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METHODS FOR THE DETECTION OF TRACE METABOLITES FROM BIOTRANSFORMATION OF POLYCYCLIC AROMATIC HYDROCARBONS BY YEAST

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ABSTRACT—The analysis of pollutants and their metabolites resulting from biotransformation is important to studies of bioremediation or toxicology of environmental toxicants. Gas chromatography-mass spectrometry (GC-MS) with solid-phase micro-extraction (SPME) has been used to determine the 9-hydroxyphenanthrene levels resulting from phenanthrene biotransformation by yeast. *Candida tropicalis* CP1-1, derived from a parent strain of *Candida tropicalis* (ATCC strain 96745), was grown in minimal media containing phenanthrene. Direct extraction of liquid media by SPME served to selectively preconcentrate the pollutants prior to GC-MS analysis. Various derivatizing reagents, including dimethyldichlorosilane, trimethylchlorosilane, acetic anhydride, and trifluoroacetic anhydride, were evaluated for their ability to enhance the volatility of metabolites containing hydroxyl groups, and improve chromatographic separation. Both dimethyldichlorosilane and trifluoroacetic anhydride gave distinctive mass spectra with both molecular and fragment ions that were suitable for selected ion monitoring of 9-hydroxyphenanthrene at trace levels. In addition, trifluoroacetic anhydride on-fiber derivatization of 9-hydroxyphenanthrene after SPME preconcentration yielded a GC-MS method capable of analysis at the parts-per-billion level. The extraction efficiencies of different SPME fiber coatings, including Carbowax™/divinylbenzene, polydimethylsiloxane, and polyacrylate, were compared for their potential application in yeast culture media metabolite analysis. The 100 μm polydimethylsiloxane provided the highest analytical sensitivity for the trifluoroacetyl derivative of 9-hydroxyphenanthrene, while the 70 μm Carbowax™/divinylbenzene provided the next best analytical sensitivity and a simpler chromatogram with less derivatization byproducts. This study demonstrates that SPME with derivatization provides a sensitive method for analyzing hydroxyl group containing metabolites in yeast cultures. The superior analytical sensitivity can be attributed to the highly efficient sample extraction process and the intense signal of the trifluoroacetyl derivative of the molecular ion in GC-MS analysis. Hence, this analytical method is desirable for studying the biochemical transformation of many environmental toxicants via hydroxylation.

Yeasts isolated from coastal sediments, including *Candida lypolytica, Candida maltosa, Candida tropicalis, Candida utilis, Debaromyces hansenii,* and *Saccharomyces cerevisiae,* have been reported to be capable of transforming polycyclic aromatic hydrocarbons (Cerniglia and Crow, 1981; Hofmann, 1986; McGillivray and Sharis, 1993). Yeasts also have been shown to exist in large numbers in petroleum contaminated soils in Alaska that were devoid of bacteria (Atlas et al., 1976). The different yeast species involved in the metabolism of aromatic compounds in a polluted estuary in Rio de Janeiro, Brazil, have been characterized (Hagler et al., 1979; Pinto et al., 1979). Since yeasts are widespread and tolerant of changes in environmental conditions, they could play an important role in the degradation of organic compounds in the environment.

Polycyclic aromatic hydrocarbons (PAHs) are common environmental contaminants that have been listed as priority pollutants by the United States Environmental Protection Agency. Because PAHs may be carcinogenic, mutagenic, and/or toxic to many organisms (Perez et al., 2001; Chiang et al., 1997; Bispo et al., 1999), there is interest in developing remedial approaches for degrading PAHs in polluted environments through PAH biotransformation by microorganisms. It also is important to develop sensitive and efficient techniques for probing the metabolites resulting from PAH biotransformation. Phenanthrene, one of the most abundant PAHs in the environment, is used as a model substrate for studying PAH biotransformation by yeasts because it shares similar molecular features with potent carcinogenic PAHs (Pothuluri and Cerniglia, 1994; Sack et al., 1997). Most microbial pathways for metabolizing phenanthrene rely on the use of dioxygenase among bacteria or cytochrome P-450 monooxygenase among yeasts and fungi to form a variety of PAH dihydrodiols and hydroxy PAHs, respectively, with different isomeric configurations. Typically, the bacteria will form the *cis*-isomers of PAH dihydrodiols before being transformed to catechols, whereas yeasts and fungi will produce the intermediate arene oxides (epoxides of PAHs) before undergoing non-enzymatic rearrangement into hydroxy PAHs or conversion into the *trans*-isomers of PAH dihydrodiols by epoxide hydrolase (Pothuluri and Cerniglia, 1994). The objective of this study was to develop an analytical method capable of characterizing phenanthrene metabolites resulting from biotransformation by *Candida tropicalis* CP1-1.

Earlier studies of microbial biotransformation were carried out using liquid/liquid extraction of culture media followed by analysis with either GC or high-performance liquid chromatography (HPLC). Selected examples of the analysis of PAHs and

their metabolites in both laboratory and field studies using the liquid/liquid extraction approach have been published (Cerniglia and Crow, 1981; Mohammed et al., 1998; Yuan et al., 2000). However, these methods involved laborious sample preparation and the use of large amounts of organic solvents for extraction due to the presence of constituents in the yeast culture media that may not be suitable for GC or HPLC analysis. In addition, the yeast metabolites may be present at very low concentrations that require an extra step of analyte preconcentration during sample preparation. To circumvent these problems, this study describes the use of solid-phase microextraction (SPME) for direct extraction of metabolites from aqueous solution containing phenanthrene or other PAHs followed by GC-MS analysis.

Solid phase microextraction techniques have been used successfully for the analysis of PAHs in a variety of sample matrices including contaminated soil (Eriksson et al., 2001), seawater (Chee et al., 1999), and suspended particulate matter and surface sediment in rivers (Wang et al., 2002). However, the analysis of hydroxy PAHs is difficult to achieve at high sensitivity with polydimethylsiloxane (PDMS) sorbent because of the lower affinity of hydroxy PAHs relative to PAHs for the non-polar PDMS coating. Although SPME/GC-MS has been used for the analysis of hydroxy PAHs in urine (Huang et al., 2002), most existing methods for the analysis of hydroxy PAHs are based on HPLC with evaporative light-scattering detection (Cebolla et al., 1997), mass spectrometric detection (Galceran and Moyano, 1996), and electrochemical detection (Galceran and Moyano, 1995). Due to the superior sensitivity and the capability for structural elucidation of PAHs by SPME/GC-MS analysis, this project investigates the use of different SPME fiber coatings such as PDMS, polyacrylate (PA), and Carbowax™/divinylbenzene (CW/DVB) for the simultaneous analysis of PAHs and hydroxy PAHs in yeast biotransformation research. Polyacrylate-coated fibers have advantages over PDMS-coated fibers since they can extract both polar and nonpolar compounds equally well and have relatively low sample carry-over effects between analyses. However, PA-coated fibers require extended extraction times due to the nature of the polymeric coating material (Buchholz and Pawliszyn, 1994).

Phenols and amines are commonly derivatized with silylating reagents prior to GC analysis to reduce their polarity for improved chromatographic separation. Derivatization has an added advantage of protecting chromatographic stationary phases against free phenols that have shown tendencies to form hydrogen bonds with phases such as Carbowax™ (Buchholz and Pawliszyn, 1994). The concept of analyte derivatization for hydroxy PAHs to enhance their extraction efficiencies using nonpolar PDMS sorbent phase is explored in the present study. Derivatization of hydroxy PAHs using reagents with trimethylsilyl groups is limited to on-fiber derivatization for SPME/GC analysis or derivatization in nonaqueous environment because the derivative hydrolyzes on contact with water. In order to develop a reliable technique of analyzing PAHs and their metabolites in the culture media, several SPME-based sample preparation schemes were considered and evaluated. The acetylation method using acetic anhydride was investigated for both on-fiber and in-matrix conversion of hydroxy phenanthrene for SPME/GC analysis (Coutts et al., 1979). Another reagent, trifluoroacetic anhydride, was used to form a trifluoroacetyl derivative that gave a prominent and unique molecular ion in GC-MS for the analysis of hydroxy PAHs while improving their analytical sensitivity. This approach has been used successfully to analyze amphetamine (Eiceman et al., 1984), 4-*o*-methylpyridoxine in serum (Fujisawa et al., 2002), and glyphosate and its metabolites (Kudzin et al., 2002).

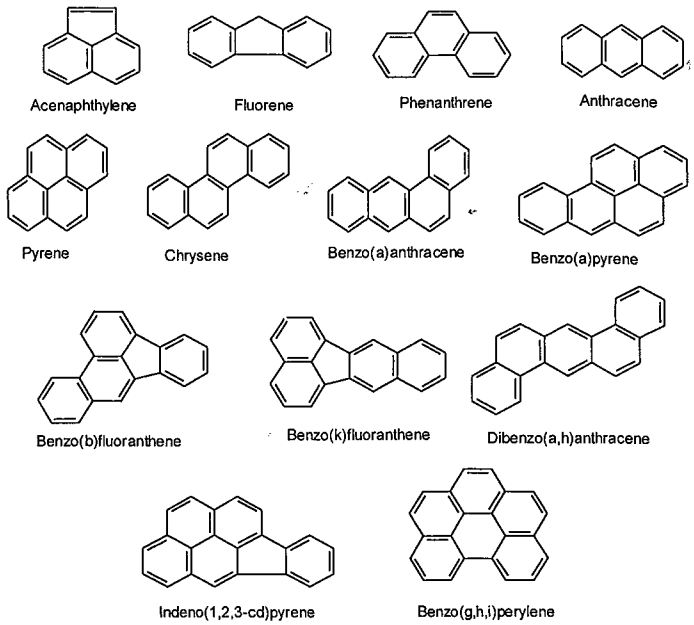

FIG. 1. The structures for a number of important polycyclic aromatic hydrocarbons.

MATERIALS AND METHODS

Chemicals and Reagents—A standard mixture of 13 PAHs at the concentration of 2000 μg/mL each (Restek, Bellefonte, Pennsylvania) and a standard of 1000 μg/mL *p*-terphenyl-d_{14} (Restek, Bellefonte, Pennsylvania) were used to prepare aqueous standards at the levels of 10 and 1.0 μg/mL, and 1.4 μg/L for the development of SPME/GC-MS methods. The 10 μg/mL and 1.4 μg/L standards were used to compare the relative sensitivity of liquid/liquid extraction and SPME methods for PAH analysis, respectively. The 1.0-μg/mL standard was used to optimize the SPME method with regard to the insertion depth of the SPME fiber in the GC injector. The names and structures of the 13 PAHs with three to six aromatic rings are shown in Fig. 1. Most of the PAHs with four or more aromatic rings have been proven to be either genotoxic or carcinogenic and were handled with gloves to prevent dermal absorption during sample preparation.

Phenanthrene, 9-hydroxyphenanthrene (technical grade), and hydroxynaphthalene were obtained from Aldrich Chemicals (Milwaukee, Wisconsin), and *trans*-1,2-dihydroxy-1,2-dihydrodiolchrysene was obtained from the National Cancer Institute. Derivatizing reagents, including acetic anhydride (AA), trifluoroacetic anhydride (TFAA), dimethyldichlorosilane (DMDCS), and trimethylchlorosilane (TMCS) as well as the three types of SPME fibers (PDMS, PA, and CW/DVB), were purchased from Supelco (Bellefonte, Pennsylvania). The internal standard, 40 μg of *p*-terphenyl, was added to samples prior to extraction or derivatization to account for slight variation in the experimental conditions. SPME fibers of 30 μm PDMS, 100 μm PDMS, and 70 μm CW/DVB in film thickness were conditioned according to procedures recommended by the manufacturer. The conditioning of 85 μm PA-coated fiber was carried out at 350°C for 4–5 h (Buchholz and Pawliszyn, 1994; Pan and Pawliszyn, 1997).

Derivatization Procedures—Fig. 2 summarizes the methods

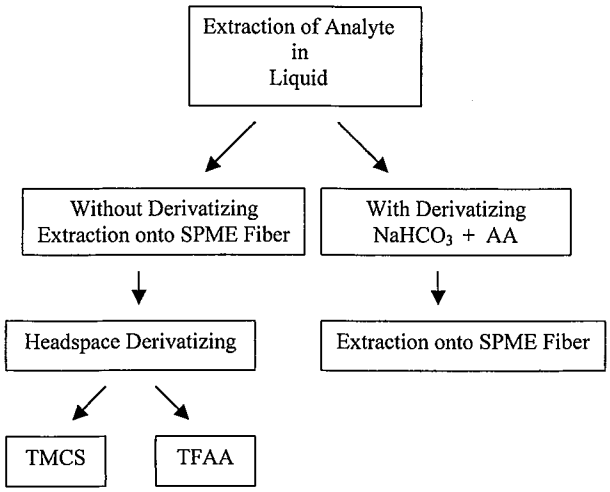

FIG. 2. This flowchart outlines our procedures for extracting analytes from liquid samples for solid-phase microextraction (SPME) and GC-MS analysis. TMCS is trimethylchlorosilane and TFAA is trifluoroacetic anhydride.

for derivatization of 9-hydroxyphenanthrene and the simultaneous extraction of both phenanthrene and the 9-hydroxyphenanthrene derivative. The 9-hydroxyphenanthrene in aqueous solution was derivatized with AA in the presence of ACS grade sodium bicarbonate (Fisher Scientific, Fair Lawn, New Jersey) according to an existing method (Coutts et al., 1979). The corresponding acetate ester product was subsequently extracted onto either the PA or the PDMS fiber. The SPME extraction was performed at room temperature for 45 min with constant stirring prior to GC-MS analysis. The high affinity of 9-hydroxyphenanthrene derivatives for the SPME sorbent phase relative to the aqueous matrix allowed efficient analyte extraction for highly sensitive analyses.

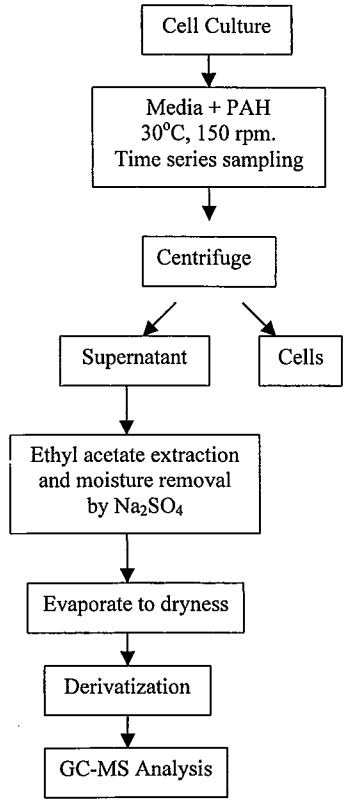

FIG. 3. This flowchart outlines our sample preparation procedures for direct GC-MS analysis without SPME.

Another approach for analyzing the metabolites was to use the SPME fiber for analyte extraction of the aqueous samples at room temperature for 45 min. The fiber was retracted and removed from the extraction vial and was subsequently exposed to the headspace above 50 μL of TMCS for 15 min or 20 μL of TFAA for 5 min. The derivatization of 9-hydroxyphenanthrene by the vapors of TMCS and TFAA on the SPME fiber was carried out in a sealed 4.5 mL vial at room temperature. The residual moisture on the SPME fiber was removed as volatile hydrolytic products of the excess derivatizing reagents.

A direct derivatization scheme without using SPME was carried out by extracting aqueous culture media with ethyl acetate followed by evaporation to dryness. The dried samples were derivatized in 10 μL of 1% TMCS or 1% DMDCS in methylene chloride at room temperature. The reaction mixture was re-dissolved in 1.0 mL of methylene chloride followed by the splitless injection of 1 μL of the derivatized samples into the GC-MS.

Yeast Strain and Growth Conditions—The *Candida tropicalis* CP1-1 was derived by selective plating of the original stock of *C. tropicalis* ATCC 96745 on 0.67% Yeast Nitrogen Base (YNB) without amino acid (Difco, Detroit, Michigan) containing 0.2 mg/mL phenanthrene followed by plating onto YNB or minimal media containing 0.2 mg/mL chrysene. A 150-mL aliquot of pre-inoculum culture containing *C. tropicalis* CP1-1 in YNB and 0.2% glucose was incubated at 30°C for 72 h at 150 rpm. The purpose of the glucose was to sustain the growth of cells that were capable of metabolizing PAHs since the yeasts cannot utilize PAHs as sole carbon sources. The cells were pelleted and placed into 150 mL of fresh YNB media with 0.2% glucose and 0.2-mg/mL phenanthrene (Cerniglia and Crow, 1981). Prior to inoculating the cells, the media was prepared by adding phenanthrene dissolved in methylene chloride to give a final media concentration of 0.2 mg/mL phenanthrene followed by incubation at 30°C and 150 rpm for at least one day to remove the methylene chloride.

The "heat-killed" control was inoculated with the same amount of cells that had been boiled for 15 minutes prior to inoculation. All cultures were incubated at 30°C and aerated by shaking at 150 rpm. At the intervals of 2 and 4 days, a 30-mL aliquot of each culture was removed and prepared for GC analysis as shown in Fig. 3. Cultures were checked for fungi and bacterial contamination by microscopic examination.

Equipment and Instrumentation—Chromatographic analysis was performed on a Hewlett Packard HP 5890 Series II GC interfaced to a HP5970 mass selective detector (MSD). The flow rate of helium carrier gas for the GC-MS was maintained at 1.0 mL/min throughout the analysis. The injector temperature was set at 280°C and the detector temperature at 300°C. The GC-MS temperature programs used for the analysis of standards or samples containing PAHs alone and PAHs plus hydroxylated PAHs, respectively, are shown in Table 1. A 30-meter SPB-5 GC column with 0.25 mm inside diameter and 0.25 μm thick stationary phase of 95% polydimethylsiloxane with 5% phenyl groups was used for the PAH analysis (Supelco, Bellefonte, Pennsylvania).

TABLE 1. Gas chromatography temperature program used for the analysis of polycyclic aromatic hydrocarbons (PAHs) and hydroxyl PAHs.

Level	Initial temp. (°C)	Rate (°C/min)	Final temp. (°C)	Hold time (min)
GC-MS temperature program for analysis of standard PAHs				
1	50	20.00	220	2.00
2	220	10.00	280	5.00
3	280	5.00	320	2.00
GC-MS temperature program for analysis of PAHs and hydroxy PAHs				
1	50	10.00	200	1.00
2	200	5.00	260	1.00
3	300	10.00	300	4.00

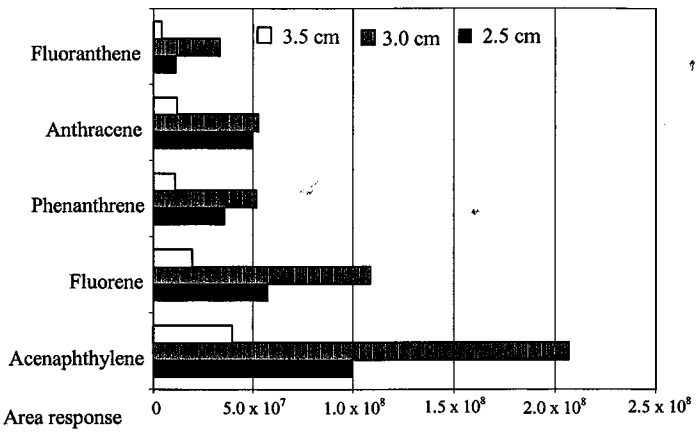

FIG. 4. Extraction efficiency at different fiber depth settings is compared for selected polycyclic aromatic hydrocarbons at concentration of 1 ppm using 100 μm polydimethylsiloxane.

RESULTS AND DISCUSSION

Different SPME fiber depths were examined to identify the optimal injector position for transferring the analytes into the GC-MS. The results for five different PAHs analyzed at 1.0 μg/mL or part per million (ppm), each using fiber depths of 2.5, 3.0, and 3.5 cm, are shown in Fig. 4. Based on the comparison of the GC-MS signals at various injector depths, the signals were the largest at 3.0 cm and smallest at 2.5 cm for all five compounds. The signal enhancement at 3.0 cm relative to 2.5 cm is especially remarkable with a 3-fold and 2-fold improvement for fluoranthene and acenaphthylene, respectively. Therefore, an SPME fiber depth setting of 3.0 cm was selected for all subsequent GC-MS analyses. The variation of GC-MS signals as a function of the SPME fiber depths showed the influence of the thermal gradient inside the GC injector on the efficiency of analyte desorption. Specifically, the analytical sensitivity was affected by the type of injector sleeve and the GC injector design.

The total ion chromatogram in Fig. 5a shows the separation of the standard PAHs and *p*-terphenyl-d_{14} at 1.4 μg/L or parts per billion (ppb), each in a 13.5-mL aqueous sample extracted by SPME with the 100 μm PDMS fiber. The sensitivity of SPME-based GC-MS analysis is clearly demonstrated by the capability to detect trace levels (parts-per-trillion), especially for anthracene, pyrene, and phenanthrene. This observation was consistent with a previous report that stated detection limits of 0.1 ng/mL were obtained for PAHs with 100 μm PDMS fibers (Doong et al., 2000; Langenfeld et al., 1996). Acenaphthylene was not shown in Fig. 5a because there were some artifact peaks from the SPME that interfered with its analysis. In comparison, the GC-MS total ion chromatogram (Fig. 5b) of the 13 PAHs obtained by direct GC-MS injection of 1 μL from a 10-ppm solution (10 ng of each PAH) showed poorer signal-to-noise ratios. The sharp contrast in the relative sensitivity of the two sample extraction techniques is due to the fact that SPME allowed the analysis of all analytes extracted from a 13.5-mL sample, whereas direct injection allowed the analysis of only 1 μL sample by the GC-MS. The typical sample injection volume of 1 to 5 μL in direct GC-MS analysis is limited by the capacity of the small diameter of the capillary columns and the requirement for maintaining the vacuum of the mass spectrometer when the GC effluents enter the ion source. On the contrary, the SPME technique is a solventless technique that allows the analysis of analytes concentrated from large sample volumes onto the sorbent phase of the SPME fiber.

Analysis of the less volatile hydroxy PAHs by GC-MS presented challenges such as chromatographic peak tailing, sample carryover between GC analyses, and reduced analytical sensitivity. Derivatization of hydroxy groups with silylating reagents has been used to overcome the problem of peak tailing and to enhance their volatility for GC analysis. In this study, DMDCS, TMCS, AA, and TFAA were investigated for their effectiveness as derivatizing agents of 9-hydroxyphenanthrene for SPME with GC-MS analysis. The chemical structures, chemical formulae, and molecular masses of the respective derivatives of 9-hydroxyphenanthrene are shown in Fig. 6. The DMDCS and TFAA derivatives, yielded more volatile derivatives compared to the 9-hydroxyphenanthrene parent compound due to the presence of the chlorine and trifluoroacetyl groups, respectively. Because DMDCS, TMCS, and TFAA are very volatile, the excess reagents can be readily removed from the sample matrix by evaporation. For AA, the excess reagent is easily broken down by hydrolysis in the aqueous samples.

GC-MS analysis of DMDCS derivatives of hydroxylated PAH standards such as 2-hydroxynapthalene, 9-hydroxyphenanthrene, and 1,2-dihydroxy-1,2-dihydrodiolchrysene gave peaks with retention times of 15.0, 22.7, and 32.0 min, respectively. The peaks for the three analytes of interest were well resolved, including the two isomeric forms of 1,2-dihydroxy-1,2-dihydrodiolchrysene. The DMDCS derivatives of these PAH metabolites in the chromatogram (Fig. 7) demonstrated the potential application of the method for studying the hydroxylation of PAHs and their subsequent biotransformation. There were no signs of underivatized hydroxylated PAHs in the chromatogram, thus indicating the highly efficient derivatizing capability of DMDCS. However, DMDCS tended to produce undesirable byproducts in the form of "artifact" peaks in the chromatogram (Fig. 7) that might complicate the analysis of phenanthrene and its metabolites. Phthalate esters were detected due to the use of culture bottles with plastic components that were avoided in subsequent experiments. Thus, it is important to rely on extracted ion chromatogram or selected ion monitoring for the simultaneous anal-

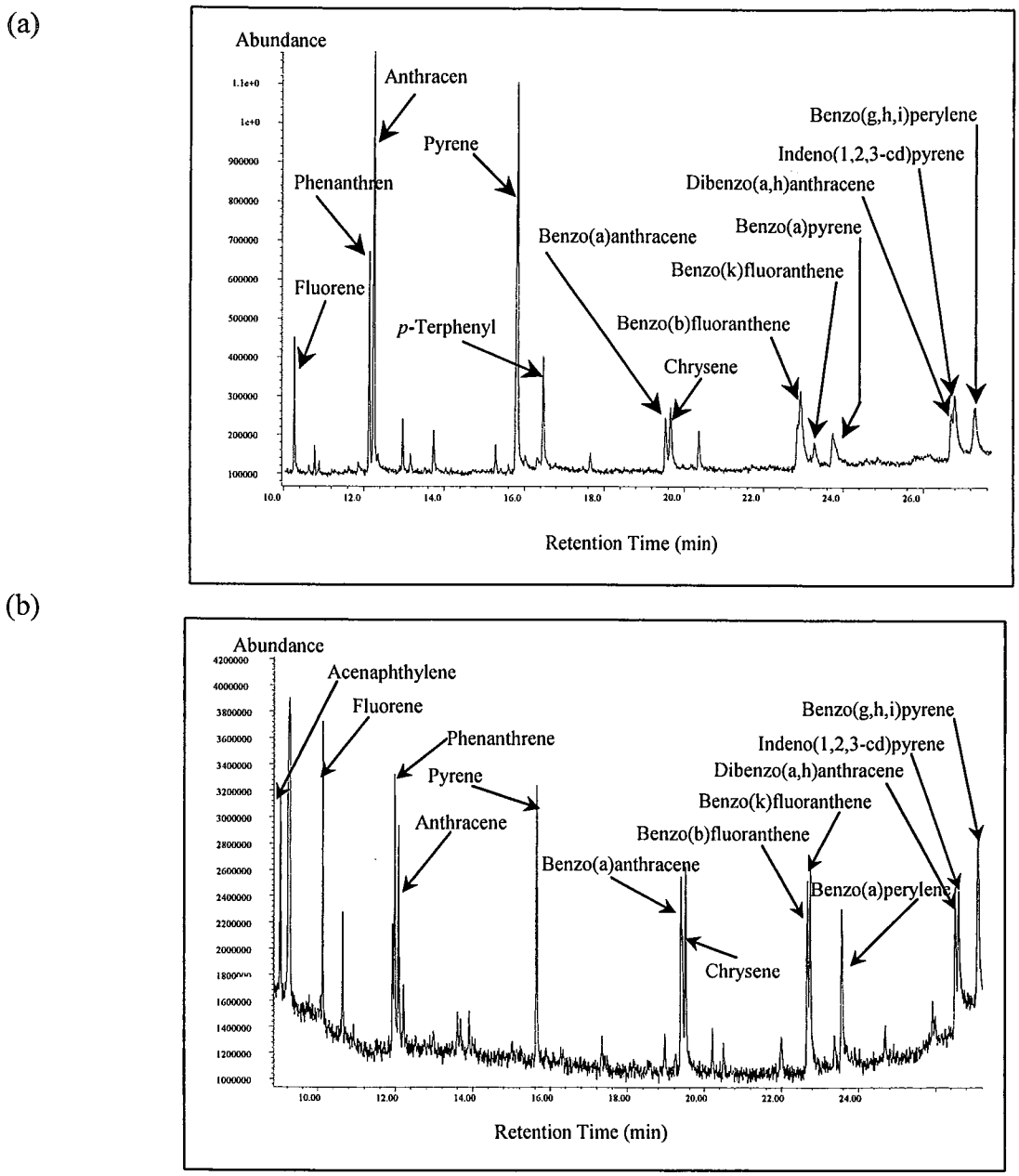

FIG. 5. GC-MS total ion chromatograms for standard polycyclic aromatic hydrocarbons (PAHs) are compared following solid-phase microextraction (SPME) or direct injection. A) SPME analysis of PAHs at a concentration of 1.4 μg/L in 13.5 mL samples. B) Direct analysis of 1 μL of standard PAHs at a concentration of 10 μg/mL.

ysis of PAHs and DMDCS derivatives of hydroxylated PAHs so that the derivatization byproducts of DMDCS are avoided.

Yeast culture samples analyzed using the DMDCS method at incubation periods of 2 and 4 days showed that the *C. tropicalis* CP1-1 was capable of metabolizing phenanthrene. Relative to the phenanthrene signal at 5.1×10^7 ion counts in the heat-killed control experiment, the extracted ion chromatograms of the phenanthrene molecular ion in the yeast culture samples showed signals of 4.3×10^7 ion counts at 2 days and 2.1×10^7 ion counts at 4 days as the phenanthrene was consumed by the yeast cells. In contrast, the extracted ion chromatogram of the molecular ion of the DMDCS derivative revealed that 9-hydroxyphenanthrene was non-detectable in the heat-killed control experiment, had ion counts of 2.7×10^7 and 5.9×10^7 in the culture samples at 2 and 4 days of incubation, respectively, and was consistent with phenanthrene metabolism to 9-hydroxyphenanthrene. The presence of 9-hydroxyphenanthrene in the yeast culture was corroborated by the analysis of 9-hydroxyphenanthrene standard that showed the same GC retention time and mass spectrum. The 9-hydroxyphenanthrene standard was chosen on the basis of the published results of phenanthrene metabolism by *Phanerochaete chrysosporium,* a fungus that shares the same P-450 monooxygenase enzyme as *Candida tropicalis* (Sutherland et al., 1991).

When TMCS was used as the derivatizing reagent, there was no appreciable formation of undesirable byproducts in the derivatization reaction. However, two major peaks were observed in the total ion chromatogram (Fig. 8a) at the retention times of

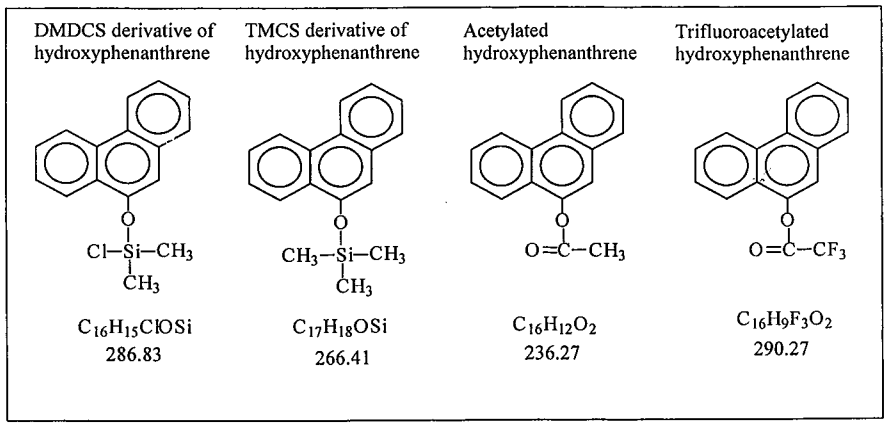

FIG. 6. The structures, formulas, and molecular weights of the various derivatives of 9-hydroxyphenanthrene are illustrated. DMDCS is dimethyldichlorosilane and TMCS is trimethylchlorosilane.

21.3 and 22.3 min that correspond to the TMCS derivative of 9-hydroxyphenanthrene and the underivatized hydroxyphenanthrene, respectively. Despite the molar excess of the TMCS reagent, a significant amount of the underivatized 9-hydroxyphenanthrene was retained. Compared to DMDCS, TMCS was less efficient in converting the hydroxyl group to the trimethylsilyl group. Application of SPME to the analysis of TMCS derivatives was limited to headspace-derivatization mode since the silylating reagent is highly susceptible to reaction with water. To circumvent this problem, AA was used as the derivatizing reagent to convert 9-hydroxphenanthrene in aqueous standard to its acetylated derivative that was then extracted by SPME with subsequent GC-MS analysis. However, the GC-MS total ion chromatogram for the analysis of 9-hydroxyphenanthrene using AA (Fig. 8b) shows two peaks at retention times of 19.0 and 19.8 min, which correspond to the underivatized 9-hydroxyphenanthrene and the acetylated 9-hydroxyphenanthrene, respectively. Therefore, both AA and TMCS are not reactive enough to quantitatively produce volatile derivatives of 9-hydroxyphenanthrene that can be easily determined by GC-MS.

Complete derivatization of hydroxy compounds is highly desirable for trace analysis of yeast culture metabolites. The 9-hydroxyphenanthrene derivatized with the TFAA reagent shows a single peak on the total ion chromatogram (Fig. 8c). The TFAA reagent is highly susceptible to reaction with water and is limited to on-fiber headspace derivatization. The advantage of using TFAA over TMCS is the TFAA derivative is more volatile than the corresponding TMCS and AA derivatives as indicated by the shorter retention time of the TFAA derivative (14.3 min) compared to the TMCS and AA derivative retention times. Furthermore, the derivatization reaction with TFAA was rapid and complete conversion of 9-hydroxyphenanthrene could be achieved in less than five minutes. Thus, the TFAA reagent was more effective than either the TMCS or the AA reagents in the reaction rate

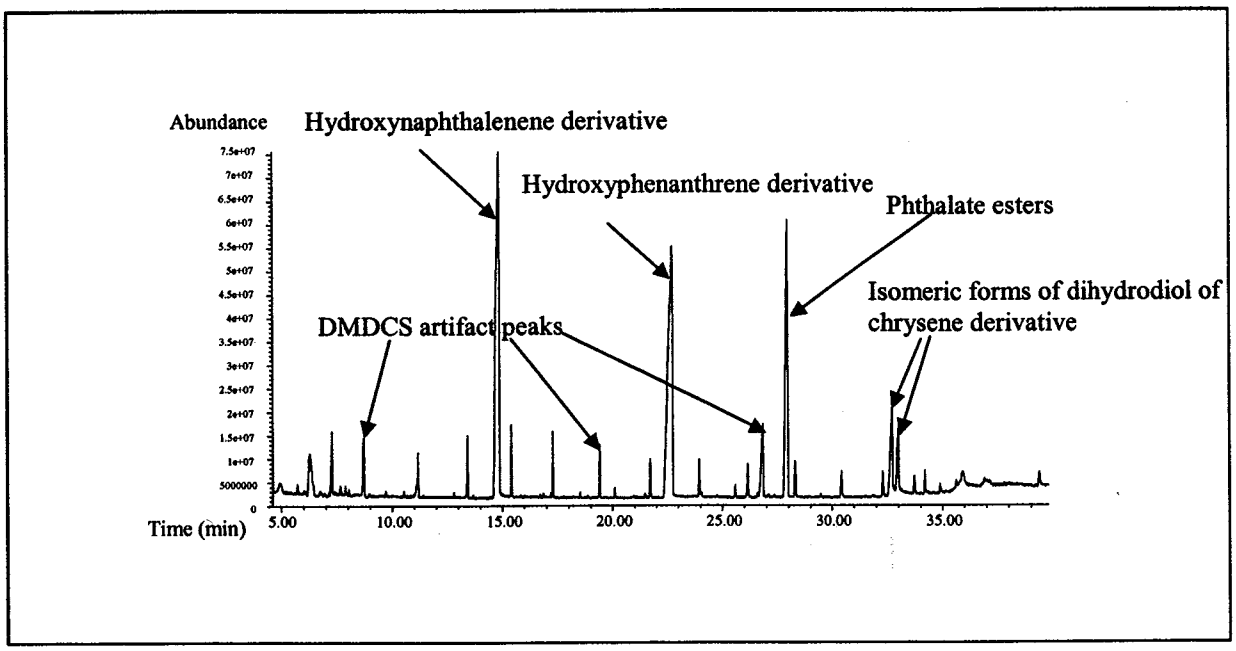

FIG. 7. Chromatographic analysis of aqueous standards containing 2-hydroxynaphthalene, 9-hydroxyphenanthrene, and 1,2-dihydroxy-1,2-dihydrodiolchrysene via liquid-liquid extraction with subsequent derivatization by dimethyldichlorosilane (DMDCS) is shown.

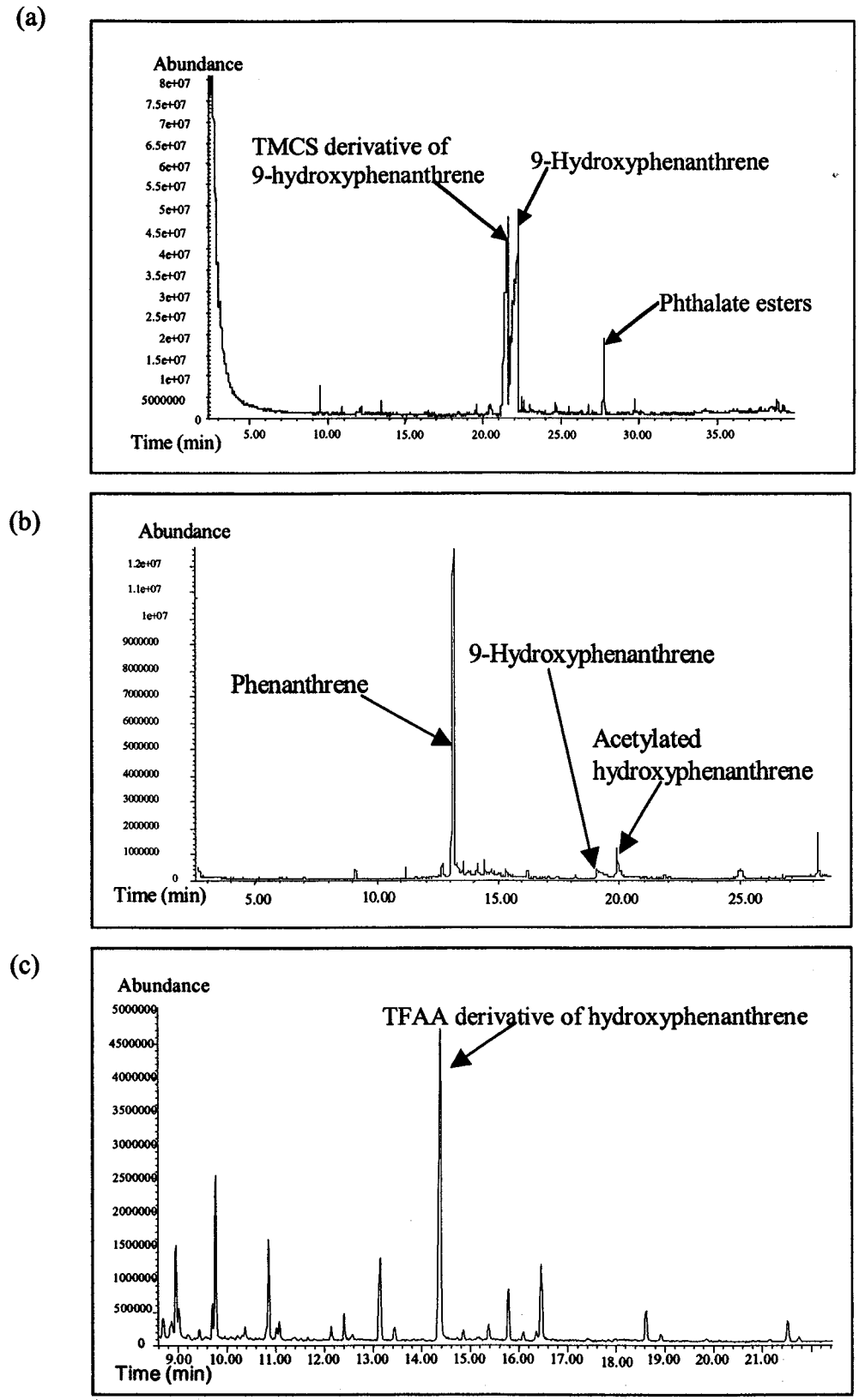

FIG. 8. Total ion chromatograms for the GC-MS analysis of 9-hydroxyphenanthrene are compared following derivatization using (a) trimethylchlorosilane (TMCS) after liquid-liquid extraction, (b) acetic anhydride (AA) with solid-phase microextraction (SPME) onto 85 μm polyacrylate fiber, and (c) trifluoroacetic anhydride (TFAA) with SPME extraction onto 85 μm PA fiber.

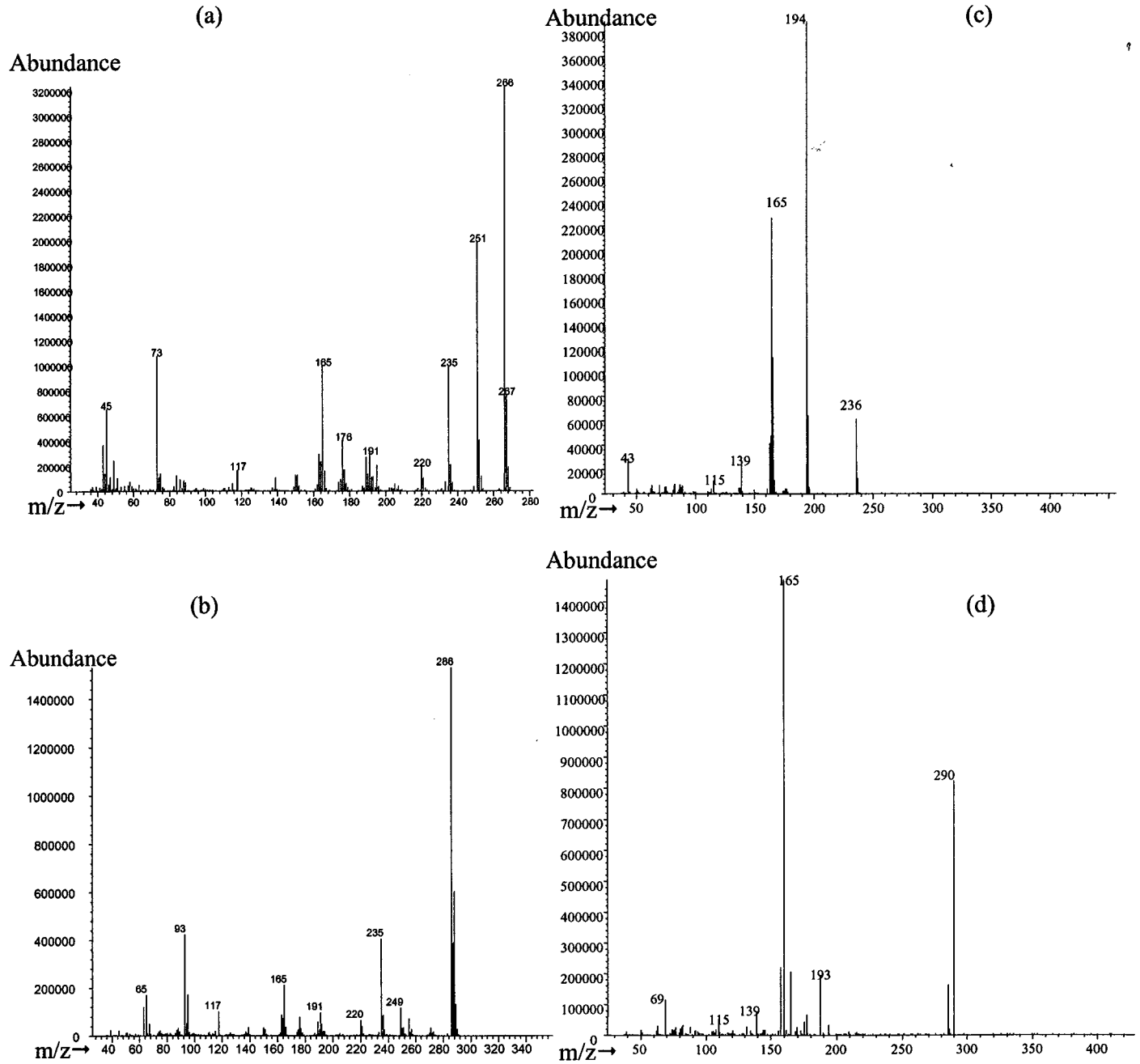

FIG. 9. Mass spectra of 9-hydroxyphenanthrene derivatized with: a) trimethylchlorosilane, b) dimethyldichlorosilane, c) acetic anhydride, and d) trifluoroacetic anhydride.

and yield of the derivatization process. The speed and efficiency of the derivatization reaction via TFAA was previously noted in a study involving the quantitative determination of amphetamine by co-injecting amphetamine and TFAA into a heated GC injector (Eiceman et al., 1984). However, on-fiber derivatization after SPME extraction in aqueous samples did not require heating for derivative formation, and was not susceptible to the need for a narrow range of the optimal injector temperature described by Eiceman and co-workers. Furthermore, the molecular ion of the TFAA derivative has a very good signal-to-noise ratio and low detection limits for hydroxy PAHs can be obtained. The superior sensitivity for the determination of 9-hydroxyphenanthrene is due to the intense molecular ion peak at m/z = 290, the excellent extraction efficiency of the TFAA derivative by SPME, and the higher volatility of the TFAA derivative relative to the underivatized 9-hydroxyphenanthrene. Due to the high electron affinity of fluorine substituents in the trifluoroacetyl derivative, the analytical sensitivity would be expected to be impressive when GC with electron-capture detection or mass spectrometry with chemical ionization in negative ion mode is used for the detection of the fluorinated derivative. Detection limits of 0.01 to 3.3 picograms have been demonstrated for the analysis of hydroxy PAHs using these two techniques (Galceran et al., 1995).

Since the mass spectra of 9-hydroxyphenanthrene derivatized with various reagents are not available in the mass spectral library of the National Institute of Standards and Technology,

TABLE 2. Characteristic molecular and fragment ions determined for three 9-hydroxyphenanthrene derivatives.

	Molecular ion	Fragment ions			
DMDCS[a] derivative	$C_{14}H_9OClSi(CH_3)_2$	$C_{14}H_9Si(CH_3)_2$	$C_{13}H_9$	$ClSi(CH_3)_2$	
m/z	286	235	165	93	
TMCS[b] derivative	$C_{14}H_9OSi(CH_3)_3$	$C_{14}H_9OSi(CH_3)_2$	$C_{14}H_9Si(CH_3)_2$	$C_{13}H_9$	$Si(CH_3)_3$
m/z	266	251	235	165	73
AA[c] derivative	$C_{14}H_9OOCCH_3$	$C_{14}H_{10}O$	$C_{13}H_9$	$C_{11}H_7$	
m/z	236	194	165	139	
TFAA[d] derivative	$C_{14}H_9OOCCF_3$	$C_{14}H_9O$	$C_{13}H_9$	$C_{11}H_7$	CF_3
m/z	290	193	165	139	69

[a] DMDCS = dimethyldichlorosilane
[b] TMCS = trimethylchlorosilane
[c] AA = acetic anhydride
[d] TFAA = trifluoroacetic anhydride

they are shown in Fig. 9, and the molecular ions and major fragment ions of these derivatives are shown in Table 2. The advantage of using SPME/GC-MS for analyzing the metabolites of yeast culture is based on the rich structural information of the mass spectra relative to data obtained by other detection techniques. For example, an electrochemical detector (Galceran and Moyano, 1995) and an ultraviolet absorption detector (Sutherland et al., 1991) had been used with liquid chromatography for characterizing hydroxy PAHs, but relied on the use of chemical standards for confirming the identity of analytes via comparison of chromatographic retention time or electrochemical/spectral information. On the contrary, the mass spectra of hydroxy PAHs are likely to yield meaningful structural information that can be interpreted by chemists and the chemical identity can be confirmed subsequently via spectral matching. For the DMDCS derivative of 9-hydroxyphenanthrene, the molecular ion is at the m/z value (mass-to-charge ratio) of 286, which is the base peak of the mass spectrum. The fragment ions with m/z values 165, 235, and 178 have the relative intensities of 13.6, 26.2, and 27.2%, respectively. The presence of chlorine and silicon in the derivative gives rise to a characteristic isotopic pattern for the molecular ion and increases the confidence level for unequivocal identification of yeast metabolites. For instance, the relative intensities of peaks for 287/286 and 288/286 m/z ratios were observed to be 24.9 and 39.0%, respectively. These values for observed relative intensity are very close to the corresponding theoretical values of 23.08 and 37.98%. The mass spectrum of the TMCS derivative of 9-hydroxyphenanthrene shows characteristic ions at m/z values of 73, 165, 235, 251, and 266. The m/z = 266 ion is both the base peak and molecular ion that shows isotopic ratios of 267/266 and 268/266 characteristic of silicon isotopic abundances. For the acetylated 9-hydroxyphenanthrene, characteristic ions are found at m/z values of 139, 165, and 236 with the base peak at 194. The base peak in the mass spectrum of the TFAA derivative of 9-hydroxyphenanthrene is at m/z = 165, with characteristic ions at m/z values of 69, 139, 193, and 290. The relative intensities associated with these four ions are 7.4, 5.6, 18.5, and 55.5%.

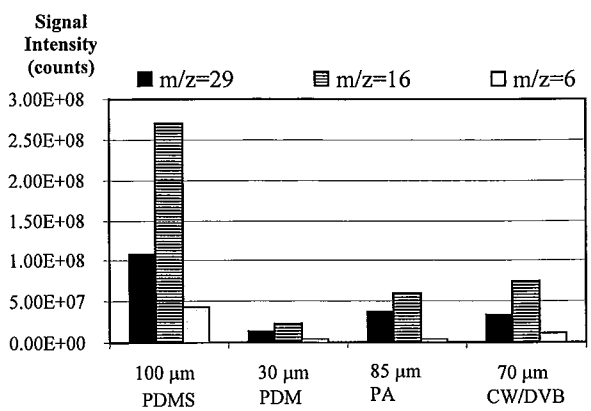

FIG. 10. The sensitivity of GC-MS ion signals depends on the types of solid-phase microextraction (SPME) fiber coatings. Fiber coatings tested included polydimethylsiloxane (PDMS), polyacrylate (PA), and Carbowax™/divinylbenzene (CW/DVB).

Different fiber types such as 30 or 100 µm PDMS, 85 µm PA, and 70 µm CW/DVB fibers were studied for their efficiency in extracting 9-hydroxyphenanthrene using the TFAA derivatization method. The ion counts for the three characteristic ions of the TFAA derivative of 9-hydroxyphenanthrene at the m/z values of 290, 165, and 69 were used to compare the analytical sensitivity of various fiber coatings (Fig. 10). The 100 µm PDMS fiber gave the best analytical sensitivity for the SPME extraction with subsequent headspace derivatization of 4.0 mL of 50 ppm 9-hydroxyphenanthrene standards. The 70 µm CW/DVB fiber had the next best analytical sensitivity with a GC-MS signal that was about one-fourth that of the 100 µm PDMS fiber. A major advantage of using the CW/DVB fiber was that it gave a simpler chromatogram that was free of undesirable derivatization byproducts compared to the other fibers tested. Both the 30 µm PDMS and 85 µm PA fibers were not suitable for the extraction of 9-hydroxyphenanthrene as shown by their weak GC-MS signals. In the case of the 85 µm PA fiber, the serious drawback of the loss of the polyacrylate coating was observed frequently. The extraction times of the various SPME fibers also were investigated, and it was found that extending the extraction time from 45 to 60 or 75 min did not improve the analytical sensitivity.

This study shows that SPME in conjunction with GC-MS analysis can be used to study biotransformation of environmental

contaminants in yeast cultures. The use of TFAA for derivatizing 9-hydroxyphenanthrene that has been extracted onto an SPME fiber greatly enhances the analytical sensitivity and facilitates the mass spectral characterization of 9-hydroxyphenanthrene. The superior sensitivity is due to the higher sample extraction efficiency and the presence of the intense molecular ion peak of the trifluoroacetyl derivative. This analytical method can be adapted for the analysis of other polar metabolites encountered in the biotransformation of pollutants by changing the SPME fiber and derivatizing reagent. Thus, the technique of SPME/GC-MS is very useful for studying metabolites in biological samples at trace levels.

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