Mushrooms: A rich source of the antioxidants ergothioneine and glutathione

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Abstract

While mushrooms are the highest dietary source for the unique sulfur-containing antioxidant ergothioneine, little is known regarding levels of the major biological antioxidant glutathione. Thus, our objectives were to determine and compare levels of glutathione, as well as ergothioneine, in different species of mushrooms. Glutathione levels varied >20-fold (0.11 to 2.41 mg/g dw) with some varieties having higher levels than reported for other foods. Ergothioneine levels also varied widely (0.15 to 7.27 mg/g dw) and were highly correlated with those of glutathione ($r=0.62$, $P<0.001$). Both antioxidants were more concentrated in pileus than stipe tissues in selected mushrooms species. *Agaricus bisporus* harvested during the third cropping flush contained higher levels of ergothioneine and glutathione compared to the first flush, possibly as a response to increased oxidative stress. This study demonstrated that certain mushroom species are high in glutathione and ergothioneine and should be considered an excellent dietary source of these important antioxidants.

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1. Introduction

Mushrooms have previously been regarded for being a low calorie, low fat food with little beneficial nutrition. However, in the past decade, research has focused on the roles of mushrooms as a nutritious component of the diet and in the prevention/treatment of chronic diseases (Roupas, Keogh, Noakes, Margetts, & Taylor, 2012). Mushrooms are an excellent source of nutrients, such as riboflavin and other B vitamins, selenium, copper and potassium, and are also rich in dietary fibre, chitin and β-glucans (key constituents of the fungal cell wall) (Feeney et al., 2014). It has also been demonstrated that mushrooms can be an abundant source of vitamin D_2 when exposed to UV light (Kalaras, Beelman, & Elias, 2012; Kalaras, Beelman, Holick, & Elias, 2012). Of particular interest, mushrooms have been found to contain high levels of antioxidants, including phenolic compounds and, in particular, the sulfur-containing amino acid ergothioneine (ERGO).

First identified in 1909 during the investigation of the Ergot fungus *Claviceps purpurea* (Tanret, 1909), ERGO is produced only by fungi and some cyanobacteria (Pfeiffer, Bauer, Surek, Schömig, & Gründemann, 2011) and mycobacteria (Genghof & Van Damme, 1964) from histidine with cysteine and methionine providing the sulfur and methyl groups, respectively (Melville, Eich, & Ludwig, 1957). ERGO is characterized by existing primarily in the thione form, rather than the thiol, in aqueous solution and at physiological pH (Hartman, 1990).

ERGO is found throughout the human body with the highest concentrations found in the red blood cells, liver, kidneys and semen. Although a specific role has yet to be elucidated, ERGO may have significance

in human health due to the presence of a dedicated transporter in many tissues (Gründemann, 2012; Gründemann et al., 2005). It has been proposed that ERGO serves as a critical biological antioxidant based on its ability to act with other antioxidants to protect against oxidative stress in the mitochondria (Paul & Snyder, 2009).

Early work demonstrated that mushrooms contain the highest ERGO levels of any dietary source (Dubost, Beelman, Peterson, & Royse, 2006; Dubost, Beelman, & Royse, 2007; Dubost, Ou, & Beelman, 2007). Other foods with high ERGO content include red beans, oat bran and liver (Ey, Schömig, & Taubert, 2007). It has been demonstrated that ERGO is bioavailable when mushrooms are consumed as part of a meal (Weigand-Heller, Kris-Etherton, & Beelman, 2012).

ERGO has been linked to that of another critical sulfur-containing antioxidant, glutathione (GSH). This ubiquitous tripeptide (γ-glutamyl cysteinyl glycine) is considered the major intracellular antioxidant in nearly all organisms and has additional functions, including detoxification of a wide range of toxins and carcinogens, post-translational regulation of protein function and maintenance of immune function (Giustarini, Rossi, Milzani, Colombo, & Dalle-Donne, 2004). It has been suggested that ERGO can help maintain GSH levels in the presence of an oxidative burden by interacting with other cellular defense systems (Arduini, Eddy, & Hochstein, 1990).

The maintenance of optimal tissue levels of GSH is critical for maintaining health and preventing diseases and even partial GSH depletion can impair immune function (Budihardjo, Oliver, Lutter, Luo, & Wang, 1999) and increase susceptibility to a wide range of xenobiotics (Jollow, 1980) and oxidative

damage (Ellouk-Achard et al., 1995). Low GSH levels are associated with increased risks for cancer (Townsend, Tew, & Tapiero, 2003), cardiovascular diseases, arthritis and diabetes (Nuttall, Martin, Sinclair, & Kendall, 1998). Maintenance of optimal GSH levels may depend, in part, on the dietary intake of GSH. In laboratory studies, dietary GSH was found to be bioavailable and an important source for maintaining intracellular GSH levels in tissues (Kariya et al., 2007; J Vina, Perez, Furukawa, Palacin, & Vina, 1989). Results from our recent clinical trial confirm the effectiveness of oral GSH at increasing body GSH stores (Richie et al., 2015). Consequently, there is a need to determine the major sources of GSH and its inducers in the diet. Previous studies on the GSH content of common foods have identified certain fruits and vegetables, including asparagus and avocados, as particularly rich sources of GSH based upon their content and usual levels of consumption (Jones et al., 1992).

In ERGO-producing organisms, ERGO biosynthesis appears to be dependent upon GSH and its precursors. GSH synthesis involves the sequential addition of Glu, Cys and Gly in a two-step enzymatically catalyzed reaction (Figure 1). The biosynthesis of ERGO involves the methylation of histidine to form hercynine followed by incorporation of sulfur derived from Cys (Figure 1) (Melville et al., 1957). In *M tuberculosis*, this latter step is thought to occur through the incorporation of the GSH precursor γ-glutamylcysteine into hercynine (Richard-Greenblatt et al., 2015). However, recent data in the cyanobacteria *Synechocystis* suggest that GSH itself is essential for EGT synthesis (Narainsamy et al., 2016). Finally, ERGO can induce GSH synthesis by induction of the Nrf2/ARE-mediated signaling pathway (Hseu et al., 2015).

Despite the links between ERGO and GSH, there is limited data regarding the impact of mushroom consumption on GSH levels. In one study, dietary intake of mushroom extracts increased GSH levels, in

various organs, in rats (Jayakumar, Thomas, Ramesh, & Geraldine, 2010). Further, there is little information available regarding mushrooms as a potential source of GSH itself. Thus, to clarify the antioxidant profile of commonly consumed mushrooms, we sought to determine and compare the levels of GSH and ERGO in a wide range of mushroom species.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, all chemicals were of reagent grade and were obtained from Sigma Chemical (St. Louis, MO).

2.1.1. Mushrooms

Fresh mushrooms were graciously donated by Kennett Square Specialties or the Penn State Mushroom Research Center (MRC) and transferred on ice for processing on the day of harvest. The mushrooms were immediately diced and frozen at -20°C. Frozen mushrooms were then lyophilized (Virtis Genesis XL) and homogenized into a fine powder. Mushrooms included brown (crimini) and white button strains of *Agaricus bisporus*, maitake (*Grifola frondosa*), gray oyster (*Pleurotus ostreatus*), shiitake (*Lentinula edodes*), yellow oyster (*Pleurotus citrinopileatus*), pioppini (*Agrocybe aegerita*), pom pom (*Hericium erinaceus*), reishi (*Ganoderma lucidum*), and portabella (*Agaricus bisporus*) (Table 1). In the case of shiitake, yellow oyster, pioppini and portabella, the mushrooms were also divided into pileus (cap) and stipe (stem) prior to freezing and lyophilization for separate analysis.

Dried mushrooms were obtained from a local grocery store and immediately homogenized into a fine powder. These included porcini (*Boletus edulis)*, morel (*Morchella esculenta)*, chanterelle (*Cantharellus cibarius*), gray oyster, and shiitake.

All mushroom powders were stored at -20°C until analyses were performed.

2.2. Methods

2.2.1. Ergothioneine analysis

ERGO analysis was carried out in triplicate for each sample, as described previously (Dubost et al., 2006) with modifications to achieve proper peak separation. Mushroom powder (0.5g) was weighed into a 15 ml, conical bottom, centrifuge tube to which 7 ml of an ethanolic extraction solution (10 mM dithiotreitol, 100 µM betaine, 100 µM 2- mercapto-1-methyl imidazole) and 3 ml deionized water were added and mixed by vortex for 10 seconds. 2 ml of a 1% SDS solution in ethanol was added and mixed by vortex for 10 seconds. Following centrifugation for 20 minutes at 4000 rpm and 25°C the supernatant was collected in a 15 ml, conical bottom, centrifuge tube and mixed by vortex. 1 ml of the supernatant was then added to a microcentrifuge tube and placed in a speed-vac for 24 hours. The dried residue was reconstituted in 0.5 ml of ultrapure water. Sonication and mixing by vortex was utilized as needed until fully dissolved. The sample was then placed in a microcentrifuge for 1 minute at 10,000 rpm. The supernatant was collected and syringe filtered (0.45 µm) into a glass HPLC vial.

HPLC analysis of ERGO was carried out on a Hewlett Packard Series 1050 system equipped with a photodiode array detector. The column used was an Agilent ZORBAX Eclipse C18 column (4.6 mm × 150 mm, 5 µm; Agilent Technolgies, Wilmington, DE) with a matching guard column. Injection volume was 10 µl. The following mobile phases were used; mobile phase A (3% acetonitrile, 0.1% TEA, 50 mM dibasic sodium phosphate in water), mobile phase B (acetonitrile), mobile phase C (water). Separation was carried out under gradient elution as follows; 0 minutes 15:85 (B:C), 0-5 minutes ramp to 5:15:80 (A:B:C), 5-6 minutes ramp to 10:90 (B:C), 6-8 minutes ramp to 3:97 (B:C), 8-15 minutes 3:97 (B:C). ERGO was detected at 254 nm at a peak retention time of approximately 5.7 minutes, with quantification based on an external standard curve prepared from authentic ERGO.

2.2.2. Glutathione analysis

Mushroom powders were prepared as described above. Powders were homogenized in 10% (w/v) metaphosphoric acid (MPA) and centrifuged at 14,000 g for 2 min. Supernatants were analyzed for free GSH using a DTNB enzymatic recycling method as described previously (J P Richie, Skowronski, Abraham, & Leutzinger, 1996; Tietze, 1969). Acid insoluble pellets were used for the analysis of protein bound GSH (GSSP) following reduction of proteins with KBH_4 and re-acidification with MPA as described previously (Muscat et al., 2004).

2.3. Statistics

All mushrooms were tested in triplicate when available. Associations between ERGO and GSH were performed by calculation of Pearson's regression coefficients. Differences between mushroom species and tissues as well as between flushes were analyzed by ANOVA.

3. Results and discussion

The GSH and ERGO contents of mushrooms varied greatly between species. On a mg/g dry weight (dw) basis, GSH levels ranged from 0.11 in chanterelles to 2.41 in maitake (Table 1). To our knowledge, this is the first comprehensive analysis of GSH levels in different mushrooms. These results demonstrate that GSH levels are high in most of the mushroom species analyzed. Limited data on GSH content in various foods is available. In two previous reports, vegetables, fruits and meats tended to be high in GSH while other foodstuffs were lower (Demirkol, Adams, & Ercal, 2004; Jones et al., 1992). The GSH content of fruits and vegetables have been shown to range primarily in the range of 0.1 to 4 mg/g dw with the highest values found in asparagus (3.9 mg/g dw) (Jones et al., 1992). In the current study, mushroom GSH levels ranged up to 7.8 mg/g dw indicating that the GSH-rich mushrooms are higher in GSH content then previously observed for any other vegetable or fruit. Thus, mushrooms are likely to be an important source of GSH in the diet, particularly in regions with high mushroom intake.

ERGO levels ranged from 0.2 to 7.3 mg/g dw with the highest concentrations being observed for yellow oyster and porcini. These levels are comparable to previous studies, including Dubost et al. (Dubost et al., 2006, 2007) who found very similar ERGO levels in crimini (0.68 and 0.40 mg/g dw, compared to 0.471 mg/g dw in the present study) and maitake (1.84 and 1.13 mg/g dw, compared to 1.11 mg/g dw in the present study). Previously, high levels of ERGO in shiitake and oyster mushrooms have been observed (Dubost et al., 2007; Ito et al., 2011), but these levels varied slightly from the present study. Variation in yields may not be unusual for such a biological commodity where crop-to-crop variation, differences in production techniques and growth substrate materials or variation due to strain

differences can play an important role. The ERGO level found in this study for porcini (7.27 mg/g dw) is among the highest for any mushroom species reported in the literature.

To compare the abundance of these two important antioxidants, ERGO and GSH, results were expressed on a µmol/g dry weight basis (Table 1). For most mushrooms, ERGO levels were equivalent to or greater than that for GSH, with yellow oyster and porcini being most notable having 4- to 7-fold higher levels of ERGO than GSH. Thus, unlike most living organisms, these mushrooms are unique in that ERGO and not GSH is the most abundant intracellular antioxidant. Based upon the very high levels of both ERGO and GSH, mushrooms clearly represent a uniquely rich dietary source for antioxidants. Given the important role of oxidative stress in disease development and the aging process itself and the well-known protective roles of antioxidants, mushrooms could play a protective role. To date, few epidemiologic studies or human intervention trials have been reported regarding the impact of mushroom intake on oxidative stress and related diseases.

In order to determine if there was a relationship between the GSH and ERGO levels in different mushrooms, a correlational analysis was performed (Fig. 2). GSH and ERGO levels were highly correlated ($r=0.62$, $P<0.001$), suggesting that mushrooms high in GSH tended to be high in ERGO as well. The nature of this relationship is not known but may reflect their linked biosynthetic pathways and a greater need for antioxidant capacity in certain strains based upon their rapid growth and high respiration rate. These results also suggest that mushrooms with high levels of both ERGO and GSH could be associated with greater health benefits when consumed based upon the high antioxidant content.

A trend was observed in all mushrooms tested that the pillei (caps) contained higher levels of both GSH and ERGO than observed in the stipe tissue (Fig. 3), although the only significant differences observed were for ERGO in yellow oyster and shiitake. One possible explanation for this trend may be that the gill and pileus tissues are where most of the metabolic and reproductive processes occur as opposed to the stipe, which functionally serves as conduit for uptake of nutrition from the growth substrate. Thus, in order to obtain the highest concentrations of antioxidants, the pileus could be selectively harvested. We observed that the pileus of yellow oysters contained very high levels of ERGO (7.18 mg/g dw). Previously, ERGO content for yellow oysters had been reported at 3.46 mg/g dw (Huang, Lin, & Tsai, 2015) and 2.85 mg/g dw (Chen, Ho, Hsieh, Wang, & Mau, 2012). These differences are likely due to the inclusion of both the pileus and stipe tissues in the latter two studies.

White button mushrooms, unlike other species, are typically harvested in 3 successive flushes (approximately one week apart) for each crop. In order to examine the potential impact of flush, white button mushrooms of different flushes were obtained from the Penn State MRC on the day of harvest. ERGO and GSH followed similar trends, the contents being similar in the 1st to 2nd flushes but were increased in the third flush (Table 2), this is in agreement with a previous study (Dubost et al., 2007). The increases during the 3rd flush may be explained as a response to stress caused by nutrient depletion, waste materials building up and available water decreasing in the compost. Consistent with this was an increase in protein bound GSH levels observed during the third flush. While the majority of GSH in cells is in the reduced form, protein-bound GSH, an oxidation product, is also present and its levels are known to be increased during periods of oxidative stress (Muscat et al., 2004). As an adaptive response to the

increasing stress levels, it may be expected that the linked synthetic pathways for GSH and ERGO are induced in an attempt to provide protection and enhance detoxification in the growing mushroom body.

4. Conclusions

Overall, the present findings have identified several types of mushrooms as being uniquely high in both GSH and ERGO. GSH plays a critical role as the master antioxidant in mammalian cells and tissues and dietary intake of GSH has been identified as an important source of GSH for the body. Recent studies have also identified ERGO as a potentially important antioxidant/nutrient with potential preventive properties. Our findings, that mushrooms (particularly the yellow oyster and porcini) are a rich dietary source of these critical antioxidants, may have important translational implications, suggesting that mushroom consumption may be associated with reductions in oxidative stress-related diseases and disorders. Thus, future studies aimed at assessing the health consequences of mushroom consumption are warranted.

Conflict of interest statement: The authors have no conflicts to report.

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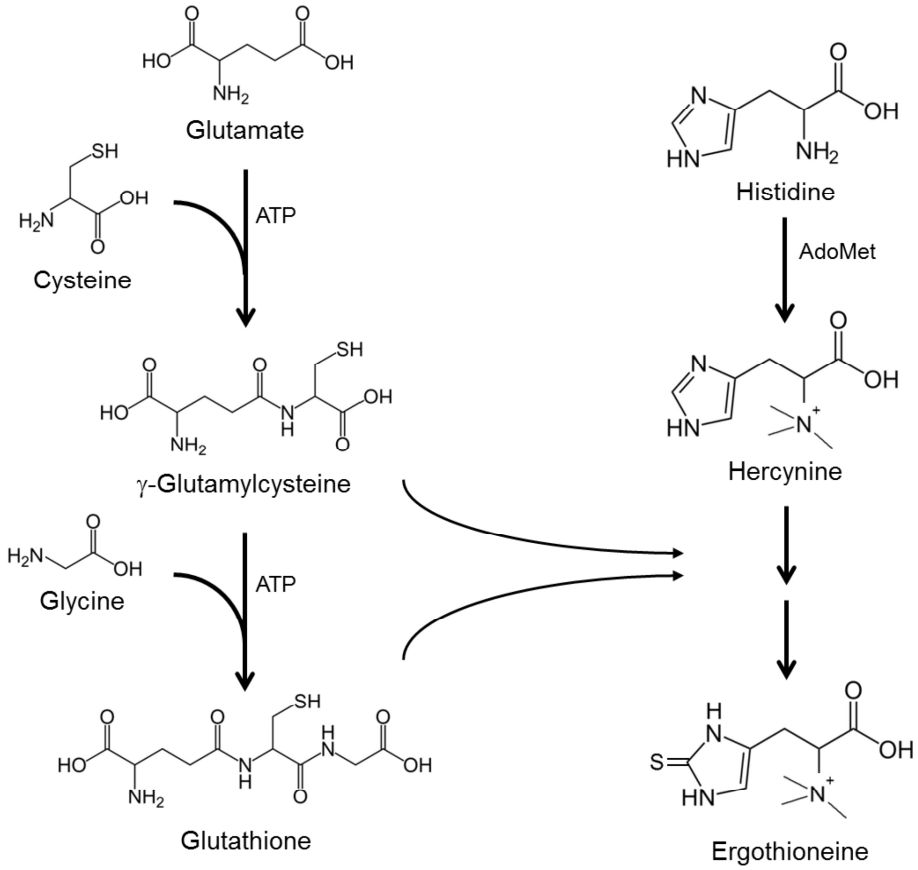
Figure Legends

Figure 1. Biosynthetic Pathway for Ergothioneine and Glutathione

Figure 2: Regression of Glutathione and Ergothioneine Contents of Mushrooms

Figure 3. Glutathione and Ergothioneine Content in Pileus and Stipe Tissues of Selected Mushroom Species. Bars represent mean ± SD. *Denotes significant difference ($p<0.05$) between pileus and stipe of same species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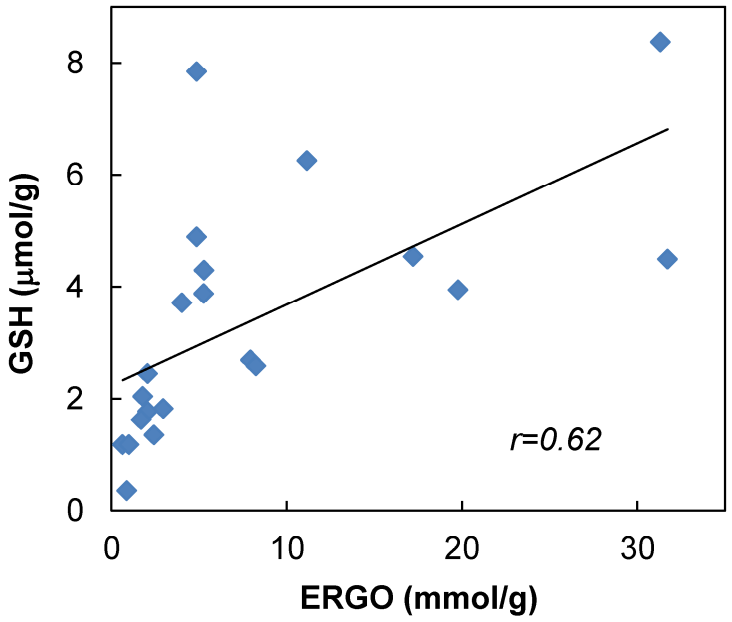

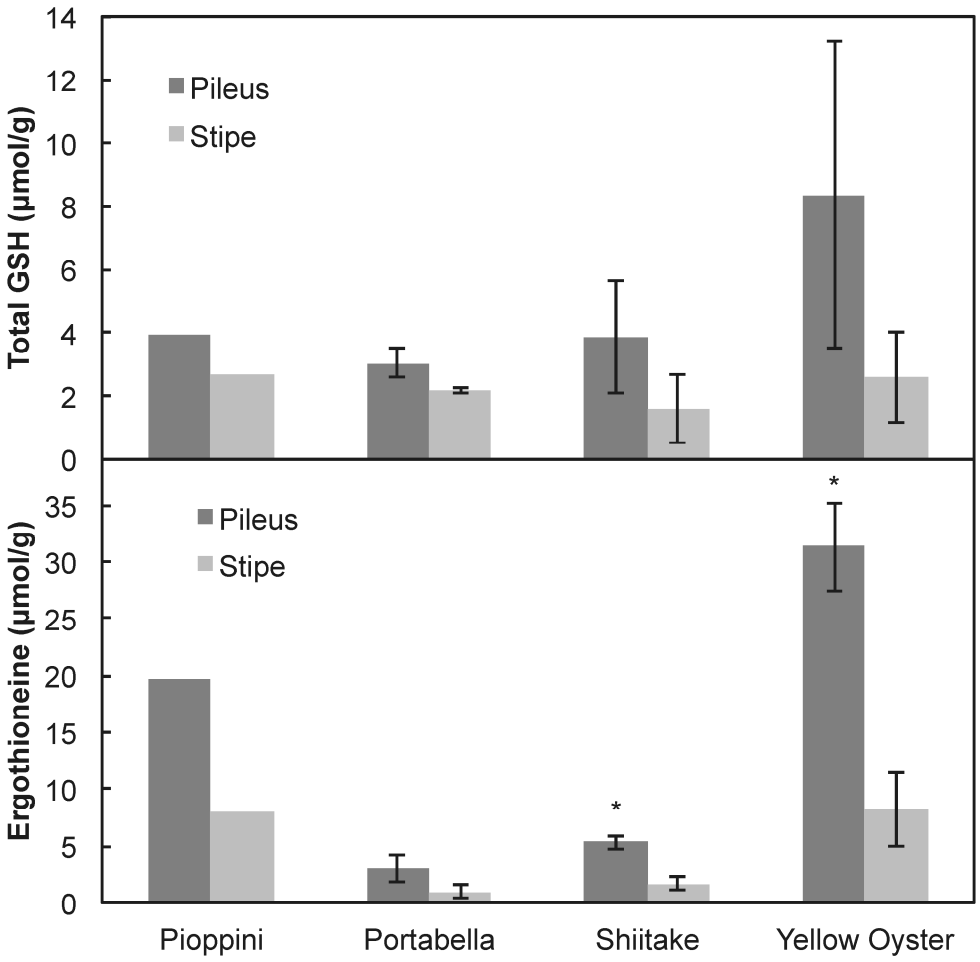

Table 1. Total Glutathione and Ergothioneine Content of Mushrooms

		Total Glutathione			Ergothioneine		
	n	(mg/g d.w.)	(umol/g d.w.)	(mg/g w.w.)	(mg/g d.w.)	(umol/g d.w.)	(mg/g w.w.)
Agaricus bisporus							
White Button	22	0.63 ± 0.57	2.04 ± 1.85	0.055 ± 0.058	0.41 ± 0.18	1.78 ± 0.77	0.034 ± 0.018
Crimini	3	0.54 ± 0.52	1.77 ± 1.70	0.034	0.47 ± 0.16	2.05 ± 0.69	0.025
Portabella	1	0.36	1.17	0.037	0.15	0.64	0.016
Pleutotus ostreatus							
Gray Oyster	5	1.32 ± 0.45	4.29 ± 1.46	0.154 ± 0..055	1.21 ± 0.25	5.27 ± 1.09	0.103 ± 0.020
Pleutotus citrinopileatus							
Yellow Oyster	1	1.39	4.54	0.138	3.94	17.21	0.417
Lentinula edodes							
Shiitake	3	1.14 ± 0.46	3.71 ± 1.48	0.101 ± 0..055	0.92 ± 0.29	4.02 ± 1.28	0.112 ± 0.071
Grifola Frondosa							
Maitake	3	2.41 ± 2.00	7.84 ± 6.52	0.341 ± 0.233	1.11 ± 0.49	4.86 ± 2.14	0.158 ± 0.025
Ganoderma lucidum							
Reishi	1	0.41	1.34	0.058	0.56	2.43	0.080
Hericium erinaceus							
Pom Pom	2	1.50 ± 0.56	4.88 ± 1.83	0.286	1.12 ± 0.12	4.87 ± 0.51	0.181
Agrocybe aegerita							
Pioppini	1	1.92	6.25	N/A	2.56	11.15	N/A

Cantharellus cibarius							
Chanterelle	1	0.11	0.35	N/A	0.20	0.86	N/A
Boletus edulis							
Porcini	1	1.38	4.49	N/A	7.27	31.73	N/A
Morchella esculenta							
Morel	1	0.75	2.44	N/A	0.47	2.06	N/A

*n denotes replicates from separate crops; N/A denotes wet weight basis not applicable, as these samples were obtained as dried material

Table 2. Glutathione and Ergothioneine Content of White Button Mushrooms by Flush

Flush	Bound GSH (μmol/g)	Total GSH (μmol/g)	ERGO (μmol/g)
1	0.18 ± 0.07	1.63 ± 1.62	1.80 ± 0.68
2	0.17 ± 0.17	1.19 ± 1.30	1.08 ± 0.33
3	0.32 ± 0.06*	3.71 ± 2.34*	2.70 ± 0.68*

* Significantly different from 2nd flush, P<0.05

Highlights:

- Glutathione levels varied >20-fold in different mushroom varieties and were highly correlated with ergothioneine levels
- Glutathione content of some mushroom species were higher than those previously found in other foods
- Mushroom species high in glutathione and ergothioneine are an excellent dietary antioxidant source