POLYCYCLIC AROMATIC HYDROCARBONS (PAHs): DEGRADATION AND FUNGAL BIOMASS (ERGOSTEROL) IN SEDIMENT WITH ADDED NITROGEN

by

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Polycyclic Aromatic Hydrocarbons (PAHs): Degradation and Fungal Biomass (Ergosterol) in Sediment with added Nitrogen

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Abstract:

White rot fungi, *Pleurotus ostreatus,* is capable of degrading a wide range of organic contaminants including PAHs. Bioremediating polycyclic aromatic hydrocarbons (PAH) contaminated river sediment with *P. ostreatus* maybe a viable and less invasive method to reduce risk of exposure. The first part of this research was to determine the degradation of PAHs from contaminated sediment. The second part was to determine if sterols (ergosterol) and PAHs can be extracted with a single extraction method. Ergosterol is produced by living fungi and can be used as a measurement of fungal biomass. Contaminated river sediments was treated with *P. ostreatus*, grown on barley, and various amendments then incubated at 25 °C. Amendments included sawdust and nitrogen supplement to stimulate fungal growth. The treatment with *P. ostreatus* showed degradation of total PAHs after 21 days using a Lipid Extraction Method and GC/MS. These results show that *P. ostreatus* is able to colonize in highly contaminated Mahoning River sediment and degrade the PAHs present. To extract ergosterol and PAHs simultaneously an Ergosterol Extraction Method (EEM) was developed. Various samples were prepared with sediment, sediment and fungi grown on barley, sediment and fungi grown on potato dextrose agar (PDA) or only fungi grown on PDA or barley. The EEM was successful in extracting ergosterol from fungi grown on barley resulting in concentrations from 17.5 - 39.94 µg/g ergosterol. Similar results were seen in the other treatments. PAHs were also found extracted in much greater amounts compared to the Lipid Extraction Method. In addition, cholesterol, usually found in animals, was detected in the fungi *P. ostreatus* at easily detectable levels. With better optimization of amendments, Ergosterol Extraction Method could be highly useful and effective in

analyzing the level of fungal biomass as well as PAH contaminants during bioremediation efforts.

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I am massively indebted to my advisor, Dr. Felicia P. Armstrong who is a great Professor. I am highly thankful for her help and advice in all through my education from the very beginning. I am facing difficulty in finding enough good words to say about her. She is not only a great professor but a great person too.

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Chapter 1: Introduction

Mahoning River History:

Contamination of soils, sediments, ground water and air with toxic materials has become a major concern today. The Mahoning River has received industrial waste since early 1900's from the steel industry. The lower branch of the Mahoning River starts at Winona, Ohio, extends towards Leavittsburg, and continues south east through Girard, Youngstown, Struthers and Lowellville into Pennsylvania (Figure 1). The Mahoning River joins with the Shenango River to form the Beaver River, which empties into the Ohio River (Figure 2).

The Mahoning River was significantly altered by the construction of numerous large reservoirs and low-head dams. The dams were constructed to provide a reservoir of cooling waters for the hot steel and machinery in the steel industries that used the Mahoning River as an 'industrial sewer' (Ohio EPA 1996). The water thus heated and filled with chemicals was directly released into the river. This release resulted in elevated river water temperatures, lower oxygen levels and high content of heavy metals thus depriving the river of any life. Throughout the years many contaminants were released into the river including heavy metals, oils, petroleum, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl compounds (PCBs) and many other carcinogenic and mutagenic compounds, thus polluting the river.

Between 1900 and the 1970's the Mahoning River received up to and over 70,000 lbs of oil and grease each day (Pabba 2008). The late 70's brought an improved look to the Mahoning River as some of the major steel industries went out of business. Although this cut down on some of the metal waste, organic waste such as PAHs and PCBs were

still being released into the river. Areas of intense PAH contamination from the industrial waste are found as "black mayonnaise" in the sediment (Pabba 2008).

In 2004, there was a drastic decrease in pollutant discharge into the river and the water quality improved appreciably. The sediment, however, remained highly contaminated with PAHs and PCBs because of their tendency to bind to the sediment particles, and their low solubility rates (Pabba 2008). When contaminants are introduced into aquatic environments, the sediments serve as a repository for the majority of these chemicals (OEPA 1996).

As the river water quality improved, the fish returned, but still a portion of them suffered from DELT (deformities, fin erosions, lesions and tumors). For these reasons, the Ohio Department of Health has issued a contact advisory for the carcinogenic contaminated sediment and a fish consumption advisory for the Mahoning River (Pabba 2008).

The Ohio EPA and US Army Corps of Engineers have estimated the contamination to a total of 750,000 cubic yards of material spread out over a span of 30 miles. Several river restoration options such as dredging, capping and bioremediation are being investigated (Pabba 2008).

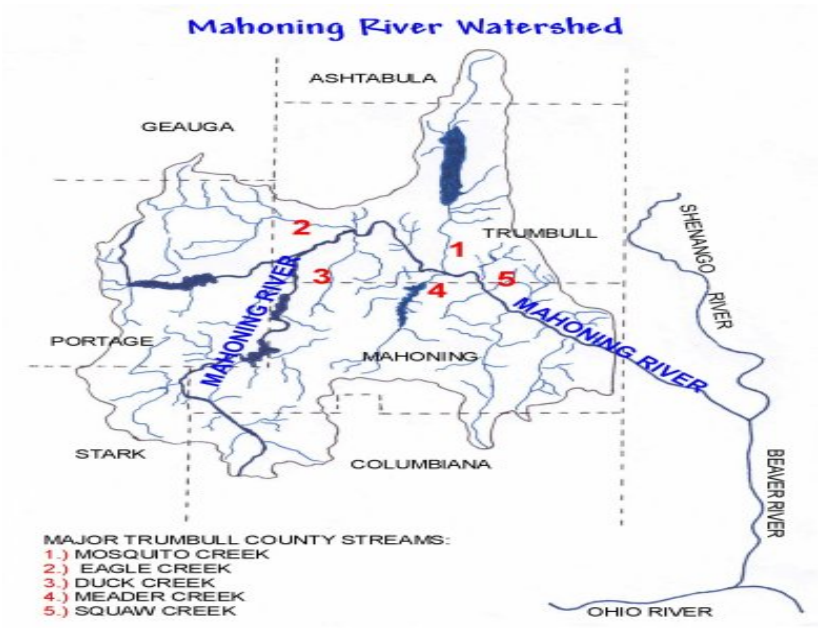


Figure 1. Picture of the Mahoning River Watershed
Courtesy of: www.swcd.co.trumbull.oh.us/.../mahoningmap.jpg

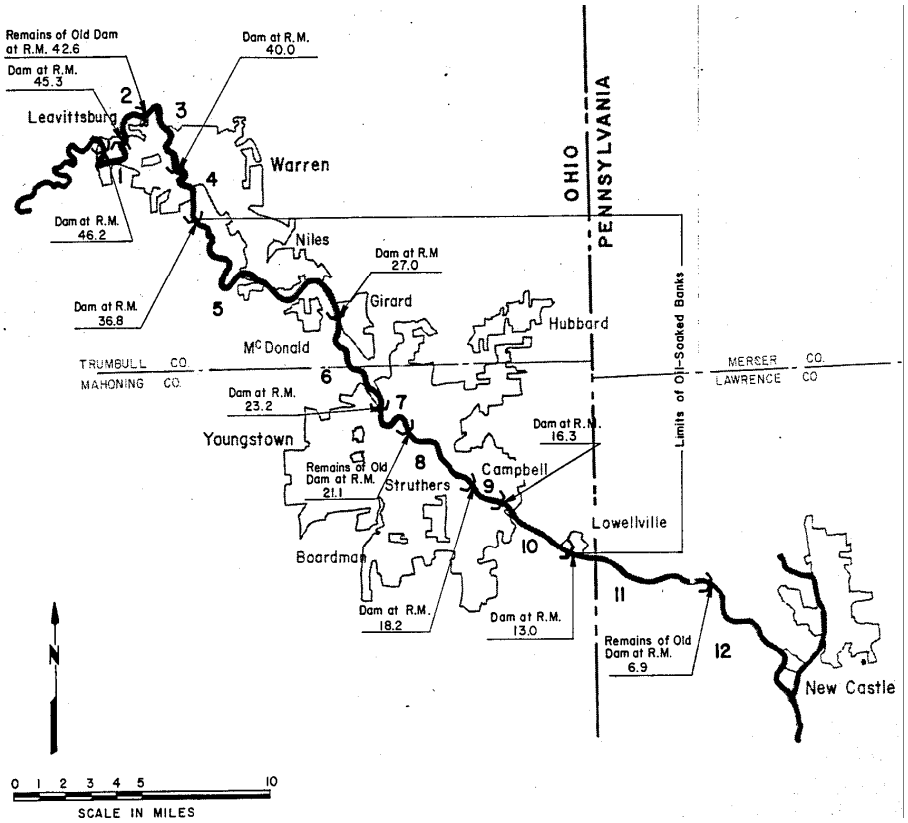
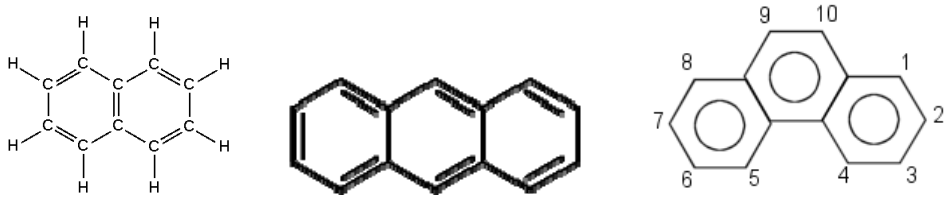


Figure 2. Map of the lower Mahoning River

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are widespread contaminants in the environment derived chiefly from the combustion of fossil fuels (Zhang et al. 2008). Polycyclic aromatic hydrocarbons constitute a large and diverse class of organic compounds and are generally described as molecules which consist of two or more fused aromatic rings in various structural configurations (Zhang et al. 2008). The smallest member of the PAH group is naphthalene (Figure 3), a two-ring compound. Three-to five ring PAHs may occur as either gases or particles in air (Figure 3). PAHs with five or more rings (Benzo(a)pyrene or B[a]P) tend to attach themselves to the surface of particles or are soot particles when formed (Figure 4) (Zhang et al. 2008, Spink et al. 2008). The sources of PAHs include (i) mobile sources (e.g. cars, buses, trucks, ships and aircraft), (ii) industrial sources (e.g. power generation, steelworks, coke ovens, aluminum production, cement kilns, oil refining and waste incineration), (iii) domestic sources (e.g. combustion for heating and cooking especially solid fuel heaters using wood and coal), (iv) fires and smoke resulting from the burning of vegetation in agricultural processes, bushfires, grilling of food, or tobacco smoke (Bixian et al. 2001, Kanaly et al. 2000, Oren et al. 2006, King et al. 2004).



Naphthalene Anthracene Phenanthrene

Figure 3. Structure of Low Molecular Weight Polycyclic aromatic hydrocarbons (PAHs)

One of the best characterized PAHs, and considered as one of the most potent PAHs from a toxicological point of view, is benzo(a)pyrene (Figure 4) **(**Zhang et al. 2008, Spink et al. 2008). The US Federal Water Pollution Control Act (1972) and the US Clean Water Act (1977) list 16 PAHs as priority pollutants based on their potential to be carcinogenic in animals and humans (Bixian et al. 2001). Current research focuses on the effects of microbial biodegradation of PAHs composed of more than three rings (Figure 4), PAHs with more than three rings are often referred to as high-molecular-weight (HMW) PAHs (Kanaly et al. 2000, Oren et al. 2006).

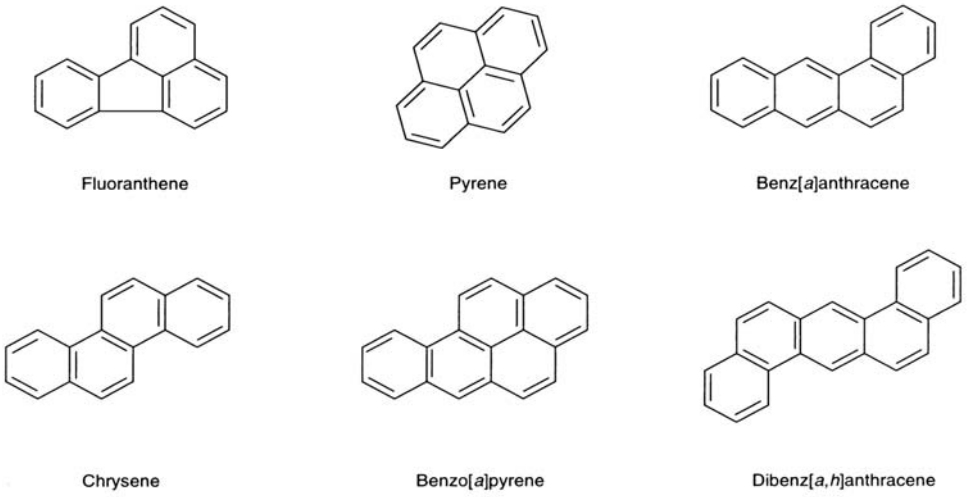


Figure 4. Representative high molecular weight (HMW) PAHs associated with the Mahoning River Sediment, Source**: (**Kanaly et al. 2000)

PAHs are lipophilic, have low volatility and are found primarily in soil, sediment and oily substances. In their pure state, PAHs generally exist as colorless, white, or pale yellow-green solids and can have a faint, pleasant odor (King et al. 2004). PAHs are ubiquitous contaminants in the environment and their fates in nature are of great environmental concern due to their potential toxicity, mutagenicity, teratogenicity and carcinogenicity (Spink et al. 2008, Luch et al. 2005). PAHs can be released into the environment, through atmospheric emissions (volcanoes, fires, exhaust from

automobiles); through water (industrial discharge, oil refining, waste water treatment plants; and into soil (atmospheric deposition, landfills). Leaking storage containers on hazardous waste sites can release concentrated PAHs into soil and water making them especially dangerous to nursing infants living near hazardous waste sites (Zhang et al. 2008, Oren et al. 2006). Foetus exposed to PAHs are at great risk and susceptibility resulting into growth retardation, low birth weight, small head circumference, low IQ, damaged DNA and disruption of endocrine systems such as estrogen, thyroid and steroids. The level of PAHs in the typical U.S. diet is less than 2 parts of total PAHs per billion parts of food (ppb), or less than 2 micrograms per kilogram of food (μg/kg; a microgram is one-thousandth of a milligram). The primary sources of exposure to PAHs for most of the U.S. population are inhalation of the compounds in tobacco smoke, wood smoke, and ambient air, and consumption of PAHs in foods (Zhang et al. 2008, Bixian et al. 2006, Kanaly et al. 2000, Oren et al. 2006, Perera et al. 2006, King et al. 2004).

The environmental fate of a PAH molecule is dependent in part upon the number of aromatic rings and the pattern of ring linkage. Generally, an increase in the size and angularity of a PAH molecule results in a simultaneous increase in hydrophobicity and electronic stability (Spink et al. 2008, Luch et al. 2005). PAH molecule stability and hydrophobicity are two primary factors which contribute to the persistence of HMW PAHs in the environment. Due to their lipophilic nature, PAHs have a high potential for biomagnifications through trophic transfers. The relationship between PAH environmental persistence and increasing numbers of benzene rings is consistent with the results of various studies correlating environmental biodegradation rates and PAH molecule size. For example, reported half life in soil and sediment of three-ring

anthracene molecule may range from 16 to 126 days while for the five-ring molecule benzopyrene range from 224 to >1400 days (Perera et al. 2006). Anthropogenic and natural sources of PAHs in combination with global transport phenomena result in their worldwide distribution. Hence, the need to develop practical bioremediation strategies for heavily impacted sites is evident. PAH concentrations in the environment vary widely, depending on the proximity of the contaminated site to the production source, the level of industrial development and the modes of PAH transport. Soil and sediment PAH concentrations at contaminated and uncontaminated sites ranging from 1 µg/kg to over 300 g/kg have been reported (Oren et al. 2006, Luch et al. 2005). A few PAHs are used in medicines and to make dyes, plastics, and pesticides. Others are contained in asphalt used in road construction (Luch et al. 2005).

Low molecular weight PAHs found in Mahoning River sediment:

(a) Naphthalene:

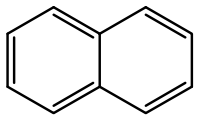


Figure 5. Structure of Naphthalene

Synonyms: Naphthalin, Naphthaline, Napthene, Tar Camphor, White tar, Albocarbon, or Antimite (MSDS).

Naphthalene is derived from coal tar and a primary ingredient of mothballs. It is used as a tanning agent and in surface active resins and dyes.

Exposure to naphthalene (>2ppb) may cause laryngeal carcinoma, damage of red blood cells (RBC) which leads to the development of hemolytic anemia. Acute exposure

causes cataracts in humans, rats, rabbits, and mice and people have an inherited condition called glucose-6-phosphate dehydrogenase deficiency (US EPA 1986).

(b) Acenaphthylene:

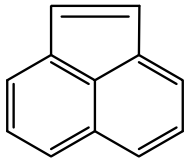


Figure 6. Structure of Acenaphthylene

Synonyms: Acenaphthalene, cyclopenta [*de*]naphthalene (MSDS).

Acenaphthylene is toxic and mostly found in crude oil, coal tar, cigarette smoke, exhaust from automobiles and wood preservatives. It is used to make dyes, plastics and pesticides.

Exposure to acenaphthylene may cause decreased red blood cell, haemoglobin, and hematocrit values and has shown decrease in platelet (males) and leukocyte counts (females), hepato cellular hypertrophy, nephropathy and related kidney lesions, decreased ovary weights, decreased ovarian and uterine activity, and smaller and fewer corpora lutea (US EPA 1989).

(c) Acenaphthene:

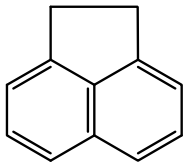


Figure 7. Structure of Acenaphthene

Synonyms: 1, 2-dihydroacenaphthylene, 1, 8-ethylenenaphthalene, peri-ethylenenaphthalene, naphthylene ethylene (MSDS).

Acenaphthene is toxic and a constituent of coal tar and is used in preparation of dyes, pesticides, and pharmaceuticals.

Exposure to acenaphthene through inhalation has been reported to lead to pathological effects in rats (12 mg/m^3) 4 hours/day; 6 days/week for five months included desquamation of alveolar epithelial cells (US EPA 1989).

(d) Fluorene:

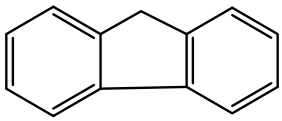


Figure 8. Structure of Fluorene

Synonyms: 9*H*-fluorene, *o*-biphenylmethane, diphenylmethane, 2, 3-benzindene (MSDS).

Fluorene is a tricyclic aromatic hydrocarbon which contains a five-membered ring. Exposure to fluorene is known to irritate skin, eye and respiratory tract. It shows evidence of mutagenic properties in laboratory animals. Agency for Toxic Substances and Disease Registry (TSDR) 1990 minimal risk level ORL 0.04 mg/kg/day.

(e) Phenanthrene:

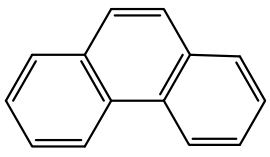


Figure 9. Structure of Phenanthrene

Synonyms: Coal tar pitch volatiles, ravatite, phenantrin (MSDS).

Phenanthrene is a toxic compound and is a polycyclic aromatic hydrocarbon composed of three fused benzene rings and provides a framework for steroids. It is usually found in vehicular emissions, coal and oil burning, wood combustion, coke plants, aluminum plants, iron and steel works, foundries, municipal incinerators, synfuel plants, and oil shale plants (US EPA 1987). It is one of a number of PAHs on EPA's priority pollutant list (ATSDR 1990). It is an irritant and causes a photosensitizing effect on skin.

(f) Anthracene:

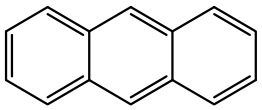


Figure 10. Structure of Anthracene

Synonyms: Paranaphthalene, Anthracin, Green oil (MSDS).

Anthracene is a linear and less stable isomer of phenanthrene and is formed as a product of incomplete combustion of fossil fuels. It is used in the production of dyes and smoke screens. It is also used as an organic semiconductor and plastic scintillator. Anthracene is toxic and causes photosensitivity, resulting in skin damage elicited by exposure to ultraviolet (UV) radiation (US EPA 1987, Dayhaw-Barker et al. 1985, Forbes et al. 1976).

High Molecular PAHs found in Mahoning River:

(a) Pyrene:

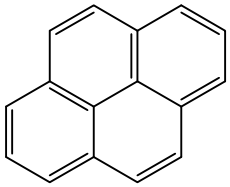


Figure 11. Structure of Pyrene

Synonyms: beta-pyrene, coal tar pitch volatiles (MSDS).

Pyrene is produced in a wider range of combustion conditions as it is much more resonance stabilized than its five-member-ring containing isomer fluoranthene.

Skin painting assay evaluations in mice showed complete carcinogenicity in mice. However, it has been reported as toxic to humans (Van Duren and Goldschmidt 1976).

(b) Benzo(a)pyrene:

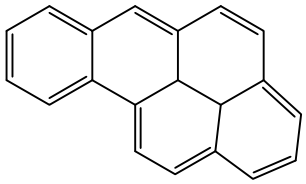


Figure 12. Structure of Benzo(a) pyrene

Synonyms: 3, 4-benzopyrene, Benzo (alpha) Pyrene (MSDS).

Benzo(a)pyrene is found in coal tar, in automobile exhaust fumes (especially from diesel engines), tobacco smoke, marijuana smoke, wood smoke, and in charbroiled food.

Benzo(a)pyrene is a probable human carcinogen, developmental toxicant, endocrine toxicant, immunotoxicant, respiratory toxicant and skin/sense organ toxicant (US EPA 1994) .

(c) Benzo(a)anthracene:

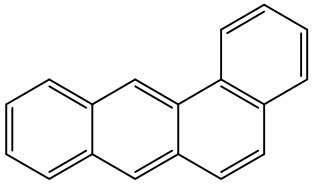


Figure 13. Structure of Benzo(a) anthracene

Synonyms: Benzo (a) phenanthrene, Tetraphene (MSDS).

Benzo (a) anthracene is natural product produced by the incomplete combustion of organic material. The arrangement of the aromatic rings in the benz(a)anthracene molecule gives it a "bay region" often correlated with carcinogenic properties (US EPA 1980, Jerina et al. 1977).

(d) Chrysene:

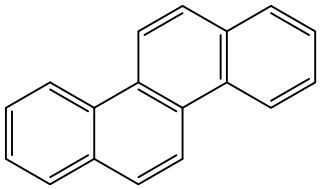


Figure 14. Structure of Chrysene

Synonyms: 1, 2-benzphenanthrene (MSDS).

Chrysene is a natural constituent of coal tar. It is formed by combustion of crude oil, burning of garbage, plant and animal material, diesel and aircraft exhaust, coke oven emissions and used in the manufacture of artificial dyes.

Chrysene is a probable carcinogen and mutagen and exposure to chrysene (> 0.2 mg/m^3) for a long time can leads to skin cancer (IARC 1983, ATSDR 1990).

(e) Dibenz (a,h) anthracene:

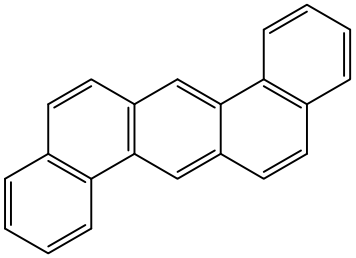


Figure 15. Structure of Dibenz (a,h) anthracene

Synonyms: 1, 2:5, 6-Dibenzanthracene (MSDS).

Dibenz (a,h) anthracene occurs as a component of coal tars and shale oils. It is found in gasoline engine exhaust, cigarette smoke, site near heavily traveled roads, surface water and soils near hazardous waste sites (ATSDR 1993, IARC 1983).

Animal studies have shown the development of pulmonary disease, pancreatic disorder and mammary carcinomas in females, thus rendering it carcinogenic and mutagenic (ATSDR 1993, IARC 1983).

(f) Benzo (ghi) perylene and Benzo (b, k) fluoranthene:

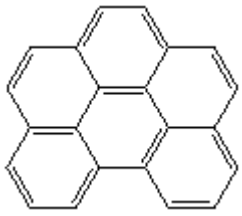


Figure 16. Structure of Benzo(ghi)perylene

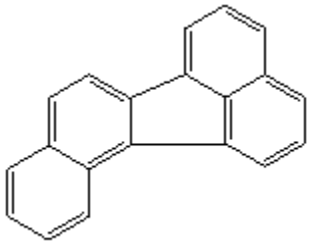


Figure 17. Structure of Benzo(b,k) fluoranthene

Benzo (ghi) perylene:

Synonyms: 1, 12-Benzoperylene; 1, 12-Benzperylene (MSDS).

Benzo (b, k) fluoranthene:

Synonynms: Benzo[b]fluoranthene, benzo[j]fluoranthene (MSDS).

These PAHs are most likely result from the incomplete combustion of a variety of fuels including wood and fossil fuels. They are found in mainstream cigarette smoke, urban air, gasoline engine exhaust, emissions from burning coal and from oil-fired heating, broiled and smoked food, oils and margarine (IARC 1983). It is classified as probable human carcinogen and exposure may result in tumors (LaVoie et al 1982).

Bioremediation by White Rot fungi (*Pleurotus ostreotus*):

Bioremediation is a managed or spontaneous technique where microbiological processes (bacteria, fungi) are used to degrade or transform contaminants to less toxic or nontoxic forms. Bacteria have been extensively used for use in the degradation of pesticides because of their ease of culture, more rapid growth rates and convenience for genetic manipulation (Pabba 2008). Bacterial remediation can be limited by the size of the PAH molecule. Low-molecular-weight PAHs are usually readily degraded, but high-molecular-weight PAHs of five or more rings resist extensive bacterial degradation in soil and sediments. This can be attributed to the limited bioavailability of PAHs strongly adsorbed onto the soil organic matter (Field et al. 1992). Studies have demonstrated that fungi, in particular white rot fungi are capable of degrading a vast number of pollutants, including PAHs (Pabba 2008). Fungi might offer some advantages over bacteria for remediation due to their rapid colonization of substrates and high tolerance of the toxin (Cerniglia et al. 1992, 1993). These fungi display an extracellular degradation system that is capable of cleaving lignin (Figure 18) (Pabba 2008). Lignins are an amorphous

and complex biopolymer with an aromatic structure that resembles the aromatic molecular structure of the pollutants like PAHs, pesticides and polychlorinated biphenyls (PCBs), (Valentin et al. 2006). Both lignin and PAHs are highly insoluble, hydrophobic and pose similar problems for catalysis by enzymes which tend to be water soluble and usually highly stereospecific. This structural resemblance makes the fungal degradation of PAHs by *Pleurotus ostreatus* or white rot fungi, feasible. The ability of these fungi to degrade multiple pollutants with large structural diversity has fuelled the interest in their use for bioremediation (Pabba 2008).

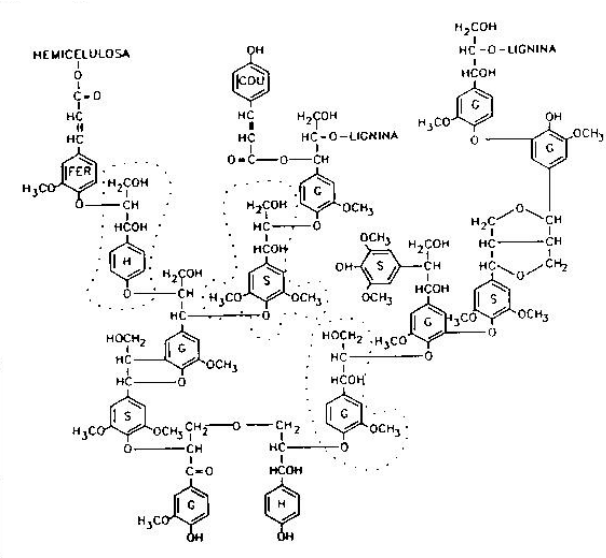


Figure 18. Chemical Structure of Lignin

Source: http://www.ibwf.de/env&enz_index.htm

The lignolytic potential of these fungi can be related to the secretion of oxidative enzymes such as lignin peroxidases, manganese peroxidases and laccases. The importance of these extra-cellular enzymes is that they are free radicals capable of cleaving lignin and other ring structures, including PAHs. *P. ostreatus* will partially

oxidize the PAHs, converting the pollutants into more soluble compounds with increased bioavailability. The principal biochemical reactions in the primarily co-metabolic degradation of pollutants by fungi include oxidation, reduction, hydroxylation, aromatic ring cleavage, hydrolysis, dehalogenation, methylation and demethylation, dehydrogenation, ether cleavage, condensation and conjugate formation. To survive in soil white rot fungi require lignocellulosic substrates as their carbon source for energy. Many studies show the use of straw and milled wheat as good substrates for fungal growth (Wolter 1997). Recent findings have shown that the toxicity of organic pollutants was found to be greatly reduced in sawdust-based media than in liquid media (Alleman et al. 1992).

Prior work has been conducted showing the effectiveness of *Pleurotus ostreatus* as a PAH degradative fungi (Baldrian et al., 2000). One study has shown that after only three days of incubation, *P. ostreatus* was capable of degrading fluorene and anthracene by 43% and 60% in liquid culture (Schutzendubel et al. 1999). A study looking at creosote contaminated soil incubated over a 70 day period using *P. ostreatus* demonstrated the two and three-ringed hydrocarbons were eradicated, leaving only small concentrations of four and five-ringed hydrocarbons (Atagana et al. 2006).

Another important reason for utilizing *P. ostreatus* for bioremediation is because it is more acceptable by the public. *P. ostreatus* is an edible species of mushroom grown commercially for human consumption. Due to the commercial availability of *P. ostreatus* it would be more acceptable by the general public to use this species for bioremediation in the Mahoning River watershed.

Steroids:

Steroids are complicated polycyclic molecules found in all plants, animals and fungal kingdom. Steroids are tetracyclic compounds that serve a wide variety of biological functions, including hormones (sex hormones), emulsifiers (bile acids) and membrane components (cholesterol). They are classified as simple lipids because they do not undergo hydrolysis like fats, oils and waxes do. Steroids are compounds whose structures are based on the tetracyclic andosterone ring system shown in (Figure 19). Steroids of interest for the growth of fungi in soil are cholesterol and ergosterol.

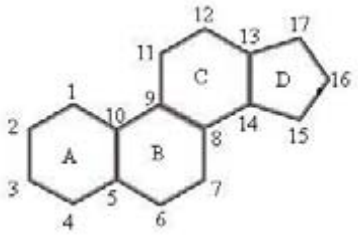


Figure 19. Andostane ring system

The four rings are designated as A, B, C and D beginning with the ring at lower left, and the carbon atoms are numbered beginning with the A ring and ending with the two "angular" axial methyl groups. Androsterone can have either *trans* or *cis* stereochemistry at each ring junction (Wade 2006).

Cholesterol:

Cholesterol is a common biological intermediate and is the biosynthetic precursor to other steroids. It was first discovered in human gall-stone, and received the name cholesterol because of its presence in bile. It is a mono-unsaturated alcohol, with the

formula $C_{27}H_{46}O$, found in all higher animals. It has three regions: a hydrocarbon tail, four hydrocarbon rings and a hydroxyl group (Javitt 1994) (Figure 20).

Cholesterol is the precursor molecule in several biochemical pathways. In the liver, cholesterol is converted to bile, which is then stored in the gallbladder. Bile contains bile salts, which solubilize fats in the digestive tract and aid in the intestinal absorption of fat molecules as well as the fat soluble vitamins, Vitamin A, Vitamin D, Vitamin E and Vitamin K (Javitt 1994). About 20–25% of total daily cholesterol production occurs in the liver; other sites of high synthesis rates include the intestines, adrenal glands and reproductive organs (Lewington et al. 2007).

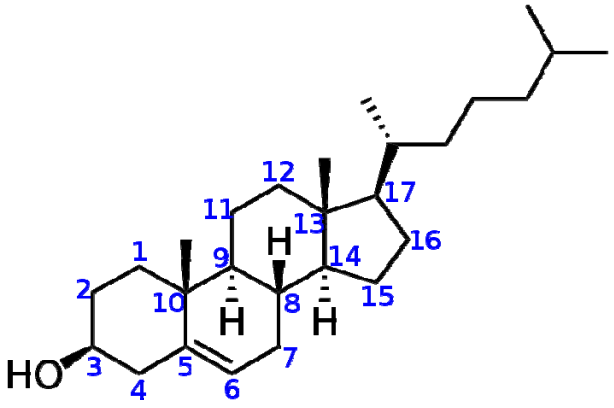


Figure 20. Chemical Structure of Cholesterol (MW 386.65)

Cholesterol is required to build and maintain cell membranes; it regulates membrane fluidity over the range of physiological temperatures. The hydroxyl group on cholesterol interacts with the polar head groups of the membrane phospholipids and sphingolipids, while the bulky steroid and the hydrocarbon chain are embedded in the membrane, alongside the nonpolar fatty acid chain of the other lipids (Olson 1998). The role of cholesterol in such endocytosis can be investigated by using methyl beta

cyclodextrin (MβCD) to remove cholesterol from the plasma membrane. Recently, cholesterol has also been implicated in cell signaling processes, assisting in the formation of lipid rafts in the plasma membrane (Haines 2001). In many neurons a myelin sheath, rich in cholesterol since it is derived from compacted layers of Schwann cell membrane, provides insulation for more efficient conduction of impulses. Within the cell membrane, cholesterol also functions in intracellular transport, cell signaling and nerve conduction (Wojciech et al. 2006). Cholesterol is an important precursor molecule for the synthesis of Vitamin D and the steroid hormones, including the adrenal gland hormones cortisol and aldosterone as well as the sex hormones progesterone, estrogen, and testosterone and their derivatives (Smith 1991).

Ergosterol:

Ergosterol, ($C_{28}H_{44}O$) (22-*E*-Ergosta-5, 7, 22-trien-3-β-ol) is a steroids which is unique to the fungal cell membrane (Figure 21). Representative fungi containing ergosterol may include mushrooms such as *Lentinus edodes*, *Grefola frondosa*, yeasts and leguminous bacteria found in roots of leguminous plants, in addition, other microorganisms containing ergosterol may include unicellular algae such as Chlorella (Boer et al. 2006).

Ergosterol is a white crystalline compound, insoluble in water and soluble in organic solvents. Assessment methods to determine the key role in fungi in terrestrial ecosystems is required to prepare bioremediation systems. Microscopic determinations of hyphal length were most widely used as an assessment method. Quantification of soil ergosterol level is increasingly used as an estimate for soil fungal biomass (Boer et al. 2006).

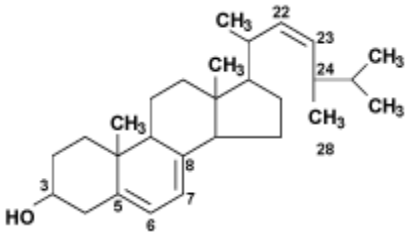


Figure 21. Chemical structure of ergosterol (M.W. 396.65)

Fungi may be the main decomposers of plant material in an aquatic system and their presence and biomass can be measured indirectly by the detection of ergosterol (Fig.21), thus making it an ideal index molecule for these microorganisms. Ergosterol is conveniently used as a measurement of living fungal biomass in environmental samples, primarily by using high-performance liquid chromatography (HPLC) (Verma et al. 2002, Larsen et al. 2004, Headley et al. 2002). Because of its inherent high degree of sensitivity and specificity, it should be advantageous to use GC-MS for measuring ergosterol. Ergosterol is believed to undergo rapid degradation after cell death thus levels of ergosterol present are generally considered to be directly correlated to living fungal biomass (Verma et al. 2002, Larsen et al. 2004, Headley et al. 2002, Axelsson et al. 1995). Most research to date has focused on fungal biomass in decaying plant leaves or grasses in salt-marshes, streams or in forest soils where there is high plant litter content. Little work has been reported for the detection of ergosterol in environmental matrices other than those noted and none in northern riparian zones.

Data indicate that the wide range in specific ergosterol content of *living* fungal biomass in soil is related to the amount of fungal hyphae in soil (Zeppa et al. 2000). Recent developments are using the detection of ergosterol in environmental samples. Numerous investigations have been published recently about the use of ergosterol as a general indicator of fungal contamination in soil. White rot fungi, ***P. ostreatus*** are most

efficient in degrading oil in contaminated soil (Axelsson et al. 1995).

Fungi are extremely important contributors to terrestrial ecosystem function. These organisms, as a group, are the heterotrophs primarily responsible for the decomposition of organic residues, represent large nutrient pools, participate in mycorrhizal symbioses with the majority of land plants, are critically involved in soil food webs, and serve to stabilize soils through their contribution to soil aggregation. In fact, fungal biomass in terrestrial environments may be surpassed only by that of plants, but problems in accurate quantification of fungi make measuring fungal biomass difficult (Stahl et al. 1996).

Fungi, like many other microorganisms, cannot readily be seen or separated from the soil matrix. Another problem is that the fungi are a highly diverse group of organisms with major morphological and physiological disparities which make it difficult to quantify their biomass with a single assay or measurement (Stahl et al. 1996).

Biochemical methods for assay of fungal marker molecules such as chitin (more properly, glucosamine) and ergosterol, may involve less observer associated variability as seen in hyphae measurements but entail other problems. There is a variable content of glucosamine in fungal tissue and specificity to fungi and glucosamine can be found in other microorganisms in soil therefore, the glucosamine technique is declining in its use (Stahl et al. 1996). The ergosterol method, which has been proposed as a measure of fungal biomass in soil more recently, is gaining in popularity but has not yet been evaluated thoroughly. Ergosterol may be a particularly useful index of fungal presence because it is indigenous only to fungi and certain green microalgae. One of the problems with this method is in converting soil ergosterol concentrations to fungal biomass

estimates. Ergosterol content of fungal tissue is known to vary with species and physiological state of the fungus and a few groups of fungi do not produce ergosterol at all (Stahl et al. 1996).

Various methods have been reported for the detection of ergosterol in environmental matrices, most of which are based on conventional high performance liquid chromatography with ultraviolet detection (HPLC-UV) and/or gas chromatography with mass spectrometry detection. For gas chromatography methods, it is common to form a trimethyl silyl derivative or methyl ester to improve the peak shape and detection (Larsen et al. 2005).

Ergosterol content has been used to estimate fungal biomass in various environments, for example in soil and aquatic systems because a strong correlation exists between ergosterol content and fungal dry mass (Parsi et al. 2006). However, the amount of ergosterol in fungal tissue is not constant. It depends on the fungal species, age of the culture, developmental stage (growth phase, hyphal formation and sporulation) and growth conditions (growth media, pH and temperature). Unfortunately, no clear trend relating ergosterol to any of these factors has yet been determined (Parsi et al. 2006).

In all these applications, the pyrolysis GC/MS system was used to liberate intact ergosterol from the sample rather than to pyrolyze it. However, other components of the sample certainly underwent pyrolysis under the conditions applied (Parsi et al. 2006).

Advantages of Ergosterol for fungal biomass:

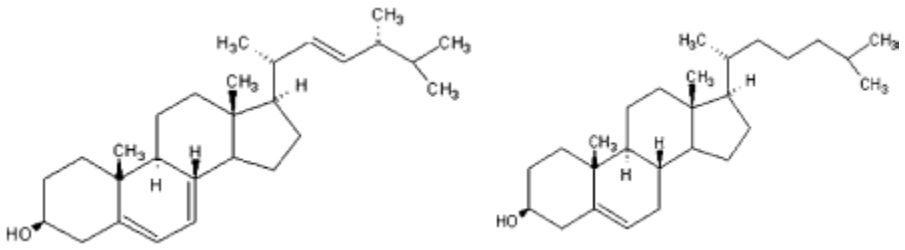
Sterols are essential structural and regulatory components of eukaryotic cell membranes (Veen et al. 2005). Ergosterol, the end product of the biosynthetic pathways

and the main sterol in yeast and other fungi, is responsible for structural membrane features, such as fluidity and permeability similar to the way cholesterol is in mammalian cells (Veen et al. 2005). Several intermediates of the ergosterol pathway are economically important metabolites. Lanosterol, the first sterol of the pathway, is used as a non-ionic, auxillary emulsifier to jellify hydrocarbons and is added to cosmetic preparations, particularly lipstick and cosmetic creams (Veen et al. 2005). Ergosterol itself is commonly known and used as a provitamin D_2. Ergosterol is a high molecular sterol when isolated in crystalline form it is an intermediate product used for preparing Vitamin D including its active metabolites and in the chemicopharmaceutical industry, for preparing steroid hormones. Ergosterol (provitamin D_2), is an unsaponifiable liquid found in ergot, yeast and other fungi. It is a white crystalline compound, insoluble in water and soluble in organic solvents. It is converted into ergocalciferol (Vitamin D_2) upon irradiation by UV light or electronic bombardment (Rajakumar et al. 2007). Sterols also function as moisturizers in skin-conditioning cosmetics and serve as the starting material of choice for the synthesis of various tetracyclic triterpenoid derivatives. Sterols act as structural components in liposomes, which are used as carriers of drugs and diagnostic substances in pharmaceutical applications. Ergosterol has anticarcinogenic effect of yeast extracts on breast cancer cells; the oxidation products of ergosterol are responsible for this effect (Veen et al. 2005).

Membrances of eukaryotic cells have several important functions. They act as barriers between the inside of the cell or the lumen of organelles and the corresponding environment. Membranes also carry proteins that relatively transport molecules or act as enzymes in different metabolic activities (Veen et al. 2005).

Ergosterol is considered as the principal sterol of fungi and it plays an important role as a cell membrane component. Therefore, it has been proposed as a global indicator of mycological quality of foods and feeds. One interesting point of this compound is that it is not affected by harsh physical treatment, allowing the detection of previous mold contamination. Ergosterol levels are commonly used as quality parameters in ecological, industrial and agronomics environments. Moreover, significant correlations were found between ergosterol and the major mycotoxins (fumonisin B_1, Zearalenone, Deoxynivalenol, Ochratoxin A, patilin) in maize, rice, tomato and wheat. Therefore, ergosterol determination can be considered as a good index of fungal development on cereals and could be an early indicator of potential mycotoxin production. Its determination can be used in industry to screen production, prior to mycotoxin analysis (Tardieu 2007).

Comparison of Cholesterol and Ergosterol:



Ergosterol Cholesterol

Figure 22. Comparison of Ergosterol and Cholesterol Structures

Source: Jedlickova et al. 2008.

Structurally, ergosterol and cholesterol (Figure 22) are related to each other but surprisingly cholesterol occurs in animals and ergosterol is present in plants and fungi. Ergosterol differs from cholesterol by having three positions of unsaturation, at carbon

atoms 5-6, 7-8 and 22-23 and containing a methyl group (carbon atom 28) substituted for a hydrogen atom at carbon atom 24. The difference in the structures of cholesterol and ergosterol is that, in ergosterol there are double bonds at the 7 and 22 positions while there are no double bonds at 7 and 22 positions in cholesterol. In ergosterol, there is an isopropyl group at the 24 position while in cholesterol there is no isopropyl group.

Objective:

The potentially deleterious effects of PAHs on human health and their microbial biodegradation in the environmental system have prompted this researcher to undertake this project as the thesis topic. The goal of this research is to extract and measure the biodegradation of polycyclic aromatic hydrocarbons for the various samples collected from the sediments of Mahoning River using GC/MS by a Lipid Extraction procedure. A new method is developed, the ergosterol extraction method, with the aim that it should be useful in the extraction of both PAHs as well as ergosterol. The importance of this study is to discover if *Pleurotus ostreatus* is capable of playing a major role in the degradation of PAHs in wet, unique, historically contaminated river sediment. *Pleurotus ostreatus*, a white rot fungus is inoculated in the contaminated sediments of Mahoning River with subsequent analysis of the effect of ergosterol as an indicator for fungal biomass. The objective of this research is to (1) determine the efficient PAH extraction procedure and (2) compare two PAH extraction methods. A most important target also is to compare the two methods and analyze which one gives most productive results. Modified ergosterol extraction method will be the most efficient method for the extraction of both PAHs and ergosterol if it gives qualitative results analyzed by GC/MS. We have inoculated the

contaminated sediments of Mahoning River by *Pleurotus ostreatus*. Our aim is to analyze the extracted ergosterol by GC/MS so that we can detect the degradation of PAHs at different time intervals by *Pleurotus ostreatus* from the sediments of Mahoning River. Further work may be explored in the future with this newly developed method.

Chapter 2: Materials and Methods

Part 1 Biodegradation of PAHs

Sample Collection

Sediment samples were collected from the shoreline of the Mahoning River in Lowellville, Ohio (Figure 23) and stored at 4 ^{o}C until use. Each sediment sample was analyzed for PAH content using both lipid extraction and ergosterol techniques to track the extraction efficiencies.

Figure 23. PAH contaminated sediment from Lowelville OH

Fungal Growth

The white-rot culture was grown by Biology graduate students on white-rot selective potato dextrose agar (PDA) plates (Dietrich and Lamar 1990). The inoculated selective plates were placed in incubators at 25 °C for 72-120 hours. Cores of the plates were taken and placed into sterile 250 mL Erlenmeyer flasks containing 125mL of Potato Dextrose broth (Sigma). Broths were placed on a shaker at 250 RPM for 48-72 hours at room temperature (detail in Appendix 9).

After growth of inocula in 125mL of Potato Dextrose broth, 500mL of grain was placed into spawn bag (Fungi Perfecti LLC) with 150 mL of deionized water. The spawn bag containing the grain and water mixture was sealed using an impulse sealer and autoclaved at 121 °C. Once autoclaved, the spawn bag was then inoculated with 125 mL of the Potato Dextrose broth containing the *P. ostreatus* inocula. Spawn bags were then placed in a 25 °C incubator for 72-120 hours.

Sediment Incubation Set-up

One liter of the PAH contaminated Mahoning River sediment was transferred into 2 liter glass containers ("fish bowls"). Eight experimental treatments were tested in triplicate in the laboratory during a 6 week period. The first treatment contained only sediment which was used as control. Treatment sediment was amended with sawdust (60% by volume), augmented with *Pleurotus ostreatus* (10% by volume) in the treatments and 10% by volume of inoculated grain was added to each bowl (containing sediment samples) as substrates for fungal growth. The second treatment (by volume) was 30% sediment with 60% sawdust augmented with *Pleurotus ostreatus* (10% by volume) and 10% of the inoculated grain as substrates for fungal growth was added to each bowl. To the treatments with saw dust (which provides carbon and nutrients to fungi), fungi (*Pleurotus ostreatus*) and additional nitrogen source (10% by volume) was added to stimulate fungal growth. A treatment free sediment sample was taken as control. The nitrogen sources included spawnmate, urea, peptone and tryptophan.

Contents of the bowl were mixed to create a homogenous mixture. A glass petri dish filled with sterile water was suspended inside of the glass bowl to keep the moisture content constant. Incubation bowls were covered first with plastic wrap then aluminium foil and placed in an incubator at 25 °C. Initially seven treatments were tested at time zero in July 2008 (Table 1); PAH extractions were done from these samples as a test of the lipid extraction method. The first controls contained sediment, saw dust (600 ml), grain (100 ml) and fungi (100 ml).

In October 2008, eight more treatments (Table 2) were prepared and PAH extractions of each treatment were done at three different times: 0, 21 and 42 days.

Table. 1 Sample treatments from July 2008

1.	Control (Sediment, Sawdust, Fungi)
2.	Control + Spawn mate (50ml)
3.	Control + Spawn mate (100ml)
4.	Control + Urea (5g)
5.	Control + Urea (50g)
6.	Control + peptone (5g)
7.	Control + peptone (10g)

Table. 2 Sample treatments from October 2008

1.	Control (Sediment only)
2.	Control+ Sawdust+Fungi
3.	Control + Sawdust+Fungi+Spawnmate (25ml)
4.	Control + Sawdust+Fungi+Spawnmate(150ml)
5.	Control + Sawdust+Fungi+Peptone (5g)
6.	Control + Sawdust+Fungi+peptone (15g)
7.	Control + Sawdust+Fungi+tryptophan (1g)
8.	Control + Sawdust+Fungi+tryptophan (10g)

Lipid Extraction Procedure

PAHs were extracted using a modified lipid extraction method from the contaminated sediments of the Mahoning River (Fang and Findlay 1996) based on the Bligh and Dyer method (Figure 24). A mixture of dichloromethane (DCM), methanol, phosphate buffer (pH 7.4) and a surrogate solution were added to the samples, mixed well and extracted (detailed procedure in Appendix 1 and 3).

The organic phase (DCM) containing PAHs was collected and purified over sodium sulfate columns. The sample was further concentrated by evaporating the solvent.

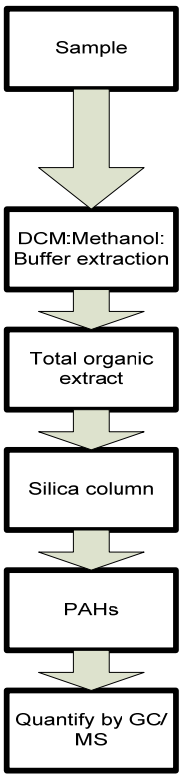


Figure 24. PAH extraction procedure flow chart (based on Fang and Findlay 1996)

Part 2 Extraction of Ergosterol and PAHs

Steroids Extraction Procedure:

Much work is done for the extraction of PAHs and ergosterol separately using high performance liquid chromatography (HPLC) which can be time consuming and create higher amounts of waste. Using a single extraction method to extract PAHs and ergosterol together is both less time consuming and produces less waste. Gas chromatography-mass spectrometry (GC/MS) is used for analysis instead of HPLC because it is more efficient and accurate due to its high degree of sensitivity and specificity (Axelsson et al. 1995, Stahl et al. 1996, Yateem et al. 1998, Parsi et al. 2006). Sediment and reference ergosterol extraction and analysis was done using a method modified from the protocol described by Brodie et al. (2003) with ethanol used as the extraction alcohol because of its greater extraction efficiency (Padgett et al. 1993). The reference standard of sterols (ergosterol and cholesterol 98% purity, Sigma- Aldrich) was prepared in dichloromethane (DCM). During the extraction procedure of samples 1.50 M KOH in 96% ethanol was used for the saponification process. This caused de-esterification of the complexed ergosterol to its free form and to facilitate its detection (detail in Appendix 7).

All extractions of sterol (ergosterol and cholesterol) was done in triplicate. The control mixtures were a combination of Sediment + Sawdust + Fungi grown on barley (Table 3). The sample mixtures were either fungi grown on PDA or barley and then mixed with sediment (Table 4). The fungi were cultivated on barley in a bio-bag for approximately 3-weeks prior to sampling. The pure fungi were grown on PDA as described previously. Sediment was collected from Lowellville, Ohio in September

2008. Fungi and sediment mixtures (controls and samples) were not incubated together; these were mixed just prior to sampling and analysis. A total of twenty-one treatments (control mixture and sample mixtures) were done in triplicate. Acetylation was used for samples containing pure fungi and any samples containing sediment. This helps to separate the sterols from other contaminants such as PAHs.

GC requires that the compound should be in the gaseous phase, therefore to extract ergosterol its melting point should be lowered (m.p.160 °C, b.p. 250 °C). Therefore, by doing acetylation its melting point is lowered and more easily detected on GC/MS. Another advantage of acetylation is that the original compound can be regenerated by hydrolysis (detail in Appendix 8).

Purification

Both the samples from the lipid extraction procedure and modified protocol from Brodie et al. for sterols were concentrated and passed over silica columns. Any unwanted sediment waste adsorbs onto the activated silica thus purifying the samples. The samples were further purified twice over the aminopropyl columns and analyzed by GC/MS after the internal standard was added (for PAH analysis only).

Table. 3 Sample treatments for control mixture **(**Sediment + Sawdust + Fungi grown on barley)

1.	Control mixture (5.1206g)
2.	Control mixture (5.2921g)
3.	Control mixture (5.1742g)
4.	Control mixture (5.0781g)
5.	Control mixture (5.0357g)
6.	Control mixture (5.0135g)

Table. 4 Sample mixtures for the extraction of sterols from fungi grown on PDA or barley then mixed with sediment

1.	Pure fungi (0.5325g)
2.	Pure fungi (0.5247g)
3.	Pure fungi (0.5225g)
4.	Pure fungi (0.2450g)+ Sediment (0.5310g)
5.	Pure fungi (0.2450g)+ Sediment (0.5310g)
6.	Pure fungi (0.2218g)+ Sediment (0.6480g)
6.	Fungi (grown on barley) (0.6673g)
7.	Fungi (grown on barley) (0.5308g)
8.	Fungi (grown on barley) (0.5343g)
9.	Fungi (grown on barley) (0.6207g)+Sediment (0.6199g)
10.	Fungi (grown on barley) (0.6143g)+Sediment (1.1886g)
11.	Fungi (grown on barley) (0.6327g)+Sediment (0.5743g)
12.	Pure Ergosterol
13.	Pure Cholesterol
14.	Acetylated Ergosterol
15.	Acetylated Cholesterol

Analysis of PAHs and steroids

Commercial ergosterol and cholesterol were analysed by GC/MS as standard references. When the samples collected from the ergosterol extraction method was analyzed using GC/MS, 16 PAHs were detected. US EPA regulated these 16 PAHs that were detected by this method.

All analysis was performed on a Hewlett Packard 5890 Gas Chromatograph/ 5989A Mass Spectrometer equipped with a DB-5 column (30 m, 0.32 mm ID, and a HP 6890 Series Injector).

The injector and the detector temperatures were set at 250 °C and 300 °C respectively. The oven temperature was held at 45 °C for 2 minutes, and then ramped at 20 °C per minute to 310 °C. The final temperature was held for 5.5 minutes.

CHAPTER 3: Results and Discussion

Part 1 Biodegradation of PAHs:

Incubations were set up over a period of 21 days at 25 °C and the extractions were performed in triplicate to confirm the PAH degradation patterns. Saw dust, substrate, and nitrogen supplements were added for fungal growth. A treatment-free control was also included in the runs to check the results without any amendments.

The data are shown (Tables 6, 7 & 8) as a comparison of degradation patterns of low and high molecular weight PAH (µg PAHs/g sediment dry weight). PAHs can be classified based on differences in their molecular weights (Table 5).

Table 5. Low and high molecular weight PAHs

LOW MOLECULAR WEIGHT PAHs	HIGH MOLECULAR WEIGHT PAHs
Naphthalene – 2 rings	Chrysene – 4 rings
Acenaphthylene – 3 rings	Pyrene – 4 rings
Acenaphthene – 3 rings	Benzo(a)anthracene – 4 rings
Fluorene – 3 rings	Fluoranthene – 5 rings
Phenanthrene – 3 rings	Benzo(b&k)fluoranthene – 5 rings
Anthracene – 3 rings	

Low Molecular Weight PAHs (LMW PAHs):

The LMW PAHs are 2/3-ring organic compounds which are hydrophobic and can be degraded by bacteria (Bouchez et al. 1999). Napthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene can be classified under the LMW category.

High Molecular Weight PAHs (HMW PAHs):

The HMW PAHs are organic compounds with 4 or more aromatic rings which makes them strongly hydrophobic. Pyrene, fluoranthene, chrysene, benzo(a)anthracene, benzo(b,k)fluoranthene are classified in this category.

PAHs analyses:

Out of eleven PAHs detected from the sediments of Mahoning River by the lipid extraction method, six of them are low molecular weight PAHs, namely naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene. Five high molecular weight PAHs were detected which include fluoranthene, benzo[a]anthracene, pyrene, chrysene and benzo[b,k]fluoranthene. The contaminated sediment had an initial PAH concentration of 342.0 µg/g dry weight. Individual concentration of PAHs ranged from 1.32 to 85.1 µg/g dry weight. Of the detected PAHs, fluoranthene was of the highest concentration with 85.10 µg/g dry weights, followed by pyrene with 69.14 µg/g dry weights (Table 6). These results are in agreement with Lee (2005) who measured the PAH concentrations of the river bottom sediment taken from Lowellville.

Table 6. PAHs detected in Mahoning river sediment by lipid extraction procedure

No	PAHs	Retention Time	Concentration µg/g dry weight
1	Naphthalene	9.05	13.03
2	Acenaphthylene	11.26	1.23
3	Acenaphthene	11.49	3.03
4	Fluorene	12.19	11.76
5	Phenanthrene	13.55	39.37
6	Anthracene	13.55	46.64
7	Fluoranthene	15.20	85.10
8	Pyrene	15.53	69.14
9	Benzo(a)anthracene	17.42	17.48
10	Chrysene	17.42	15.10
11	Benzo(b&k)fluoranthene	19.79	40.19
12	Benzo(a)pyrene	0	N.D
13	Dibenz(ah)anthracene	0	N.D
14	Indeno(1,2,3-cd)pyrene	0	N.D
15	Benzo(ghi)perylene	0	N.D

N.D – Not detected

Four treatments were done: (1) Control (2) Sawdust (3) Sawdust + fungi (4) Sawdust + fungi + nitrogen. By the end of the experiment (42 days) nine of the eleven PAHs detected showed degradation in the sample with *Pleurotus ostreatus* + sawdust (Figure 25). The total PAH concentration was reduced by 58.6% from 253 µg/g of dry weight on day 0 to 173 ug/g on day 21 and day 42 was estimated to be reduced to 106 µg/g of dry weight after 42 days. The samples from day 42 were lost due to lab accident therefore degradation rates were estimated using Pabba (2008) rates.

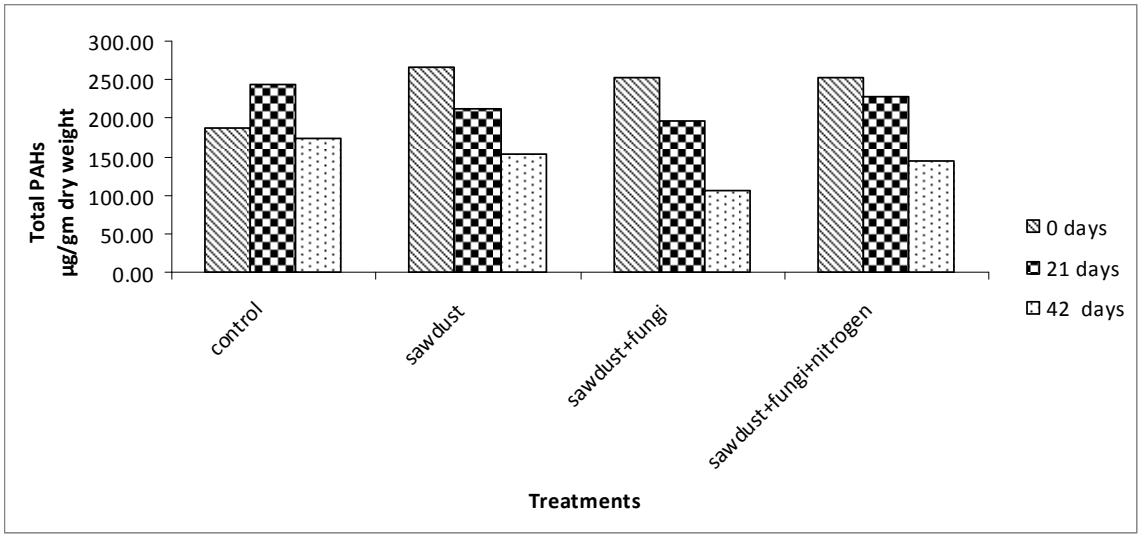


Figure 25. Total PAH concentrations in all the treatments at 0 days, 21 days and (estimated) 42 days of incubation.

When extraction of PAHs was done by lipid extraction method, degradation occurred in 9 out of 11 compounds after 21 days of incubation (Figure26). The two aromatic structures that did not show degradation were benzo(a)anthracene and chrysene.

When extractions were carried out in triplicate by the lipid extraction method for PAHs, only seven PAHs were detected, at time zero (Table 7) and only nine PAHs were detected, at time 21 days (Table 8) by GC/MS. The quantity of low molecular weight

PAHs and high molecular weight PAHs varies from sample to sample when analyzed by GC/MS at time 0 and 21 days.

Table 7. PAHs detected in Mahoning river sediment by lipid extraction procedure at time 0 days

No.	PAHs	Retention Time	Concentration ug/g dry weight
1	Naphthalene	9.05	N.D
2	Acenaphthylene	11.26	N.D
3	Acenaphthene	11.49	N.D
4	Fluorene	12.19	N.D
5	Phenanthrene	13.55	15.51
6	Anthracene	13.55	18.92
7	Fluoranthene	15.20	39.63
8	Pyrene	15.53	26.37
9	Benzo(a)anthracene	17.42	N.D
10	Chrysene	17.42	19.89
11	Benzo(b&k)fluoranthene	19.79	17.55
12	Benzo(a)pyrene	0	14.11
13	Dibenz(ah)anthracene	0	N.D
14	Indeno(1,2,3-cd)pyrene	0	N.D
15	Benzo(ghi)perylene	0	N.D

N.D – Not detected

Table 8. PAHs detected in Mahoning river sediment by lipid extraction procedure at time 21 days

No.	PAHs	Retention Time	Concentration ug/g dry weight
1	Naphthalene	9.05	N.D
2	Acenaphthylene	11.26	0.48
3	Acenaphthene	11.49	N.D
4	Fluorene	12.19	8.65
5	Phenanthrene	13.55	12.09
6	Anthracene	13.55	24.72
7	Fluoranthene	15.20	19.23
8	Pyrene	15.53	3.65
9	Benzo(a)anthracene	17.42	8.32
10	Chrysene	17.42	5.92
11	Benzo(b&k)fluoranthene	19.79	13.78
12	Benzo(a)pyrene	0	N.D
13	Dibenz(ah)anthracene	0	N.D
14	Indeno(1,2,3-cd)pyrene	0	N.D
15	Benzo(ghi)perylene	0	N.D

N.D – Not detected

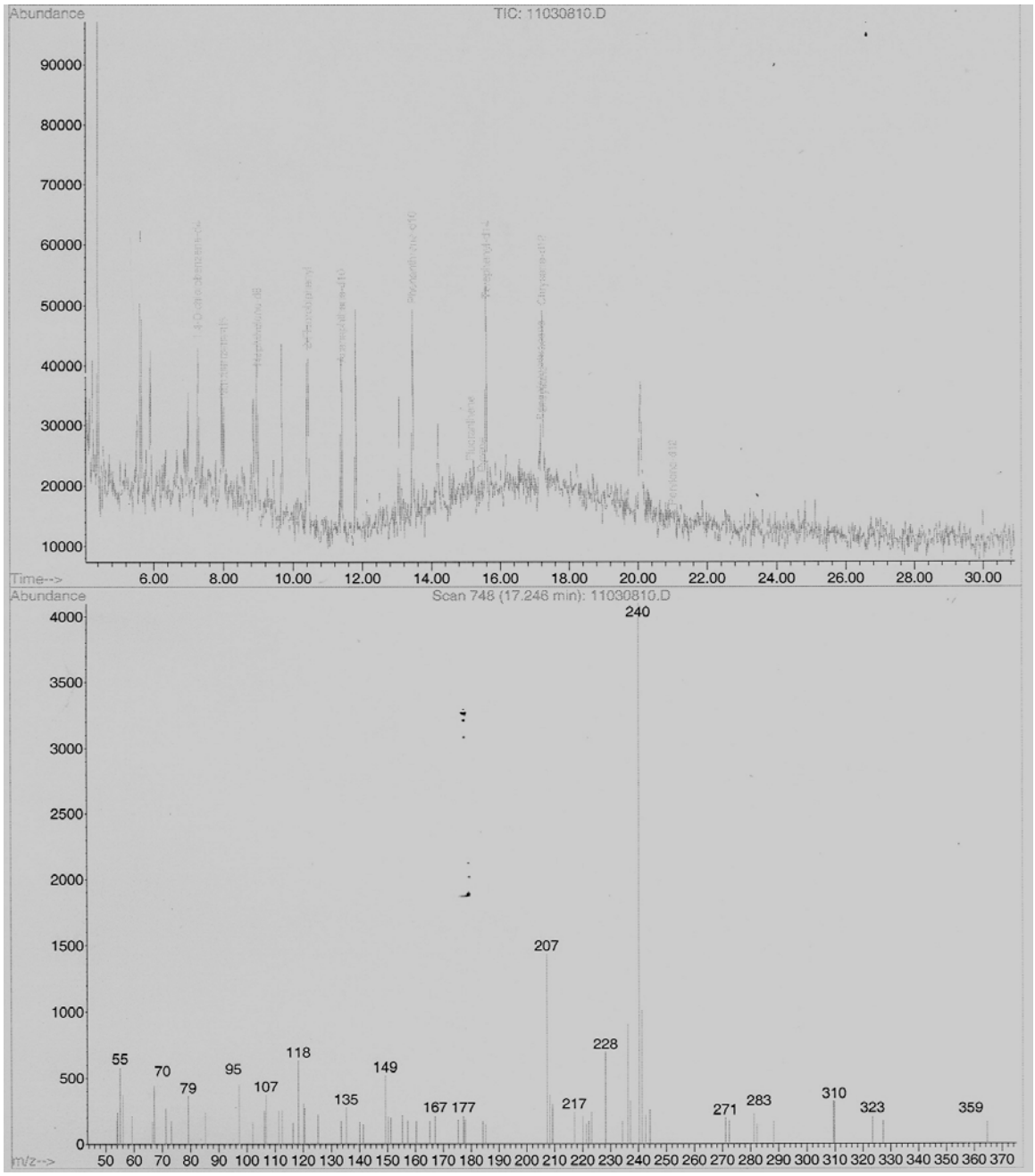


Figure 26. Chromatogram of PAHs from sediment samples at time 21 days

Part 2 Extraction of Ergosterol and PAHs

Sixteen PAHs were extracted using the modified ergosterol extraction method (Table 7), six of them being low molecular weight PAHs, namely naphthalene,

acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene. Ten high molecular weight PAHs were detected which include fluoranthene, benzo[a]anthracene, pyrene, chrysene, benzo[b,k]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, bibenzo[a,h]anthracene, indeno[1,2,3-cd]pyrene and benzo(ghi)perylene. The quantity of PAHs extracted from this method is much more than that extracted by the lipid extraction method (Table 9, Figure 27).

Table 9. Average PAHs detected in Mahoning river sediment (Controls) by Ergosterol Extraction Method

No.	PAHs	RetentionTime	Concentration ug/g dry weight
1	Naphthalene	9.71	7.36
2	Acenaphthylene	11.06	53.72
3	Acenaphthene	11.21	26.22
4	Fluorene	11.60	43.51
5	Phenanthrene	12.43	235.76
6	Anthracene	12.46	183.12
7	Fluoranthene	13.43	1013.57
8	Pyrene	13.63	858.76
9	Benzo(a)anthracene	14.66	385.04
10	Chrysene	14.70	224.34
11	Benzo(b)fluoranthene	15.83	375.29
12	Benzo(k)fluoranthene	15.83	328.76
13	Benzo(a)pyrene	16.17	336.57
14	Dibenz(ah)anthracene	18.05	68.71
15	Indeno(1,2,3-cd)pyrene	18.06	42.63
16	Benzo(ghi)perylene	18.58	140.90

The PAHs were extracted by the modified ergosterol extraction method done in triplicate (Figure 28) from the samples having the treatment: 1) fungi grown on barley (0.6207g) + sediment (0.6199g), 2) fungi grown on barley (0.6143g) + sediment (1.1886g) (Figure 28), 3) fungi grown on barley (0.6327g) + sediment (0.5743g). The

extraction of PAHs from these samples were analyzed by GC/MS without acetylation. (Figure 28).

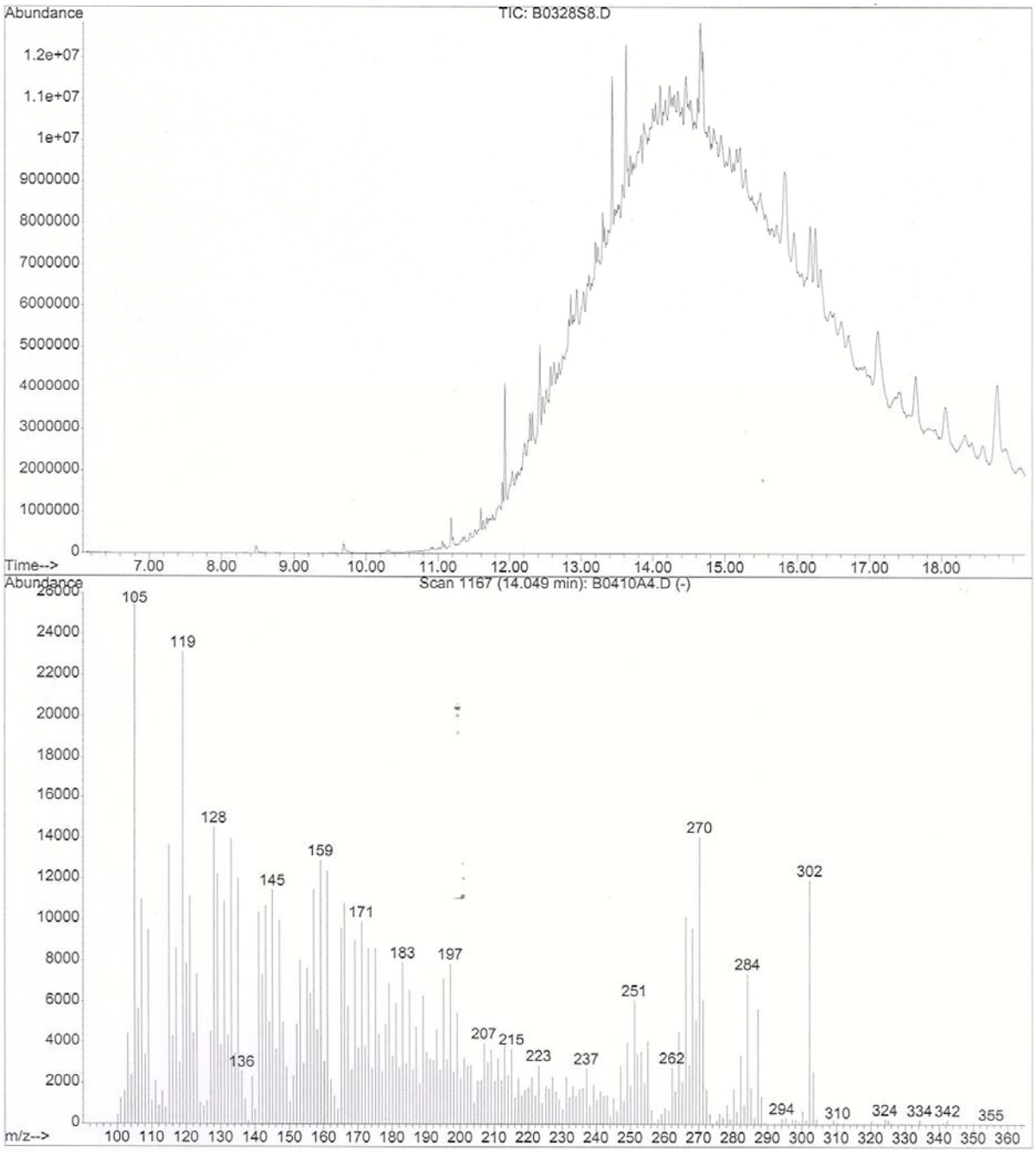


Figure 27. Chromatogram of PAHs extracted by Ergosterol Extraction Method from the fungi grown on barley plus sediment

The concentration of PAHs in the contaminated sediment samples were calculated by:

$$\frac{D * X}{M} = C$$

Where: D = Amount of DCM with suspended PAHs extracted from the sample (ml)

X = Concentration of the sample given by GC/MS (µg/ml)

M = Mass of the sample before extraction (g)

C = Concentration of PAHs in the sample (µg/g or ppm)

The Ergosterols were extracted and analyzed by GC/MS from the samples of pure fungi grown on PDA done in triplicate: 1) pure fungi (0.5325g), 2) pure fungi (0.5247g) (Figure 28), 3) pure fungi (0.5225g). The peaks were detected at 397 a.m.u. which correspond with ergosterol (Figure 28). Acetylation of ergosterol was done after extraction of ergosterol.

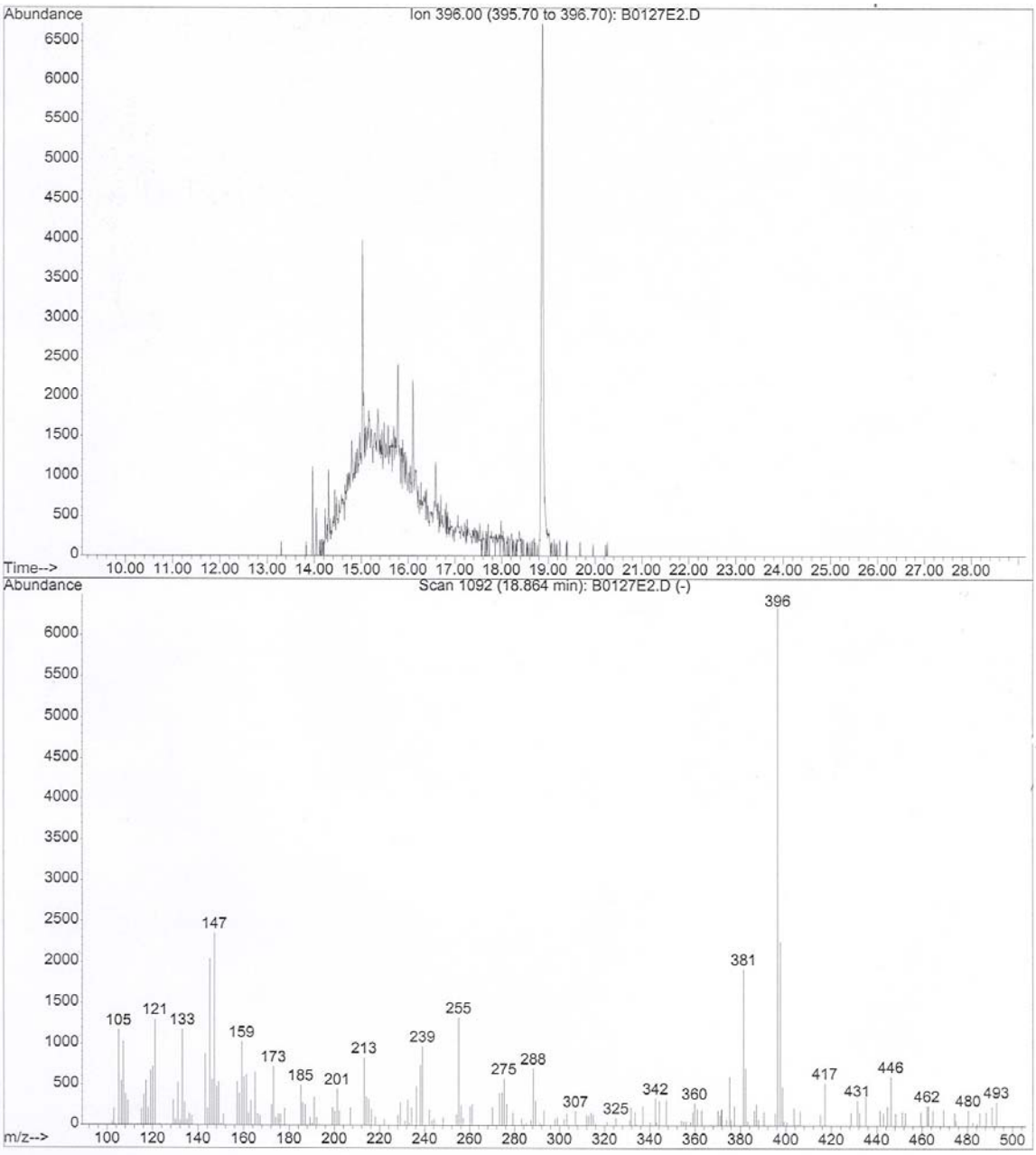


Figure 28. Chromatogram of ergosterol by modified Ergosterol Method from fungi grown on potato dextrose agar.

The extraction of samples done in triplicate taking pure fungi grown on PDA and sediment added separately: 1) pure fungi (0.5283g) + sediment (0.5880g), 2) pure fungi (0.54480g) + sediment (0.5132g) (Figure 29 and 30), 3) pure fungi (0.6184g) + sediment (0.5368g). After the extraction, cholesterol and PAHs were detected from GC/MS, this is

the first report for the occurance of cholesterol (Figure 30 and 31). Cholesterol was seen after acetylation of sterol by GC/MS (Figure 30).

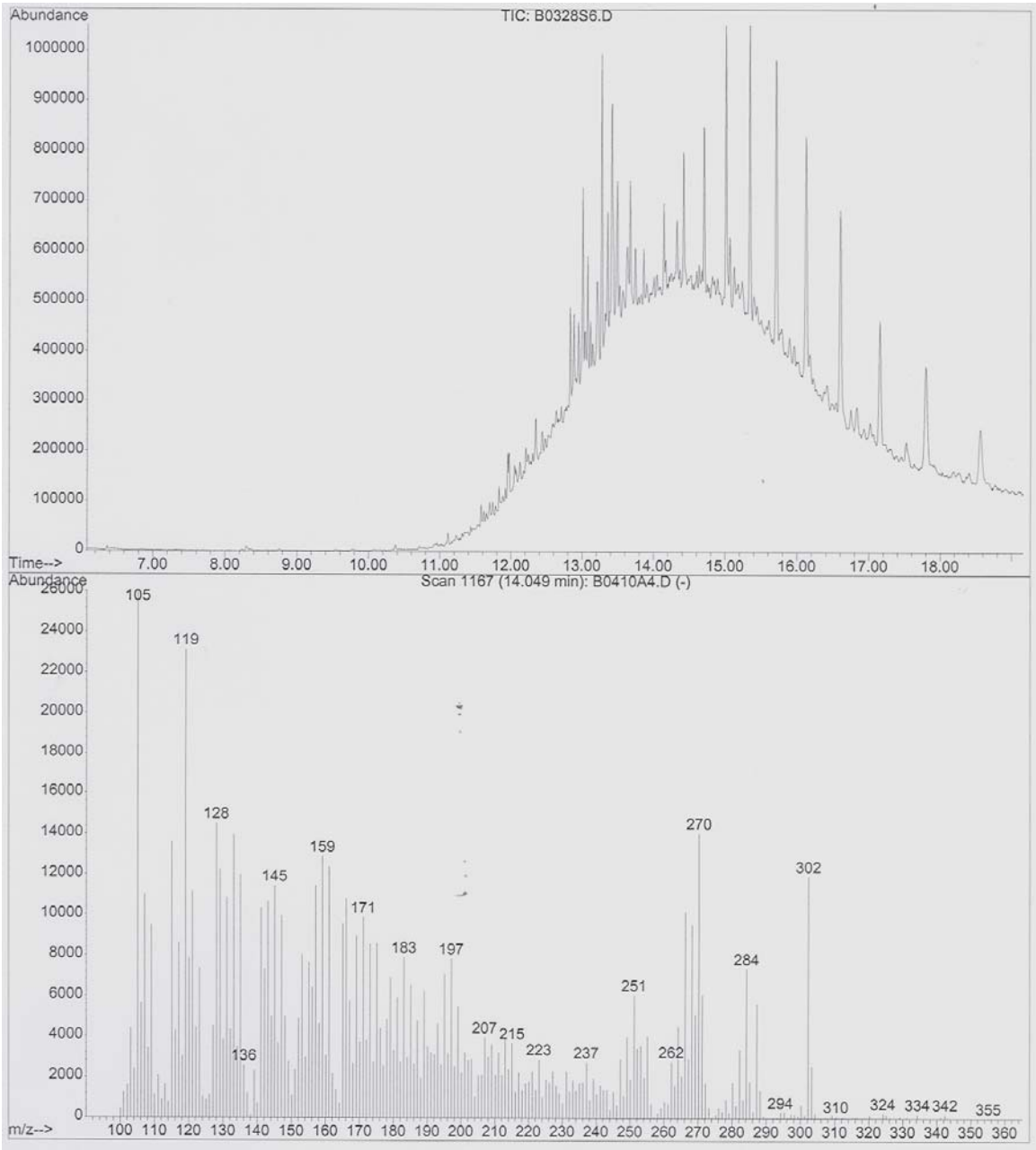


Figure 29. Chromatogram of PAHs by Ergosterol Extraction Method from pure fungi plus sediment

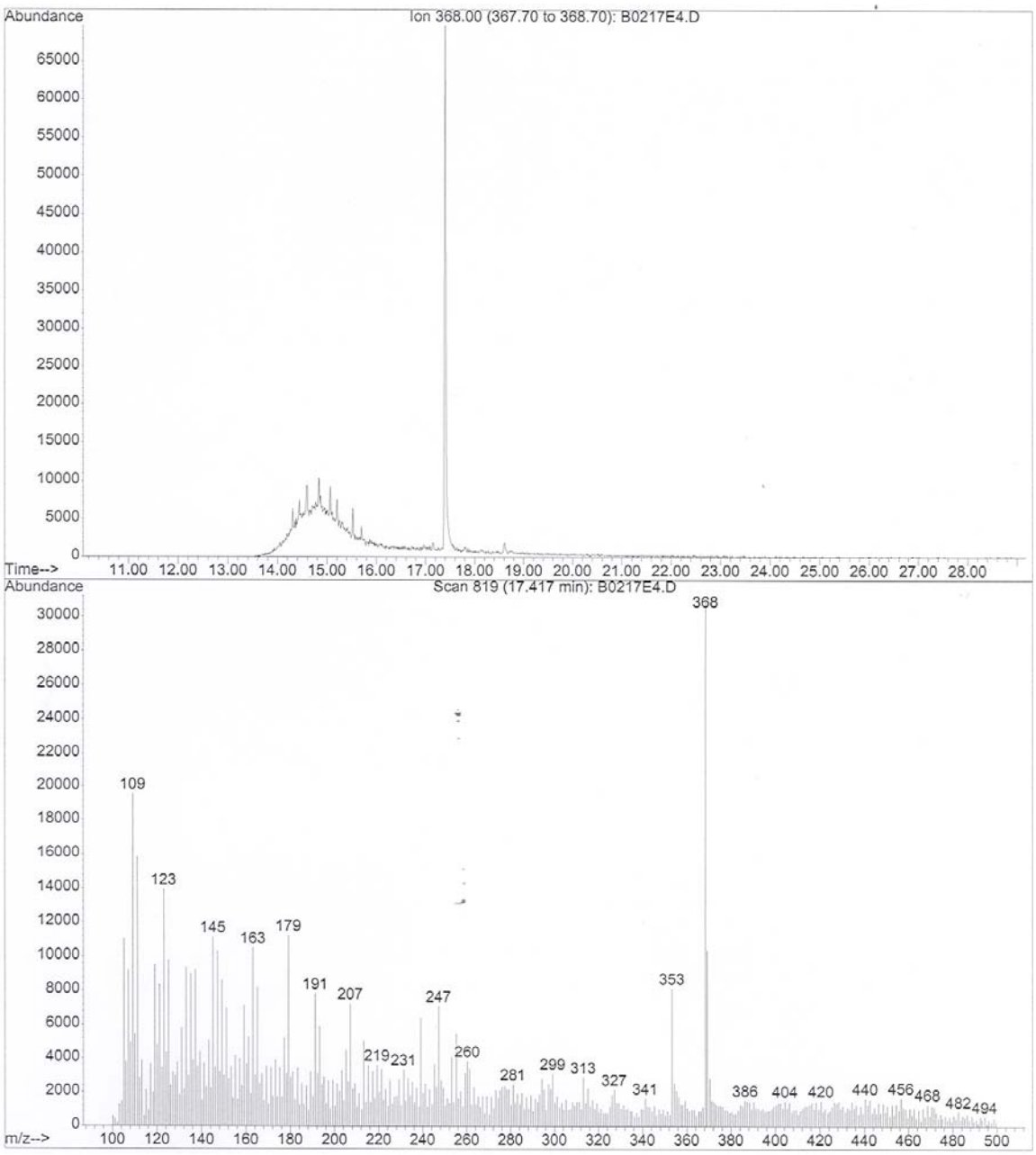


Figure 30. Chromatogram of cholesterol by Ergosterol Extraction Method from pure fungi plus sediment

The extraction of pure fungi grown on PDA was done without acetylation and analyzed by GC/MS. Cholesterol and ergosterol were detected in high amounts with high intensity of peaks being observed for both sterols (Figures 31 and 32). The significant

result is that cholesterol are usually found in animals but was detected in fungi *P. ostreatus* in good amount (Figure 31).

Figure 31. Chromatogram of cholesterol by Ergosterol Extraction Method from the fungi *P. ostreatus* grown on potato dextrose agar.

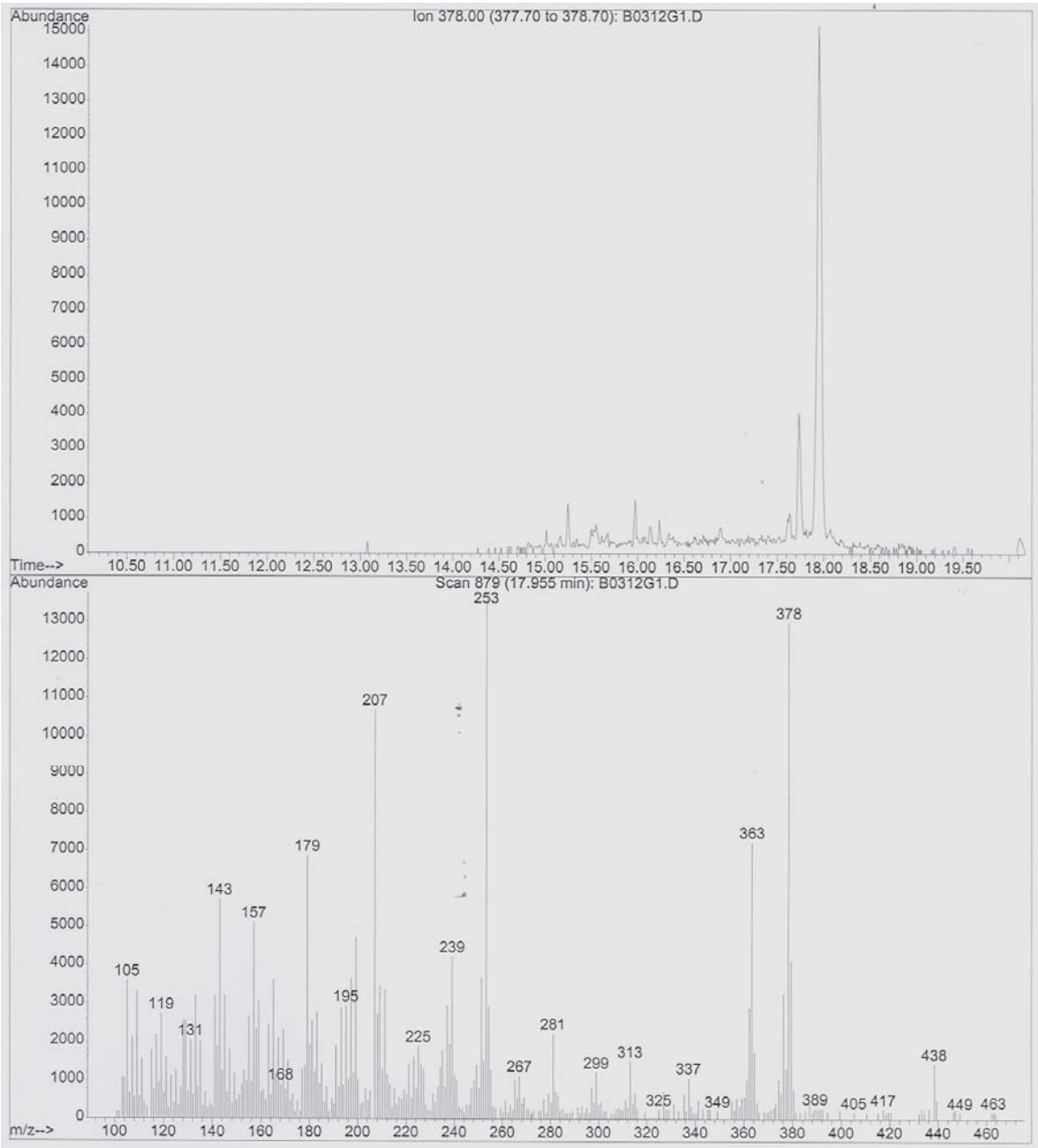


Figure 32. Chromatogram of ergosterol extracted by Ergosterol Extraction Method from the fungi *P. ostreatus* grown on potato dextrose agar.

The detection of cholesterol and ergosterol in fungi by GC/MS was analyzed from the peaks of standard reference spectra of commercial ergosterol and cholesterol (Figures 33 and 34). The cholesterol and ergosterol in fungi was also confirmed by the standard

reference peaks of acetylated cholesterol and ergosterol (Figures 35 and 36), which was acetylated from the commercial cholesterol and ergosterol.

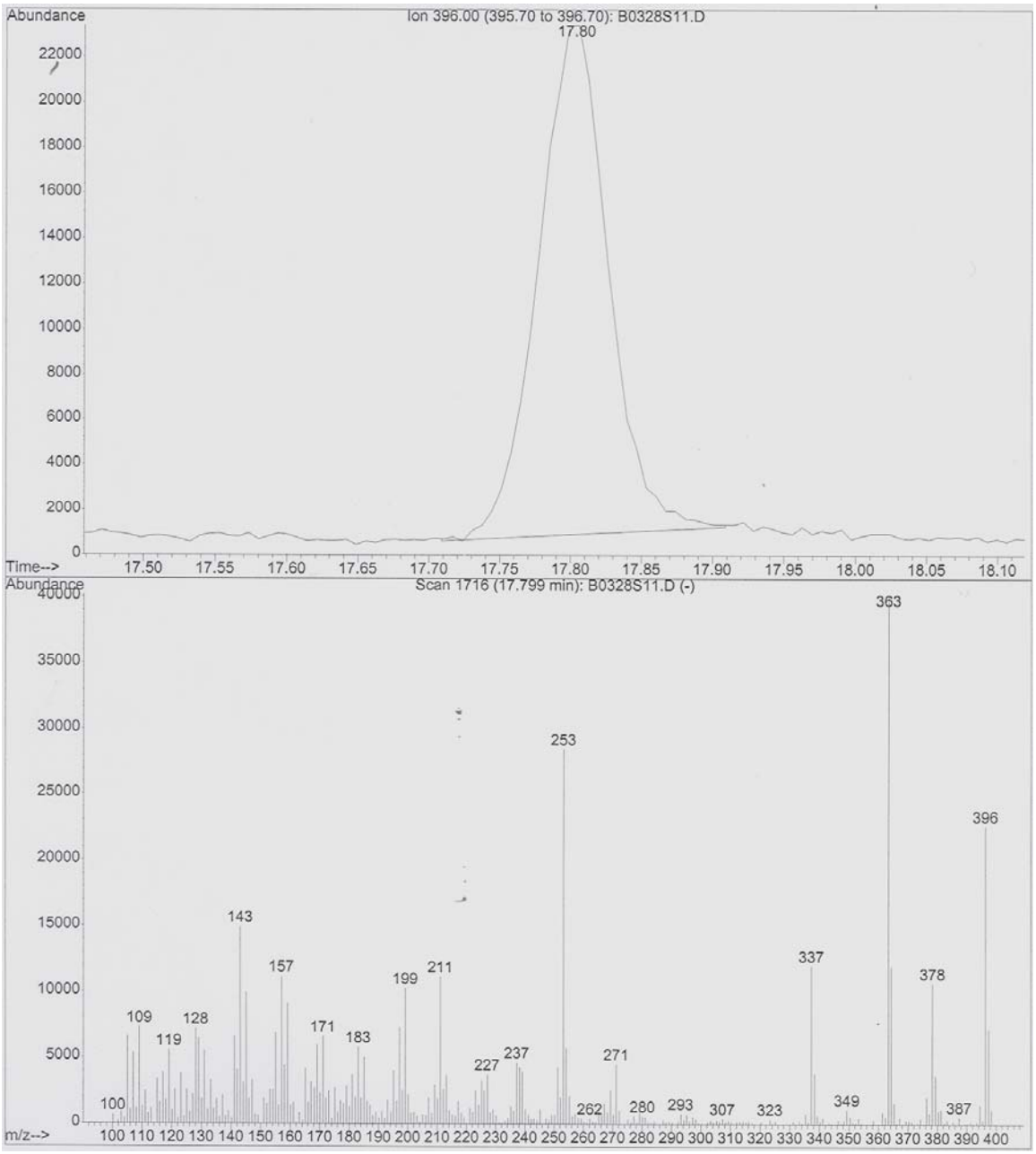


Figure 33. Chromatogram of standard reference spectra from commercial ergosterol

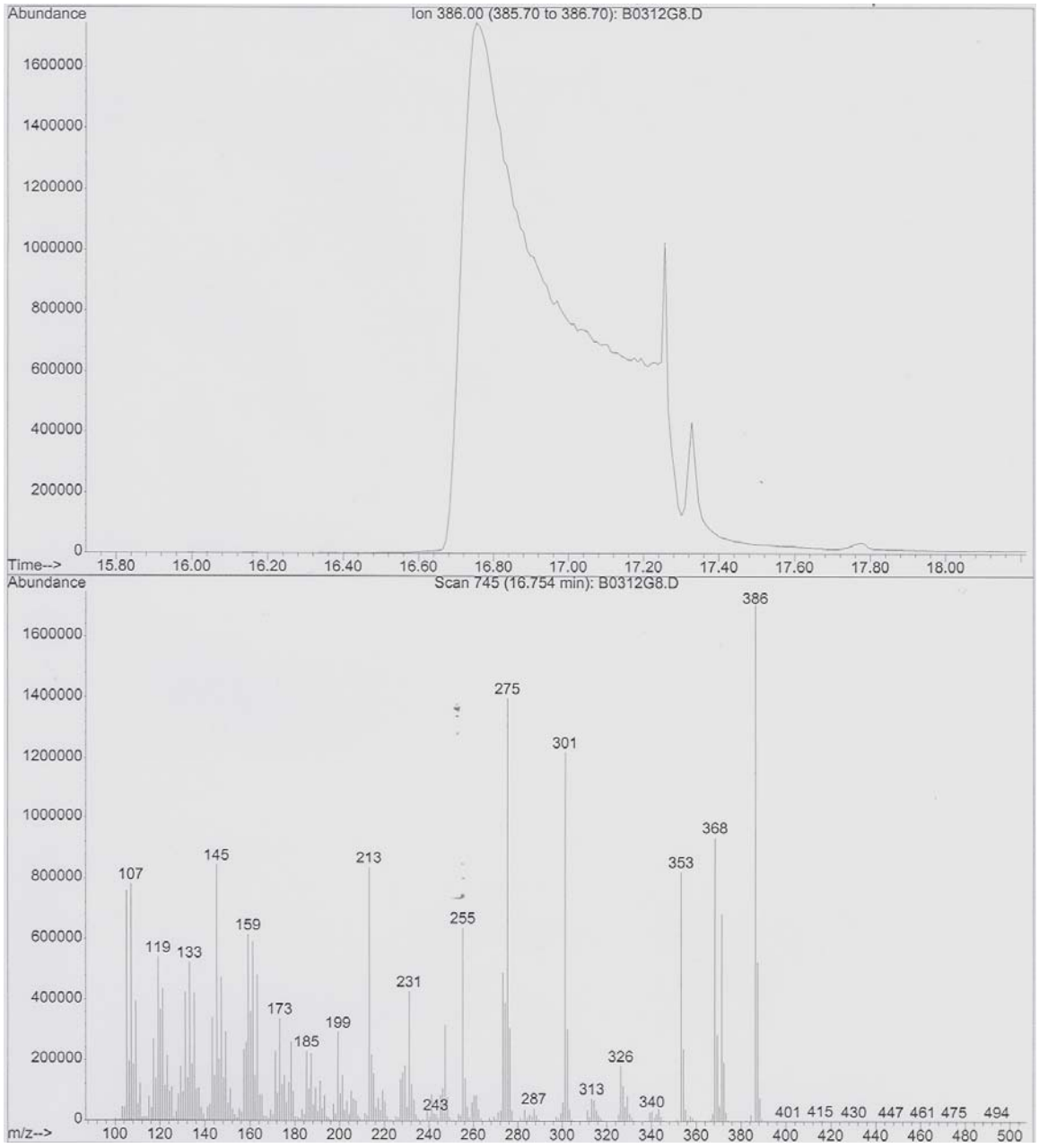


Figure 34. Chromatogram of standard reference spectra from commercial cholesterol

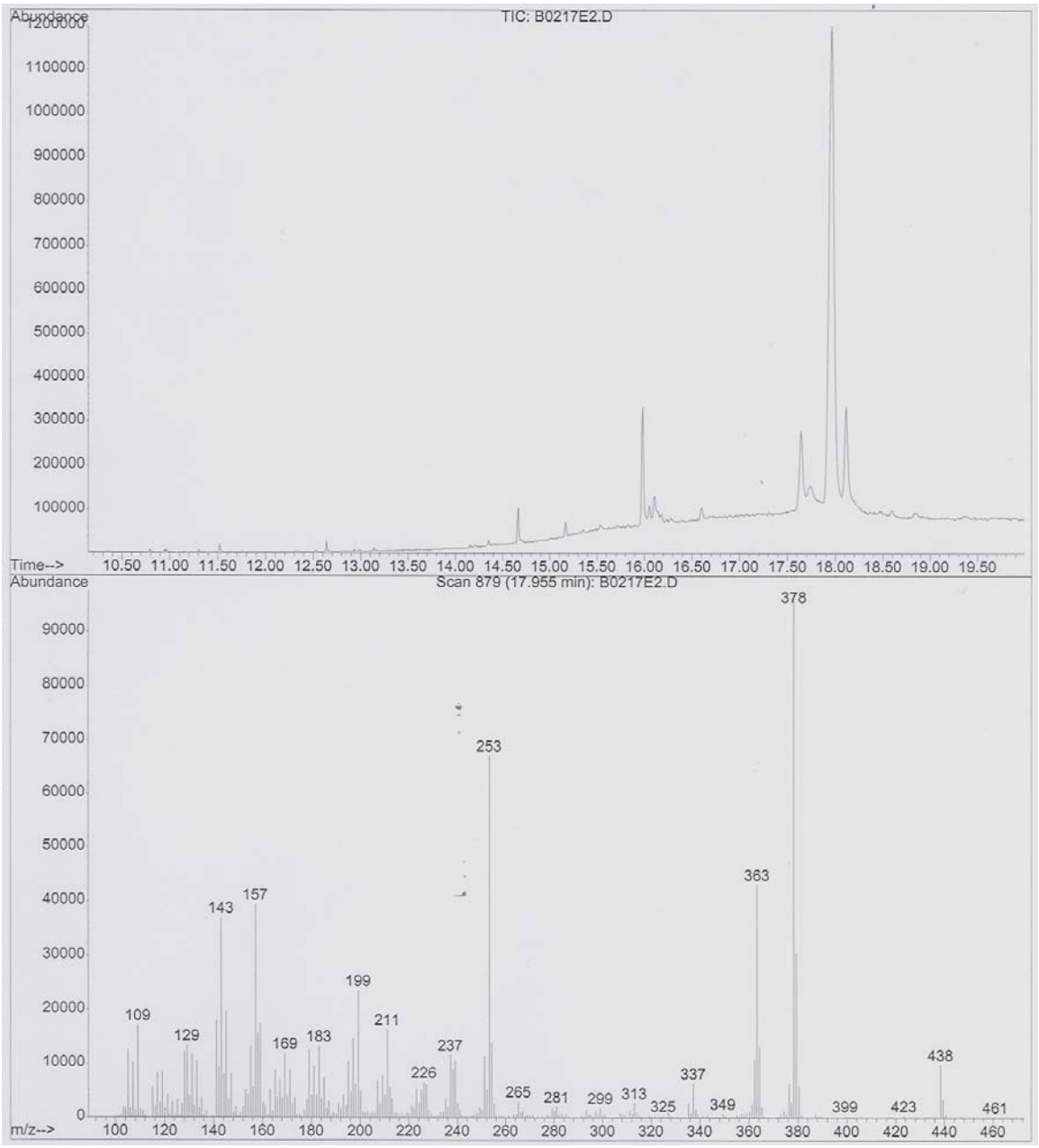


Figure 35. Chromatogram of standard reference spectra of ergosterol acetate

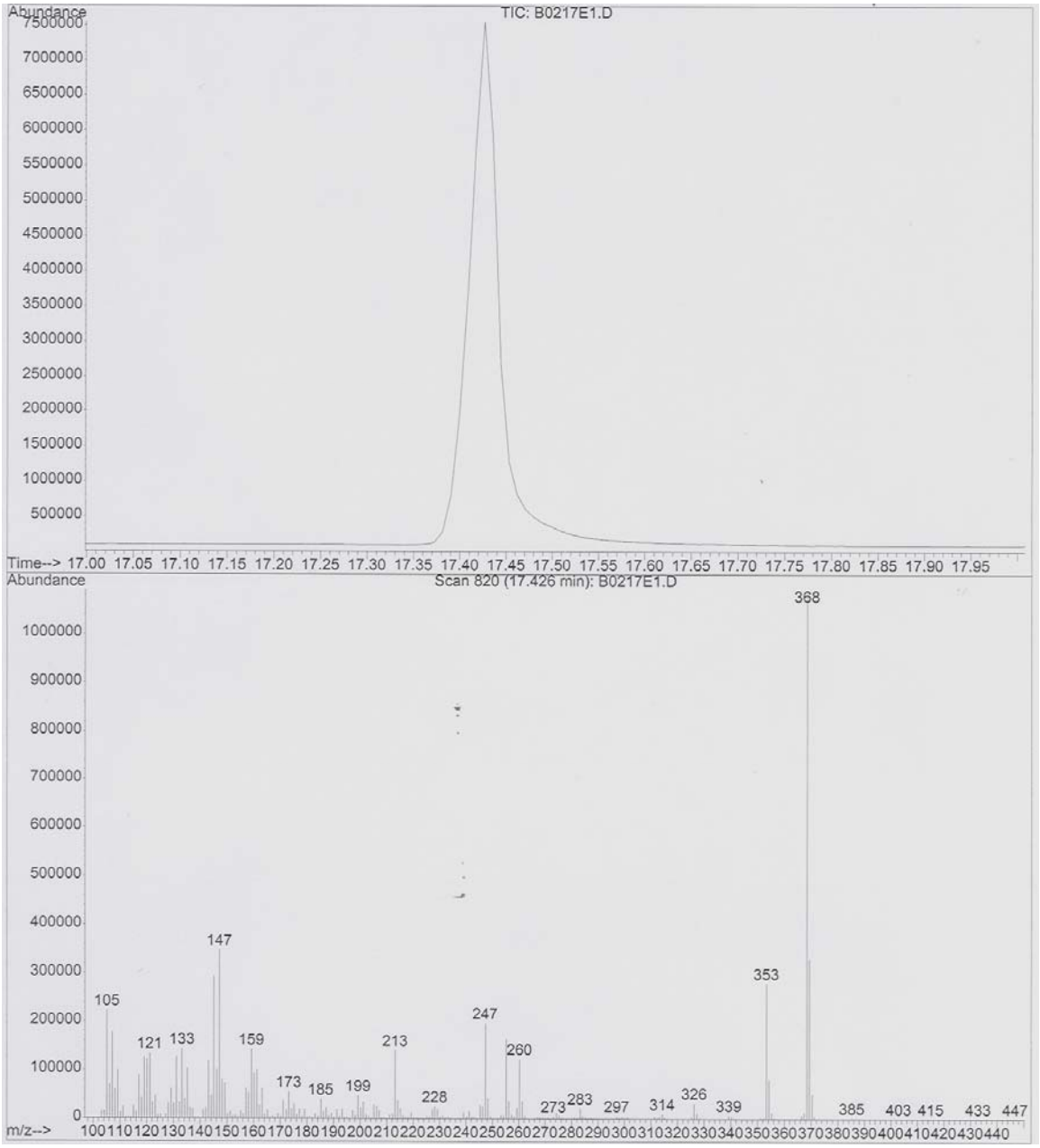


Figure 36. Chromatogram of standard reference spectra of cholesterol acetate

The detection of ergosterol by GC/MS from the samples done in triplicate and the amounts of ergosterol per gram of sample was analyzed after calculation: fungi grown on barley1) 0.67g sample, 17.5µg/g of ergosterol were present (Figure 37); 2) 0.53g sample, 39.94 µg/g of ergosterol were present (Figure 38); 3) 0.53g) sample, 21.58 µg/g of ergosterol were present (Figure 39).

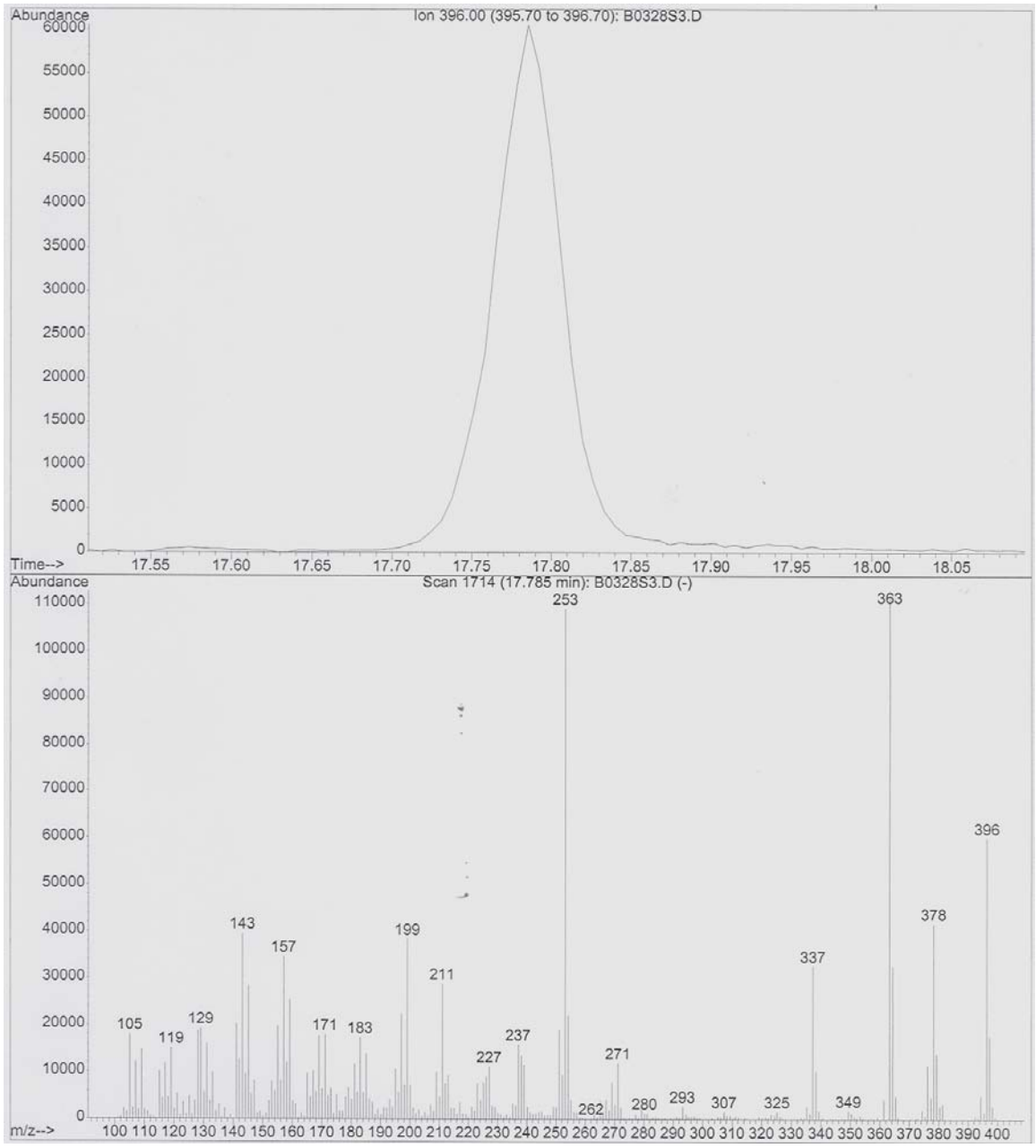


Figure 37. Chromatogram of 17.5µg/g ergosterol in *P. ostreatus* grown on barley

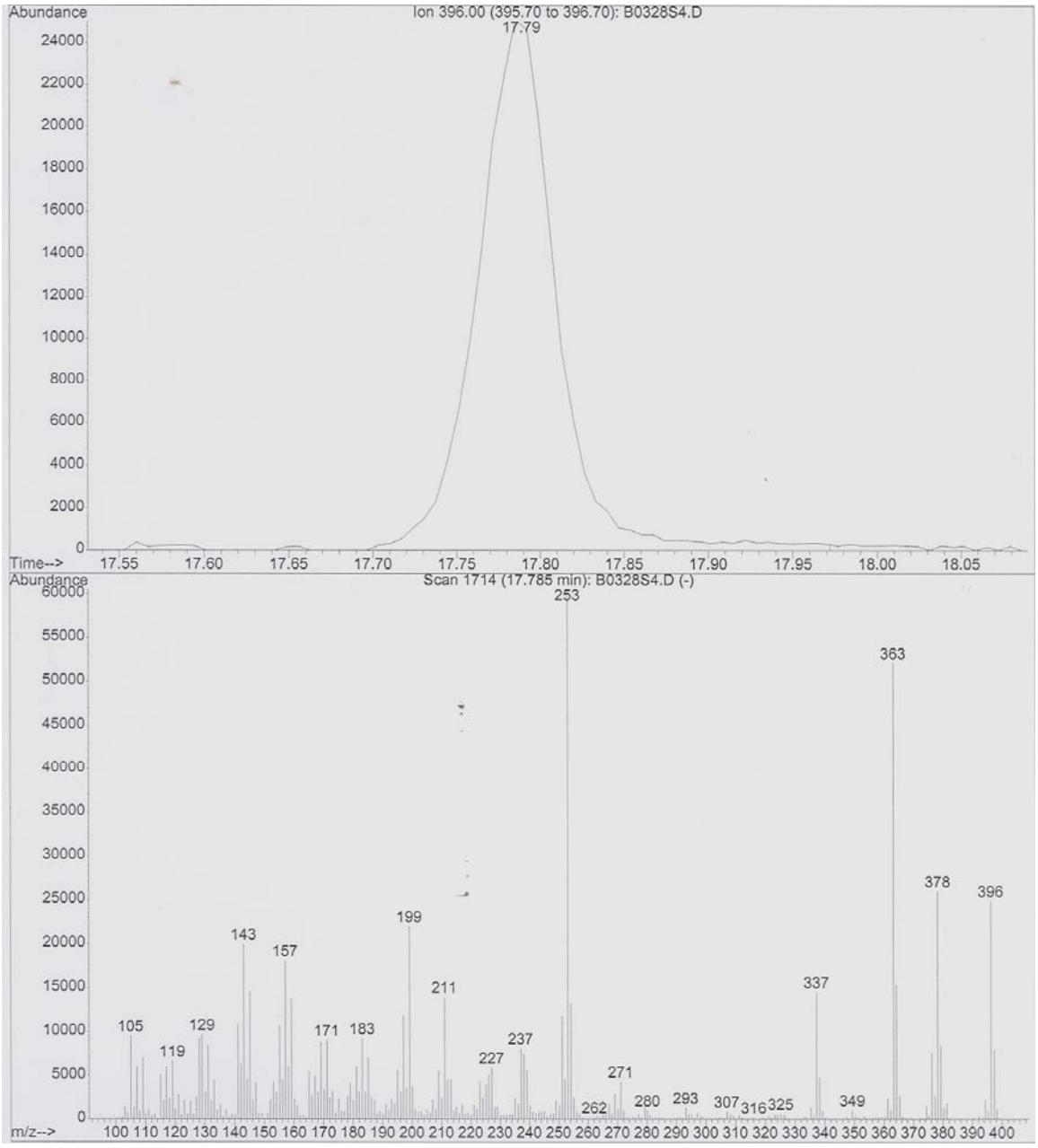


Figure 38. Chromatogram of 39.94µg/g ergosterol in *P. ostreatus* grown on barley

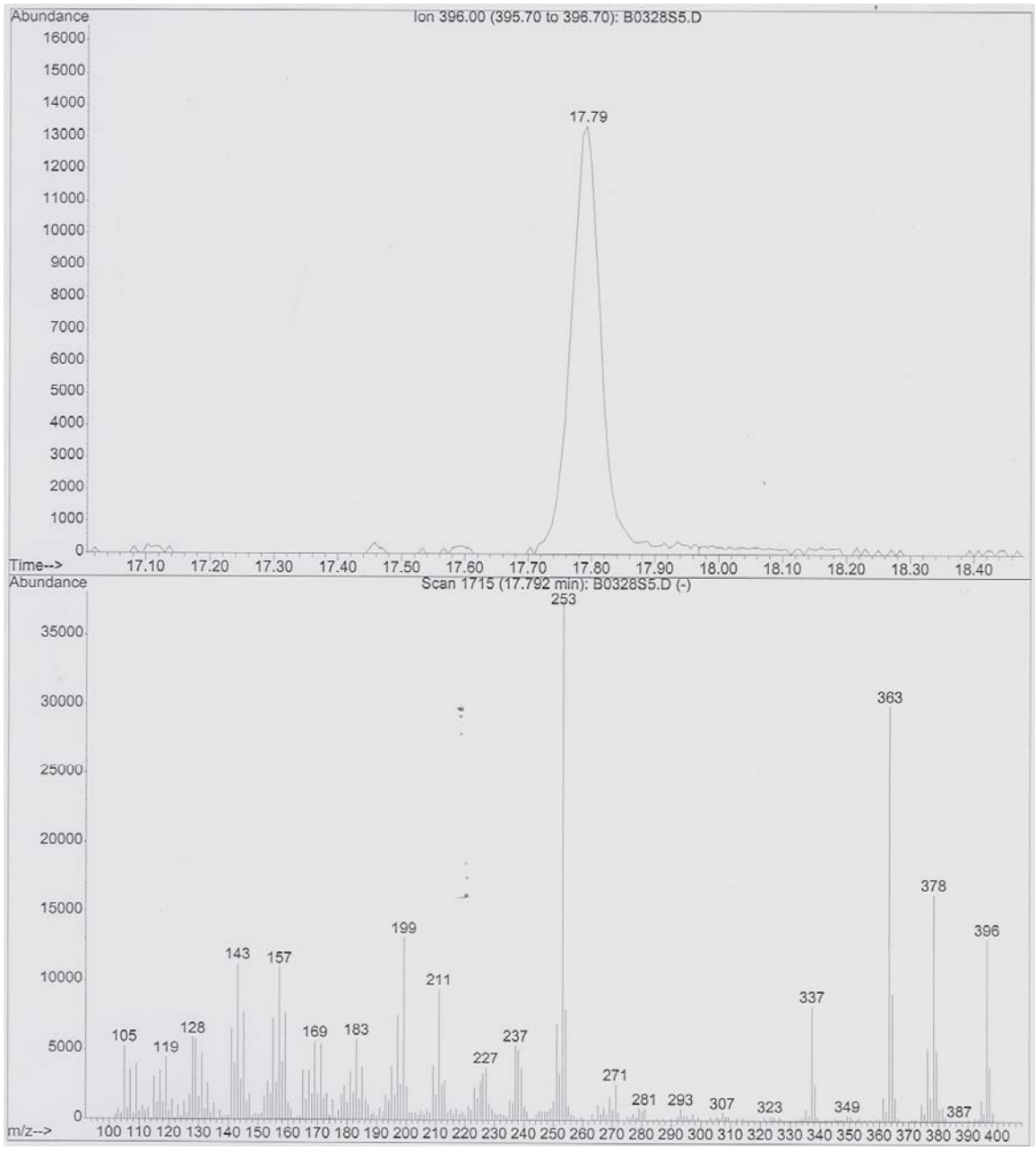


Figure 39. Chromatogram of 21.58µg/g ergosterol in *P. ostreatus* grown on barley

The peaks of ergosterol were identified on the basis of retention times and spectral characteristics as compared to those of standard materials. The concentration of ergosterol samples (µg/g or ppm) were calculated using the formula as:

Calibration Std.:

Calibration Factor (CF) = $\frac{\text{Area counts of std. ergo sterol}}{\text{Concentration of std. ergosterol (µg)}}$

For Sample:

$$\frac{\text{Area count of ergosterol sample}}{\text{CF}} \times \frac{\text{Volume of extract (ml)}}{\text{Mass of sample (g)}} = \text{µg/g (ppm)}$$

This method is useful for measuring the PAHs and ergosterol with great precision and accuracy without any hindrance. The extracts of the extractions for the samples which include sediments such as: pure fungi plus sediment and fungi (grown on barley) plus sediment, sterols were detected only after acetylation of the reaction mixture. This is because, acetylation was necessary to separate sterols from contaminants such as PAHs, these contaminants act as hindrance in analyzing extracts on GC/MS.

Comparison of Lipid Extraction Method and modified Ergosterol Extraction Method:

The solutes obtained from the extractions of the samples from these two methods were both analyzed by GC/MS. Eleven PAHs were detected in Mahoning River sediment using the modified Fang and Findlay lipid extraction procedure (Figure 40). A total of sixteen PAHs were detected by modified ergosterol extraction method of Brodie et al. 2003 (Figure 41). Ergosterol and cholesterol are also extracted and detected from this method. By modified ergosterol method both PAHs and sterols were detected with great efficiency and ease. It is also less time-consuming and does not require many

solvents. Therefore, the modified Ergosterol Extraction Method is the most effective method both qualitatively and quantitatively simultaneous analysis of PAHs and ergosterol.

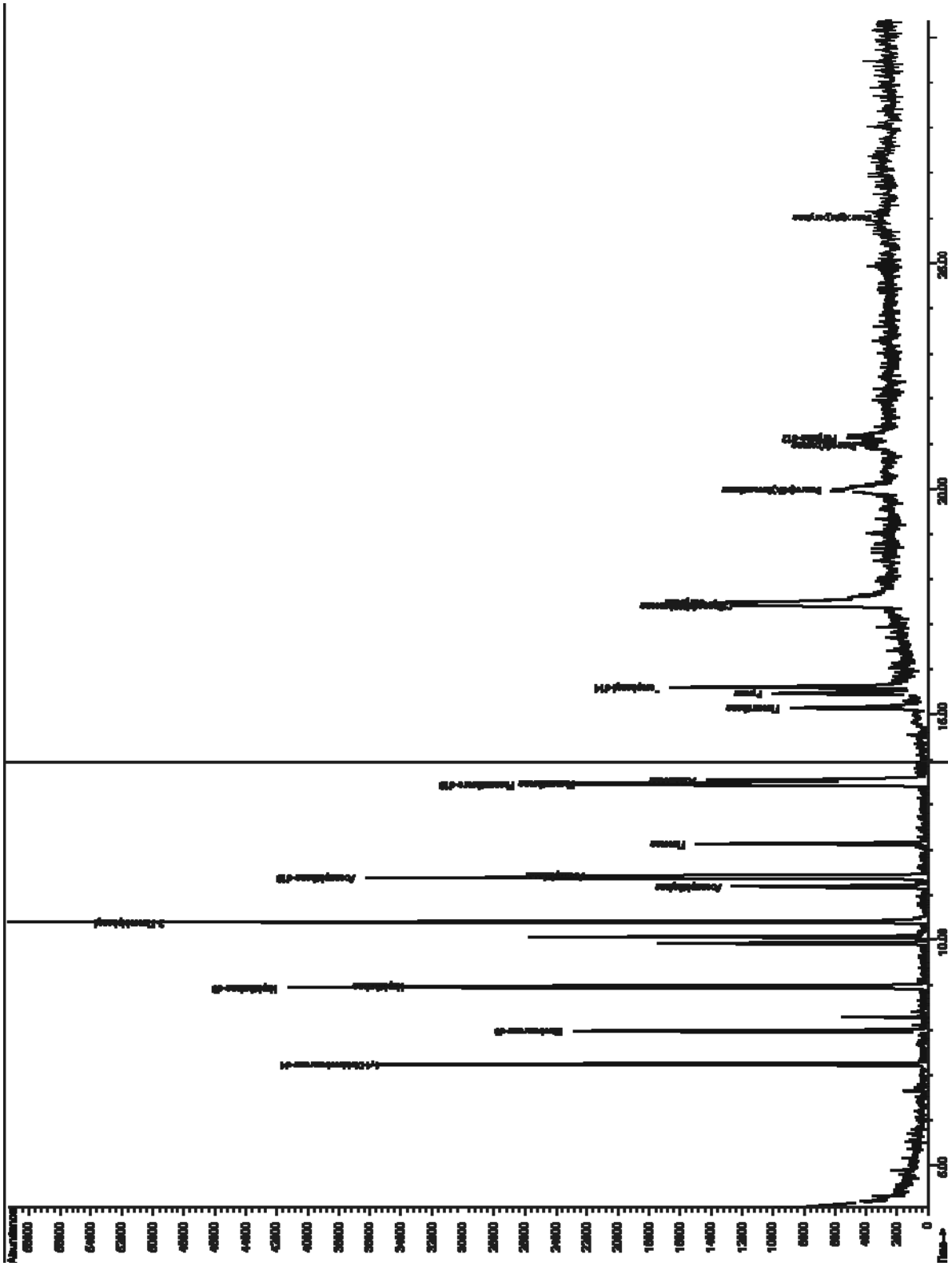


Figure 40. Chromatogram of PAHs from sediment sample by Lipid extraction method

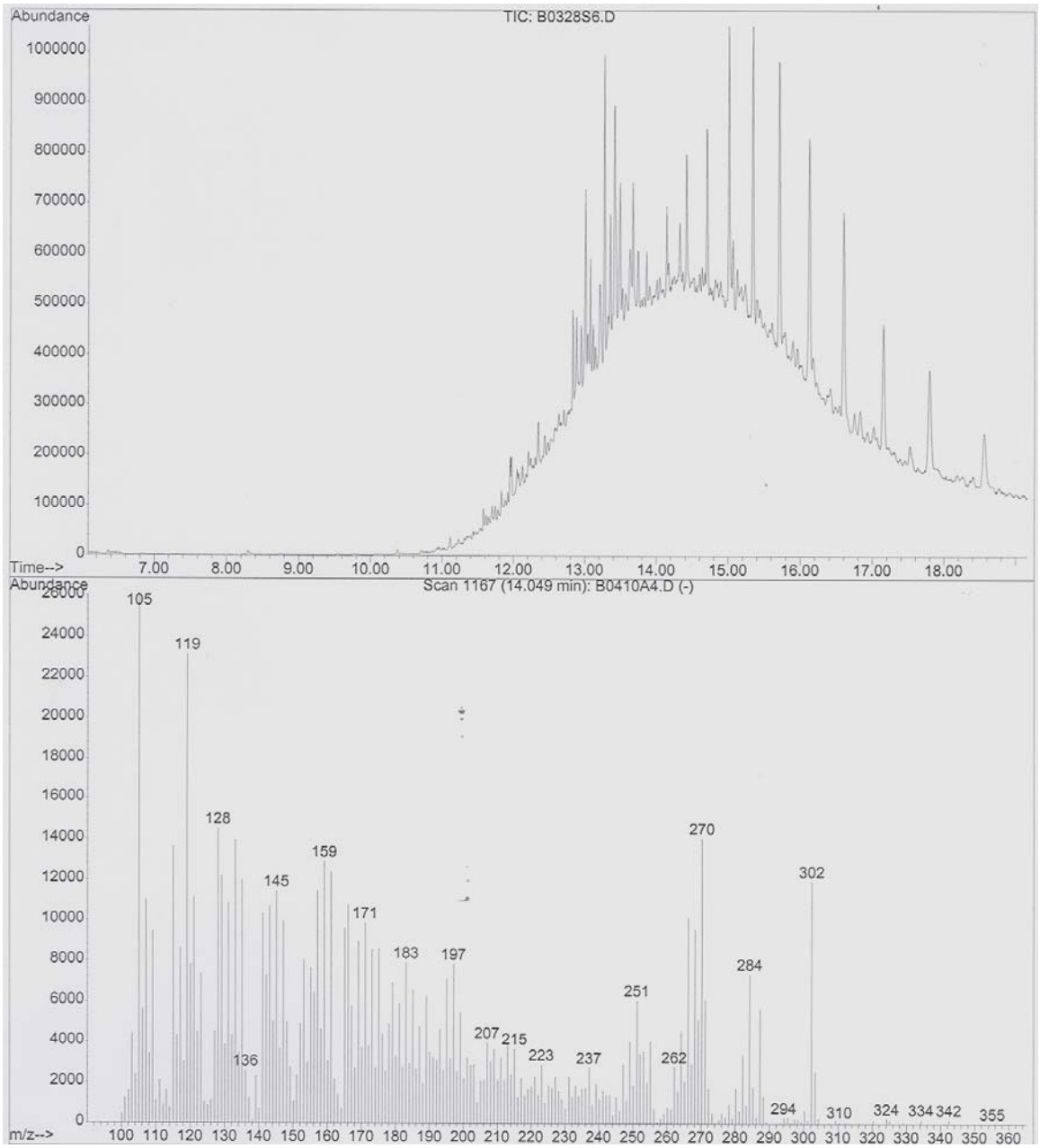


Figure 41. Chromatogram of PAHs from sediment sample by Ergosterol Extraction Method

CHAPTER 4: Conclusion

More PAHs were obtained by the Ergosterol Extraction Method than with the lipid extraction method. Sediments should be inoculated with *P.ostreatus,* extractions of PAHs and ergosterol should be done by Ergosterol Extraction Method. Ergosterol extraction method can be used as an effective tool for the extraction of ergosterol from *P.ostreatus* and bioremediation of contaminated sediment from PAHs by adding *P.ostreatus*. The detection of good amounts of ergosterol in white rot fungi, *P.ostreatus* confirms the fact that it acts as bioindicator of fungi. The growth of fungal mycelia can be increased by inoculating the treatment with a nitrogen source which serves as nutrients for fungi.

The ergosterol extraction method seems particularly adapted for studying ergosterol in various treatments and matrixes. Ergosterol extraction method should be used to quantitate and analyze the biodegradation of PAHs and the change in the amount of ergosterol in *P.ostreatus* at different treatments, at different times such as 0 day, 21 days and at 42 days should be analyzed. For Egosterol extraction, different treatments should be set up with various amendments for at least three runs and every extraction should be done in triplicate at three different times for these runs.

Degradation of PAHs should be seen in the future work by Ergosterol Extraction Method at times 0, 21 and 42 days, also the change in the amounts of ergosterol must be seen at these time periods. The quantity of ergosterol should increase over a period of time as PAHs are used as nutrients by *P.ostreatus*.

A preliminary analysis without acetylation would be necessary to indicate if any interfering peaks co-eluting with ergosterol were present. If interfering material was not present, the procedure could be shortened by elimination of the acetylation step.

The use of relatively volatile solvents such as dichloromethane and hexane presents hazards to laboratory workers from both flammability and inhalation toxicity. Therefore, additional fume hood space would be needed for solvent preparation and extra care should be taken while doing the work.

Ergosterol Extraction Method can be optimized by using different solvent combinations such as (3:7 DCM:Hexane). For triplicate incubations, sediments from the same site and the same spot sampled at same time are recommended. Triplicate extractions of each treatment would accommodate the inherently large sample variation better and improve statistics. *Pleurotus ostreatus* was found to be effective in removing PAHs from the contaminated sediment. It is recommended to repeat the study with the most effective treatment.

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Appendices

Appendix 1:
PAH extraction

A) Reagents for PAH extraction

1. Dichloromethane(DCM) optima grade
2. Methanol optima grade
3. 50 mM phosphate buffer (add 8.7 g of KH_2PO_4 to 700 ml milli-Q water, stir and adjust he pH to 7.4 with 1 N HCl, complete to 1000 ml with milli-Q water)
4. Sodium chloride
5. Chloroform optima grade preserved with 0.75% ethanol
6. Anhydrous sodium sulfate for preparation of sodium sulfate columns
7. Unisil (activated silica, 100-200 mesh size) for construction of silica columns
8. Aminopropyl columns
9. Copper filings

B) Preparation of Sodium Sulfate columns

1. Use 6 ml glass columns with Teflon frits in the bottom.
2. Prepare the columns just before the run, so that the DCM does not dry out.
3. Load the columns with 1 g of anhydrous sodium sulfate.
4. Saturate the columns with DCM.
5. Add 2 ml of DCM to the assembled columns.
6. Allow DCM to drip through, stopping when the meniscus is just above the Na_2SO_4.
7. Discard the DCM and the waste collection tubes and replace the waste tubes with clean round bottom evaporating flasks.

C) Preparation of silica columns.

1. Weigh 0.5 g of Unisil in the glass columns with frits.
2. Heat the columns with Unisil at 100 °C for 2 hours to activate Unisil (to get rid of any moisture present in the Unisil).
3. Place glass columns in Visiprep apparatus and close valves.
4. Add 4 ml of chloroform to Unisil in the glass columns.
5. Open valves and let chloroform drip through at 1 drop per second, do not let the column dry.
6. Rinse the glass column with 2 ml of chloroform.
7. Stop the flow when the meniscus is just above the silica.
8. Add copper filings (20-30) per column- This is done to get rid of any sulfur present within the glass column.(cleaned in 2 rinses of 1N HCl, 2 rinses methanol, 2 rinses of DCM, 2 rinses of hexane and dried under nitrogen).

D) Preparation of Aminopropyl Columns

1. Use 3 ml aminopropyl columns (prepacked).
2. Rinse columns with 1 ml chloroform, rinse again with 2 ml and pull through with vacuum one drop per second.
3. Rinse with 2 ml of hexane pull through with vacuum 1 drop per second but do not let the column dry.

E) Extraction of Polycyclic Aromatic Hydrocarbons by Lipid Extraction:

1. Take 0.65g of sediment and 0.5 ml milli-Q water in a 50 ml glass tube.
2. At this point a treatment can be done.
3. Add 7.5 ml of dichloromethane (DCM) and 15 ml of methanol, followed by

5.3 ml of phosphate buffer.

4. Mix the contents by shaking and venting, and check for any leaks.
5. Place samples on platform shaker for about 2 hours at 320 rpm and cover them with an aluminum foil to protect them from light exposure.
6. Remove the samples from the shaker, add 7.5 ml of DCM, 7.5 ml phosphate buffer, shake and vent again.
7. Add a pinch of sodium chloride, shake and vent again.
8. The samples should be placed in the dark at 4 °C for 24 hours.

After 24 hours,

9. The sample shows 2 distinct phases.
10. Remove the upper water/methanol phase with a pipette and discard.
11. Remove the lower phase using another clean pipette to a 15 ml conical tube.
12. The amount of sample recovered should be recorded at this point.
13. To recover more sample, add 1 ml of DCM to original tube, vortex and wait for 5 minutes, see if any organic phase can be recovered.
14. Repeat 2 more times without vortex.(if sample cannot be seen in organic phase discard it)
15. Transfer all organic phase from the conical tubes to sodium sulfate columns on the Supelco visiprep apparatus.
16. Samples should be collected in 50/100 ml round bottom evaporating flasks under the Supelco visiprep apparatus.
17. Rinse conical tubes three times using 3 ml of DCM and transfer the wash to the sodium sulfate columns.

18. Rinse column with two1 ml aliquots of DCM, then pull to dryness using vacuum.
19. Use evaporating flasks to rotovap and concentrate the sample to around 1 ml.
20. Transfer sample from evaporating flask to conical tube using a clean pipette.
21. Rinse evaporating flask with two 1 ml aliquots of DCM and add to the conical tube.
22. Concentrate the sample to one drop under nitrogen at 37 °C, but don't let the sample dry.
23. Bring volume of the sample to 1 ml- 1.5 ml using chloroform.
24. Record the amount of sample
25. Samples can be stored at -20 °C for short term storage or at -70 °C for long term storage. The sample at this stage can be used for the extraction of PAHs.

F) PAH Fraction

1. Silica columns should be prepared as outlined in appendix 1 (c)
2. Transfer the sample (1.0 – 1.5 ml) in chloroform to 200 µl hexane using solvent exchange (do not let the sample dry as this will reduce PAH recovery).
3. Concentrate sample to 100 µl under nitrogen evaporator, then add 1 ml hexane. Concentrate sample to 100 µl again. Dope sample with one drop of chloroform, vortex and transfer to silica column. Draw sample through, but do not let column dry.
4. Repeat step 3 two more times using two aliquots 100 µl hexane
5. Rinse conical tube with 1 ml, 2 ml and 2 ml aliquots of hexane to recover more of the sample. Use this hexane to rinse sides of the silica column after rinsing conical tubes. Draw between each aliquot but do not let the column dry.

6. Rinse silica column once more with 100 µl hexane to finish recovery of PAH fraction.
7. Store PAH fraction in hexane without drying at -20 °C for short term storage or at -70 °C for long term storage until ready to clean with aminopropyl columns.

G) Cleanup of PAH Fraction on Aminopropyl Columns

1. Aminopropyl columns should be prepared prior to cleanup.
2. Concentrate PAH fraction to one drop under nitrogen evaporator.
3. Bring volume to 200 µl with hexane.
4. If water is present in the sample add methanol until clear. Pipette off top PAH fraction and discard bottom methanol fraction.
5. Dope with one drop of chloroform, vortex sample and add to column.
6. Rinse conical tube three more times using 300 µl hexane in three aliquots of 100 µl, dope with one drop of chloroform, vortex and add to column each time.
7. Draw sample through column.
8. Wash PAH fraction from column using 5 ml hexane in three aliquots: 1 ml, 2 ml, 3 ml and let go to dryness.
9. The sample is then concentrated to 1.0 ml under nitrogen evaporator.
10. If the sample evaporates more than that accidentally, make up the volume to the 1.0 mL mark with DCM and then transfer to an auto sampler vial.
11. Label the vial appropriately.
12. Add 20 µl of internal standard before being read on the GC-MS.

Appendix 2:
Solutions for the extraction of PAHs

Optima Grade Chloroform (Fisher): preserved with 0.75% ethanol.

Optima Grade Methylene chloride (DCM)

50 mM Phosphate buffer: add 8.7 g of K_2HPO_4 (Sigma) to approximately 950 ml of Millipore water. Adjusted pH to 7.4 with 1N hydrochloric acid (HCl) and then adjusted to 1000 ml final volume in 1L volumetric flask with Millipore water.

Saturated potassium persulfate solution: Add 10 g of $K_2S_2O_8$ (Sigma) and 2 ml of conc. sulfuric acid (Fisher) to 200 ml volumetric flask and fill to 200 ml with Milli-Q water. This mixture is light sensitive and must be stored in the refrigerator until use. Before use, it must be warmed up to room temperature.

Sodium sulfate (Na_2SO_4) columns: Add 1 g of dry Na_2SO_4 (Fisher) to clean 6 ml glass column. The columns were then packed with 2 ml of DCM without letting the packing go dry.

Solvent exchange: samples in DCM were concentrated to 100 µl using a nitrogen evaporator. Hexane (1 ml) was added and the samples again concentrated to 100 µl. This was repeated two more times.

Unisil (100 – 200 mesh) activated silica columns (Clarkson Chromatography): 0.5 g of unisil were placed into 10 ml tubes and heated at 100 °C for 2 hours to activate. The activated unisil was dissolved in 2 ml of chloroform and was transferred to the glass

column. The tube was rinsed 4 times with 1 ml portions of chloroform and the solution transferred to the column. The chloroform was pulled through at 1 drop/second without allowing the unisil go dry. The sides of the column were rinsed with two 1 ml aliquots of chloroform and 2 ml of hexane. Copper filings (cleaned in 2 rinses of 1 N HCl, methanol, DCM, and hexane and dried under nitrogen) were added to the column. The columns were then ready to use.

Aminopropyl (NH_2) columns (VWR): 1 ml of optima grade chloroform, then another 2 ml were added to the column before pressurizing it and letting it drip. Hexane in the amount of 2 ml was added and pulled through at 1 drop per second, without letting the packing go dry.

Appendix 3: Standard Curve for PAHs

A standard curve was performed using 10.0, 20.0, 30.0, 40.0 and 50.0 ug/mL concentrations of the calibration mix, 20 uL of internal standard, and 50 uL of surrogate solution were added and the volume adjusted to 1.0 mL with hexane. 2 mL autosampler vials were used.

Table 23. Concentrations for standard curve- PAH analysis

Surrogate mix (µL)	PAH mix (µL)	Internal Standard (µL)	Solvent (Hexane) (µL)
50µL	10 µL	20 µL	920 µL
50 µL	20 µL	20 µL	910 µL
50 µL	30 µL	20 µL	900 µL
50 µL	40 µL	20 µL	890 µL
50 µL	50 µL	20 µL	880 µL

Appendix 4: Standards for PAHs

Surrogate Solution: Restek B/N surrogate mix

2-fluorobiphenyl

nitrobenzene-d5

p-terphenyl-d14

1,000 µg/ml each in methylene chloride, 1ml/ampul

Calibration Mix: Restek SV Calibration Mix #5 / 610 PAH Mix

acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorine, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, pyrene

2,000 µg/ml each in methylene chloride, 1ml/ampul

Internal Standards: Restek SV Internal Standard Mixes

acenaphthene-d10, chrysene-d12, 1,4-dichlorobenzene-d4, naphthalene-d8, perylene-d12, phenanthrene-d10

2,000 µg/ml each in methylene chloride, 1ml/ampul

Appendix 5: Correlated Internal Standards with PAHs and Surrogates

Internal Standards:	Correlating PAHs and Surrogates
Napthalene-d8:	Nitrobenzene-d5 (surrogate), Napthalene
Acenaphthene-d10:	2-fluorobiphenyl (surrogate), Acenaphthylene, Acenaphthene, Fluorene
Phenanthrene-d10:	Phenanthrene, Anthracene, Fluoranthene, Pyrene,
Chrysene-d12:	Terephenyl-d14 (surrogate), Benzo(a)anthracene, Chrysene, Benzo(b,k)fluoranthene, Benzo(a)pyrene
Perylene-d12:	Dibenz(ah)anthracene, Ideno(1,2,3-cd)pyrene, Benzo(ghi)perylene

Appendix 6: Standard Curve for PAHs

A standard curve was performed using 10.0, 20.0, 30.0, 40.0, and 50.0 µg/ml concentrations of the PAH mix, 20 µl of internal standard, and 50 µl of surrogate solution were added and the volume adjusted to 1.0 ml with hexane. Two ml autosampler vials were used.

Table 38. Standard curve for PAHs

Surrogate mix (µL)	PAH mix (µL)	Internal Standard (µL)	Solvent (Hexane) (µL)
50µL	10 µL	20 µL	920 µL
50 µL	20 µL	20 µL	910 µL
50 µL	30 µL	20 µL	900 µL
50 µL	40 µL	20 µL	890 µL
50 µL	50 µL	20 µL	880 µL

B/N Surrogate mix, PAH mix and Internal Standard mix solutions are warmed and sonicated prior to use. They are stored at 4^0C after use.

Internal Standard: An *internal standard* is a chemical substance that is added in constant amounts to samples, the blank and calibration standards in a chemical analysis, to correct the loss of analyte during sample preparation or sample inlet. The internal standard is a compound that matches in many aspects, with the chemical species of interest in the samples, as the effects of sample preparation should, relative to the amount of each species, be the same for the signal from the internal standard as for the signal(s) from the species of interest in the ideal case.

Surrogate: *Surrogate compounds* are organic compounds which are similar to the analytes of interest chemically, but which are not normally found in environmental samples. Surrogates are added to samples to monitor the effect of the specific sample matrix on the accuracy of the analysis.

PAH mix: Prepared solution of known PAH concentrations. Preparing mixtures of differing concentrations help to calibrate the GC/MS and generate a standard curve for the instrument due to the known peaks of the PAHs being tested.

Reference:
http://www.groundwateranalytical.com/qual_quality_assur.htm

Appendix 7. Extraction of PAH and Ergosterol by Ergosterol extraction method

1. Weigh 5g of fresh sieved (2mm) soil

2. Place in a 50ml centrifuge tube

3. Add 15ml of cold methanol and 5ml of a fresh KOH solution (40g KOH / L ethanol)

4. Vortex for 30 seconds and sonicate (1min output 6 on a Cell disruptor Model W-370, Heat Systems-Ultrasonics, Inc.)

5. Place the tubes in a water bath pre-heated at 85°C

6. Remove after 15min, mix manually for 1min, and replace for a further 15min

7. Cool in a fridge for 20min

8. Add 10ml of HPLC grade pentane, and hand shake for 1min

9. Centrifuge at 3000xg for 3min (to separate the pentane layer from soil)

10. Remove the pentane layer and transfer to a new tube

11. Carry out steps 8-10 three times for each sample and combine the pentane extracts

12. Condense extracts under a stream of nitrogen to 2 ml

13. Pass the extract through a silica gel column using hexane and DCM in the ratio of 6:4 as solvent system.

14. The eluent is again condensed under a stream of nitrogen to 1 ml

15. At that point, dry extracts can be either stored at -20 °C until analysis and the reaction mixture is used for acetylation. The acetylated ergosterol is prepared for NMR or GC/MS for analysis.

Appendix 8. Acelylation of Ergosterol

1. Dry the reaction mixture after extraction of ergosterol.
2. Dissolve compound in excess amount of pyridine and same quantity of acetic anhydride.
3. Leave it in magnetic stirrer overnight
4. Add DCM (approximately 20 ml)
5. Transfer to separating funnel
6. Wash with 5% H_2SO_4 (20 ml)
7. Wash with H_2O (20 ml, cold)
8. Wash with NaCl (10ml, saturated)
9. Dry with $MgSO_4$, filter into a round-bottomed flask
10. Dry the residue and analyze by NMR or GC/MS.

Appendix 9 – Fungal Cultures and Media

White-Rot Selective Media – White-rot selective media (Dietrich, Lamar, 1990) was prepared by first adding 19.5 g of potato dextrose agar (PDA) to 500 ml of Milli-Q water in a 1,000 ml Erlenmeyer flask. The mixture was then autoclaved at 121^{o}C to put the PDA into solution. Benomyl solution (15 ppm) and streptomycin solution (550 ppm) are added from stock solutions. The stock solution of benomyl consists of 1,000 mg of benomyl per liter of acetone. The stock solution of streptomycin consists of 4,000 mg of streptomycin per liter of sterile water. Media was then poured in deep Petri dishes made for fungal cultures.

Fungal Culturing – White-rot mycelia was scrapped from inoculated sediment or prior inoculated media using a sterile swab. The swab was then streaked onto the white-rot

selective media. The Petri dishes containing the media were place upside down in a 25°C incubator for 72 – 96 hours.

Potato Dextrose Broth – Potato dextrose broth was made by adding 12 g of potato dextrose broth to 500 ml of Milli-Q water in a 1,000 ml Erlenmeyer flask. The flask is then autoclaved at 121°C to bring the media into solution.

Potato Dextrose Broth Culturing – 125 ml of potato dextrose broth is added to an autoclaved 250 ml Erlenmeyer flask. To the flask, a core from an inoculated white-rot selective media plate is added and place on a rotary shaker at 250 rpm at room temperature. The flasks remain on the shaker for 72 – 96 hours.

Grain Preparation – 500 ml of grain is placed into a new spawn bag. To the spawn bag, 150 ml of Milli-Q water is added and the bag is sealed on an impulse sealer set to no. 4 setting. The spawn bag is then autoclaved at 121°C and then cooled to room temperature.

Grain Inoculation – To the autoclaved grain in the spawn bag, 125 ml of white-rot inoculated potato dextrose broth is added and the spawn bag is resealed on an impulse sealer on setting no. 4. The spawn bag is then placed in a 25°C incubator with the filter facing upward for 72-120 hours and then kept refrigerated until use.

Procedure for Incubation Set-up – 1,000 ml of sediment is added to a 1.9 L glass bowl. For treatments containing a fungal nutrient source, 600 ml of sawdust is added and the contents of the bowl are mixed. For the treatments containing *P. ostreatus*, 100 ml of inoculated grain is added to the bowl and the contents are mixed. Treatments are then sampled for time 0 data and remixed to create a homogenous mixture. The bowls are then incubated for 21 days at 25 °C in an incubator. At 21 days, half of the mixture in the bowl is mixed by hand to see the effect of tilling as a treatment. To incubations

containing sawdust, half of the newly mixed sediment was also mixed with 25 ml of a nitrogen source to see the effects of nitrogen. Sampling is down accordingly at 21 days, and bowls are incubated for another 21 days at 25 ^{o}C. Sampling is performed once more at 42 days.