Supercritical Fluid Extraction and Supercritical Fluid Chromatography of the Fungal Metabolite Ergosterol†

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Novel techniques for the CO_2 supercritical fluid extraction and supercritical fluid chromatography of the fungal metabolite ergosterol in its free (nonconjugated) form were developed and applied to samples of flour, moldy bread, and mushrooms. Ergosterol was extracted directly, and subsequent chromatography on a Spherisorb Amino 3 μm column with ultraviolet detection at 282 nm was accomplished without any further sample cleanup. The procedures are simple, rapid, and reliable. The overall method showed an 83% recovery of free ergosterol for a spiked bread flour. Sensitivity on a 1.0-g sample of flour was about 0.05 μg/g. Observed levels of ergosterol ranged from 0.08 μg/g (fresh weight basis) in a cake flour to 14.3 mg/g (freeze-dried basis) in mushroom caps.

INTRODUCTION

Ergosterol is the major sterol constituent of most fungi and is either absent from or only a minor constituent of higher plants (Weete, 1974; Nes, 1977). Seitz et al. (1977) suggested that this metabolite could be used as an indicator of fungal contamination. Since then, it has become widely used for such a purpose in a diversity of matrices. These include the following: cereal seeds or heads during growth (Miller et al., 1983; Miller and Young, 1985; Young and Miller, 1985), after harvest (Miller et al., 1985; Seitz and Bechtel, 1985; Cahagnier, 1988; Hamilton et al., 1988; Jambunathan et al., 1991), in storage (Seitz et al., 1979, 1982; Cahagnier et al., 1983; Naewbanij et al., 1986), after milling (Young et al., 1984), and in feeds (Schwadorf and Mueller, 1989; Ramakrishna et al., 1990); cereal plant leaves (Griffiths et al., 1985); other plants such as broad beans (Al-Shabibi and Al-Mashikhi, 1987), malt (Mueller et al., 1991), tobacco (Bindler et al., 1988), and spruce needles (Osswald et al., 1986; Simmleit and Schulten, 1989); decaying plant material (Newell et al., 1988); decaying wood (Nillson and Bjurman, 1990); soil (West et al., 1987; Zelles et al., 1987); study of plant–root–soil interactions (Salmanowicz and Nylund, 1988; Johnson and McGill, 1990), house dust (Miller et al., 1988), fungi (Newell et al., 1987), mushrooms (Yokakawa and Mitsuhashi, 1981; Huang et al., 1985), and yeast (Arnezeder et al., 1989); and monitoring fermentations (Degranges et al., 1991).

The methods used most frequently for analysis of ergosterol are based on that of Seitz et al. (1977, 1979) and involve methanolic extraction, alkaline saponification, and C18 reversed-phase high-performance liquid chromatographic (HPLC) separation with ultraviolet (UV) detection at 282 nm. Reported modifications include methanolic extraction under saponifying conditions (Griffiths et al., 1985; Schwadorf and Mueller, 1989) and use of Si-60 normal-phase HPLC columns (Zill et al., 1988; Schwadorf and Mueller, 1989). Gas chromatographic (GC) separations have been followed by flame ionization detection (Johnson and McGill, 1990), photoionization detection (Krull et al., 1985), or mass spectrometry (Miller et al., 1988). Screening methods using thin-layer chromatography (TLC) (Naewbanij et al., 1984; Sashidhar et al., 1988; Xu et al., 1988; Ramakrishna et al., 1990) have also been developed and employed. Prior extraction of ergosterol was not required in a temperature-programmed pyrolysis/mass spectrometric study (Simmleit and Schulten, 1989).

Supercritical fluid extraction (SFE) is emerging as a valuable technique (Lee and Markides, 1990; Hawthorne, 1990) for the isolation of solutes from solid samples, using supercritical fluids as the extraction media. While supercritical fluids exhibit solvation powers approaching those of liquids, they have both lower viscosities and higher diffusivities (Games et al., 1988), which lead to more rapid and efficient extractions of analytes. Moreover, the solvent strength of a supercritical fluid increases with increasing density, allowing modifications of the extraction selectivity simply by changing the pressure or temperature. Finally, carbon dioxide, the supercritical fluid most frequently used in SFE, is nontoxic and available in a pure form at a reasonable cost. Hence, it represents an excellent alternative to the potentially hazardous solvents currently used in sample preparation.

This paper reports the results of studies to determine the efficacy of SFE and supercritical fluid chromatography (SFC) in the analysis of ergosterol. While the SFC of ergosterol has been demonstrated on a fused silica capillary column (White et al., 1988), this study investigated the use of a standard packed HPLC column.

MATERIALS AND METHODS

Reagents. All reagents and solvents were of analytical reagent grade. Ergosterol was recrystallized from ethanol. Instrument grade carbon dioxide supplied in cylinders with a dip tube (BOC, London) and glass-distilled methanol were used for mobile phases.

Samples. Flour was provided by Kristy's Bakery, Swansea, U.K. Moldy bread was obtained by placing several slices of several day old white bread into a clear plastic bag and allowing the bread to stand for several weeks at room temperature until it was well covered with mold; the bread was then air-dried and ground into a fine powder. Mushrooms were obtained from a local grocery store; caps were separated from stems, and each was cut into 2–3 mm sized pieces, freeze-dried, and ground into powder. Backyard garden soil was air-dried, and stones and other extraneous materials were removed.

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Supercritical Fluid Extraction. Samples, typically 1.00 g in a 7-mL stainless steel extraction thimble, were placed into a Hewlett-Packard 7680A SFE module (Hewlett-Packard, Avondale, PA). Initial studies were conducted with Celite spiked with ergosterol (20 μg/g). Ultimately, the best operating conditions were chosen as follows: extraction chamber temperature, 40 °C; extraction conducted with supercritical CO_2 at a density of 0.90 g/mL (pressure 281 bar) for 11.4 min at a flow rate of 3.3 mL/min (equivalent to 5.5 thimble volumes); analytes from the extraction chamber were trapped on an octadecylsilyl (ODS) column at 40 °C; the trap was then heated to 50 °C and rinsed successively with 0.5, 1.0, and 1.5 mL of methanol. These collected fractions were evaporated to dryness and redissolved in dichloromethane immediately prior to chromatographic analysis.

Chromatographic Analyses. SFC analyses were conducted on a Hewlett-Packard 1084B liquid chromatograph modified for SFC (Gere et al., 1982). The liquid carbon dioxide and the pump heads of the chromatographs were cooled to −25 °C using a Neslab RTE-4Z refrigerated bath (Neslab Instruments, Portsmouth, NH). Separations were achieved on a 250 × 4.6 mm i.d. stainless steel column filled with Spherisorb Amino 3 μm with the eluant supercritical CO_2 containing 10% methanol at an oven temperature of 50 °C and pressure of about 300 bar. At a flow rate of 4.0 mL/min, ergosterol eluted at 1.45 min. Ergosterol was detected with a Hewlett-Packard VWD-79875 variable-wavelength detector set at 282 nm. Estimations of ergosterol were made by a comparison of peak heights or areas, as appropriate, with those of external standards. Confirmation of ergosterol was accomplished by comparison of retention times with the external standard, by coinjection with a standard, or by using a moving belt interface to connect the SFC effluent to a VG-7070E (VG Analytical, Manchester, U.K.) mass spectrometer operating in the electron impact mode at 70 eV.

Recovery Studies. Bread flour (1.00 g, in duplicate) was spiked with ergosterol at 2.5, 5.0, and 10.0 μg/g and extracted as above. Garden soil (5.00 g, in triplicate) was spiked with ergosterol at 10 μg/g and extracted as above.

RESULTS AND DISCUSSION

Supercritical Fluid Extraction of Ergosterol. Because the solubility of a given substance in supercritical CO_2 depends upon the density of the fluid, one can achieve partitioning by density programming. In the initial stages of this study, extractions were conducted using stepwise increases in supercritical CO_2 densities. Ergosterol was partially extracted at a density of 0.70 g/mL, and complete extraction was achieved at 0.90 g/mL. Thus, pre-extraction below 0.70 g/mL could be used to remove potentially interfering substances, although this was not found to be necessary for the analyses reported below.

Another opportunity for fractionation was available in the elution of substances from the ODS trap. When 3 mL of methanol was used, ergosterol eluted in the fraction between 0.5 and 1.5 mL. The initial 0.5 mL was found to remove many of the nonpolar coextractives and resulted in a cleaner chromatogram.

Supercritical Fluid Chromatography of Ergosterol. Under SFC conditions on a Spherisorb Amino 3 μm column, ergosterol can be readily eluted. Figure 1 shows the effect of methanol modifier on retention time. At a modifier concentration of 10%, ergosterol eluted quickly (ca. 1.5 min). Reversed-phase HPLC retention times are up to 10 times longer (Young et al., 1984). Use of the SFC modifier methanol as solvent for standards and samples resulted in peaks that were quite broad (6.7-s half-height peak width). However, when dichloromethane was used as solvent, sharp (2.6-s half-height peak width) and higher peaks were obtained and resolution was improved.

Figure 2 shows that SFC with UV detection at 282 nm gives a linear calibration curve over nearly 3 orders of magnitude. The detection limit for the optimum conditions was about 20 ng. Smaller amounts might be detectable with a more sensitive detector; Arnezeder et al. (1989) and Colin et al. (1979) reported detecting 6 and 0.7 ng, respectively, at 282 nm under reversed-phase HPLC conditions. Coefficients of variation for 3–5 manual injections per sample for 15 determinations averaged 4.8 and 5.4% for area count and peak height determinations, respectively.

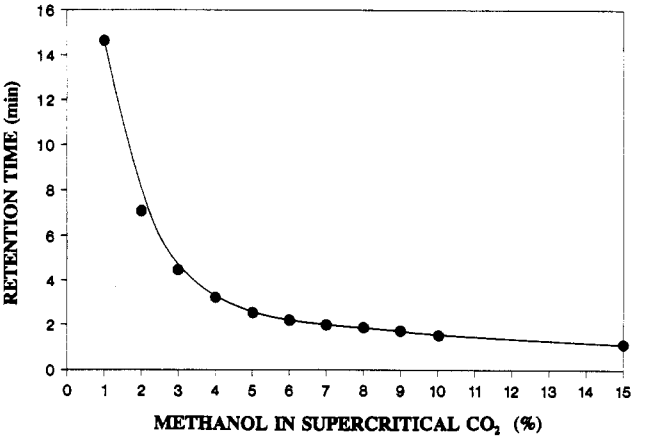

Figure 1. Effect of modifier concentration on retention time of ergosterol separated by SFC with supercritical CO_2 containing methanol at 50 °C and a pressure of 291 bar on a 250 × 4.6 mm i.d. stainless steel column of Spherisorb Amino 3 μm. Ultraviolet detection was at 282 nm.

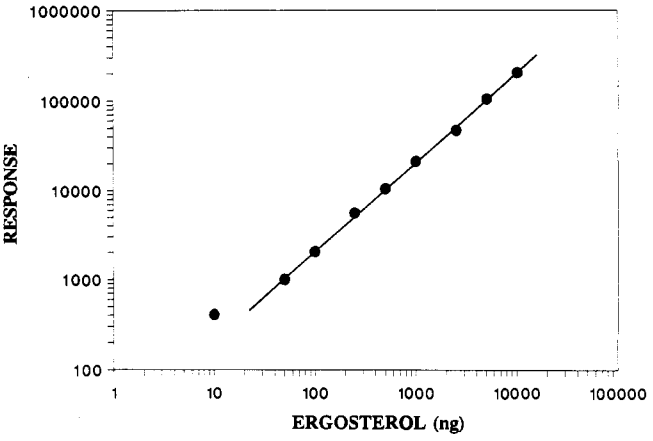

Figure 2. Standard curve for ergosterol analyzed by SFC with supercritical CO_2 containing 10% methanol at 50 °C and a pressure of 291 bar on a 250 × 4.6 mm i.d. stainless steel column of Spherisorb Amino 3 μm. Ultraviolet detection was at 282 nm. Values represent averages of triplicate determinations.

Analysis of Ergosterol. The appropriate sample size taken for SFE depended upon the level of ergosterol present. If the samples were too large, than all of the ergosterol might not be extracted from the sample or the ODS trap could be overloaded. The maximum sample sizes, for the volume of supercritical CO_2 employed, were 1000, 200, and 15 mg for flour, moldy bread, and mushrooms, respectively. On the basis of detection limits and background noise, the practical limit of detection for a 1.0-g sample of flour was about 0.05 μg/g. Recovery of ergosterol from bread flour with an initial ergosterol content of 1.09 μg/g ranged from 78 to 86% for the three levels of ergosterol added. Figure 3 shows a linear response of recovered vs added ergosterol and corresponds to an average recovery of 83%. Recovery of ergosterol from a garden soil that did not show any background ergosterol was about 73%.

The initial 0.5-mL methanol rinse of the SFE ODS trap afforded a mixture that showed numerous peaks at 282 nm with SFC retention times of up to 8.0 min. In addition,

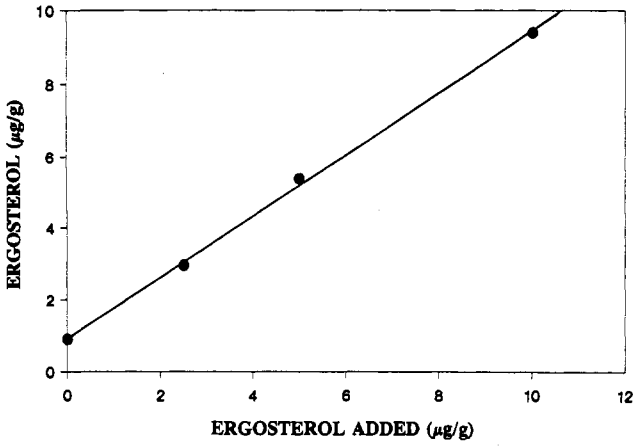

Figure 3. Recovery of ergosterol added to bread flour, extracted by SFE and analyzed by SFC. The least-squares regression line was $Y = 0.83X + 1.09$, where Y is the ergosterol concentration in the flour (μg/g) and X is the concentration of the ergosterol added (μg/g). Values represent averages from duplicate determinations.

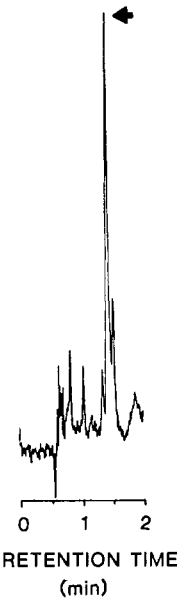

Figure 4. Chromatogram of a CO_2 supercritical fluid extract of whole meal flour obtained from a commercial bakery. Injection of 20 μL was equivalent to 100 mg of flour. Analysis was performed with supercritical CO_2 containing 10% methanol at 50 °C and a pressure of 302 bar on a 250 × 4.6 mm i.d. stainless steel column of Spherisorb Amino 3 μm. Ultraviolet detection was at 282 nm.

several other peaks were observed when the eluant was monitored at shorter wavelengths (e.g., 210 nm). The next methanol rinse (1.0 mL) contained the desired analyte. A typical chromatogram for the second methanolic rinse fraction from an SFE of whole meal flour is illustrated in Figure 4. Ergosterol eluted at 1.43 min and the final peak at about 2.0 min. The first rinse removed other components that would have increased the time needed for analysis.

Table I shows the levels of free ergosterol found in a variety of flours used in a commercial bakery in the United Kingdom. They ranged from 0.08 μg/g in a soft cake flour to 34.9 μg/g (as-is basis) in a granary flour. The flours with the higher levels were those containing the outer layers of the kernel, which tend to have the higher proportion of fungal contamination (Young et al., 1984). Free ergosterol levels as determined according to this method are of the same order as those reported elsewhere for total

Table I. Ergosterol Concentrations in Commercial Bakery Flour, Moldy Bread, and Mushroom Caps and Stems

sample	sample weight, mg	ergosterol,[a] μg/g
cake flour	1000	0.08
plain flour	1000	0.70
bread flour	1000	1.09
mid flour	1000	1.59
whole wheat flour	1000	1.92
rye flour	1000	2.54
brown flour	1000	3.82
whole meal flour	1000	8.51
granary flour	1000	34.9
moldy bread	200	152[b]
mushroom stems	15	12 800[c]
mushroom caps	15	14 300[c]

[a] Determined by CO_2 supercritical fluid extraction and supercritical fluid chromatography with ultraviolet detection at 282 nm. Unless noted otherwise, values are means for duplicate determinations on a fresh weight basis and are not corrected for recovery. [b] Air-dried weight basis. [c] Freeze-dried weight basis.

ergosterol (Young et al., 1984; Miller et al., 1985; Ramakrishna et al., 1990). A sample of moldy bread contained 152 μg/g (air-dried basis) and the mushrooms contained 14.3 and 12.8 mg/g (freeze-dried basis) in the caps and stems, respectively.

Ergosterol levels in various fungi typically range from about 0.1 to 15 mg/g of dry weight (Newell et al., 1987, and references cited therein). For a given fungal species, environmental factors such as age, medium, moisture, and temperature may affect the yield of ergosterol produced per unit of fungal mass. Thus, caution must be used when one attributes absolute amounts of fungal biomass on the basis of measured ergosterol levels. West et al. (1987) suggest that it may be more appropriate to use ergosterol for measuring *changes* in fungal populations (authors' emphasis).

This method as presented gives levels of free ergosterol. Most of the methods for determining ergosterol cited under Introduction involved liquid extraction and saponification to give total ergosterol (free plus conjugated). Hamilton et al. (1988) reported that free ergosterol was highly correlated ($r = 0.98$) with total ergosterol in contaminated corn over the range 1–200 μg/g. Salmanowicz and Nylund (1989) observed that free ergosterol accounted for about 65–85% of the total ergosterol in mycorrhizal plants. Given that it may be difficult to correlate absolute levels of ergosterol and fungal biomass, levels of free ergosterol may give sufficient information for a particular investigation.

Other methods of extraction typically require larger samples (e.g., 20 g) and larger volumes of reagents (up to 300 mL), are labor intensive (at least 12 sample manipulations), and are longer (at least an hour) [see Schwadorf and Mueller (1989) and references cited therein]. The method of analysis reported herein requires smaller samples and smaller reagent volumes (3 mL of methanol, 40 mL of supercritical CO_2), and therefore is more economical with respect to reagent purchase and disposal costs, is not labor intensive, is more rapid (SFE completed in less than 30 min and SFC in less than 3 min), and is less hazardous. The use of such small amounts of material requires that special attention be paid to ensuring that samples be homogeneous and representative of the whole.

Conclusions. It has been shown that CO_2 supercritical fluid technology can be effectively applied to the extraction and/or analysis of the fungal metabolite ergosterol in a variety of matrices including flour, bread, mushrooms, and soil. Compared with classical extraction procedures, the SFE conditions employed required smaller samples and reagent volumes, are quicker, are not labor intensive, and

are safer (no hazardous solvents or waste). This study also demonstrates for the first time the efficacy of SFC using a standard packed HPLC column for the separation and quantitation of ergosterol.

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