The Pennsylvania State University

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Department of Food Science

THE BIOAVAILABILITY OF ERGOTHIONEINE FROM MUSHROOMS AND THE ACUTE EFFECTS ON ANTIOXIDANT CAPACITY AND BIOMARKERS OF INFLAMMATION IN HUMAN PARTICIPANTS

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AuBrei Jayne Weigand

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The thesis of AuBrei Jayne Weigand was reviewed and approved* by the following:

Robert B. Beelman
Professor of Food Science
Thesis Advisor

Penny M. Kris-Etherton
Distinguished Professor of Nutrition

Devin G. Peterson
Associate Professor of Food Science

John D. Floros
Professor of Food Science
Head of Department of Food Science

*Signatures are on file in the Graduate School.

ABSTRACT

The growing demand of functional foods in the market is leading research on bioactive components found in food. Recently mushrooms have been discovered to contain ergothioneine, a known antioxidant. Ergothioneine was discovered in 1909 and although it has been widely studied, its bioavailability from mushrooms in humans is still unknown. The overall goal of this research was to assess the bioavailability of ergothioneine from mushrooms and evaluate its bioactive effect as part of a small pilot study. An HPLC method for the separation and quantification of ergothioneine from red blood cells and plasma was developed. Healthy men (n=10) ages 23-50 years were recruited to assess the bioavailability of ergothioneine through a dose-response time-course study. A randomized crossover design was employed in which each subject consumed a test meal containing 0g, 8g, and 16g of brown button mushroom powder. At baseline (t=0) and at the subsequent time points (0.5, 1, 2, 4, 6 hours) ergothioneine concentrations were measured. In addition, plasma Cu, plasma Se, glucose, triglycerides, HDL, LDL, total cholesterol, $ORAC_{total}$ and C-reactive protein were also monitored. No statistically significant differences were observed among the various end points ($p<0.05$). An ergothioneine response was observed in 90% of the subjects. Of those that responded 67% had an ergothioneine response after the 8 g dose and 100% had a response after the 16 g dose. The postprandial triglyceride response was blunted after the mushroom treatments when compared to the control meal of 0 g. A negative correlation ($R^2=0.9957$) was found for the peak ergothioneine concentration and the percent increase in postprandial triglycerides. $ORAC_{total}$ values decreased with the 8 g and 16 g doses compared to the control meal.

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CHAPTER 1

INTRODUCTION

Problem Statement

The growing demand of functional foods in the market is leading research on active components found in food. Current research performed in this field has shown that many of the functional components found in food provide health benefits beyond basic nutrition. Mushrooms recently have been discovered to contain ergothioneine, a known antioxidant. Ergothioneine was discovered in 1909 and although it has been widely studied, its bioavailability in humans is still unknown. In order to promote the idea of eating a well-balanced diet, rich in fruits and vegetables, it is important to understand the physiological effects due to the consumption of functional foods.

Hypothesis

Our hypothesis is that the human consumption of dried brown button mushroom powder will have a time-dose response on the erythrocyte levels of ergothioneine over a six hour time frame. As the ergothioneine level in the blood increases, inflammation and oxidative stress will decrease.

Objectives

The long-term goal of our research is to assess the bioavailability of ergothioneine from mushrooms and evaluate its bioactive effect. The objectives, designed to establish that mushrooms are a bioactive food that when consumed optimize health, are: (1) to separate and quantify ergothioneine in human plasma and erythrocytes by developing a new analytical technique using High Performance Liquid Chromatography (HPLC); (2) to evaluate the bioavailability of ergothioneine in 10 healthy men ages 23-50 years old through a dose-response

time-course study over 6 hours; (3) to evaluate ergothioneine as an antioxidant using $ORAC_{TOTAL}$ (4) to evaluate ergothioneine as an anti-inflammatory compound by measuring C-reactive protein (CRP) in blood during the postprandial period.

CHAPTER 2

LITERATURE REVIEW

Functional Foods and Phytochemicals

The International Food Information Council defines a functional food as a food or dietary component that may provide a health benefit beyond basic nutrition. Functional foods can be derived from a variety of ingredients such as amino acids, herbal extracts, plant sterols, beta-glucans, and many others (Shahidi, 1997). Foods that provide antioxidant properties, especially those that are natural organic compounds found in plant products, known as phytochemicals, represent the latest trend in functional food development (Maletto, 2007). According to the 2007 USA Health Focus Trend report, 79% of consumers believe that certain foods contain substances that can aid in their current health problems. Consumers have become aware of the benefits of phytochemicals and are aware that they can have antioxidant properties that can result in certain health benefits (Sloan, 2007). The functional food market has been on the rise since consumers gained awareness of the health benefits associated with functional foods. A report titled, *The Functional, Fortified and Inherently Healthy Foods and Beverages: The US Phood Market*, estimated that the functional food market reached 25 billion dollars in 2006, a growth of 30% between the years of 2002-2006, and is predicted to grow to 39 billion by 2011 (just-food.com).

With the growing demand for functional foods, research on the active components within the food has also grown. The Food and Drug Administration requires scientific evidence of the health benefits associated with a functional food ingredients in order for health claims to be made. Companies wishing to make health claims on their products are required to perform scientific research on the ingredients they wish to use. The physiological role that antioxidants play within the body must also be identified to rationalize their importance in the diet.

Inflammation

The biological response or protective reaction of tissues to harmful chemical, physical, or biological stimuli is known as inflammation. Two types of inflammation exist, acute and chronic. Acute inflammation is a short term response with rapid onset, short duration, and obvious signs and symptoms. The initial onset of acute inflammation causes dilation, increased permeability, and flow stagnation of blood vessels. The slow blood flow allows for the accumulation of the leukocytes. Neurtophils and monocytes accumulate in acute inflammation and mainly monocytes and lymphocytes in chronic inflammation. The term chronic inflammation is used when inflammation occurs over a long duration of time with slow onset. Chronic inflammation often occurs when the stimulus that triggers acute inflammation persists or when there is a failure in the mechanisms required to stop the inflammatory response. *De novo* chronic inflammation can occur with persistent infection or autoimmune disease (Crawford, 2008).

A chronic low-level of inflammation is thought to play an important role in many chronic diseases due to the increased release of pro-inflammatory cytokines such as, tumor necrosis (TNF-α), interleukin (IL)-6, and IL-8. Through experimental studies and clinical observations pro-inflammatory cytokines have been linked to the pathogenesis of type 2 diabetes (Guest, 2008), renal disease (Vielhauer, 2007), HIV (Decrion, 2005; Williams, 2008), cardiovascular disease (Ridker, 2000), and Alzheimer's disease (Kamer, 2007; Steinman, 2008). The release of cytokines during inflammation allows them to serve as measurable biomarkers of the response. The production of inflammatory cytokines also leads to the release of acute phase proteins. Nuclear factor kappa-B (NF-κB) present in monocytes, endothelial cells, and smooth muscle cells is activated by hypercholesterolemia, hyperglycemia, oxidized LDL, and elevated levels of

angiotensin II. Activated NF-κB triggers the activation of TNF-α, interleukins, interferons, and adhesion molecules. TNF-α promotes the induction of IL-1 and IL-6 and the production of acute phase proteins such as CRP (Patel, 2001).

Diets high in omega-3 fatty acids, fruits, vegetables, nuts, and whole grains, and low in saturated and trans fat have been associated with decreasing inflammation (Giugliano, 2006). Regular exercise can also have an anti-inflammatory response by reducing the circulating levels of pro-inflammatory cytokines (Wilund, 2007).

Measuring Inflammation

Many markers of inflammation and assay kits to measure them exisit. The most commonly measured include cytokines such as IL-6 and TNF-α, inflammatory response related proteins such as serum amyloid A (SAA) and CRP are also commonly used. A high sensitivity C-reactive protein assay(hs-CRP) has been developed to detect small changes in CRP levels. This assay is useful for detecting and measuring low-grade inflammation in healthy individuals and has been refered to as the "golden marker" of inflammation (Patel, 2001). The hs-CRP assay is widely used in the prediction of cardiovascular disease due to the role of inflammation in the pathogenesis. One study evaluted 12 markers of inflammation as predictors of the risk of cardiovascular events. Of these makers hs-CRP and the ratio of total cholesterol to HDL cholesterol were the only two that independently predicted the risk of cardiovascular events (Ridker, 2000). In addition, CRP has an absence of diurnal variation in healthy subjects (Meier-Ewert, 2001) unlike IL-6 which has been shown to be low in the morning and high before bedtime (Sothern, 1995; Gerbig, 1998). Overall, hs-CRP is gaining popularity as a inflammatory biomarker due to its powerful predictive power of cardiovascular risk.

Free Radicals and Antioxidants

Atoms or compounds that contain at least one unpaired electron are known as free radicals. Reactive oxygen species (ROS), such as superoxide ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2), hydroxyl ($\cdot OH$) and hypochlorite ion (OCl^-), are the free radicals that are most commonly formed in the body due to various physiological or non-physiological mechanisms. Physiologically, ROS can be formed by the auto-oxidation of electron transport carriers, lipid peroxidation, and oxygen metabolism among other mechanisms. The formation of ROS can also be due to air pollutants, irradiation from gamma rays, x-rays, and UV radiation (Moller, 1996). Cell signaling, gene expression, and phosphorylation all depend on the existence of free radicals; hence, a certain amount of free radicals are needed for normal biological functioning (Hancock, 2001). The body is equipped with enzymatic antioxidants, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, to defend against free radicals; however, under increased amounts of environmental stress, or a biological imbalance of oxidation product and antioxidant species, oxidative stress occurs (Matés, 1999). Oxidative stress is defined as the body's disability to balance the free radicals being produced and the degeneration of the reactive species.

The high reactivity of free radicals can cause damage to cells within the body under oxidative stress. Tissue damage is caused by ROS through lipid peroxidation, enzyme oxidation, and protein or DNA damage (Chapple, 1997). Biomolecule damage via oxidation has been linked to many diseases, such as rheumatoid arthritis (Chapman, 1989), muscular dystrophy (Murphy, 1989), amytrophic lateral sclerosis (Bowling, 1993), Alzheimer's (Harris, 1995), respiratory distress syndrome (Gladstone, 1994), and cancer (Turker, 2000), along with many others.

Increasing the enzymatic and non-enzymatic reactions that occur in the body to quench free radicals can be a daunting task. Therefore, the role of antioxidants from other sources has gained much interest in the medical world. Antioxidants, such as vitamin C, vitamin E, thiol compounds and phenolic compounds, can be found in dietary sources. The ability of these compounds to quench free radicals has been found to be useful in maintaining good health and preventing disease. Natural antioxidants have been isolated from common food sources, like fruits, vegetables, teas, spices, seeds, and roots (Shahidi, 1997). Mushrooms, are the primary source of the antioxidant L-ergothioneine (Figure 2.1), and this compound will be the focus of this study. Dietary sources of healthful components are of great importance and were described by Hippocrates over two thousand years ago when he said, "Let food be thy medicine and medicine be thy food".

Measuring Antioxidants

Evaluating an antioxidant's effect on oxidative stress cannot solely depend on concentration measurements. Due to various efficacies among antioxidants, the reductive capacity to inhibit oxidation needs to be assessed as well. Total antioxidant potential is commonly measured, since many antioxidants work together to produce an effect. Antioxidants can be classified as enzymes, large molecules, or small molecules. Numerous methods have been developed, most of which are based on the potential of reducing free radicals.

A standard method for measuring the antioxidant capacity of a food is the oxygen radical absorbance capacity (ORAC). This assay measures the oxidative degradation of fluorescein when reacted with peroxy radical. The degradation of the antioxidant of interest is compared to trolox, a standard antioxidant. ORAC values are reported in units of Trolox Equivalent (TE) / g

food product. $ORAC_{TOTAL}$ values are found by analyzing both the lipophilic and hydrophilic antioxidant components of a food.

Choosing a method to measure antioxidant potential can pose a challenge due to the lack of correlation between methods. An extensive meta-analysis on oxidative stress concluded that there is not one single method that can be used universally, due to the different types of oxidative stress. Strong correlation was found between methods when peroxidation products were measured; however, there was a lack of correlation to antioxidant concentration. Due to the various types of oxidative stress, it is suggested that a combination of methods should be used (Dotan, 2004).

Discovery and Properties of Ergothioneine

Ergothioneine [2-mercaptohistidine trimethylbetaine] was first isolated from rye grain that was infected with ergot fungus, *Claviceps purpurea* (Tanret, 1909). Hunter and Eagles (1925) later discovered an unknown compound in human blood that was identified by Eagles and Johnson (1927) as ergothioneine. The discovery of ergothioneine and its presence in human blood made it a prevalent focus of scientific research from the 1920s to the 1950s.

Ergothioneine, the betaine of thiolhistidine, has the empirical formula $C_9H_{15}N_3SO_2$, it is soluble in water up to 0.9M at room temperature, and is nearly insoluable in hot methanol, acetone, and hot ethanol (ed. Budavari, 1996). The pK_{SH} ranges from 10.4 to 10.5 (Motohashi, 1976) and the λ_{max} is 258 nm (Heath, 1958). Ergothioneine is the only naturally occurring 2-thio-imidazole known at this time.

Ergothioneine is synthesized in fungi and mycobacteria from three amino acids, cysteine, methionine, and histidine. Histidine serves as a precursor of ergothioneine with cysteine

supplying the sulfhydryl moiety and methoionine providing the three methyl groups (Melville, 1956). The reaction sequence for the biosynthesis of ergothioneine begins with the methylation of histidine to form a hercynine intermediate which then undergoes sulfhydration to form ergothioneine (Askari, 1962). Ergothioneine readily tautomerizes between the thiol and thione forms (Figure 2.1) however, in aqueous solutions it is predominantly present as the thione rather than the thiol. The thione form is predominating in aqueous solution due to its ability to resonance stabilize. At physiological pH the thione form also dominates.

Dietary Sources of Ergothioneine

Animal species are not capable of synthesizing ergothioneine; therefore, the presence of ergothioneine in animal and man originates from dietary sources (Melville, 1954). Red beans, oat bran, and meat products, such as liver and kidney, are major dietary sources of ergothioneine. Another good source of ergothioneine can be found in mushrooms (Dubost, 2006; Ey, 2007). It was reported that ergothioneine content in mushrooms ranged from 0.4-2.0 mg/g (dry weight). Within the most commonly consumed strain, *Agaricus bisporus*, white button mushrooms contain the least amount of ergothioneine (0.41 mg/g (dry weight) and portebellas contain the most ergothioneine(0.68 mg/g (dry weight). Specialty mushrooms, such as, *Pleurotus eryngii, Grifola frondosa, Pleurotus ostreatus,* and *Lentinus edodes* contain significantly more ergothioneine than the commonly consumed button mushrooms (Dubost, 2006).

Antioxidant Properties of Ergothioneine

Antioxidants are described as molecules that slow or inhibit the oxidation of molecules. Ergothioneine exerts antioxidant properties through multiple mechanisms, including; (1)

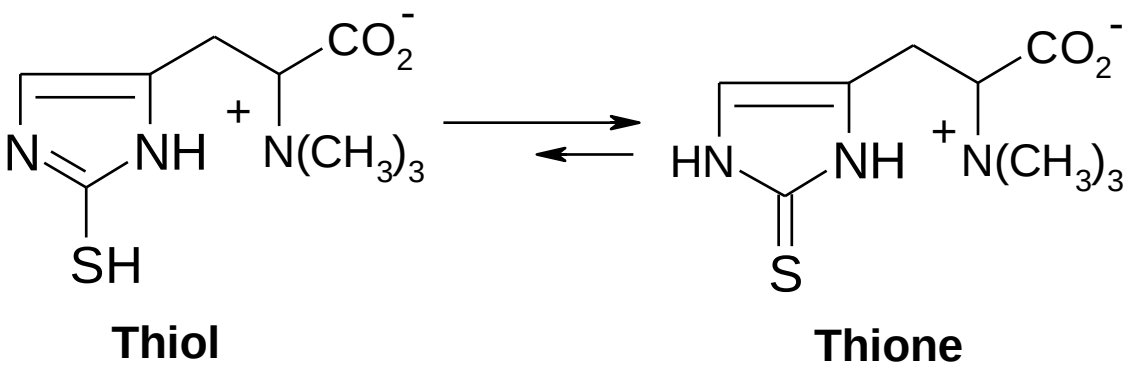


Figure 2.1 Thiol and Thione Structures of Ergothioneine

scavenges free radicals (2) chelates metal ions (3) interferes with the release of pro-inflammatory cytokines (4) affects the oxidation of hemoproteins (5) activates or inhibits enzymes. Ergothioneine has also been shown to have neuroprotective and photoprotective properties. The presence of antioxidants like ergothioneine in the body decreases the production of free radicals and can be preventative for some diseases.

An *in vitro* study revealed that ergothioneine, when compared to classic antioxidants such as, glutathione, uric acid, and trolox, is better at scavenging peroxyl (ROO·), hydroxyl (HO·), and peroxynitrite (HOONO·) radicals (Franzoni, 2006). The ability of ergothioneine to scavange peroxynitrite has been attributed to inhibiting pheochromocytoma (PC12) cell death in rats induced by β-Amyloid. The peptide β-Amyloid is a major component of senile plaques which contributes to the development and progression of Alzheimer's disease (Jang, 2003). Ergothioneine has also been shown to inhibit apoptosis induced by H_2O_2 in PC12 cells. At ergothioneine concentrations of 0.25 mM and 1.0 mM the viability of cells incubated with 0.25 mM H_2O_2 for 1h was 13% and 34% in respect to ergothioneine concentration. A challenge study also displayed the protective effect of ergothioneine. PC12 cells were incubated for 1 hour with 0.25 mM and 1.0 mM of ergothioneine and challenged against a 23 hour H_2O_2 incubation. Cell viability increased 21% and 42% in respect to ergothioneine concentration (Colognato, 2006). Aside from Alzheimer's disease the basic ability of ergothioneine to scavenge free radicals aids in the prevention of many diseases associated with free radical damage.

Ergothioneine has also been shown to inhibit the ion-dependent generation of the hydroxyl radical by iron and copper through its ability to chelate metal ions. Through its interaction with $Cu^{2+,}$ ergothioneine also inhibits the copper-ion dependent oxidation of oxyhemoglobin (Akanmu, 1991). Metal complexes of ergothioneine are formed with Cu(II),

Hg(II), Zn(II), Cd(II), Co(II) and Ni(II), the relative stability of the metal-ergothioneine complex is in decreasing order respectively (Motahashi, 1974). Since the ergothioneine-Cu(II) complex was found to be the most stable the interaction between the two was investigated further. Through the use of optical electron spin resonance (ESR) and nuclear magnetic resonance (NMR) it was concluded that the coordination of the complex was established through the sulfur atom of ergothioneine (Motohashi, 1976). The importance of metal chelation by ergothioneine was shown when Akanmu et al. found that ergothioneine was able to inhibit the generation of hydroxyl radicals when water or ascorbic acid was exposed to copper ions. The ability of ergothioneine to chelate and inhibit the production of hydroxyl radicals shows its importance in the prevention of oxidative damage (Akanmu, 1991). Through the chelation of metal ions with substances like ergothioneine, the body's burden of toxic metal ions can be reduced. Site specific oxygen radical damage can result when copper ions bind to albumin, carbohydrates, enzymes, or DNA (Halliwell, 1990).

A chronic low-level of inflammation is thought to play an important role in many chronic diseases due to the increased release of pro-inflammatory cytokines such as, tumor necrosis (TNF-α), C-reactive protein (CRP), interleukin (IL)-6, and IL-8. Pro-inflammatory cytokines have been linked to the pathogenesis of many diseases which has been discussed above. Oxidants and pro-inflammatory cytokines can activate the release of transcription factors like nuclear factor- κβ (NF-κβ). Nuclear factors such as NF-κβ has been shown to activate the transcription of IL-8 and therefore, plays an important role in inflammation (Rahman, 2002). The effect of ergothioneine on the activation of NF- κβ by the release of TNF-α was studied in alveolar epithelial cells (A549). Ergothioneine was reported to inhibit the activation of NF- κβ when cells were exposed to H_2O_2 and TNF-α. The release of IL-8 from A549 cells was also

inhibited by ergothioneine when exposed to H_2O_2 and TNF-α (Rahman, 2003). The role of ergothioneine in these cases shows its importance as an anti-inflammatory compound.

The oxidation of myoglobin (Mb) by H_2O_2 produces ferryl myoblobin (Mb^{IV}) and other free radicals which are known to cause tissue damage due to their ability to oxidize cellular constituents. The cellular damage of hypervalent myoglobin has been associated with cardiac ischemia/reperfusion. Ergothioneine was discovered to efficiently reduce Mb^{IV} to Mb^{III} and therefore, protect against cellular damage associated with ischemia and reperfusion when oxygen is reintroduced (Arduini, 1990).

Lipid peroxidation refers to the degradation of lipids through oxidation reactions which produce free radicals that cause cell membrane damage. Lipid peroixidation can occur through a non-enzymatic pathway or through a NADPH-dependent enzymatic reaction. It was found that ergothioneine not only inhibits NADPH-cytochrome c reductase *in vitro* by 50% using 5 mM of ergothioneine but also increases glutathione peroxidase and glutathione reductase which have an inhibitory effect on lipid peroxidation by inhibiting the deposition of lipid peroxides. Heating ergothioneine in boiling water for 5 minutes had no effect on its ability to protect against lipid peroxidation (Kawano, 1983). An *in vivo* study performed in rats using a Fenton chemistry model, which makes use of the iron complex of the chelating agent nitrilotriacetic acid (Fe-NTA), demonstrated that ergothioneine protects against lipid oxidation in both the kidney and liver. Ergothioneine protected the kidney by 50% and the liver by 26% against Fe-NTA induced lipid peroxidation (Deiana, 2003). Protecting organs against lipid oxidation by dietary supplementation with ergothioneine aid in preventing diseases caused by ROS. A recent virus gene-based assay showed that beta-galactosidase (beta-gal) activity increases when the long terminal repeat of HIV-1 is enhanced. The presence of ergothioneine was shown to inhibit the

beta-gal activity, suggesting that its antioxidant properties may be beneficial for HIV patients (Xiao, 2006).

Ergothioneine has also been shown to be neuroprotective in an *in vivo* rat retinal model. Injury to neurons may be caused by an overstimulation of the neuron receptors. A receptor that plays a key role in normal brain function is the *N*-methyl-D-aspartate (NMDA) receptor. Over stimulation of the NMDA receptor is known to produce excess free radicals; therefore, causing cell death within the neuron. Injections of ergothioneine into the vitreous body of the rat eye protected neurons against induced cell degeneration/loss by NMDA excitotoxicity. (Moncaster, 2002). The ability to protect neurons from apoptosis or degeneration is important when considering conditions such as Alzheimer's, a chronic neurodegenerative disease.

Ergothioneine can also act as a photoprotectant. DNA lesions due to exposure to ultraviolet A (UVA, 315-400 nm) and visible light may lead to carcinogenesis. At a concentration of 0.5 mM ergothioneine was found to effectively reduce DNA lesions, induced by light, by 97.9% against visible light and 59.8% against UVA/visible light. When a concentration of 0.25 mM of ergothioneine was used there was a 44% protection against visible light and 40.4% protection against UVA/visible light. The dose-dependent photoprotectant effect of ergothioneine against both UVA and visible light can be attributed to its ability to act as an antioxidant and protect against oxidative damage caused by solar radiation (Botta, 2008).

After the discovery of the presence of ergothioneine in various mushrooms the total antioxidant capacity was measured using the $ORAC_{TOTAL}$ method. White button and portabella mushrooms contain 586 and 823 $ORAC_{TOTAL}$/serving (µmol of TE), respectively (Dubost, 2006).

Uptake and Metabolism of Ergothioneine

The biological distribution of ergothioneine in many species has been investigated. Mellville analyzed the distribution of ergothioneine in various tissues of species, including rabbit, cat, dog, and rat. The tissue concentrations vary among species; however, concentrations were highest in the liver, kidney, and red blood cells. Radioactive labeled ergothioneine (synthesized with ^{35}S) was used to study the metabolism of ergothioneine in rats. Rats were allowed a 25 mg/100 mL solution of ergothioneine in tap water for 21 days *ad libitum.* The presence of radioactive labeled ergothioneine was reported in all organs; however, the highest accumulations present in the liver, kidney, blood cells, and bone marrow. The ergothioneine concentration present in urine dropped from 180 mg/100 mL to 12 mg/100 mL after 5 days of discontinuing the ergothioneine/water solution. Unlike thiolhistidine, ergothioneine was concluded to be absorbed and retained by rats after consumption (Heath, 1953).

Another *in vivo* rat study was performed by injecting rats with, 0.8 or 1.6 mg per 100 g body weight, of ^{3}H-ergothioneine solution into the femoral vein. The retention of radioactive ergothioneine in various tissues and the rate of metabolism were measured.. The decrease of radioactivity due to ^{3}H-ergothioneine was faster in plasma than in whole blood. The half life of ergothioneine in whole blood and whole-body was found to be 18 minutes and 1 month respectively, with the metabolic turnover rate in various organs (liver, kidney, spleen, lung, blood) being low. Ninety percent of the ^{3}H-ergothioneine was found to be incorporated into the red blood cells after 24 hr regardless of the level of ergothioneine administered (Mayumi, 1978).

Ergothioneine is known to be present in the blood of humans but early studies found that humans are unable to synthesis the compound (Melville, 1954). The ability of human erythrocytes to take up and retain ergothioneine *in vitro* is time and concentration dependent, human erythrocytes contain 2 to 9 fold more ergothioneine than plasma. A maximum

intracellular uptake of ergothioneine was observed; however, the effect was not consistent among blood donors (Mitsuyama, 1999). The concentration variation of ergothioneine in human blood with respect to age was investigated in 400 healthy Saudi Arabian males. The study revealed that the concentration of ergothioneine in the red blood cells is lowest at the early stages of life (1-10 years) and increases gradually between years 11-18 and reaches a maximum value of 3.7 mg/100mL at age 18. The concentration gradually decreases (2.3-3.0 mg/100 mL) from ages 19-50; however, increases again at 51^{+} years (Kumosani, 2001). Urban (2007) reported broad variations in the amount of ergothioneine observed in human erythrocytes and suggested it was due to a genetic variation in OCTN1, a multi-specific transporter, proposed to be responsible for transporting ergothioneine.

Gründemann (2005) renamed the transporter formally known as OCTN1to the ergothioneine transporter (ETT) after determining its high specificity for the physiological substrate, ergothioneine. Ergothioneine is transported over a hundred times more efficiently than tetraethylammonium or carnitine and twenty-five folds better than hercynine; hence, the transporter with gene symbol SLC22A4 (formally known as OCTN1) was named the ergothioneine transporter (ETT). Cells containing ETT reached an intracellular concentration of 850 µmol/liter after 60 minutes of incubation with a 10 µmol/liter ergothioneine solution. Cells lacking ETT only reached an intracellular concentration of 2.6 µmol/liter under the same conditions, demonstrating that cells lacking ETT are impermeable to ergothioneine. (Gründemann, 2005). Specific uptake of ergothioneine by ETT is strongly stimulated by extracellular sodium ions. The expression of ETT by quantitative real time PCR in human tissues revealed that expression is high in bone marrow, kidney, mammary gland, ovary, and prostate tissue (Gründemann, 2005; Grigat, 2007).

Measuring Ergothioneine in Erythrocytes

The quantitative determination of ergothioneine in human blood has been investigated since 1926. One of the first reported quantification of ergothioneine was in 1931. A method based on the color development when ergothioneine reacted with uric acid reagents was used to estimate ergothioneine in blood (Salt, 1931). Later, a semi-quantitative method using paper chromatography was developed (Mackenzie, 1956). Emerging technology led to a spectrophotometric titration method based on the nature of ergothioneine to resist Cu^{2+} catalyzed oxidation and its rapid reaction with excess 2,2'-dipyridyl disulphide which releases 2-thiopyridone, the moiety responsible for color observation (Carlsson, 1973). A limited number of HPLC methods have been developed for the quantification of ergothioneine in blood. The methods that exist do not focus on the determination in blood alone and those that do require tedious and time consuming preparation techniques and analyses (Mayumi, 1978; Muda, 1988; Newton, 1993). A more time efficient and reproducible HPLC method, specific for its use in human blood, would be a beneficial tool for studying and quantifying ergothioneine.

Mushroom Consumption and the Benefits

The consumption of mushrooms has risen in the United States over the past several decades with the greastest per capa consumption being in the West and Midwest. Consumer demands for fresh produce has led the fresh-market mushrooms to make up two-thrids of the total market. The United States is the second largest producer of mushrooms, acounting for 16% of the world output. Within the United States, Pennsylvania and California are the leading producers of mushrooms (Lucier, 2003).

Mushrooms are a low calorie, low fat food that are commonly misconcieved to have little to no nutritional value. Although mushrooms are over 90% water, they provide many nutrients, such as selenium, potassium, copper, vitamin D, fiber, and B vitamins. According to the United States Department of Agriculture (USDA) edible mushrooms contain 15 IU per serving of vitamin D, and contain complex B vitamins such as, riboflavin (B_2), niacin (B_3), and pathothnic acid (B_5) (USDA, 2008).

Current research has investigated mushrooms and their link to overall human health. An animal study has indicated that white button mushrooms enhance natural killer (NK) cell activity which are related to innate and cell-mediated immunity. The same study also indicated that mushroom supplementation increases the expression and function of dendritic cells which play a role in anti-microbial and anti-tumor defense within the body (Wu, 2007). American's obesity problem has also been addressed by investigating the substitution of white button mushrooms for meat entrees. Substituting mushrooms in place of meat at one meal reduces total calorie intake by 379. Protein intake remained within the RDA range and the feeling of satiety was not compromised. The researchers suggests that making this substitution for one meal each day for one year can lead to a potential loss of 29 pounds (Cheskin, 2007). Mushrooms are also the primary source of the antioxidant ergothioneine ranging from 0.4-2.0mg/g (dry weight) (Dubost, 2006). Within the most commony consumed strain *Agaricus bisporus* white button mushrooms contain about 0.41 mg/g (dry weight) of ergothioneine while portebellas contain 0.68 mg/g (dry weight). Specialty mushrooms, such as, *Pleurotus eryngii, Grifola frondosa, Pleurotus ostreatus,* and *Lentinus edodes* contain significantly more ergothioneine than the commonly consumed button mushrooms. In summary, even though mushrooms contain over 90% water they still provide many vital nutrients and physiological benefits for humans.

CHAPTER 3

SEPARATION AND QUANTIFICATION OF ERGOTHIONEINE IN HUMAN PLASMA AND ERYTHROCYTES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

ABSTRACT

The quantitative determination of ergothioneine in human blood has been investigated since 1926. Many methods have emerged including color estimation, paper chromatography, spectrophotometric titration, and a limited number of High Performance Liquid Chromatography (HPLC) methods. Due to the inaccuracy and consuming preparation techniques that present using methodologies of the past, the objective was to develop a more time efficient and reproducible extraction method and HPLC method for the separation and quantification of ergothioneine in human blood. Red blood cell and plasma extracts from human blood were analyzed with a C18 column using an isocratic mobile phase of aqueous sodium phosphate buffer with 0.2% triethylamine. The lowest limit of quantification and the detection limit was found to be 0.07 mg/100 mL and 0.05 mg/100 mL respectively. Liquid Chromatography-Mass Spectroscopy (LC-MS) was used to verify the identity and purity of ergothioneine from the extraction and quantification techniques employed. Through stability, accuracy, precision, sensitivity, specificity, response function, and reproducibility experiments we ensured that our methodology is acceptable for extracting and quantifying ergothioneine in human blood.

Introduction

Ergothioneine [2-mercaptohistidine trimethylbetaine] was first isolated by Tanret (1909) from rye grain that was infected with ergot fungus, *Claviceps purpurea*. Animal species are not capable of synthesizing ergothioneine; therefore, the presence of ergothioneine in animal and man originates from dietary sources (Melville, 1954). Red beans, oat bran, and meat products, such as liver and kidney, are major dietary sources of ergothioneine. Another good source of ergothioneine is mushrooms (Ey, 2007). Dubost et al. (2006) suggested that mushrooms are the best source of ergothioneine. The ability of human erythrocytes to take up and retain ergothioneine *in vitro* is time and concentration dependent, and human erythrocytes contain 2 to 9 fold more ergothioneine than plasma (Mitsuyama, 1999).

The quantitative determination of ergothioneine in human blood has been investigated since 1926. Early methods involved an estimation technique based on the color developed when ergothioneine reacted with uric acid reagents (Salt, 1931). Paper chromatography was also used as a semi-quantitative method in rat blood (Mackenzie, 1956). A spectrophotometric titration method based on the nature of ergothioneine to resist Cu^{2+} catalyzed oxidation was developed in 1973 (Carlsson, 1973). A limited number of HPLC methods have been developed for the quantification of ergothioneine in blood. The methods that exist do not focus on the determination in blood alone and those that do require tedious and time consuming preparation techniques (Mayumi, 1978; Muda, 1988; Newton, 1993). A more time efficient and reproducible HPLC method, specific for its use in human blood, would be a beneficial tool for studying and quantifying ergothioneine. The goal of this research is to identify and quantifiy ergothioneine in human blood by modifying a HPLC method developed by Dubost et. al (2006) to quantify ergothioneine in edible mushrooms.

Materials and Methods

Chemicals

Ergothioneine was generously donated by OXIS international (Foster City, CA). Dithiotreitol (DTT), acetic acid, acetonitrile (HPLC grade) and sodium phosphate dibasic anhydrous were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium phosphate monobasic anhydrous and triethylamine (TEA) were purchased from Fisher Scientific (Pittsburg, PA).

Standard Solution and Standard Curve

A 0.5 mg/mL stock solution of ergothioneine was prepared by dissolving the pure compound in the extraction solution (10 nM dithiothreitol in 0.1 mM phosphate buffer; pH 8). The stock solution was serially diluted, using the extraction solution, to concentrations ranging from 0.001 mg/mL to 0.2 mg/mL to create a standard curve.

Preparation of Samples

Dubost et. al (2006) developed a method for the identification and quantification of ergothioneine in cultivated mushrooms. The extraction procedure from the mushroom analysis was modified for the use of blood samples. Whole blood was collected from subjects in sodium heparin. Whole blood was centrifuged to separate the red blood cells from the plasma at 2,000 g for 5 minutes. Red bloods cells (0.75mL) were washed two times with 0.75 mL of 0.9% NaCl solution to remove plasma proteins, and centrifugation at 1,700 g for 7 minutes after each wash, pipetting off the supernatant. The extraction solution (10nM dithiothreitol in 0.1mM phosphate buffer pH 8) in the amount of 0.75mL was added to the washed cells. Dithiothreitol was used due to its ability to reduce the disulfide bonds of proteins and prevent the formation of disulfide bonds. Red blood cells were lysed by placing the samples in a 90°C water bath for three

minutes. The samples were then vortexed, and placed back in the water bath for three additional minutes. The samples were then micro-centrifuged for 7 minutes at 12,000 g. The supernatant was filtered through a 0.45 micron syringe filter prior to injection into the HPLC.

Plasma (1.0 mL) samples were extracted in the same manor; however, the wash step was skipped and samples were centrifuged for 40 minutes at 12,000 g after being placed in the 90°C water bath. The high temperature water bath was used to denature plasma proteins. The supernatant was collected and 0.8 mL was added to a clean Eppendorf tube. The liquid was evaporated in a speed vacuum for 7.5 hours. The dried samples were re-suspended in 0.25 mL of extraction solution and filtered through a 0.45 micron syringe filter prior to injection into the HPLC.

Red blood cell samples spiked with ergothioneine were prepared for the identity verification and recovery experiments. The spiked extraction solution was made by diluting the 0.5 mg/mL stock solution with 4.5 mL of the extraction solution (0.1543 g dithiotretol in 0.1 M phosphate buffer). Plasma samples were extracted with an extraction solution spiked with 0.005 mg/mL of ergothioneine. The spiked extraction solutions were spiked with a concentration of ergothioneine equal to the normal ergothioneine concentration found in the unspiked samples of red blood cell and plasma. Red blood cells and plasma samples were extracted using the ergothioneine spiked extraction solutions following the methodology previously described. Identity verification was made by comparing the peak areas of the spiked samples and the unspiked samples. Percent recovery was calculated by quantifying and comparing spiked and unspiked samples by the use of a standard curve.

The stability of the extracted red blood cells was measured by extracting red blood cells as previously described and injecting one sample at time points: 0, 60, 120, 240, and 360 minutes. Stability was also measured by analyzing freeze-thawed samples. The initial (freshely extracted) sample was analyzed as normal. Excess red blood cell extract was stored at -80 °C, for 30 days, thawed and analyzed. The freeze-thaw cycle was repeated for a total of 2 cycles. Samples were separated and quantified by HPLC UV-VIS under the same conditions described above.

Quantification of Ergothioneine by HPLC

The quantification of ergothioneine from extracted human blood was carried out using high performance liquid chromatography (HPLC). A HP1050 series HPLC equipped with a quaternary pump, autosampler, helium degasser, and ChemStation analysis and control software was used for the analysis. A Grace (Deerfield, IL) econosphere C18 column, (250mm x 4.6mm, 5 micron) with an isocratic mobile phase of 0.3M phosphate buffer at pH 7.3 and 10% methanol, run at a flow rate of 0.5mL per min was employed. Injection volume was set at 10 µL and the column temperature was ambient. Absorbance was measured at 254 nm using a HP1050 series variable wavelength diode array detector (DAD). ChemStation analysis software was used for peak area integration. Quantification of ergothioneine in human red blood cells and plasma was determined using the integrated peak areas and the standard curve.

LC-MS

Liquid Chromatography-Mass Spectroscopy was used to verify the identity and purity of ergothioneine from the extraction and quantification technique. The analysis was performed by a Shimadzu HPLC (Shimadzu, Columbia, MD) equipped with a LC-104D pump, a degasser (DGU-14A), an autosampler (SIL-10AD), and a Grace econosphere C18 column (250mm x

4.6mm, 5 micron). The effluent was split 3:2 post column (zero-dead volume Tsplitter; Sepelco, Bellfonte, PA) with 2 parts (200 µL/min) directed to the MS and 3 parts (300µL/min) directed to the UV-VIS. The HPLC conditions were as follows: isocratic mobile phase of 30% acetonitrile and 1% acetic acid in distilled water, flow rate was 0.5 mL/min., ambient column temperature, and injection volume was 10 µL. The mass spectroscopy conditions were as follows: electrospray ionization in positive ion mode, capillary voltage (3.5 kVolts), cone voltage (20 V), extractor voltage (3 V), scan range (130-600 Da), probe temperature (250°C).

Statistical Analysis

All statistical analyses were performed using Excel. Coefficients of variation (CV) were calculated for standard concentrations of ergothioneine and red blood cell and plasma samples during method development.

Results and Discussion

The quantification, of ergothioneine in human blood samples, was validated by performing multiple tests. The reproducibility of the HPLC method utilized for quantification was tested by injecting three standard concentrations of ergothioneine ten times each. The average peak area and standard deviation for each concentration was used to calculate the CV. The CV for all standard concentrations was found to be less than 5%. The lowest limit of quantification was determined by identifying the smallest concentration with a CV less than 5%. The lowest limit of quantification was found to be 0.0007 mg/mL. The detection limit, defined as the smallest concentration able to be distinguished from the absence of the compound, was found to be 0.0005 mg/mL. The calculated CV for the detection limit concentration was 10.2%. The reproducibility of the extraction technique applied to extract ergothioneine from human red blood cells and plasma was measured by performing five extractions of each, red blood cells and

plasma, from whole blood collected from a single blood draw. The CV for the reproducibility or precision of the extraction of ergothioneine was calculated to be 1.6% and 9.7% for human red blood cells and plasma, respectively.

A typical HPLC chromatograph of extracted red blood cells and a sample extracted with a spiked extraction solution helps prove the identity of ergothioneine through the co-elution of the natural ergothioneine found in red blood cells and the added standard (Figure 3.1). Percent recovery, of the extraction method for ergothioneine in human red blood cells and plasma was determined by using a spiked extraction solution. The percent recovery was 107% for red blood cells and 114% for plasma.

The stability of the red blood cell extracts was measured by injecting the same sample at time points 0, 1, 2, 4, and 6 hours. Between injections the sample was held at room temperature to mimic the conditions during normal analysis. The CV among ergothioneine concentrations at the various time points was 1.3%. Low variability among injection time up to 6 hours indicates the stability of ergothioneine within the red blood cell extract. A second stability study was performed by analyzing the difference between a sample that was freshly extracted, and the same sample after two freeze-thaw cycles. The variability was found for the fresh extract, the extract after one freeze-thaw cycle, and the extract after the second freeze-thaw cycle. The calculated CV was 3.9% indicating the stability of ergothioneine through freeze-thaw cycles.

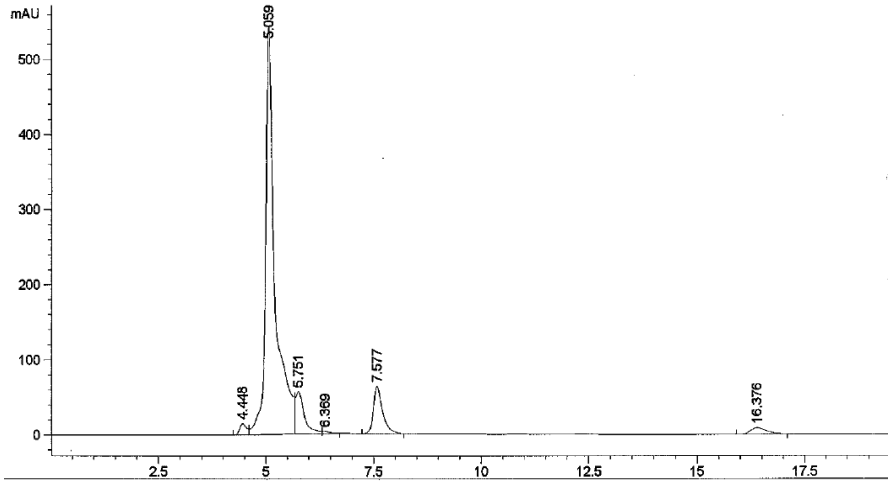
The ability of human erythrocytes to take up and retain ergothioneine *in vitro* is time and concentration dependent, human erythrocytes were previously found to contain 2 to 9 fold more ergothioneine than plasma (Mitsuyama, 1999). In our preliminary studies we found the erythrocyte concentration of ergothioneine to be 8 times greater than the plasma concentration.

LC-MS results both verified the identification of ergothioneine in the extracted red blood cell samples and verified its purity (no co-elution). Figure 3.2 illustrates the MS spectra of ergothioneine (m/z 230) in a sample of extracted red blood cells. Ergothioneine was identified by comparing the retention time of the UV-VIS output and the MS output for species having a mass of 230 m/z. Figure 3.3 indicates that ergothioneine alone is responsible for the UV-VIS output retention time 9.3 minutes.

Conclusions

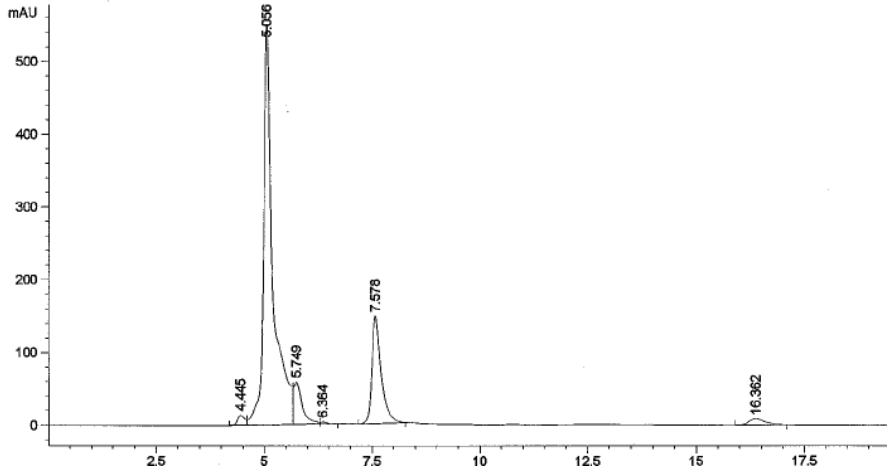
We have shown and verified a HPLC method for the separation and quantification of ergothioneine in red blood cells and plasma. Shah et al. (1992) suggests that there are essential parameters to ensure the acceptability of an analytical method. They include stability, accuracy, precision, sensitivity, specificity, response function, and reproducibility. Through our validation experiments we are able to ensure that our methodology is acceptable for extracting and quantifying ergothioneine in human blood.

[A]



Retention Time (min.)

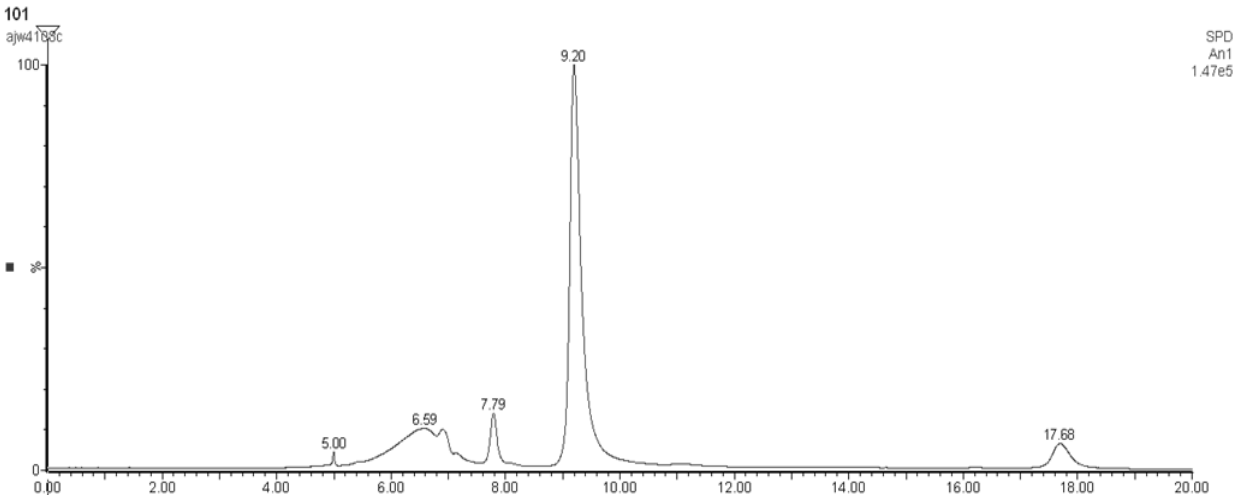
[B]



Retention Time (min.)

Figure 3.1 HPLC chromatograms with UV-VIS detection at 254 nm of red blood cell extract (A) and red blood cell extract spiked with pure ergothioneine (B).

[A]



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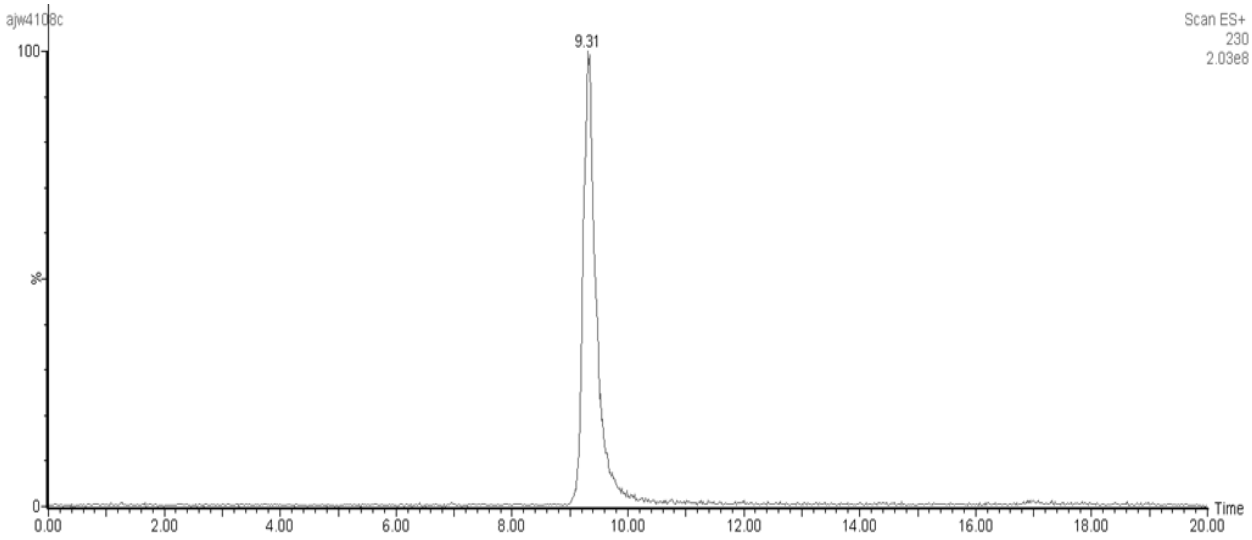

Figure 3.2 LC-MS chromatograms of UV-VIS output of red blood cell sample (A) and Mass spectra output for 230 m/z (B).

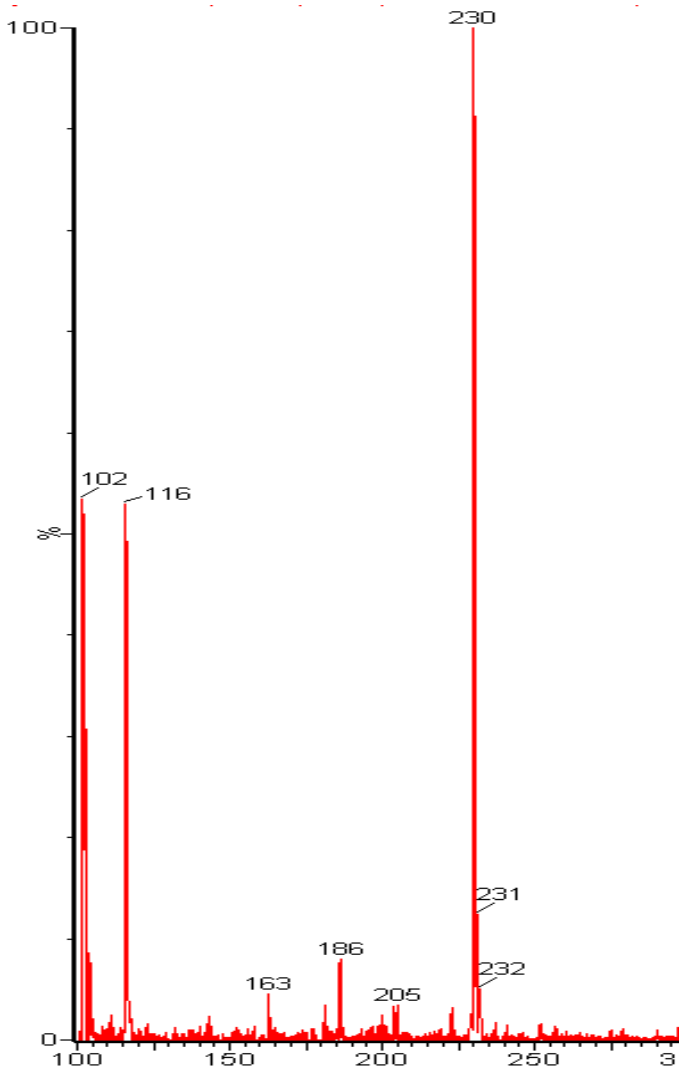

Figure 3.3 Ion spectra of ergothioneine (230 m/z) and fragmentation ions.

CHAPTER 4

DETERMINING THE BIOAVAILABILITY OF ERGOTHIONEINE IN HUMANS

ABSTRACT

Ergothioneine was discovered in human blood in 1927 however early studies showed that animal species are unable to synthesis the compound. The presence of ergothioneine in animals and humans originates from dietary sources. The ergothioneine content in mushrooms ranges from 0.4-2.0 mg/g (dry weight). Within the most commonly consumed strain, *Agaricus bisporus,* white button mushrooms contain the least amount of ergothioneine (0.41 mg/g (dry weight)) and portabellas contain the most (0.68 mg/g (dry weight)). Although the antioxidant properties of ergothioneine have been widely studied its bioavailability in humans is unknown. Thus, the objective was to evaluate the bioavailability of ergothioneine in healthy men (n=10) ages 23-50 years through a dose-response time-course over 6 hours. A randomized crossover design was employed in which each subject consumed a test meal containing 0g, 8g, and 16g of mushroom powder. At baseline (t=0) and at subsequent time points (0.5, 1, 2, 4, 6 hours) blood samples were collected to monitor the time course of ergothioneine in red blood cells. In addition, Cu, Se, glucose, triglycerides, HDL, LDL, total cholesterol, $ORAC_{total}$ and C-reactive protein were also monitored. The ergothioneine concentration in red blood cells was higher after the 8g and 16 g dose meals and the postprandial triglyceride response was blunted after the mushroom meals when compared to the control meal. A negative correlation (R^2=0.9957) was found for the peak ergothioneine concentration and the percent increase in triglycerides. $ORAC_{total}$ values decreased with the 8 g and 16 g doses compared to the control meal.

Introduction

Ergothioneine is known to be present in the blood of humans but early studies found that humans are unable to synthesis the compound (Melville, 1954). The ability of human erythrocytes to take up and retain ergothioneine *in vitro* is time and concentration dependent, and human erythrocytes contain 2 to 9 fold more ergothioneine than plasma (Mitsuyama, 1999). The concentration variation of ergothioneine in human blood with respect to age was investigated in 400 healthy Saudi Arabian males. The study revealed that the concentration of ergothioneine in the red blood cells is lowest at the early stages of life (1-10 years) and increases gradually between years 11-18 and reaches a maximum value of 3.7 mg/100mL at age 18. The concentration gradually decreases from ages 19-50; however, increases again at 51^{+} years (Kumosani, 2001). Broad variations in the amount of ergothioneine observed in human erythrocytes was reported to be due to a genetic variation in OCTN1, a multi-specific transporter, proposed to be responsible for transporting ergothioneine (Urban, 2007).

Red beans, oat bran, and meat products, such as liver and kidney, are major dietary sources of ergothioneine. Another good source of ergothioneine can be found in mushrooms (Ey, 2007). The ergothioneine content in mushrooms ranges from 0.4-2.0 mg/g (dry weight) (Dubost, 2006).

Although it is know that mushrooms contain the antioxidant ergothioneine, the bioavailability along with the absorption kinetics and metabolism of ergothioneine from mushrooms has not been characterized in humans. The purpose of this study was to conduct a dose response time course study to evaluate the bioavailability of ergothioneine from mushrooms.

Materials and Methods

Experimental Design

Healthy men (n=10), 23-50 years of age, with a body mass index (BMI) between 20-35 kg/m2, LDL cholesterol < 150 mg/dL, TG < 200 and BP < 140/80 were studied. The study involved three postprandial study days in which subjects consumed a placebo dose and two levels (8 g and 16 g) of dried mushroom powder. Each study day was preceded by a three day low-antioxidant diet, and subjects were instructed to complete food logs for each day to evaluate compliance. Subjects were also instructed to refrain from consuming any vitamins, minerals, or supplements for one month prior to beginning the study and throughout the entire course of the study. The mushroom powder was incorporated into a test meal of mashed potatoes, gravy, and biscuits, instructions as follows:

Mashed Potatoes
251.4 g water
15 g butter
60 g potato buds
Boil water with butter. Remove from heat and add potato buds stir and let stand for 2 minutes.

Gravy
100 g gravy
10 g water (when using 8 g dose)
30 g water (when using 16 g dose)
In a microwave safe dish heat gravy in microwave on high for about 30-40 seconds. Add mushroom powder in desired dose slowly to the gravy and mix thoroughly using a wire whisk. Add water and mix thoroughly.

Meal
On one large dinner plate
150 g of potatoes
1 ½ ready-to-eat biscuits (two halves split side up, one half brown side up)
Mushroom powder/gravy mixture poured over potatoes, and two halves of biscuits (split side up)

The 8 g and 16 g doses of mushroom powder provided 4.4 mg and 8.8 mg of ergothioneine respectively. The 8 g dose is equivalent to 1 serving of fresh mushrooms while the 16 g dose is equivalent to 2 servings of fresh mushrooms. A randomized crossover study design was employed (Figure 4.1). At baseline (t=0) and at the subsequent time points (30 min, 1 hr, 2 hr, 4 hr, 6 hr) 30 mL of blood was drawn to monitor the time course of ergothioneine in red blood cells. In total 180 mL of blood was drawn on each study day. Subjects were given the choice to have repeated venipunctures or an indwelling catheter. If the indwelling catheter was chosen the line was washed with saline at time points 3 and 5 hours. In addition to monitoring ergothioneine levels, plasma Cu and Se levels were also measured. Ergothioneine levels were measured at time points 0, 0.5, 1, 2, 4, and 6 hours. Copper and selenium were measured at time points 0, 1, 2, and 6 hours. Both copper and selenium are found in large quantities in mushrooms; therefore, serving as a marker that nutrients and bioactive components in mushrooms were absorbed. Glucose, triglycerides, HDL, LDL, total cholesterol, $ORAC_{TOTAL}$, and C-reactive protein (CRP) were monitored at time points 0, 1, 2, 4, and 6 hours. Lymphocytes were isolated and stored from 4 of the subjects for future genetic studies. This study was approved by the Pennsylvania State University Institutional Review Board (IRB#29956) and subjects gave their written consent.

Mushroom Powder Preparation and Micronutrient Analysis

Agaricus bisporus (brown crimini) mushrooms were obtained from Modern Mushroom Farms Inc., Avondale, Pennsylvania. Mushrooms were transported by vehicle and immediately sliced and stored in a walk-in cooler. The mushrooms were freeze-dried (Model 15 SRC-X Virtis Genesis Co. Inc., Gardiner, New York) and ground into a powder (Grindomix GM100, Retsch, Haan, Germany). The mushroom powder was collected in Ziploc bags and stored in the dark at -6 °C.

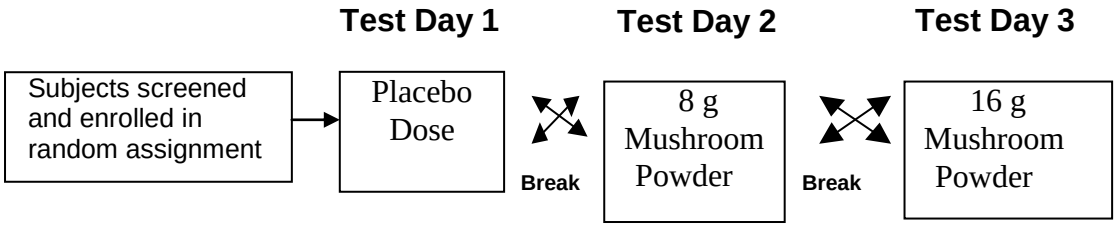

Figure 4.1 Study Scheme: Randomized Cross-Over Design

Mineral analysis was performed on the prepared mushroom powder by the Agricultural Analytical Services Laboratory at Pennsylvania State University. The micronutrient analysis included P, K, Ca, Mg, Mn, Fe, Cu, B, Al, Zn, Na, and Se and were run in triplicate. Average values are presented in table 4.1.

Ergothioneine Analysis of Mushroom Powder

The ergothioneine concentration of the mushroom powder was quantified using the extraction and HPLC method developed by Dubost et al. (2006). The mushroom powder used in this study contained 0.55 mg/g (dry weight).

Subject Recruitment

10 healthy men, age 23 to 50 years, were recruited to participate in this study. Potential subjects were initially screened by telephone through a series of questions concerning their medical history and current lifestyle. Subjects were ineligible after the telephone screening if they did not fit within the following criteria:

- 23 to 50 years of age
- BMI between 20 and 35 kg/m^2
- Non-Smoker
- Weight loss not greater than 10% of body weight in the past 6 months
- Not currently on a weight-loss diet or program
- Not allergic to latex
- Not allergic to mushrooms
- Do not consume mushrooms more than once or twice a week
- No major medical conditions
- Not on any cholesterol lowering medication

Based on the telephone screening, eligible subjects were invited to participate in the final screening. Final screenings took place at the General Clinical Research Center (GCRC) at

Table 4.1 Micronutrient Analysis of Mushroom Powder

P	K	Ca	Mg	Mn	Fe	Cu	B	Al	Zn	Na	Se
%				µg/g							
1.23	4.5	0.037	0.13	7	30.3	54.7	3.07	1.33	67	671	1.45

Pennsylvania State University to determine participant eligibility. The visit consisted of filling out forms (medical history, personal information, and informed consent); measuring height and weight in order to calculate body mass index (BMI) and measuring blood pressure. If BMI measurement was between 20 and 35 kg/m^2 and blood pressure was 90/140 or below a blood sample of approximately 15 mL was taken for a blood test for complete blood count, to check liver and kidney function and for blood lipid panel. Potential participants also met with a study dietitian to discuss the background low-antioxidant diet to be followed 3 days prior to each test day. Subjects were instructed to abstain from taking any vitamins or minerals for three weeks before the first test day and during the course of the study. Following the second screening, subjects were ineligible if they did not fit within the following criteria:

- BMI between 20 and 35 kg/m^2
- Average blood pressure below or equal to systolic blood pressure 140 mmHg and diastolic 90 mmHg
- LDL less than 150 mg/dL
- Triglycerides less than 200 mg/dL

Eligible subjects were instructed to schedule their three test days over a six week time period with 7-10 days between each test day. Subjects were randomized to each test meal using balanced permutations.

Mushroom Feeding Test Day

After consuming a low antioxidant diet for 3 days and no vitamins/minerals for 1 week, and following a 12 hour fast subjects were admitted for their test day. On each test day subjects arrived at the GCRC and were given the option of having an indwelling catheter inserted or repeated venipunctures at each time point (0, 0.5, 1, 2, 4, and 6 hour). If the catheter was chosen the option of having LMX-4 anesthetic cream was also given. If the subject had the anesthetic

cream applied catheter insertion was postponed for 30 minutes. The test meal was served after a baseline blood draw was taken and the subject was asked to consume the meal in 15 minutes.

Ergothioneine Analysis

Green topped tubes containing sodium heparin were used to collect 10 mL of whole blood at time points 0, 0.5, 1, 2, 4, and 6 hours. Samples were stored at 3°C until analysis which occurred within 48 hours. At time of analysis whole blood was centrifuged to separate the red blood cells from the plasma at 2,500 g for 5 minutes. Red bloods cells (0.75mL) were washed two times with 0.75 mL of 0.9% NaCl solution and centrifuged at 1,700 g for 7 minutes after each wash, pipetting off the supernatant. The extraction solution (10nM dithiothreitol in 0.1mM phosphate buffer pH 8) in the amount of 0.75mL was added to the washed cells. The samples were then placed in a 90°C water bath for three minutes, vortexed, and put back in the water bath for three additional minutes. The samples were then micro-centrifuged for 7 minutes at 12,000 g. The supernatant was filtered through a 0.45 micron syringe filter prior to injection into the HPLC.

Plasma (1.0 mL) samples were extracted in the same manor; however, the wash step was skipped and samples were centrifuged for 40 minutes at 12,000 g after being placed in the water bath. 0.8 mL of the supernatant was collected into a clean vial and placed in a speed vacuum for 7.5 hours to evaporate off the liquid portion leaving a dry pellet. The dried samples were stored at -80°C for potential future analysis.

The quantification of ergothioneine from extracted human blood was carried out using high performance liquid chromatography (HPLC). A HP1050 series HPLC equipped with a quaternary pump, autosampler, helium degasser, and ChemStation analysis and control software

was used for the analysis. A Grace (Deerfield, IL) econosphere C18 column, (250mm x 4.6mm, 5 micron) with an isocratic mobile phase of 0.3M phosphate buffer at pH 7.3 and 10% methanol, run at a flow rate of 0.5mL per min was employed. Injection volume was set at 10 µL and the column temperature was ambient. Absorbance was measured at 254 nm using a HP1050 series variable wavelength UV-VIS detector. ChemStation analysis software was used for peak area integration. Quantification of ergothioneine in human red blood cells was determined using the integrated peak areas and a standard curve.

C-Reactive Protein (CRP), Glucose, and Lipid Analysis

A 7.5 mL tiger top tube containing a clot activator and a double gel for transport was used to collect blood at time points 0, 1, 2, 4, and 6 hours. Tubes were held at room temperature until clotting was observed and then separated by centrifugation at 4°C for 15 minutes at 1000g in a Eppendorf centrifuge 5702R (Westbury, New York). The processed blood was sent to Quest Diagnostic (Pittsburgh, Pennsylvania) for the analysis of cardio CRP (hs-CRP1), glucose, cholesterol, triglycerides, HDL cholesterol, and LDL cholesterol.

CRP is an acute phase protein found in the blood. Increases in blood CRP levels are due to the body's inflammatory response; therefore, it serves as a biomarker for underlying cardiovascular inflammation. The relative risk categories of cardiovascular risk are as follows: <1.0 mg/L low risk, 1.0-3.0 mg/L average risk, >3.0 mg/L high risk.

$\mathbf{ORAC_{total}}$

Extracted red blood cells, extracted in the same manner as the ergothioneine samples, were used for the evaluation of $ORAC_{TOTAL}$ at time points, 0, 1, 2, and 6 hours. The extracted red blood cells were stored at -80 °C until all samples were collected. Samples were analyzed by

Brunswick Laboratories. The $ORAC_{TOTAL}$ analysis measures the scavenging capacity of antioxidants against the peroxyl radicals. A water-soluable Vitamin E analog known as Trolox is used as a standard. The acceptable precision of the assay is reported to be 15% relative standard deviation (Ou, 2001).

IL-6 and TNF-α Analysis

Blood was collected in 7.0 mL lavender tubes containing EDTA at time points 0, 1, 2, 4, and 6 hours. Samples were separated by centrifugation at 4°C for 15 minutes at 1000 g in Eppendorf centrifuge 5702R (Westbury, New York). The serum was allocated, in 0.5 mL portions, into six vials and stored at -80°C for future analysis.

Copper and Selenium Analysis

Two 5 mL royal blue topped tubes containing EDTA were used to collect blood at time points 0, 1, 2, and 6 hours. Tubes were wrapped in aluminum foil to prevent light exposure upon collection. Blood was separated by centrifugation at 4°C for 15 minutes at 1000 g in Eppendorf centrifuge 5702R (Westbury, New York). Serum was allocated, in 2 mL portions, into two element free vials provided by Quest Diagnostic, and stored at -80°C for 24-48 hours. Samples were shipped to Quest Diagnostics Nichols Institute (Chantilly, Virginia) on dry ice for analysis. Copper analysis was performed by Inductively Coupled Plasma-Mass Spectrometry. Selenium samples were analyzed using spectrophotometric atomic absorption.

Lymphocyte Cell Isolation for Future Studies

Prepared samples are to be used for future analysis. Isolation of lymphocytes was performed by the method of Ann Skulas-Ray. The method is as follows: Excess blood collected

in EDTA was poured into a 50 mL tube. Blood tubes were washed with saline. Histopaque 1077 (about 10 mL) was placed in a second 50 mL tube. The blood and saline mixture was layered on top of the Histopaque® and centrifuged at 815 g for 10 minutes. The cell layer above the Histopaque® was transferred to a 50 mL tube and diluted with about 45 mL of saline and centrifuged at 200 g for 10 minutes. The supernatant was decanted and 200 µL of RNAlater® was added to suspend the cells. The suspended cell solution was stored at -80°C for future gene expression experiments. It should be noted that this protocol was neither sterile nor quantitative.

Statistical Analysis and Calculations

All statistical analyses were performed using SAS 9.1 software. Distribution of the data was checked for normality. Skewed data was square root or log transformed. Repeated measures analysis of covariance using PROC MIXED was used for exploratory analysis ($\alpha = 0.05$). Change scores (Δ) were calculated by subtracting values at time 0 hours from each proceeding time point. Area under the curve (AUC) was computed for the triglyceride response using the integration method. Data are presented as means ± standard deviations unless otherwise noted.

Results and Discussion

Baseline characteristics, compliance, and adverse effects

Baseline characteristics of the study participants are presented in Table 4.2. All subjects complied with the 3 day low antioxidant diet prior to each test day monitored by food logs. Any deviations from the diet were pointed out by a research dietician and the subject was asked to refrain from eating those foods prior to future test days. All ten subjects completed the entire study and no adverse effects were reported.

TABLE 4. 2. Baseline characteristics of subjects (n=10) reported as means ± standard deviations.

Variable	Mean ± SD
Age, *y*	26.5 ± 3.3
Body Weight, *kg*	83.9 ± 16.7
Body Mass Index, *kg/m2*	25.6 ± 4.2
Cholesterol, *mg/dL*	148.7 ± 32.9
HDL, *mg/dL*	47.8 ± 7.5
LDL, *mg/dL*	85.0 ± 30.5
Triglycerides, *mg/dL*	78.7 ± 27.9
Baseline ERGO *mg/dL*	3.4 ± 1.8

Glucose

The postprandial glucose response was measured to determine if the mushroom treatments would reduce the glucose response after eating the provided meal. Change scores were square root transformed to establish a normal distribution. A significant quadratic trend ($p<0.05$) was observed; however, no significant difference in treatment dose was determined. The lack of significance is due to the small sample size (n=10) and the large standard errors. Figures 4.2 and 4.3 illustrate the glucose response as absolute numbers and changes respectively.

Plasma Concentrations of Selenium and Copper

Due to the natural presence of copper and selenium in mushrooms, postprandial levels within plasma were measured. The amount of copper ingested in the 8 and 16 g test meals was 440 µg and 880 µg respectively. The recommended daily allowance for copper is between 1500 and 3000 µg. No significant differences in plasma copper levels were observed among the treatment doses. Copper data are presented in table 4.3.

Copper absorption is highly dependent on dietary copper intake. Studies have shown that the percent of copper absorbed from a meal decreases as the copper content of the meal increases (Turnlund, 1989). Copper stores and indexes of copper status have been show to be resistant to change, except under extreme dietary conditions (Turnlund, 1990). The absorption balance of copper is important for preventing deficiencies and toxicity. It is suspected that the copper concentration present in both test meals did not challenge the subjects copper status; therefore, leading to no significant changes in absorption or excretion.

Selenium concentrations were 11.6 µg and 23.2 µg for the 8 g and 16 g mushroom test meals respectively. For adults, the recommended daily allowance (RDA) for selenium is 55 µg,

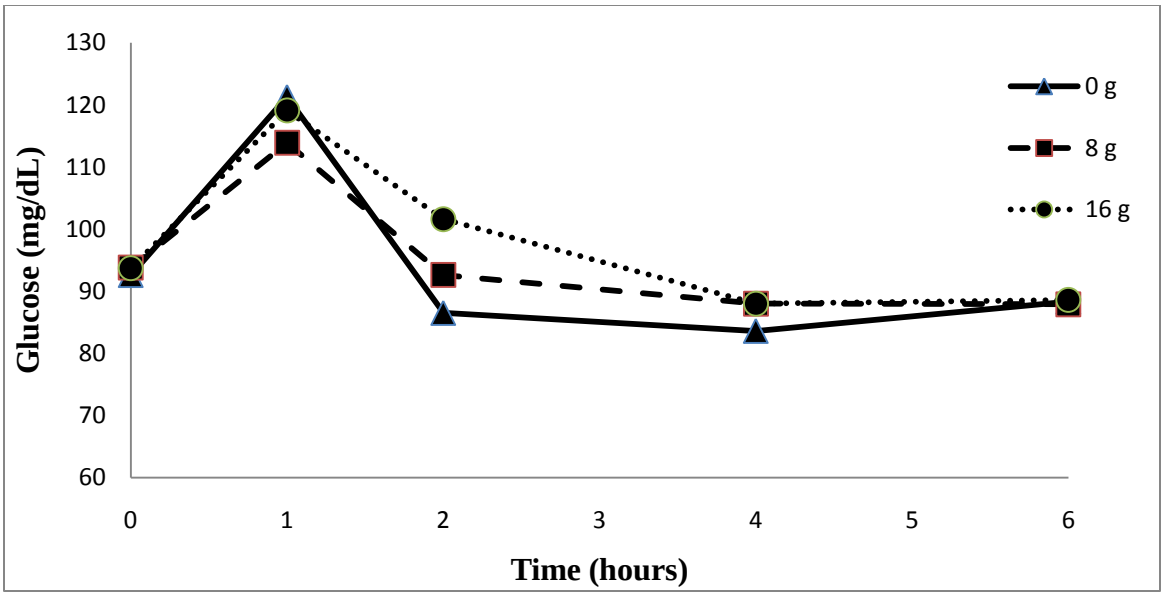

Figure 4.2 Glucose response (mg/dL) over the time course of 6 hours with mushroom treatments of 0 g, 8 g, and 16 g

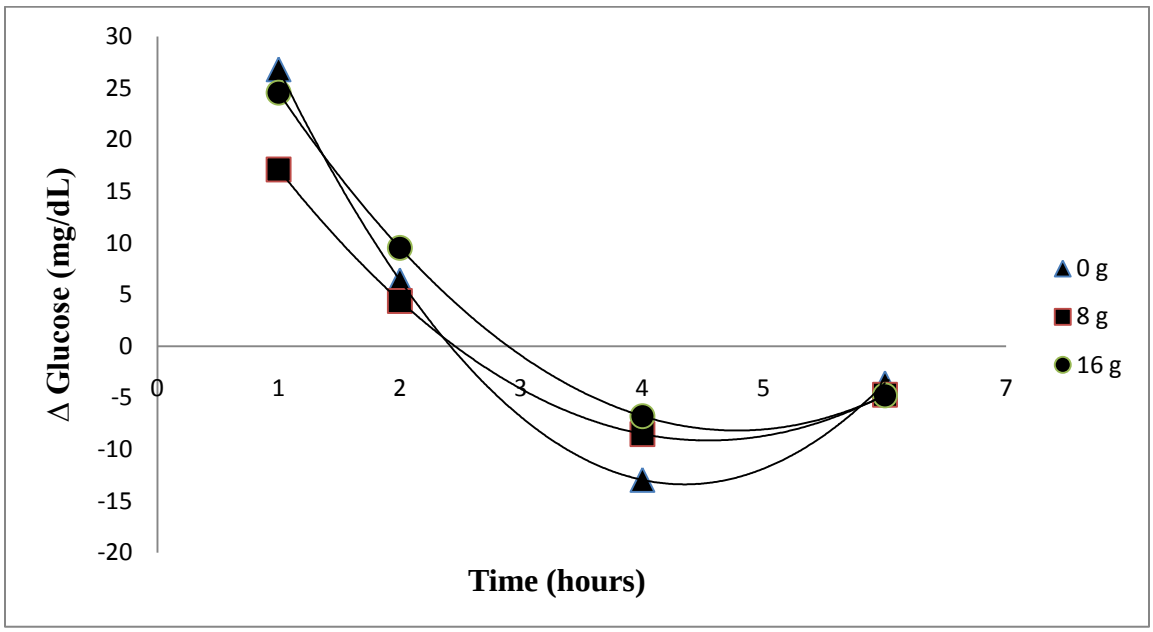

Figure 4.3 Glucose response (mg/dL), as change scores, over the time course of 6 hours with mushroom treatments of 0 g, 8g, and 16 g.

with intake not to exceed 400 µg. No significant differences in plasma selenium levels were observed among the three treatment doses (table 4.4).

Selenium absorption isdependent on the form of selenium ingested (inorganic and organic). Organic forms of selenium have been shown to be more bioavailable and selenium is better absorbed from a high protein meal (Robinson, 1978, Greger, 1981). *Agaricus bisporus* mushrooms have been shown to contain selenium primarly in the forms selenocystine and selenium disulfide (Se(IV)), 25% and 42% respectively (Stefanka, 2001). Another study analyzing the selenium speciation in *A. bisporus* mushrooms showed that four selenium species are present, selenocystine, selenomethionine, methylselenocysteine, and inorganics selenium. However, inorganic selenium compounds and selenocystine were the primary species detected (Gergely, 2006). The lack of significant changes in the plasma selenium levels over the 6 hour time course of this study is suggested to be due to the high concentration of inorganic selenium species present in *A. bisporus* mushrooms.

Lipid Panel: Cholesterol, HDL, LDL, and Triglycerides

No treatment or time effects were observed for cholesterol, HDL, or LDL, data presented in tables 4.5-4.7. Change scores were calculated for triglyceride levels to determine if the mushroom treatment blunted the postprandial triglyceride response. A significant quadratic trend ($p<0.05$) was observed; however, no significant difference in treatment dose was determined by evaluating area under the curve (AUC) ($p=0.1660$). The lack of significance may be due to the small sample size ($n=10$) and the large standard errors. Although treatment doses were not statistically significant in blunting the triglyceride response a trend can be observed. In Figure 4.4 mushroom doses of 8 g and 16 g are shown to have some blunting effect on the postprandial triglyceride response compared to the 0 g dose. It has been suggested that

Table 4.3 Copper response (µg/dL) after mushroom consumption. Values are means of 10 subjects ± standard deviations. The symbol (*) indicates a significant difference from the control (0 g dose) ($p<0.05$).

	Mushroom Dose (g)		
Time (hr)	0	8	16
0	81.9 ± 12.6	79.4 ± 14.2	85.3 ± 16.5
1	81.5 ± 13.2	79.2 ± 13.6*	85.4 ± 13.7
2	79.4 ± 11.7	77.4 ± 11.9	84.7 ± 13.9*
6	83.9 ± 14.6	81.9 ± 14.9	86.9 ± 14.2

Table 4.4 Selenium response (µg/L) after mushroom consumption. Values are means of 10 subjects ± standard deviations. No statistically significant differences were observed between control and treatments ($p<0.05$).

	Mushroom Dose (g)		
Time (hr)	0	8	16
0	125.8 ± 14.83	120.6 ± 14.68	126.6 ± 21.22
1	121.1 ± 18.86	119.7 ± 14.29	126.1 ± 20.52
2	125.0 ± 15.62	122.6 ± 13.08	124.9 ± 18.26
6	126.6 ± 21.09	122.0 ± 15.31	130.6 ± 19.44

postprandial triglyceride levels may be a superior assessment of cardiovascular risk compared to fasting triglyceride measurements (Bansal, 2007). Postprandial triglycerides can contribute to the formation of foam cells, which play an important role in the progression of atherosclerosis. By blunting the triglyceride response or clearing postprandial triglyceride faster the development of foam cells may be prevented leading to the prevention of heart disease.

C-Reactive Protein

High sensitivity CRP (hs-CRP) was measured to follow the postprandial inflammation response after each treatment dose. No treatment or time effects were found to be statistically significant. Table 4.8 presents the CRP data as means ± standard deviations. It is expected that no effects on inflammation were observed due to the small sample size and the fact that healthy subjects were recruited for this study. A postprandial study encompassing a longer time period would better indicate any effect mushroom consumption has on inflammation.

Red Blood Cell Concentration of Ergothioneine

The main focus of this research was to determine if ergothioneine was bioavailable from mushrooms. Raw ergothioneine levels and change scores were analyzed. Change scores shown graphically in Figure 4.5 indicate that the 0 g dose had little effect on the ergothioneine concentration within the red blood cells while the 16 g and 8 g dose increased the ergothioneine concentration. The ergothioneine response to the 16 g mushroom dose was statistically higher after the 1 hour and 4 hour time points ($p<0.1$). At the 2 hour time point the response to the 16 g dose was significantly higher ($p <0.05$). It should be noted that 90% of the subjects did have an ergothioneine response (increased red blood cell ergothioneine concentrations) after the 8 g or the 16 g dose. The 8 g dose resulted in the highest ergothioneine concentration in 4 out of the

Table 4.5 HDL response (mg/dL) after mushroom consumption. Values are means of 10 subjects ± standard deviations. No statistically significant differences were observed between control and treatments ($p<0.05$).

	Mushroom Dose (g)		
Time (hr)	0	8	16
0	47.6 ± 7.66	47.7 ± 3.95	49.3 ± 6.43
1	46.1 ± 7.13	46.4 ± 5.04	47.8 ± 5.90
2	46.4 ± 7.50	46.3 ± 5.08	47.3 ± 6.50
4	45.6 ± 8.41	46.9 ± 5.57	47.7 ± 6.17
6	48.4 ± 7.09	49.4 ± 5.56	49.9 ± 5.63

Table 4.6 LDL response after mushroom consumption. Values are means of 10 subjects ± standard deviations. No statistically significant differences were observed between control and treatments ($p<0.05$).

	Mushroom Dose (g)		
Time (hr)	0	8	16
0	85.0 ± 29.2	88.4 ± 32.2	90.4 ± 35.8
1	84.4 ± 28.6	84.5 ± 26.9	88.6 ± 34.5
2	79.0 ± 30.9	84.0 ± 29.9	82.9 ± 33.3
4	80.4 ± 22.6	85.5 ± 29.5	89.3 ± 33.3
6	89.4 ± 29.9	91.3 ± 35.0	93.0 ± 34.8

Table 4.7 Total cholesterol response (mg/dL) after mushroom consumption. Values are means of 10 subjects ± standard deviations. The symbol (¶) indicates a missing value due to a missed blood draw.

	Mushroom Dose (g)		
Time (hr)	0	8	16
0	147.2 ± 28.8	152.8 ± 37.4	154.6 ± 41.4
1	149.3 ± 29.1	152.1 ± 33.2	154.2 ± 39.6
2	134.6 ± 56.6¶	154.5 ± 35.8	151.3 ± 38.6
4	143.8 ± 24.9	151.3 ± 35.3	154.3 ± 39.0
6	151.5 ± 30.5	156.0 ± 38.3	158.0 ± 39.1

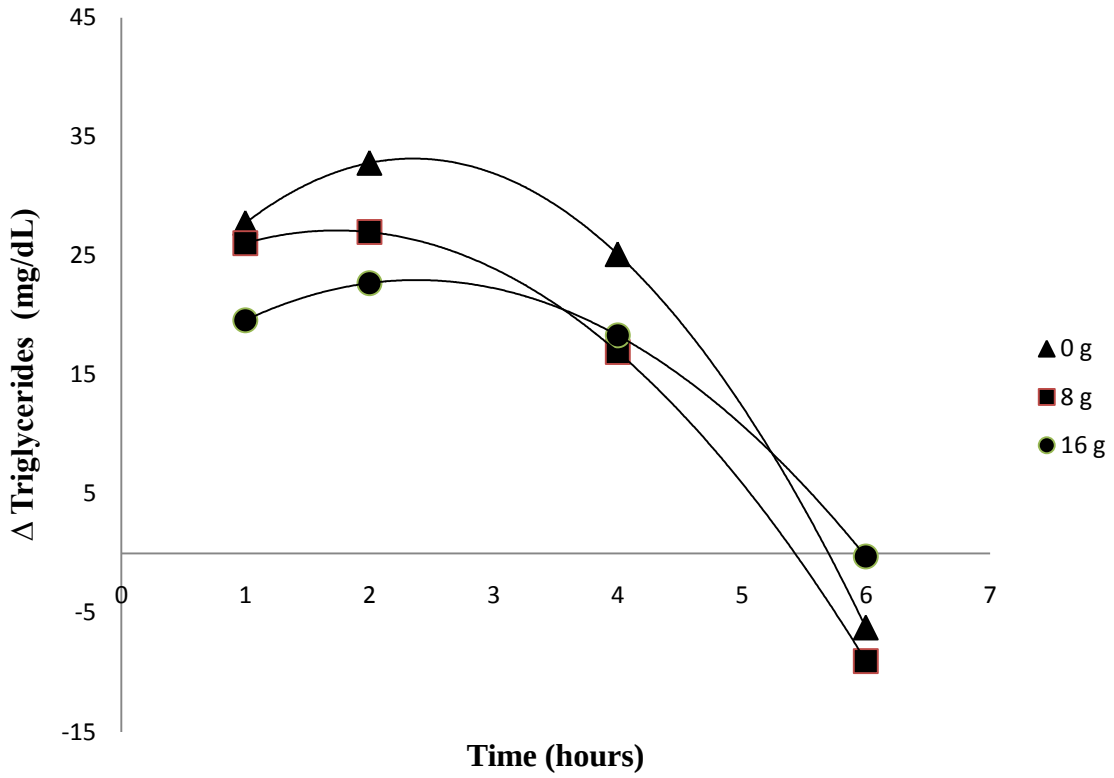

Figure 4.4 Triglyceride response, as change scores, over the time course of 6 hours with mushroom treatments of 0 g, 8 g, and 16 g

Table 4.8 CRP response (mg/L) after mushroom consumption. Values are means of 10 subjects ± standard deviations. No statistically significant differences were observed between control and treatments ($p<0.05$).

	Mushroom Dose (g)		
Time (hr)	0	8	16
0	0.66 ± 0.54	0.89 ± 0.77	0.98 ± 0.63
1	0.68 ± 0.57	0.91 ± 0.78	0.95 ± 0.62
2	0.66 ± 0.53	0.90 ± 0.78	1.01 ± 0.55
4	0.64 ± 0.47	0.89 ± 0.76	1.08 ± 0.61
6	0.70 ± 0.67	0.92 ± 0.75	1.09 ± 0.59

9 subjects that responded to treatment, while 5 out of the 9 responded higher after the 16 g dose. Only 6 of the 9 responded to the 8 g dose; however, all 9 responded to the 16 g dose. A treatment response was determined if the peak ergothioneine response after treatment was >10% from the 0 g dose. This level was chosen based upon the coefficient of variation within the HPLC method (<5%).

Broad variations in the amount of ergothioneine observed in human erythrocytes is suggested to be due to a genetic variation in OCTN1, a multi-specific transporter, proposed to be responsible for transporting ergothioneine (Urban, 2007). The variation within our results is consistent with Mitsuyama et al. who reported that the intracellular uptake of ergothioneine is observed; however, the effect is not consistent among donors (Mitsuyama, 1999). Raw ergothioneine data is presented in Appendix A.

Comparing the Triglyceride and Ergothioneine Response

A correlation between peak ergothioneine values and percent increase in triglycerides was found to be negatively correlated (R^2=0.9957). The correlation is shown in Figure 4.6. Although a correlation is observed showing that as the ergothioneine concentration increases in the red blood cells the triglyceride response is blunted, further investigation is needed to determine if ergothioneine alone is causing this response. Other single components of mushrooms or mushrooms as a whole may be responsible for the triglyceride response.

$ORAC_{TOTAL}$ Response

Total ORAC was measured at time points 0, 1, 2, and 6 hours to follow the postprandial antioxidant scavenging response. Although ergothioneine has been reported to have the capability of scavenging free radicals the postprandial $ORAC_{TOTAL}$ values were significantly

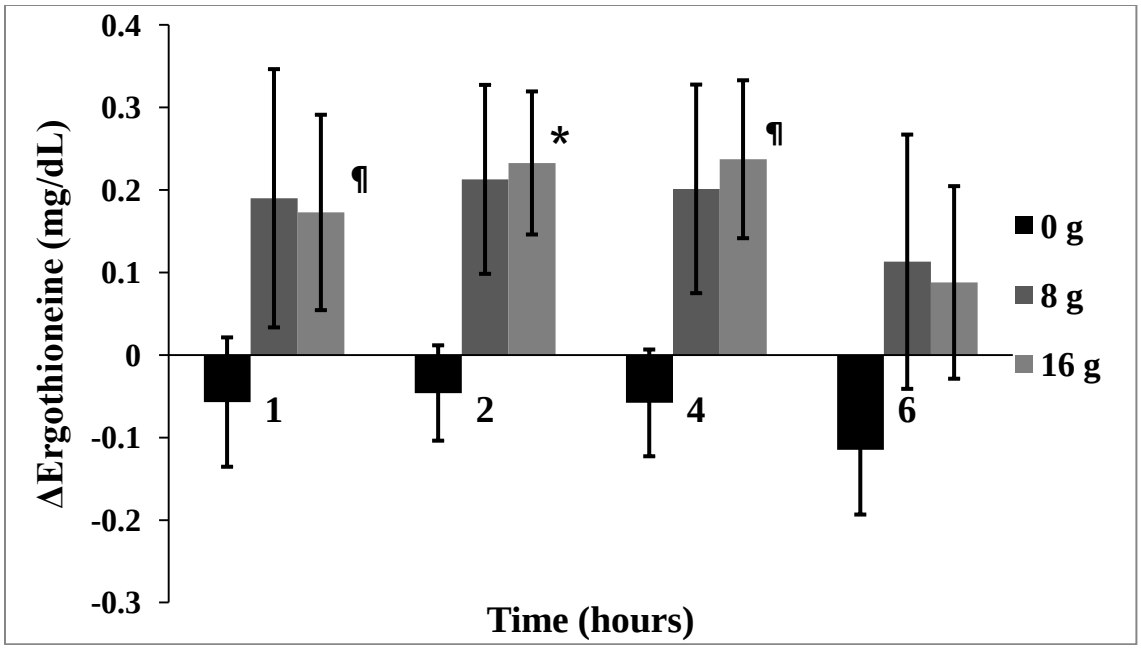

Figure 4.5 Ergothioneine response, as change scores, over the time course of 6 hours with mushroom treatments of 0 g, 8 g, and 16 g. The * symbol represents statistical significance from the 0 g dose with $p<0.05$. The ¶ symbol represents statistical significant from the 0 g dose with $p<0.1$.

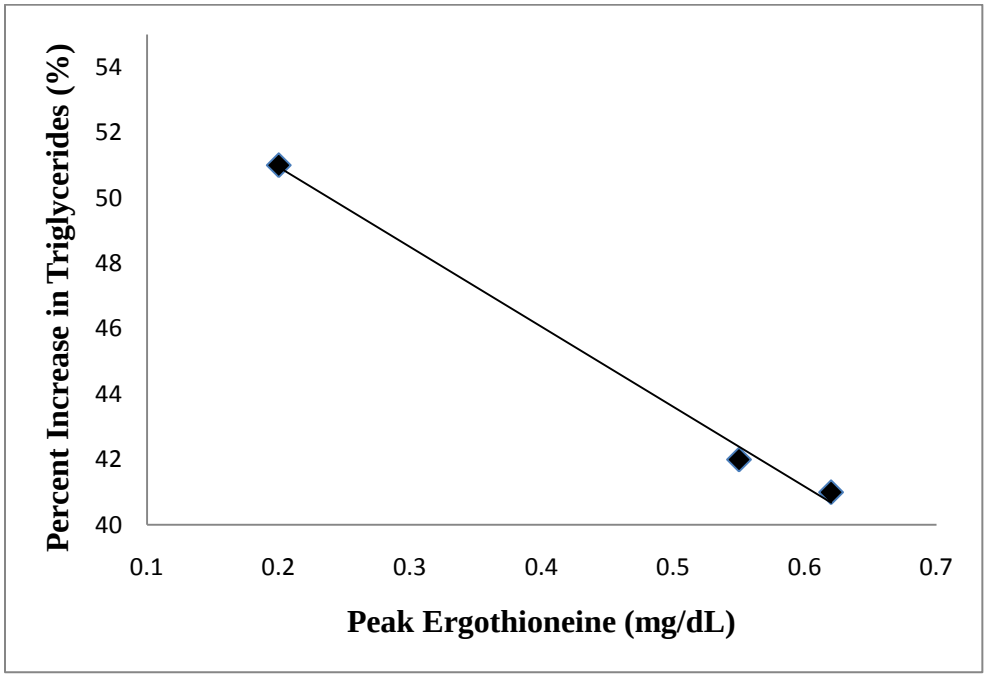

Figure 4.6 Correlation (R^2=0.9957) between peak ergothioneine value and percent increase in triglycerides.

lower after the 8 g and 16 g dose of mushroom powder ($p<0.05$). Results are shown in Figure 4.7. This is counterintuitive due to the vast majority of research that has shown ergothioneine to be a powerful antioxidant.

Recently it was found during a study evaluating the effect of wild blueberry consumption on the postprandial serum antioxidant status that the control meal had an effect on the $ORAC_{total}$ and $ORAC_{acetone}$ measurements. Results of the study indicated a correlation between serum triglycerides and $ORAC_{total}$ and $ORAC_{acetone}$. It was speculated that the response was due to the ORAC assays inability to differentiate between fatty acid oxidation and free-radical quenching (Kay, 2002). Our results agree with this speculation in that as the postprandial triglycerides are highest in the control meal the $ORAC_{TOTAL}$ measurements are highest. However, when the postprandial triglyceride response is blunted after mushroom consumption the $ORAC_{TOTAL}$ measurements are lower as well. These observations agree with the speculation that the ORAC assay may not be able to differentiate between fatty acid oxidation and free-radical quenching.

Conclusion

It is known that mushrooms contain the antioxidant ergothioneine; however, the bioavailability from mushrooms has not been characterized in humans. The purpose of this study was to conduct a dose response time course study to evaluate the bioavailability of ergothioneine from mushrooms. A postprandial time course study of varying mushroom doses (0 g, 8 g, and 16 g) was used to evaluate the bioavailability of ergothioneine. This study convincingly indicated that ergothioneine is bioavailable in humans through the consumption of mushrooms. No changes in Se, Cu, HDL, LDL, total cholesterol, glucose or CRP were observed over the 6 hour time course. The percent increase in postprandial triglycerides after the 8 g and

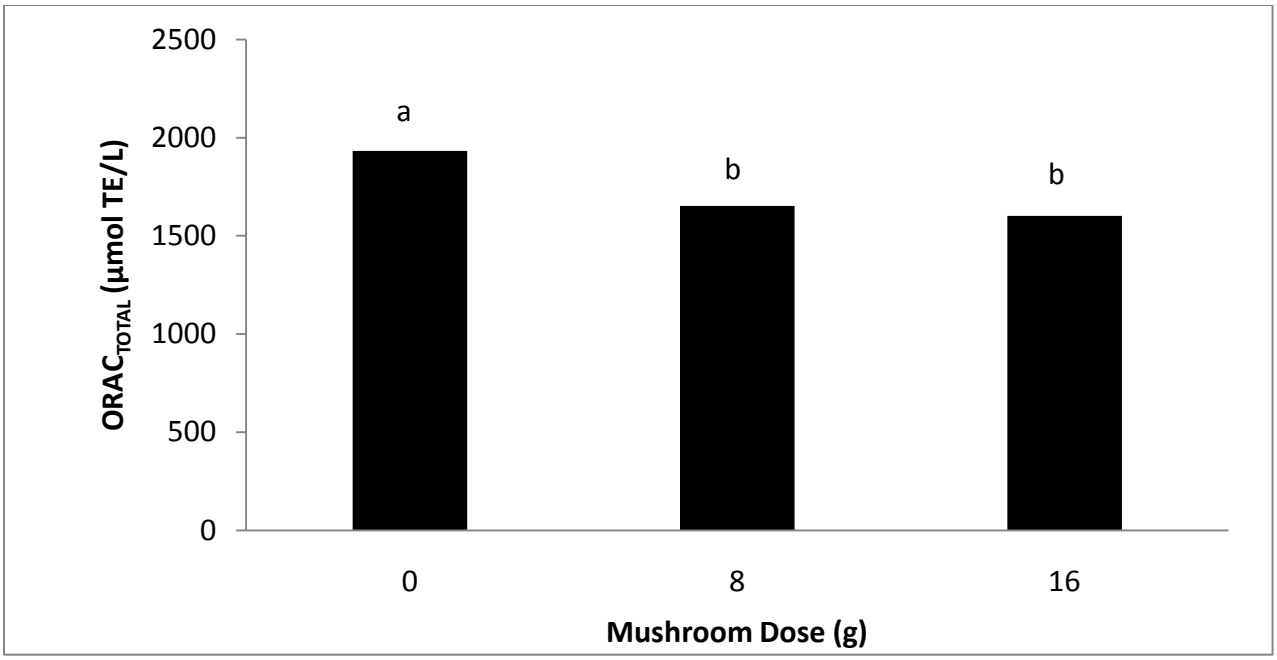

Figure 4.7 ORAC$_{TOTAL}$ values (n=10) for mushroom treatment doses 0 g, 8 g, and 16 g. Different lower case letters represent statistical significance ($p < 0.05$).

16 g mushroom dose was approximately 10% less than the control meal containing no mushroom powder. Postprandial $ORAC_{TOTAL}$ values were significantly lower after the 8 g and 16 g dose of mushroom powder. Although this is counterintuitive it may be due to the inability of the ORAC assay to differential between fatty acid oxidation and free radical quenching. Further studies, containing a larger sample size are needed to determine if any statistically significant effects are observed after the consumption of mushrooms.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The growing demand of functional foods in the market is leading research on active components found in food. Current research performed in this field has found that many of the functional components found in food provide health benefits beyond basic nutrition. Mushrooms have recently been discovered to contain ergothioneine, a known antioxidant. Ergothioneine was discovered in 1909 and although it has been widely studied, its bioavailability in humans is still unknown. The primary objective of this project was to assess the bioavailability of ergothioneine from mushrooms and evaluate its bioactive effect in humans.

The quantitative determination of ergothioneine in human blood has been investigated since 1926. Early methods involved an estimation technique and semi-quantitative methods. A limited number of HPLC methods have been developed for the quantification of ergothioneine in blood. The methods that exist do not focus on the determination in blood alone and those that do require tedious and time consuming preparation techniques (Mayumi, 1978; Muda, 1988; Newton, 1993). Therefore, the first objective of this research was to identify and quantifiy ergothioneine in human blood by modifying a HPLC method developed by Dubost et. al (2006) to quantify ergothioneine in edible mushrooms. The quantification, of ergothioneine in human blood samples, was validated by performing multiple tests. The reproducibility of the HPLC method utilized for quantification was found to be less than 5%. The lowest limit of quantification and the detection limit were found to be 0.0007 mg/mL and 0.0005 mg/mL respectively. The CV of the reproducibility of the extraction technique applied to extract ergothioneine from human red blood cells and plasma was calculated to be 1.6% for red blood cells and 9.7% for plasma. The percent recovery was 107% for red blood cells and 114% for

plasma. Various validation experiments demonstrated that our methodology was acceptable for extracting and quantifying ergothioneine in human blood.

A randomized cross-over dose response time course study was used to evaluate the bioavailability of ergothioneine from mushrooms in humans. Ten subjects between the ages of 23 and 50 years were recruited and consumed three doses of mushroom powder on three separate days with a 7-10 day break between study days. The mushroom doses were equivalent to 1 and 2 servings of fresh mushrooms (equivalent to 85 and 170 g fresh mushrooms). Each subject served as their own control by consuming a test meal containing no mushrooms. The postprandial study determined that ergothioneine is bioavailable in humans through the consumption of mushrooms. No changes in Se, Cu, HDL, LDL, total cholesterol, glucose or CRP due to the consumption of mushrooms were observed over the 6 hour time course. The percent increase in postprandial plasma triglycerides after the 8 g and 16 g mushroom dose was approximately 10% less than the control meal at 0 g.

Overall, the results of this research project indicate that ergothioneine can be extracted and quantified from human blood. It was also determined that ergothioneine is bioavailable to humans through the consumption of *A. bisporus* mushrooms. In order to promote the idea of eating a well-balanced diet, rich in fruits and vegetables, it is important to understand the physiological effects due to the consumption of functional foods. From these research findings the consumption of mushrooms is recommended due to the absorption of the powerful antioxidant, ergothioneine.

CHAPTER 6

SUGGESTIONS FOR FUTURE STUDIES

The result of this small study has laid the ground work for future clinical and nutrigenomics studies. It has been determined that ergothioneine is bioavailable from mushrooms; however, a study involving a larger sample size should be conducted using the current study for power calculations in order to generate statistically significant results. During the course of the current study plasma samples were stored at -80 °C for future analysis. These additional plasma samples should be used to determine if an inflammation response, other than CRP, using the inflammation markers IL-6 and TNF-α. Lymphocytes were also isolated from 4 out of the 10 subject and stored at -80 °C. The stored lymphocytes should be used to investigate the genetic factors that predispose people to a higher or lower level of ergothioneine. Genetic factors may also affect the postprandial uptake of ergothioneine. Studies should be performed that investigate the efficacy of ergothioneine uptake, by red blood cells, in relation to genetic variations within the ergothioneine transporter.

The current study also showed that there is a negative correlation between the postprandial triglyceride response and the peak ergothioneine concentration. Further investigation is needed to establish if ergothioneine alone or other components within the mushroom are responsible for the response.

Future studies should also examine the absorption and retention of ergothioneine over a long period feeding study. These studies would allow for the determination of maximal absorption within the red blood cells. It is also possible that ergothioneine absorption is similar to the absorption of copper in that the body adapts to a certain concentration and excretes any

excess that is ingested. However, it may be possible, through continual feeding of ergothioneine that the body can adapt to higher concentrations.

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APPENDIX

Raw ergothioneine data for subjects (n=10) at time points 0, 1, 2, 4, and 6 hours with treatments of 0 g, 8 g, and 16 g mushroom powder.

0 GRAM DOSE			8 GRAM DOSE			16 GRAM DOSE		
SUBJECT	TIME (hr)	ERGO (mg/mL)	SUBJECT	TIME (hr)	ERGO (mg/mL)	SUBJECT	TIME (hr)	ERGO (mg/mL)
MUSH001	0	0.045	MUSH001	0	0.038	MUSH001	0	0.036
MUSH001	0.5	0.043	MUSH001	0.5	0.035	MUSH001	0.5	0.040
MUSH001	1	0.042	MUSH001	1	0.037	MUSH001	1	0.035
MUSH001	2	0.040	MUSH001	2	0.044	MUSH001	2	0.033
MUSH001	4	0.042	MUSH001	4	0.034	MUSH001	4	0.034
MUSH001	6	0.042	MUSH001	6	0.037	MUSH001	6	0.032
MUSH002	0	0.033	MUSH002	0	0.031	MUSH002	0	0.031
MUSH002	0.5	0.031	MUSH002	0.5	0.038	MUSH002	0.5	0.029
MUSH002	1	0.039	MUSH002	1	0.033	MUSH002	1	0.027
MUSH002	2	0.040	MUSH002	2	0.031	MUSH002	2	0.031
MUSH002	4	0.040	MUSH002	4	0.035	MUSH002	4	0.027
MUSH002	6	0.035	MUSH002	6	0.030	MUSH002	6	0.027
MUSH006	0	0.036	MUSH006	0	0.027	MUSH006	0	0.028
MUSH006	0.5	0.031	MUSH006	0.5	0.026	MUSH006	0.5	0.024
MUSH006	1	0.039	MUSH006	1	0.029	MUSH006	1	0.033
MUSH006	2	0.030	MUSH006	2	0.028	MUSH006	2	0.030
MUSH006	4	0.035	MUSH006	4	0.027	MUSH006	4	0.028
MUSH006	6	0.032	MUSH006	6	0.026	MUSH006	6	0.026
MUSH008	0	0.029	MUSH008	0	0.038	MUSH008	0	0.035
MUSH008	0.5	0.030	MUSH008	0.5	0.039	MUSH008	0.5	0.039
MUSH008	1	0.029	MUSH008	1	0.041	MUSH008	1	0.038
MUSH008	2	0.028	MUSH008	2	0.040	MUSH008	2	0.038
MUSH008	4	0.028	MUSH008	4	0.037	MUSH008	4	0.038
MUSH008	6	0.027	MUSH008	6	0.039	MUSH008	6	0.036
MUSH009	0	0.027	MUSH009	0	0.013	MUSH009	0	0.019
MUSH009	0.5	0.027	MUSH009	0.5	0.009	MUSH009	0.5	0.022
MUSH009	1	0.024	MUSH009	1	0.011	MUSH009	1	0.029
MUSH009	2	0.024	MUSH009	2	0.011	MUSH009	2	0.030
MUSH009	4	0.024	MUSH009	4	0.010	MUSH009	4	0.029
MUSH009	6	0.020	MUSH009	6	0.010	MUSH009	6	0.031

MUSH010	0	0.074	MUSH010	0	0.060	MUSH010	0	0.063
MUSH010	0.5	0.074	MUSH010	0.5	0.065	MUSH010	0.5	0.070
MUSH010	1	0.074	MUSH010	1	0.071	MUSH010	1	0.058
MUSH010	2	0.072	MUSH010	2	0.071	MUSH010	2	0.069
MUSH010	4	0.074	MUSH010	4	0.065	MUSH010	4	0.072
MUSH010	6	0.074	MUSH010	6	0.080	MUSH010	6	0.072
MUSH011	0	0.020	MUSH011	0	0.005	MUSH011	0	0.039
MUSH011	0.5	0.021	MUSH011	0.5	0.005	MUSH011	0.5	0.037
MUSH011	1	0.019	MUSH011	1	0.005	MUSH011	1	0.050
MUSH011	2	0.020	MUSH011	2	0.005	MUSH011	2	0.042
MUSH011	4	0.024	MUSH011	4	0.005	MUSH011	4	0.043
MUSH011	6	0.024	MUSH011	6	0.005	MUSH011	6	0.034
MUSH012	0	0.031	MUSH012	0	0.029	MUSH012	0	0.021
MUSH012	0.5	0.028	MUSH012	0.5	0.032	MUSH012	0.5	0.021
MUSH012	1	0.027	MUSH012	1	0.027	MUSH012	1	0.022
MUSH012	2	0.029	MUSH012	2	0.036	MUSH012	2	0.018
MUSH012	4	0.029	MUSH012	4	0.019	MUSH012	4	0.021
MUSH012	6	0.028	MUSH012	6	0.019	MUSH012	6	0.015
MUSH013	0	0.037	MUSH013	0	0.027	MUSH013	0	0.016
MUSH013	0.5	0.039	MUSH013	0.5	0.023	MUSH013	0.5	0.014
MUSH013	1	0.037	MUSH013	1	0.041	MUSH013	1	0.016
MUSH013	2	0.034	MUSH013	2	0.039	MUSH013	2	0.016
MUSH013	4	0.036	MUSH013	4	0.042	MUSH013	4	0.020
MUSH013	6	0.036	MUSH013	6	0.041	MUSH013	6	0.020
MUSH016	0	0.046	MUSH016	0	0.035	MUSH016	0	0.044
MUSH016	0.5	0.047	MUSH016	0.5	0.038	MUSH016	0.5	0.048
MUSH016	1	0.049	MUSH016	1	0.035	MUSH016	1	0.046
MUSH016	2	*	MUSH016	2	0.035	MUSH016	2	0.045
MUSH016	4	0.046	MUSH016	4	0.034	MUSH016	4	0.047
MUSH016	6	0.046	MUSH016	6	0.034	MUSH016	6	0.047