Ergosterol Profiles, Fatty Acid Composition, and Antioxidant Activities of Button Mushrooms as Affected by Tissue Part and Developmental Stage

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This article investigated the mycochemical profiles and the antioxidant activities of the lipophilic extracts of the white and brown button mushrooms. We found that only free ergosterols were present in both mushrooms at 2.04–4.82 mg/g dry matter (DM). Ergosterol concentration was higher in early growth stages but decreased as the mushrooms grew, and it distributed evenly between the caps and stems during early developmental stages but accumulated more in the caps after maturation. The photochemiluminescence (PCL) values of the two mushrooms were 5.49–10.48 nmol trolox equivalent/mg DM, and the EC_{50} values of 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay ranged 20.19–41.49 mg DM/μg DPPH. The ergosterol content positively correlated with the antioxidant activities ($r^2 > 0.89$). The total fatty acid content was 8.7 mg/g DM in the white and 5.1 mg/g DM in the brown button mushroom and contained mainly linoleic, palmitic, and stearic acids. Our data provide guidance for optimized harvesting time of mushrooms and maximized health benefits.

KEYWORDS: Mycochemicals; ergosterol; vitamin D_2; fatty acids; mushroom; *Agaricus bisporus*; antioxidant; PCL; DPPH

INTRODUCTION

Mushrooms are known for many bioactive compounds. These compounds have been mistaken as phytochemicals by some researchers in the literature; however, strictly speaking these must be termed mycochemicals. Ergosterol (**Figure 1**), a principle sterol of the cell membrane to which it is strongly bound in fungi, is able to activate the expression of a number of defense genes and increase the resistance of plants against fungal pathogens (*1*). Ergosterol is mostly found in fungi and is present both as free and esterified ergosterols, with relative ratios of free to esterified ergosterol varying among different species (*2*). Studies have demonstrated that ergosterol and its peroxidation products may contribute to potential health benefits and significant physiological functions that include reduction in pain related inflammation, incidences of cardiovascular disease, inhibition of cylcooxygenase (COX) enzymes, and antimicrobial, anticomplementary, and antitumor activities (*3–6*). The antitumor activity of ergosterol may be due to direct inhibition of angiogenesis induced by solid tumors (*6*). Dietary ergosterol is absorbed in the alimentary tract, accumulated in the adrenals and other organs, and can be metabolized in vivo to generate newer bioactive products, such as 17,24-dihydroxyergosterol, which has been found to inhibit the proliferation of abnormal skin cells in culture, as demonstrated in human keratinocytes and melanoma cell lines (*7*).

When exposed to UV light, ergosterol undergoes photolysis to yield a variety of photoirradiation products, principally previtamin D_2's, tachysterol, and lumisterol. Furthermore, the previtamin D_2's undergo spontaneous thermal rearrangement to vitamin D_2 (*8*). Vitamin D_2 has been known for its role in regulating levels of calcium and phosphorus (*9*). Clinical studies have shown a relationship between vitamin D levels and cardiovascular health, and low levels of vitamin D metabolites have been associated with higher incidence of congestive heart failure and increases in mortality (*10*). Additionally, a growing body of evidence has demonstrated the protective relationship between sufficient vitamin D status and lower risk of colorectal, breast, prostate, and ovarian cancers (*11, 12*).

Mushrooms contain high levels of ergosterol and have been used as foods and medicines for centuries. Although there are more than 2000 edible species of known mushrooms, white and brown button mushrooms (*Agaricus bisporus*) make up about 90% of those presently grown and consumed in North America (http://www.foodproductiondaily.com/Supply-Chain/A-mushrooming-industry). The concentration of ergosterols in button mushrooms have been studied by several groups, with reported values ranging from 0.446 mg/g fresh matter in white button mushrooms and 0.395 mg/g in brown button mushrooms (*13*); the dry matter (DM) concentration was reported to be 6.54 mg/g in the white button and 6.02 mg/g in the brown button by Mattila et al. (*14*). A significantly lower concentration (2.73 mg/g DM) was reported by Mau et al. for the white button mushroom (*15*). The most

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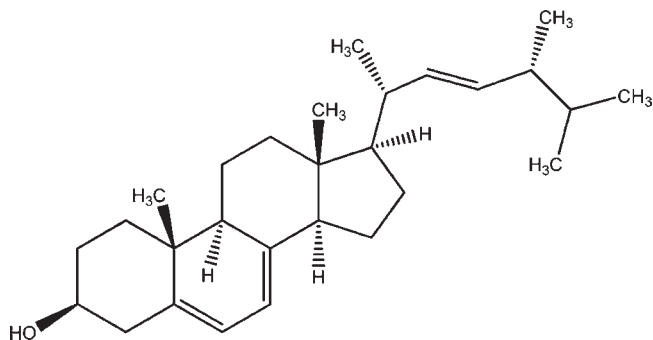
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Figure 1. Structure of ergosterol.

frequent analytical method used for ergosterol analysis in mushroom is AOAC method 2002.05 (*16*), which involves alkaline saponification in ethanol solution, extraction with hexane, followed by C18 reversed-phase HPLC separation with UV detection at 282 nm. Reported modifications of the method include combining the methanolic extractions and saponification, i.e., extraction with methanol under saponifying conditions (*17*). Since ergosterol may be present in both free and esterified forms in fungi, this information on the composition of these two forms will be lost by saponification. Recently, Yuan et al. reported an HPLC method for the simultaneous analysis of free and esterified ergosterols in ganoderma spore lipids (*18*, *19*).

Aqueous and methanolic extracts from mushrooms have been studied to evaluate their antioxidant and free radical scavenging activities that have been attributed to the polyphenol component in the extract (*20*, *21*). However, besides analyzing the antioxidant activity of the lipophilic components of mushrooms, only one report was found that further investigated the inhibition of liposome peroxidation by ergosterol from the fruiting body of an edible mushroom *Agrocybe aegerita* showing an inhibition rate of 43% (*22*).

Very few studies have reported the tissue distribution of bioactive metabolites in mushrooms during development, despite growing evidence that maturation affects the concentration of natural compounds in mushrooms. Jasinghe et al. (*23*) found that the distribution of ergosterol was different in different parts of shiitake mushrooms, accumulating more in the gills than in the stalks. Ribeiro et al. (*24*) studied the phenolics and organic acids in different parts of mushrooms and found the amount of both groups of compounds were tissue dependent. Tsai et al. (*25*) showed that the antioxidant activity was higher during early developmental stages in button mushrooms. To the best of our knowledge, no report has been published on the tissue distribution and developmental accumulation of ergosterol and other lipophilic contents, the antioxidant activity of the different tissues at different developmental stages, and the relationship between ergosterol and antioxidant activity in button mushrooms.

The objectives of this study were to investigate the ergosterol profile, the antioxidant activity of the lipophilic extract of the white and brown button mushrooms (*Agaricus bisporus*), and to determine how the growth stage affects the quantity and quality of ergosterol in the caps and stems of the two button mushrooms. The antioxidant activity of the lipophilic extracts and how ergosterol contributes to the activity was also evaluated and compared between the two varieties. To better understand the composition of the lipophilic components, we also examined the fatty acid composition in the two mushrooms to provide complete mycochemical profiles of these mushrooms. Results for the hydrophilic extract of the button mushrooms will be reported separately.

Table 1. Descriptions of Mushrooms at Different Developmental Stages[a]

		dry weight per mushroom (g)	diameter of cap (cm)	length of stem (cm)
white button	S1[b]	0.18 ± 0.06	2.2 ± 0.2	0.8 ± 0.1
	S2[b]	1.00 ± 0.17	3.5 ± 0.1	1.1 ± 0.1
	S3[b]	2.32 ± 0.20	5.0 ± 0.1	1.4 ± 0.1
brown button	S1[b]	0.42 ± 0.04	3.0 ± 0.2	1.2 ± 0.2
	S2[b]	4.33 ± 0.35	8.2 ± 0.3	4.8 ± 0.3
	S3[b]	8.34 ± 0.42	11.5 ± 0.5	5.0 ± 0.2

[a] Values are mean of at least 10 mushrooms; S1[b], stage 1; S2[b], stage 2; S3[b], stage 3.

MATERIALS AND METHODS

Chemicals. Ergosterol standards, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium ascorbate, Trolox, NaOH, and KOH were purchased from Sigma Chemical Co. (St. Louis, MO). The fatty acid methyl ester (FAME) mixture #463 was from Nu-Check Prep (Elysian, MN). Reagents for the photochemiluminescence (PCL) were purchased from Analytik Jena AG (Berlin, Germany). All solvents were of HPLC grade from Caledon Laboratories (Georgetown, Ontario, Canada).

Mushroom Sample Collection and Preparation. White button mushrooms of different developmental stages (**Table 1**) were freshly picked at the experimental mushroom farm of University of Guelph, Vineland Campus, Ontario, Canada. Brown Button mushrooms were collected fresh from Whitecrest Mushrooms Ltd., Putnam, Ontario, Canada. Both species were grown using a standard tray system under standardized, controlled conditions typical of commercial operations at mushroom farms across the country and would therefore be representative of mushrooms available to consumers. Freshly collected mushrooms were flash-frozen in liquid nitrogen, kept frozen, and stored at −20 °C. The mushrooms were then freeze-dried and manually separated into caps and stems, and the surface dust was gently brushed off with a spatula. Dried mushroom caps and stems were then ground into powder using a mortar and pestle. All operations were conducted under subdued light. Mushroom powder was collected in centrifuge tubes and stored in the dark at 4 °C prior to analysis. Each value consisted of at least 10 mushrooms.

Saponification of Mushroom Powder for Ergosterol Analysis. This analysis was based on AOAC method 2002.05 (*16*). Briefly, freeze-dried mushroom powder (0.2 g) was accurately weighted into a 40 mL amber glass vial and mixed with 1.6 mL of sodium ascorbate solution (17.5 g of sodium ascorbate in 100 mL of 1 M NaOH), 20 mL of ethanol (95%), and 4 mL of 50% KOH. The mixture was saponified at 80 °C for 1 h. After cooling to room temperature, the solution was transferred into an amber separatory funnel, to which 7.5 mL of water and 7.5 mL of ethanol were added. The mixture was then extracted successively with three volumes of hexane (25, 25, and 10 mL). The pooled hexane phase was transferred into a round-bottom flask and evaporated to dryness at a temperature below 40 °C under vacuum. The residue was immediately dissolved in 2 mL of ethanol, filtered through a 0.45 μm PTFE filter (Whatman, Sanford, ME, USA), and then subjected to HPLC analysis.

Extraction of Native Ergosterols from Mushrooms. A direct extraction method was developed by Yuan et al. (*18*, *19*) that permitted the investigation of native ergosterols (free and esterified ergosterols). This protocol involved no saponification. Mushroom powder (0.2 g) was homogenized in methanol (2 mL) by vortex mixing for 2 min, followed by the addition of 2 mL of hexane, and further vortexing for an additional 1 min. One milliliter of saturated NaCl solution was added followed by vortexing for 1 min, and centrifugation for 5 min at 4000 rpm. The clear upper layer was transferred into a vial, and the aqueous phase was re-extracted twice with 2 mL of hexane. The combined hexane extract was then dried under a steam of nitrogen and redissolved in 2 mL of ethanol. The ethanol solution was subjected to HPLC analysis after filtration through a 0.45 μm PTFE filter.

Direct Extraction of Native Ergosterols without Partitioning. A simplified direct extraction method using hexane was developed in the present study and compared to the two aforementioned methods. In this procedure, mushroom powder (0.2 g) was vortexed with 6 mL of hexane for 1 min, centrifuged at 4000 rpm for 10 min, and the supernatant (hexane phase) transferred into a vial. The mushroom residue was further extracted

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twice with 6 mL of hexane. The hexane phases were then pooled and dried under a steam of nitrogen. The extract was dissolved in 2 mL of ethanol and filtered through a 0.45 μm PTFE filter before analysis by HPLC.

Sample Preparation for Fatty Acid Analysis. Mushroom powder (0.5 g) was extracted according to the modified procedure of Bligh and Dyer (*26*, *27*). One aliquot of extracted lipids (7−15 mg) was methylated in a 15 mL culture tube equipped with a Teflon-lined screw cap under nitrogen. First, 3 drops of toluene were added to solubilize the fat followed by 2 mL of anhydrous 5% HCl/methanol (w/v) and then heating at 80 °C for 1 h. After the tubes were cooled to room temperature, 0.5 mL of distilled water was added, and the total fatty acid methyl esters (FAME) were extracted two times with 2 mL of hexane. The hexane layer was reduced in volume and then applied directly onto prewashed (with chloroform/methanol, 1:1) silica gel G thin-layer chromatography (TLC) plates (Fisher Scientific, Ottawa, ON) for purification using the developing solvent system of hexane/diethyl ether/acetic acid (85:15:1, v/v/v). The FAME band was visualized after the TLC plates had been sprayed with 2′,7′-dichlorofluoroscein/methanol (0.001% w/v) and viewed under UV light (254 nm). The corresponding FAME band was removed and eluted with chloroform. After the removal of chloroform using a stream of nitrogen, the FAMEs were dissolved in an appropriate volume of hexane (1 μg/μL) and analyzed by GC.

The base-catalyzed methylation involved the addition of 3 drops of toluene to another aliquot of extracted mushroom lipids (7−15 mg) followed by 1 mL of a 1 M NaOH/95% ethanol solution. After mixing, the solution was heated for 15 min at 80 °C. The solution was then neutralized with 1.0 mL of 3 M aqueous HCl, and the free fatty acids were extracted two times with 2 mL of hexane. The hexane layers were combined in a 15 mL test tube and taken to dryness in a steam of nitrogen. The free fatty acids were dissolved in 0.8 mL of benzene and 0.2 mL of methanol followed by the dropwise addition of trimethylsilyl diazomethane (TMS-DAM) (TCI America, Portland, OR) until a yellow color persisted (*28*). The test tube was shaken lightly and occasionally over a 60-min period at room temperature (excessive mixing should be avoided; TMS-DAM is explosive). Excess TMS-DAM was destroyed by the cautious addition of glacial acetic acid until the yellow color disappeared. Water was then added (0.5 mL), and the FAMEs were extracted 2 times with 2 mL of hexane. Hexane was reduced and the FAMEs purified by TLC as described above.

Analysis of Ergosterol by HPLC. An HPLC system (Agilent Technologies 1100 Series, Palo Alto, CA) equipped with a quaternary pump, an inline degasser, a thermostatic autosampler, and a diode array detector (DAD) was used. A Phenomenex Luna 5 μm C18 column (250 × 4.6 mm) with a C18 guard column (Torrance, CA) was used for the separation. The binary mobile phase consisted of solvent A (methanol/water, 80:20 v/v) and solvent B (methanol/dichloromethane, 75:25, v/v), that were programmed as follows: 0−5 min, 0% B; 5−25 min, 0−100% B; 25−45 min, 100% B; 45−50 min, 100−0% B. The flow rate was 1.0 mL/min, and the injection volume was 20 μL for the standard and samples. The DAD collected data from 190 to 900 nm, and absorbance at 280 nm only was used to monitor and quantify ergosterol. Ergosterol in mushrooms was tentatively identified by a combination of the retention time in HPLC chromatograms and UV spectra.

Analysis of FAMEs by GC. FAMEs were analyzed using a GC (model 5890; Hewlett-Packard. Palo Alto, CA) equipped with a splitless injection port (flushed after 0.3 min), a flame ionization detector (FID), an autosampler (Hewlett-Packard, Model 7673), a 100 m SP2560 fused capillary column (Supelco Inc., Mississauga, ON), and a Hewlett-Packard ChemStation software system (Version A.10.1). The injector and detector temperatures were both set at 250 °C, and H$_2$ served as carrier gas at a flow of 1 mL/m. The FAMEs were analyzed using two separate temperature programs on the same GC column and equipment; see Kramer et al. (*29*). The GC reference standard FAME mixture #463 was used spiked with the 4-positional CLA isomer mixture #UC-59M, and 21:0, 23:0, and 26:0 FAME all from Nu-Chek Prep (Elysian, MN). Quantification of the FAMEs was based on the FID response expressed as percent of total FAMEs, and total lipids were quantitated using 23:0 as the internal standard.

Photochemiluminescence (PCL) Assay. The PCL assay followed the procedure described by Li et al. (*30*) with minor modifications. A commercially available ACL-Kit (antioxidant activity of lipid-soluble

compounds) from the manufacturer was used. A mixture containing 0.2 mL of reaction buffer, 2.3 mL of methanol, 25 μL of 1.0 mM luminal, and 10 μL of a standard or a sample solution was subjected to PCL measurement. The antioxidant activity of the sample was calculated as equivalent units of Trolox.

Radical Scavenging Capacity: DPPH Assay. The DPPH assay was carried out according to the procedure of Brand-William et al. (*31*) with minor modifications. In this study, different volumes (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μL) of hexane extract were mixed with a methanolic solution of DPPH radicals (2.2 mg/L, 200 μL) in a 96 well microplate. The final volume of each well was made up to 300 μL by adding appropriate amounts of methanol. The mixture was shaken gently on a UV−vis plate reader (EL 340, Bio-Tek Instruments, Inc., Winooski, VT), and the absorbance at 515 nm was taken every 2 min for 30 min or until the absorbance reached its minimum value. The DPPH concentration in the reaction medium was calculated on the basis of a calibration curve derived from serial dilutions of the DPPH standard. The percentage of remaining DPPH was then plotted against the concentrations and calculated EC$_{50}$ values. EC$_{50}$ is defined as the amount of dry mushroom necessary to decrease the initial DPPH concentration by 50%, and the value was normalized to 1 μg of DPPH. A lower EC$_{50}$ value indicates a higher antiradical activity (*30*, *31*).

Statistical Analysis. All samples had three replicates. Data were analyzed by the standard analysis of variance (ANOVA) techniques, followed by a least significant difference (LSD) comparison of mean data values at a *P*-value of 0.05, using Statistix software (V2.0, Analytical Software, Tallahassee, FL).

RESULTS AND DISCUSSION

Parameters Used for the Three Defined Developmental Stages of Mushrooms. The developmental stages of mushrooms (stage 1, S1; stage 2, S2; and stage 3, S3) were determined according to their sizes (cap diameter and stem length) and their dry weight (**Table 1**). S3 is similar to the size for commercial harvest.

Ergosterol Profiles of Button Mushrooms. To obtain a full picture of ergosterol profiles of button mushrooms, we used both the AOAC 2002.05 protocol (total ergosterol content, with alkaline hydrolysis) and the more recently reported direct HPLC method for direct and simultaneous analysis of free ergosterol and ergosteryl esters (*18*, *19*). Using the latter method, we found no esterified ergosterols in either of the button mushrooms studied (**Figure 2**). There were no ergosteryl ester peaks which would have been expected to elute about 10 min after free ergosterol according to Yuan et al. (*18*). The values from the two known extraction procedures and our direct extraction gave essentially the same total ergosterol concentration, which supports the results from the saponification method that these samples contained no esterified ergosterol (**Figure 2** and **Table 2**).

This is the first report of the complete ergosterol profile in button mushrooms using direct solvent extraction and HPLC methods. The fact that nearly all ergosterols in the button mushrooms are free ergosterol and its nonester analogues suggests that such a simplified method is preferred for analyzing total ergosterols in these mushrooms. This simplified protocol eliminates the use of alkaline treatment and subsequent partitioning which could possibly contribute to error and is certainly more cost-effective. Neither the white nor the brown mushroom in this study contained vitamin D$_2$, a result consistent with findings by others (*14*). The lack of vitamin D$_2$ in the mushrooms is attributed to the absence of light in commercial growth conditions.

The nature of peak 3 in the HPLC chromatogram (**Figure 3**) was further investigated. Mattila, et al. (*14*), reported the presence of trace amounts of other sterols in mushrooms using GC/MS, which included ergosta-7,22-dienol, ergosta-5,7-dienol and fungisterol. Our results showed that peak 3 in **Figure 2** had an UV spectrum nearly identical to that of ergosterol (**Figure 3**), which suggests that this peak is an ergosterol analogue and not a sterols

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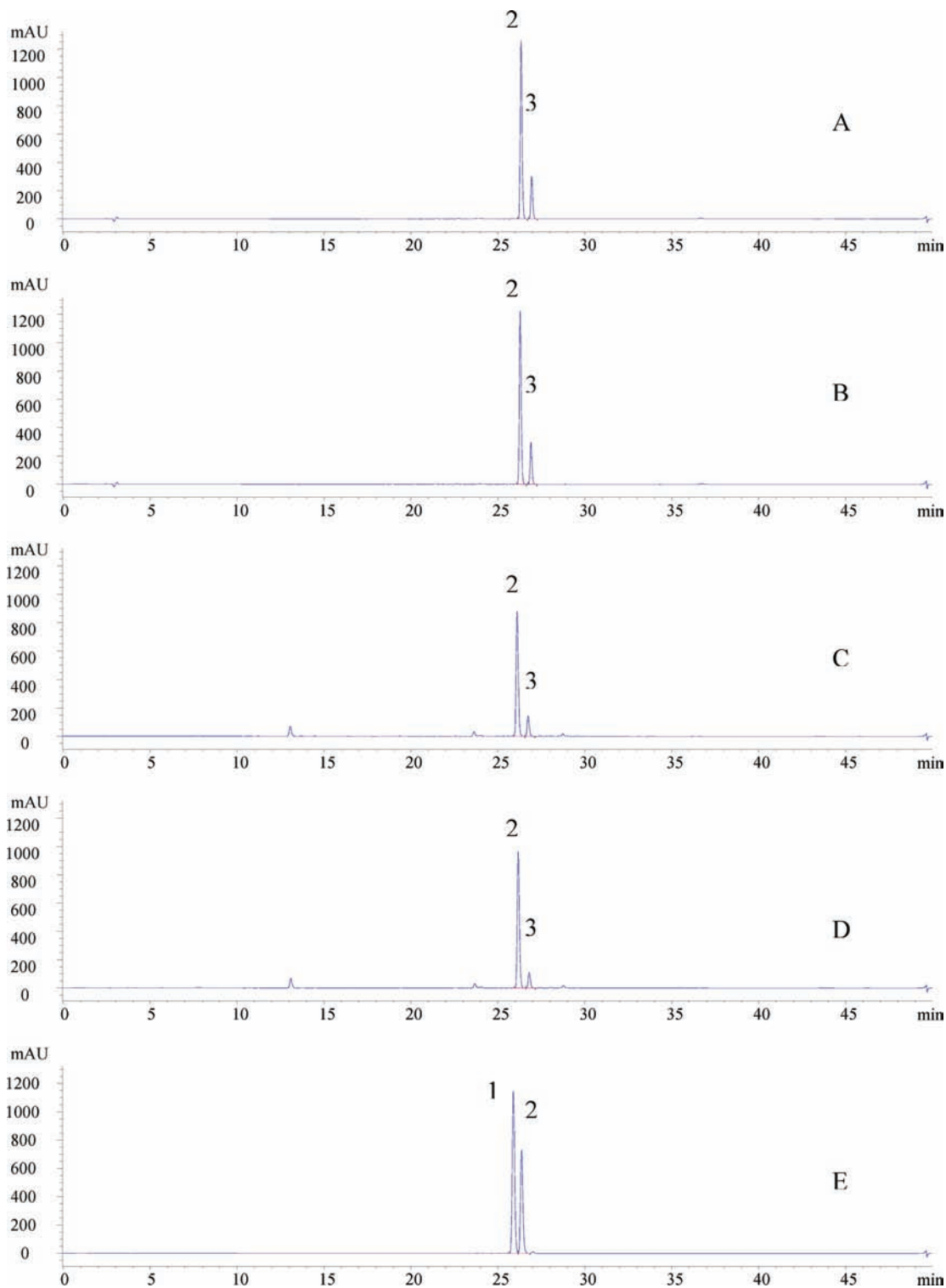
Figure 2. (**A**) Chromatograph of saponified white button mushroom extract (caps at stage 3). (**B**) Chromatograph of white button mushroom solvent extract (caps at stage 3). (**C**) Chromatograph of saponified brown button mushroom extract (caps at stage 3). (**D**) Chromatograph of brown button mushroom solvent extract (caps at stage 3). (**E**) Chromatograph of ergosterol and vitamin D_2 standard. Peak identity: peak 1, vitamin D_2; peak 2, ergosterol 3; peak 3, ergosterol analogue.
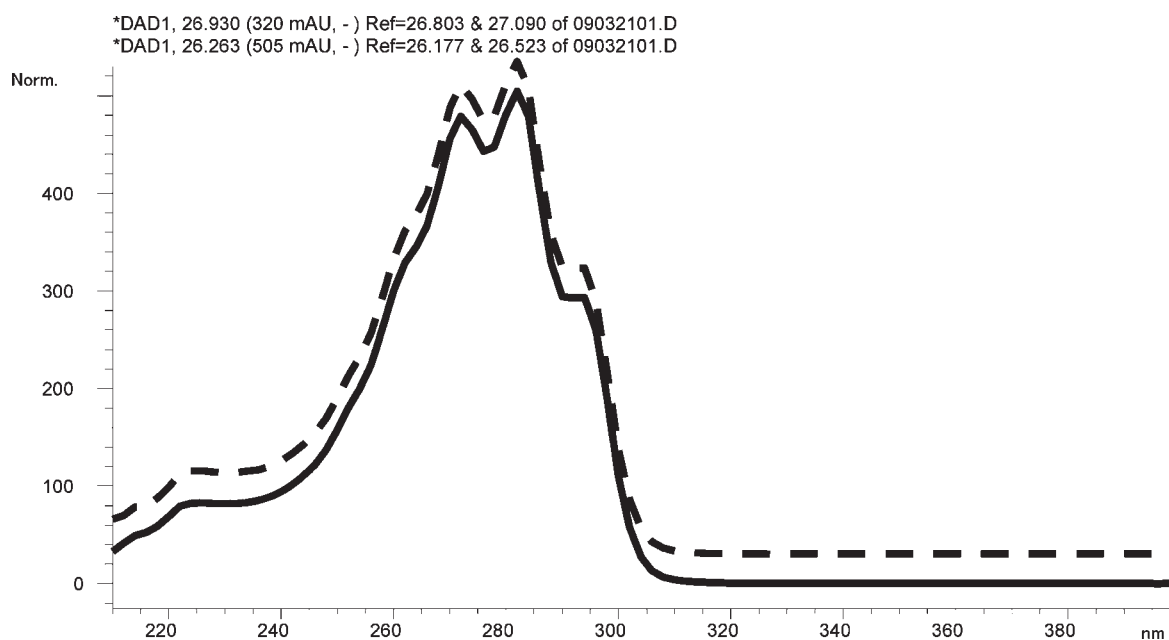
as found by Mattila et al. (*14*). A reduced sterol would have a significantly different UV spectrum than ergosterol (*32*). In addition, peak 3 was present in both the saponified products and the direct solvent extract indicating it was not an esterified ergosterol (**Figure 2**). The amount of ergosterol analogue was calculated using the calibration curve of ergosterol and expressed as ergosterol equivalent values as shown in **Table 2**. This analogue

was 12−14% of the total ergosterols in the white button mushroom regardless of its developmental stage and tissue type; however, in the brown button mushroom, the percentage of the ergosterol analogue ranged from 5.6% to 13%, with significant differences between different tissues and growth stages. Stems of the brown button mushroom had lower concentrations of this analogue compound, and the concentration was higher in

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Table 2. Amount of Ergosterol and Ergosterol Analogue in Different Parts of Mushrooms at Different Developmental Stages (mg/g Dry Matter)

|		saponified extraction[a]			hexane/methanol/water partition[a]			hexane extract[a]		
		ergosterol	ergosterol analogue	total ergosterols	ergosterol	ergosterol analogue	total ergosterols	ergosterol	ergosterol analogue	total ergosterols
white button	S1S[b]	3.35 ± 0.06 b	0.46 ± 0.02 b	3.81 ± 0.07 b	3.29 ± 0.08 b	0.46 ± 0.02 b	3.75 ± 0.08 b	3.32 ± 0.12 b	0.46 ± 0.02 b	3.78 ± 0.12 b
	S1C[b]	3.46 ± 0.08 b	0.49 ± 0.01 a	3.95 ± 0.09 b	3.39 ± 0.08 b	0.48 ± 0.01 a	3.87 ± 0.08 b	3.43 ± 0.16 b	0.48 ± 0.01 a	3.91 ± 0.16 b
	S2S[b]	2.94 ± 0.08 de	0.40 ± 0.01 cd	3.34 ± 0.09 c	2.94 ± 0.07 de	0.40 ± 0.02 cd	3.34 ± 0.07 c	2.95 ± 0.10 de	0.40 ± 0.01 cd	3.35 ± 0.10 c
	S2C[b]	3.76 ± 0.10 b	0.45 ± 0.02 b	3.76 ± 0.11 b	3.27 ± 0.09 b	0.44 ± 0.01 b	3.71 ± 0.09 b	3.29 ± 0.12 b	0.44 ± 0.02 b	3.73 ± 0.12 b
	S3S[b]	2.33 ± 0.04 h	0.30 ± 0.01 e	2.63 ± 0.05 e	2.38 ± 0.07 h	0.30 ± 0.01 e	2.68 ± 0.07 e	2.36 ± 0.17 h	0.30 ± 0.01 e	2.66 ± 0.17 e
	S3C[b]	3.30 ± 0.07 bc	0.41 ± 0.01 c	3.71 ± 0.08 b	3.33 ± 0.06 bc	0.42 ± 0.02 c	3.75 ± 0.06 b	3.36 ± 0.06 bc	0.42 ± 0.02 c	3.78 ± 0.06 b
brow button	S1S[b]	4.82 ± 0.10 a	0.27 ± 0.02 f	5.09 ± 0.12 a	4.75 ± 0.10 a	0.27 ± 0.01 f	5.02 ± 0.10 a	4.77 ± 0.03 a	0.27 ± 0.01 f	5.04 ± 0.03 a
	S1C[b]	4.56 ± 0.05 a	0.38 ± 0.01 d	4.94 ± 0.05 a	4.49 ± 0.09 a	0.38 ± 0.02 d	4.87 ± 0.09 a	4.51 ± 0.02 a	0.38 ± 0.02 d	4.89 ± 0.02 a
	S2S[b]	2.67 ± 0.08 g	0.20 ± 0.01 g	2.87 ± 0.08 d	2.72 ± 0.06 g	0.20 ± 0.01 g	2.92 ± 0.06 d	2.70 ± 0.14 g	0.20 ± 0.01 g	2.90 ± 0.14 d
	S2C[b]	3.06 ± 0.08 cd	0.40 ± 0.02 cd	3.46 ± 0.08 c	3.12 ± 0.08 cd	0.40 ± 0.02 cd	3.52 ± 0.08 c	3.11 ± 0.13 cd	0.40 ± 0.02 cd	3.51 ± 0.13 c
	S3S[b]	2.04 ± 0.06 i	0.12 ± 0.01 h	2.16 ± 0.06 f	1.98 ± 0.07 i	0.12 ± 0.01 h	2.10 ± 0.07 f	1.99 ± 0.07 i	0.12 ± 0.01 h	2.11 ± 0.07 f
	S3C[b]	2.71 ± 0.09 ef	0.30 ± 0.02 e	3.01 ± 0.09 d	2.76 ± 0.06 ef	0.31 ± 0.01 e	3.07 ± 0.06 d	2.74 ± 0.05 ef	0.31 ± 0.02 e	3.05 ± 0.05 d

[a] Saponified extraction, the mushroom sample was saponified by alkaline and then extracted using hexane before HPLC analysis; hexane/methanol/water partition, the mushroom sample was vortexed with a mixture of hexane/methanol/water, and the hexane phase was collected for HPLC analysis; hexane extract, the mushroom sample was directly extracted by hexane, and the hexane phase was collected for HPLC analysis. S1S[b], stem at stage 1; S1C[b], cap at stage 1; S2S[b], stem at stage 2; S2C[b], cap at stage 2; S3S[b], stem at stage 3; S3C[b], cap at stage 3. Each value is the mean and standard deviation of three independent runs. Within the same column, values with the same superscript letter are not significantly different from each other by general ANOVA followed by an LSD comparison of means ($P > 0.05$).

*DAD1, 26.930 (320 mAU, -) Ref=26.803 & 27.090 of 09032101.D
*DAD1, 26.263 (505 mAU, -) Ref=26.177 & 26.523 of 09032101.D

Figure 3. UV spectrum of peak 2 (solid line, ergosterol) and peak 3 (dashed line, ergosterol analogue).

younger tissues (**Table 2**). The structure of the ergosterol analogue is currently under investigation.

Distribution of Ergosterol in Different Tissues of Mushrooms and at Different Stages of Development. As shown in **Table 2**, the total ergosterol content of the consumable parts of a mushroom (cap and stem) decreased as they grew. While the decrease was relatively modest among the white button mushrooms, averaging 3.41, 3.32, and 3.01 mg/g DM for S1, S2, and S3 mushrooms, respectively (averaged from the total concentration of ergosterols in the caps and stems by taking into consideration the stem/cap ratio), it was highly significant among the brown button mushrooms, averaging 4.66, 2.93, and 2.49 mg/g DM for S1, S2, and S3 mushrooms, respectively. S3 mushrooms are of typical commercially harvested size or growth stage, and contained 3.30 and 2.71 mg ergosterol/g DM in the caps of the white and brown mushrooms, respectively, and 2.33 and 2.04 mg ergosterol/g DM in the stems, respectively (**Table 2**). These data agree well with those reported for commercial button mushrooms which typically range between 2.70 and 7.8 mg/g DM (*14*, *15*, *23*). **Table 2** also

shows how ergosterols are distributed in different parts of mushrooms and how maturity (growth stage) affects the concentration and distribution pattern. Ergosterol concentration in the cap of the white button mushroom did not change over the 3 growth stages; however, in stems the values were significantly different, decreasing from S1 to S3 (**Table 2**). In the brown button mushroom, the ergosterol concentration decreased significantly from S1 to S3 in both the cap and the stem (**Table 2**). There was no significant difference between the concentrations of ergosterol in the cap and the stem at S1 for both the white and the brown mushrooms; however, as the mushrooms grew, the ratio of ergosterol between the cap and stem increased significantly (**Table 2**). Our results also showed that ergosterol concentration of the brown button mushroom was significantly higher than that of the white button mushroom at S1; however, this was reversed as the mushrooms become more mature (**Table 2**). Varietal differences and the effect of developmental stage on ergosterol production in mushrooms have not received much attention even though one paper by Jasinghe and Perera (*23*) showed the
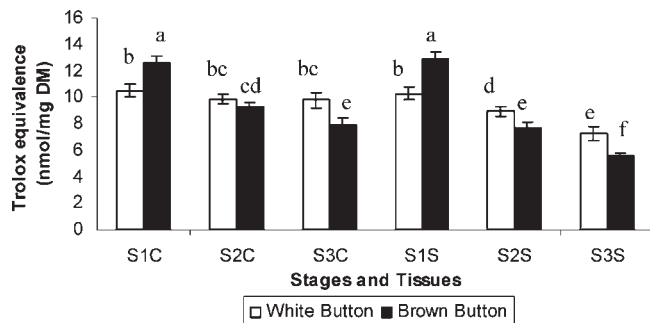
Figure 4. Antioxidant activity of hexane extract from mushrooms using the PCL assay. S1C, cap at stage 1; S2C, cap at stage 2; S3C, cap at stage 3; S1S, stem at stage 1; S2S, stem at stage 2; S3S, stem at stage 3.

distribution pattern of ergosterol in different tissues of shiitake mushroom, being higher in caps than in stems. To the best of our knowledge, the findings in the present study are the first to report not only differences in ergosterol distribution in the edible parts of mushrooms but also significantly higher concentrations during the early developmental stages. In brown button mushroom, the average concentration of ergosterol at S1 was nearly twice as much as that of S3 mushroom (**Table 2**). This is particularly important for developing ergosterol (precursor of vitamin D_2)-rich mushrooms or related functional food products.

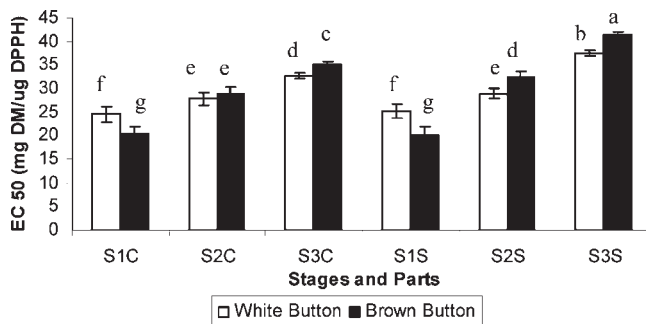**Antioxidant Activity in Hexane Extracts from Mushroom.**
Antioxidant activities present in aqueous and methanol extracts of mushrooms have been well documented (*20*); however, little is known for the lipophilic components in mushrooms. In this study, we tested the antioxidant activities of the hexane extracts using two different assays, namely, the PCL-ACL and DPPH assays. This is the first report of antioxidant activity measured using the PCL assay of mushroom extracts. The PCL assay is based on the photoinduced autoxidation inhibition of luminol by antioxidants, mediated from the radical anion superoxide ($O_2 \cdot ^-$) and is suitable to measure the radical scavenging properties of single antioxidants as well as more complex systems in the nanomolar range. Luminol works as both a photosensitizer and a chemiluminogenic probe for free radicals. The antioxidant potential is measured by means of the lag phase at different concentrations, calculated and expressed as nmol trolox equivalents (*30, 33*). As shown in **Figure 4**, the PCL values of the hexane extracts present in the different mushroom parts at different developmental stages ranged from 5.49 to 10.48 nmol trolox equivalence/mg DM. These values correlated well with the total ergosterol content in the corresponding parts and stages of mushrooms ($r^2 = 0.91$). The PCL-ACL antioxidant activity of the mushroom samples followed a pattern similar to that of the ergosterol content (**Table 2** and **Figure 4**), i.e., the PCL-ACL antioxidant activity in the cap of the white button mushroom did not change over the 3 growth stages but significantly decreased in the stem from S1 to S3 (**Figure 4**). In the brown button mushroom, the antioxidant activity decreased significantly from S1 to S3 in both the cap and the stem (**Figure 4**). Again, the antioxidant activity of the S1 hexane extract of the cap was similar to that of the stem for both the white and brown button mushrooms, but the ratio of the antioxidant activity became significantly in favor of the caps as the mushrooms grew to S2 and S3 stages (**Figure 4**). The PCL-ACL antioxidant activity of S1 brown button extract was significantly higher than that of the S1 white button mushroom; however, this trend was reversed as the mushrooms became more mature (**Figure 4**).

The PCL value of 1 μg of pure ergosterol was 2.46 nmol trolox equivalent. The mechanism behind the antioxidant activity of

Table 3. Percent Contribution of Ergosterol to the Total Antioxidant Activity of Mushroom Hexane Extracts[a]

	white button						brown button					
	S1S[b]	S1C[b]	S2S[b]	S2C[b]	S3S[b]	S3C[b]	S1S[b]	S1C[b]	S2S[b]	S2C[b]	S3S[b]	S3C[b]
PCL assay	91	92	92	93	90	95	96	95	93	93	94	95
DPPH assay	86	86	86	93	97	100	91	89	83	89	80	94

[a] Percent contribution by ergosterol to the total antioxidant activity in a particular assay. In the PCL assay, % contribution = (PCL value of ergosterol × ergosterol concentration)/PCL value of hexane extract) × 100; in DPPH assay, % contribution = (μg of ergosterol in the amount of EC_{50} of mushroom/μg EC_{50} of ergosterol) × 100. PCL value for 1 μg of pure ergosterol, 2.46 nmol trolox equivalent; DPPH EC_{50} for pure ergosterol, 0.112 mg/μg. S1S[b], stem at stage 1; S1C[b], cap at stage 1; S2S[b], stem at stage 2; S2C[b], cap at stage 2; S3S[b], stem at stage 3; S3C[b], cap at stage 3.

Figure 5. Antioxidant activity of hexane extract from mushroom using DPPH assay. S1C, cap at stage 1; S2C, cap at stage 2; S3C, cap at stage 3; S1S, stem at stage 1; S2S, stem at stage 2; S3S, stem at stage 3.

ergosterol is not known; however, the conjugated double bond in its structure might have contributed to the total antioxidant activity found with the PCL and other methods used in this study (**Figure 1**). Using the PCL value of ergosterol, we calculated the percent contribution of total ergosterol content to the total antioxidant activity of the hexane extracts. As shown in **Table 3**, 90−96% of the total PCL-ACL antioxidant activity in the extracts was from ergosterols. The percent contribution was calculated by multiplying the PCL value of standard ergosterol by the total ergosterol content of a sample, followed by dividing by the PCL value of the hexane extract of the same sample, and then multiplied by 100 (**Table 3**). The hexane extracts of mushroom powders may contain lipophilic components other than ergosterols including fatty acids (*34*). Fatty acids did not contribute to the PCL-ACL antioxidant activity in a study by Li et al. (*30*). The high contribution rates of the ergosterol and its analogue to the total antioxidant activity of mushroom extracts suggest that at least with this assay, fatty acids appear to have a very limited independent role (**Table 3**).

DPPH is an alternative antioxidant assay, which has been widely used for the determination of antioxidant activity in the aqueous or methanolic extract of mushroom (*24*). Gopalakrishnan et al. applied this assay to the hexane extract of the white button mushroom but did not establish EC_{50} values (*35*). By using a relatively larger amount of extract in the assay, we were able to calculate the EC_{50} values of the hexane extracts of mushroom samples, which were found to range from 20.19 to 41.49 mg DM for 1 μg of DPPH (**Figure 5**). These values were higher (lower antioxidant activity) compared to the EC_{50} values of the aqueous or methanolic extracts of other mushrooms (*24*). Nevertheless, the DPPH antioxidant activity of the hexane extracts had a trend similar to that found in the PCL-ACL assay, i.e., the higher the ergosterol content, the stronger the antioxidant activity, and the more immature the mushroom (S1), the stronger the antioxidant

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Table 4. Fatty Acid Contents in White Button and Brown Button Mushrooms at Developmental Stage 1[a]

FA (%)	WBcap[b]	WBstem[b]	BBCap[c]	BBstem[c]
10:0	0.13	0.17	0.12	0.1
12:0	0.01	0.02	0.03	0.06
14:0	0.39	0.58	0.33	0.41
15:0 iso	0.03	0.02	0.02	0.03
15:0 ai	0.03	0.03	0.02	0.04
15:0	0.56	0.61	0.85	1.45
16:0	14.1	14.93	12.15	13.63
17:0	0.42	0.53	0.54	0.74
18:0	3.82	4.56	3.71	4.6
19:0	0.05	0.07	0.07	0.13
20:0	2.11	2.33	1.91	2.54
21:0	0.19	0.29	0.26	0.46
22:0	1.35	1.43	1.2	1.52
24:0	1.24	0.91	0.73	0.52
25:0	0.03	0.03	0.03	0.02
26:0	0.02	0.02	0.03	0.01
$\sum$SFA	**24.47**	**26.53**	**22.05**	**26.28**
7c-16:1	0.02	0.03	0.02	0.02
9c-16:1	0.18	0.2	0.25	0.22
10c-16:1	0	0	0	0.02
11c-16:1	0.2	0.08	0.25	0.15
7c-17:1	0.002	0.002	0.002	0.02
9c-17:1	0.08	0.08	0.06	0.06
11c-17:1	0	0	0.01	0.01
6−8c-18:1	0.003	0.01	0.01	0.01
9c-18:1	3.28	1.96	1.5	1.32
10c-18:1	0.02	0.02	0.03	0.02
11c-18:1	0.64	0.41	0.9	0.37
12c-18:1	0.03	0.03	0.04	0.03
13c-18:1	0.01	0	0.01	0
7c-20:1	0.01	0.01	0.02	0.01
9c-20:1	0.05	0.05	0.04	0.04
11c-20:1	0.18	0.16	0.13	0.11
13c-22:1	0.03	0.03	0.05	0.03
15c-24:1	0.26	0.39	0.13	0.1
$\sum$c-MUFA	**5.01**	**3.46**	**3.45**	**2.59**
6−8t-18:1	0.03	0.03	0.04	0.03
9t-18:1	0.13	0.14	0.12	0.12
10t-18:1	0.04	0.04	0.06	0.02
11t-18:1	0.04	0.04	0.06	0.05
12t-18:1	0.03	0.02	0.03	0.02
13t/14t-18:1	0.06	0.06	0.07	0.08
$\sum$t-MUFA	**0.34**	**0.34**	**0.38**	**0.33**
9t12t-18:2	0.02	0.04	0.02	0.02
9c13t-/8t12c-18:2	0.03	0.03	0.04	0.03
8t12c-/9c12t-18:2	0.12	0.11	0.08	0.09
9t12c-/11t15c-18:2	0.07	0.07	0.03	0.05
$\sum$c/t-dienes	**0.27**	**0.26**	**0.2**	**0.26**
$\sum$c/c-18:2	**0.08**	**0.07**	**0.05**	**0.04**
$\sum$unusual PUFA	**0.13**	**0.14**	**0.16**	**0.22**
16:2n-6	0	0	0	0.001
18:2n-6	68.59	67.49	72.99	68.74
20:2n-6	0.12	0.13	0.12	0.09
22:2n-6	0.01	0	0.01	0
20:4n-6	0.01	0	0	0.01
22:2n-6	0.01	0	0.01	0
$\sum$n-6 PUFA	**68.73**	**67.61**	**73.12**	**68.84**
16:3n-3	0.03	0.02	0.02	0.07
18:3n-3	0.06	0.07	0.08	0.14
20:4n-3	0.005	0.01	0.01	0
22:5n-3	0.01	0.01	0	0.02
$\sum$n-3 PUFA	**0.12**	**0.11**	**0.11**	**0.23**
9c,11t-CLA	0.01	0.03	0.02	0.01
9t11c-CLA	0.01	0.01	0.01	0.01
10t12c-CLA	0.02	0.02	0.02	0.02

Table 4. Continued

| FA (%)	WBcap[b]	WBstem[b]	BBCap[c]	BBstem[c]
9t11t-CLA	0.04	0.03	0.03	0.02
$\sum$**CLA**	**0.1**	**0.13**	**0.08**	**0.07**

[a] The values are the mean of at least 4 replicates followed by standard deviation; WB[b], white button; BB[c], brown button.

activity according to the DPPH assay (**Figure 5**). The EC_{50} value of ergosterol by the DPPH assay was 0.112 mg/μg of DPPH, ranking ergosterol a much weaker antioxidant compared to phenolic compounds and tocopherols (*20, 21, 30*), hence the weak free radical scavenging capacity of the mushroom hexane extract. The EC_{50} values in mushroom samples inversely correlated with the ergosterol contents ($r^2 = 0.89$), again indicating that ergosterol is the principle contributor to the antioxidant activity of the mushroom hexane extract. The contribution of ergosterols to the antioxidant activity of the lipophilic extracts of mushrooms is listed in **Table 3**. The percent contributions of ergosterol were calculated similarly to that stated above for the PLC-ACL assay. The ergosterol contribution was between 80 and 100%, confirming that ergosterols were the principle antioxidant components in the mushroom hexane extract.

Although the antioxidant activity is mainly derived from ergosterol, we also examined the contents of tocopherols, β-carotene, and fatty acids in the mushroom hexane extract, which have been found in some mushrooms. Tocopherols and β-carotene have been found to be 33.33−10.92 mg/g DW in a white mutant of the mushroom *Hypsizigus marmoreus* (*36, 37*), and the total fat content was 0.6−4.7% of the dry weight in the fruiting bodies from 23 species of Indian naturally grown mushrooms (*34*). However, neither tocopherols nor β-carotene was found in the white and brown button mushrooms in the present study.

Fatty Acid Profiles. The fatty acid composition of many types of mushrooms has been investigated (*34, 38, 39*), but only recently some have reported the presence of highly unsaturated PUFAs of up to five double bonds in mushrooms (*39*), while others reported the occurrence of conjugated fatty acids (CLA) (*40*). To analyze such lipids requires methods that protect the integrity of labile lipids and prevent their degradation. For this reason, mushrooms were immediately frozen in liquid nitrogen, freeze-dried, and pulverized to prevent lipolysis of lipids (*41*), and isomerization and/or reaction of fatty acid components during the methylation procedure (*42*). It should be noted that such prior procedures are also mandatory for the analysis of intact antioxidants in mushrooms. Methylation procedures were selected to retain native fatty acid structures and assess possible artifact formation. An acid-catalyzed procedure (HCl/methanol) was used to ensure complete methylation of all lipids, recognizing that it could destroy some lipid components (*42−44*). However, a mild base-catalyzed procedure (saponification followed by methylation using TMS-DAM) was used to ensure the retention of labile lipids, specifically CLA (*42−44*). A similar approach was used to analyze the alkenyl ether lipids and CLA in beef lipids (*43, 44*). The FAMEs produced were then resolved using two separate GC temperature programs to confirm their identity besides the use of authentic standards (*29, 45*).

The total lipid content was greater in white button (caps 10.6 and stems 6.8 mg/g DM) than in brown button mushrooms (caps 6.9 and stems 2.9 mg/g DM). The difference in lipid content between the varieties and parts were similar to the distribution of ergosterol in these mushrooms. The average lipid content of the whole mushroom body was 8.7 mg/g DM for the white button mushroom and 5.1 mg/g DM for the brown button mushroom (at growth stage 2 in this study). These values are similar to those

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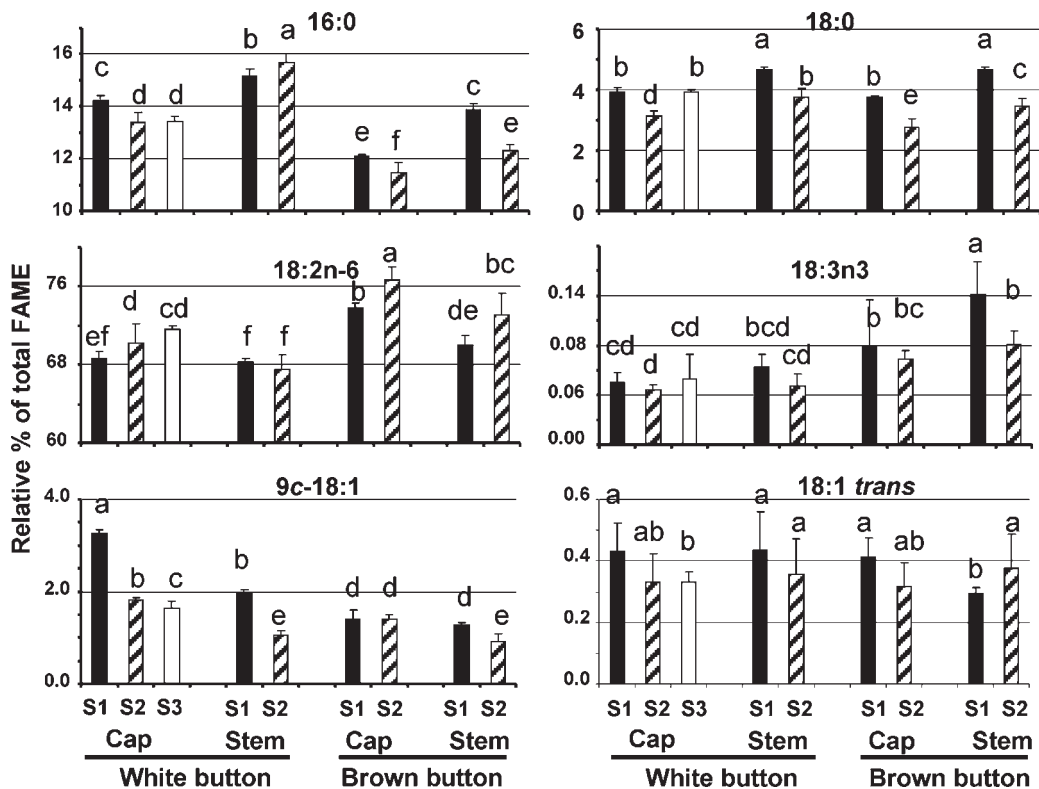
Figure 6. Comparison of the major fatty acids and fatty acid groupings (as a % of total lipids) in the two mushroom varieties (white and brown button), mushroom parts (caps and stems), and stages of development (stage 1, S1; stage 2, S2; and stage 3, S3). Standard error bars and significant differences ($P < 0.05$) are indicated.

reported by Hiroi and Tsuyuki (*38*) but higher than those found in several wild edible mushrooms (*34, 39*). Using chloroform/methanol (ref *38* and the present study) rather than petroleum ether (*34*) will also extract the polar components, which may explain the higher lipid content in some studies.

In the present study, both methylation procedures yielded similar results, which indicate that mushroom lipids do not contain acid labile lipids. The fatty acid composition of immature button mushrooms (S1) presented in **Table 4** represent the average of the two methylation procedures and the two GC temperature programs. The total saturated fatty acid (SFA) content ranged from 22.1 to 26.5% of total lipids and was consistently higher in stems than in caps. Palmitic acid (16:0) was the major SFA at about 14% followed by stearic acid (18:0) at about 4% (**Table 4**). Long-chain SFAs up to 26:0 were detected in these mushrooms, but no short-chain SFAs below 10:0 were found, as previously reported (*46*). In general, the SFA content decreased in both cap and stem lipids as the mushrooms matured, with the exception of white button stems that showed an increase in 16:0 from S1 to S2 (**Figure 6**).

The predominant fatty acid in these mushrooms was linoleic acid (18:2n-6) which ranged from 67 to 76% of total lipids (**Figure 6**). With few exceptions, brown button mushrooms had a higher content than white button mushrooms, higher levels in caps than in stems, and increased with maturation (**Figure 6**). The other essential fatty acid, linolenic acid (18:3n-3), was present only in small amounts (0.06 to 0.14%). However, it was also present in higher amounts in brown mushroom than in white mushroom, but generally there were no differences between mushroom parts and maturation (**Figure 6**). Trace amounts of desaturated and chain elongated products of 18:2n-6 (20:2n-6, 22:2n-6, and 20:4n-6) and 18:3n-3 (18:4n-3, 20:3n-3, 20:4n-3, and 22:5n-3) were detected in these mushroom lipids (**Table 4**), which

is consistent with a previous report (*39*). The pattern of PUFA metabolites appears to be a random formation of desaturated and chain-elongation products rather than the main desaturated and chain-elongated products seen in mammalian systems.

Oleic acid (9c-18:1) was the major monounsaturated fatty acid (MUFA) present at about 1.5% of total lipids. In general, there were only minor differences in the 9c-18:1 content between the varieties, except during the early stage in the caps of white button mushrooms, and stem lipids showed a decrease during maturation (**Figure 6**). It is of interest to note that all MUFAs of chain length 16:1, 17:1, 18:1, and 20:1 contained mainly the 9c- and 11c-isomers (**Table 4**). The formation of the 9c isomers could be explained by the action of Δ9-desaturase on the corresponding SFA analogues, while 7c-16:1 and 11c-20:1 could be explained by peroxisomal chain-shortening and chain-elongation of the more abundant 9c-18:1 isomer, respectively (*29*). However, this does not explain the distribution of the *cis*-16:1 isomers, specifically the origin of 11c-16:1.

Trace peaks corresponding to several CLA isomers were detected in all of the GC chromatograms from both GC separation conditions; total CLA ranged from 0.08 to 0.13%. This random distribution of CLA isomers was observed using both acid- or base-catalyzed methylation procedures, which suggests that these isomers were not specifically the product of acid-catalyzed isomerization during methylation. Unlike the CLA distribution present in ruminant products that show mainly specific isomer formation, such as 9c11t-CLA (*27, 44*), the formation of CLA isomers from PUFA in mushrooms do not appear to be specific. This less specific formation of CLA isomers appears similar to the pattern PUFA metabolites formed, as noted above. We were unable to confirm the presence of larger concentrations of 9c11t-CLA in the same variety of white button mushrooms (*Agaricus bisporous*) as previously reported (*40*), despite using appropriate preparation and methylation conditions

to preserve the CLA structures. Aqueous fractions are currently being investigated as described by Chen et al. (*40*) to see whether this CLA isomer is present in these concentrates.

The amount and distribution of the *trans*-18:1 isomers was of interest since these isomers may indicate improper methodology or specific PUFA metabolism, as in ruminants (*27, 29, 44*). Trace amounts of *trans*-18:1 were observed in both mushrooms ranging in concentration from 0.33 to 0.38% of total lipids, with 9*t*-18:1 as the major isomer followed by 11*t*-18:1 (**Table 4**). There were no significant differences in the total *trans*-18:1 and among the *trans*-18:1 isomers between methylation procedures or between cultivars, mushroom parts, and maturation (**Table 4**). The distribution of *trans*-18:1 isomers does not appear to be a biosynthesis of specific isomers, much the same as the PUFA and CLA isomers distribution.

In conclusion, results obtained in the present study clearly indicated that free ergosterol was the only form present in button mushrooms and the principle contributor to the antioxidant activity of the lipophilic fraction (hexane extract) of the button mushrooms. Concentration of mycochemicals such as ergosterols in the mushrooms depended on the tissue part and growth stage. The ergosterol content and antioxidant activity decreased as the mushroom ages, and in general, the caps contained higher concentrations of ergosterols than the respective stems of mushrooms at the same growth stage. This study also revealed that button mushrooms are poor in tocopherols and β-carotenes but do contain the essential fatty acid, linoleic acid, minor amounts of linolenic acid, and trace amounts of CLA. Results on hydrophilic mycochemicals will be reported in a sequel paper in this journal. Information obtained in this study will provide guidance for mushroom growers to optimize harvesting time for nutritionally superior mushroom products and for the consumers to increase the intake of mushrooms for added health benefits.

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