a rapid one wherein each grain sample can be screened for fungal contamination within 2 h (unlike earlier methods). This method could be put to use by regulatory agencies and food industries for

determining the quality of cereal grains in relation to mould damage. The advantage of the present method over the earlier reported methods of screening is its simplicity, rapidity, specificity and application to a large number of samples in minimal time.

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REFERENCES

AOAC (1984). *Official Methods of Analysis* (14th edn). Association of Official Analytical Chemists. Arlington, Virginia, pp. 928–35.

Booth, C. (Ed) (1971). *Methods in Microbiology*. Academic Press, London, pp. 42–47.

Donald, W. W. & Mirocha, C. J. (1977). Chitin as a measure of fungal growth in stored corn and soyabean seed. *Cereal Chem.*, **54**, 466–74.

Naewbanji, M., Seib, P. A., Burroughs, R., Seitz, L. M. & Chung, D. S. (1984). Determination of ergosterol using thin layer chromatography and ultraviolet spectroscopy. *Cereal Chem.*, **61**, 385–8.

Nes, W. R. (1977). *The Biochemistry of Plant Sterols. Advances in Lipid Research.* Vol. 15. Academic Press, London, New York, p. 233.

Ride, J. P. & Drysdale, R. B. (1972). A rapid method for the chemical estimation of filamentous fungi in plant tissue. *Physiol. Plant pathol.*, **2**, 7–15.

Seitz, L. M., Mohr, H. E., Burroughs, R. & Sauer, D. B. (1977). Ergosterol as an indicator of fungal invasion in grains. *Cereal Chem.*, **54**, 1207–17.

Subrahmanyam, C. & Rao, S. L. N. (1987). An enzymatic method for the determination of Chitin and Chitosan in fungal cell walls. *J. Biosci.*, **12**, 125–9.