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HOLOMETRIX, INC.

ENZYMATIC DESULFURIZATION OF COAL

Third Quarterly Report

Holometrix Report No. 2465
Holometrix Project No. DOE-12
DOE Contract No. DE-AC22-88PC88855

Submitted to:

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Project Manager,
U.S. Department of Energy
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Prepared by:

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Principal Investigator
Boston University School of Medicine
and
Judith P. Kitchell, Ph.D.
Holometrix, Inc.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Section 1

INTRODUCTION

1.1 Review of Program Goals

Numerous studies are underway to develop biological processes for the removal of both mineral and organic sulfur from coal. To remove the organic sulfur which is covalently bound, various research groups are studying strains of bacteria and fungi which can be induced to utilize organic sulfur compounds as feedstocks.

A consideration of industrial scale-up and operational requirements indicates that microbial ingestion of sulfur may produce technical difficulties that can be circumvented by the use of extracellular (i.e., secreted) or purified enzymes rather than whole microbes. For example, a 20,000 ton/day coal process would require about 200 tons of microbes to achieve a 1 percent removal of organic sulfur. If this sulfur is incorporated into the microbe, the daunting task of separating the fuel from the sulfur-enriched organisms presents added cost and process requirements.

Our current efforts to develop clean coal technology emphasize the advantages of enzymatic desulfurization techniques and have specifically addressed the potential of using partially-purified extracellular microbial enzymes or commercially available enzymes. Our work is focused on the treatment of "model" organic sulfur compounds such as dibenzothiophene (DBT) and ethylphenylsulfide (EPS). Furthermore, we are designing experiments to facilitate the enzymatic process by means of a hydrated organic solvent matrix.

During the first nine months of this project, our laboratories have pursued primarily the multi-step, enzymatic breakdown of DBT and the development of the Klibanov-type hydrated solvent reaction system. Previous studies with the aromatic sulfur compound DBT have shown that there are two general biological pathways for the oxidative breakdown of this compound. In the reaction most frequently observed in microbial oxidative pathways,

DBT is oxidized at a ring carbon, and the reaction is accompanied by a considerable decrease in the free energy of the compound. Our work is focused on oxidation at the sulfur with consequent liberation of inorganic sulfate. The identification of this multi-step ("4S") reaction pathway has led us to examine each of the oxidized sulfur intermediates, as well as the desulfurized product. These compounds are illustrated in Figure 1.1.

1.2 Review of the First Half Results

Our technical progress in the first quarter can be summarized as follows. We have worked with laccase and horseradish peroxidase in buffer and in aqueous organic solvents. After establishing the activity of our enzymes in buffer, many tests of activity against standard substrates in hydrated dioxane and hydrated DMF media were made. In both solvents, conditions were found for obtaining activity. We obtained some evidence of activity against dibenzothiophene [DBT]. We also investigated spectral and chromatographic methods of identification of the compounds in the "4S" pathway.

In the second quarter, the screening of media for the enzyme reactions with DBT was expanded. Changes in buffer were examined and several more hydrophobic solvents were utilized. An extensive amount of data was obtained by gas chromatography, utilizing a method which identifies the products of the "4-S" pathway. Particular success was noted with peroxidase in new solvents. It seemed that the high concentrations of DBT often utilized for easy detection with the GC might inhibit enzyme activity. The reactivity of DBT with H_2O_2 at varying concentrations was measured and it was shown that at the levels utilized, little if any oxidation occurred.

1.3 Summary of Third Quarter Results

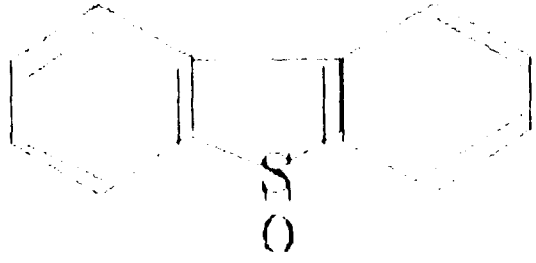
This report covers the period of December 16, 1988 to March 15, 1989.

In the third quarter we obtained important results both with the development of our understanding of the enzyme reaction systems and also with the microbial work at Woods Hole. In the latter case, we have received

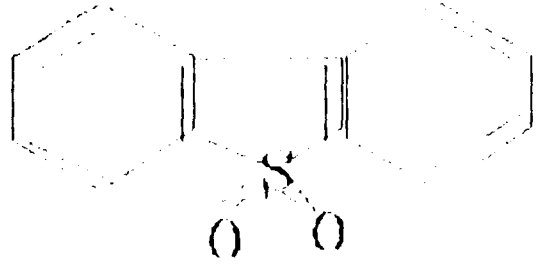
Figure 1.1

THE "4S" PATHWAY

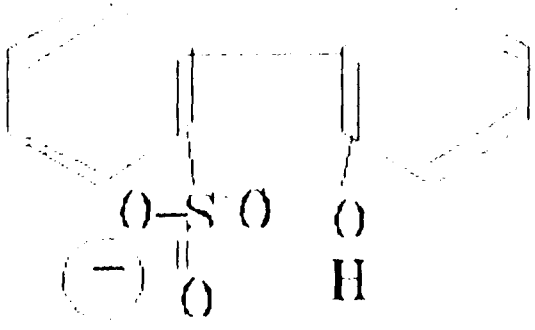
DIBENZOTHIOPHENE



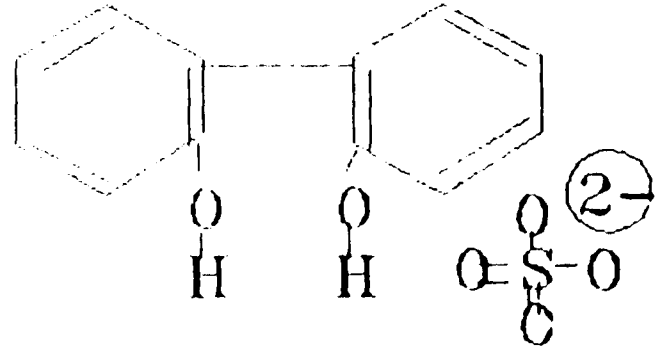
DIBENZOTHIOPHENE SULFOXIDE



DIBENZOTHIOPHENE SULFONE



2-HYDROXY,2'SULFONIC-BIPHENYL



O - O'-BIPHENOL and SULFATE

from Dr. Bazylinski (from Dr. Jannasch's group) two pure cultures which thrive in the presence of DBT. One of these produces a colored product indicative of DBT oxidation.

In Dr. Marquis' laboratory at Boston University, kinetic studies with three enzymes (laccase, horseradish peroxidase, and sulfatase) were made to evaluate the inhibition of these enzymes by our model coal compounds and their sulfur oxidation products. The inhibitions observed, interpreted tentatively as a measure of binding in the substrate active site, have implications for the planning of efficacious coal processing.

Section 2

EXPERIMENTAL RESULTS AND DISCUSSION

2.1 Microbial Studies

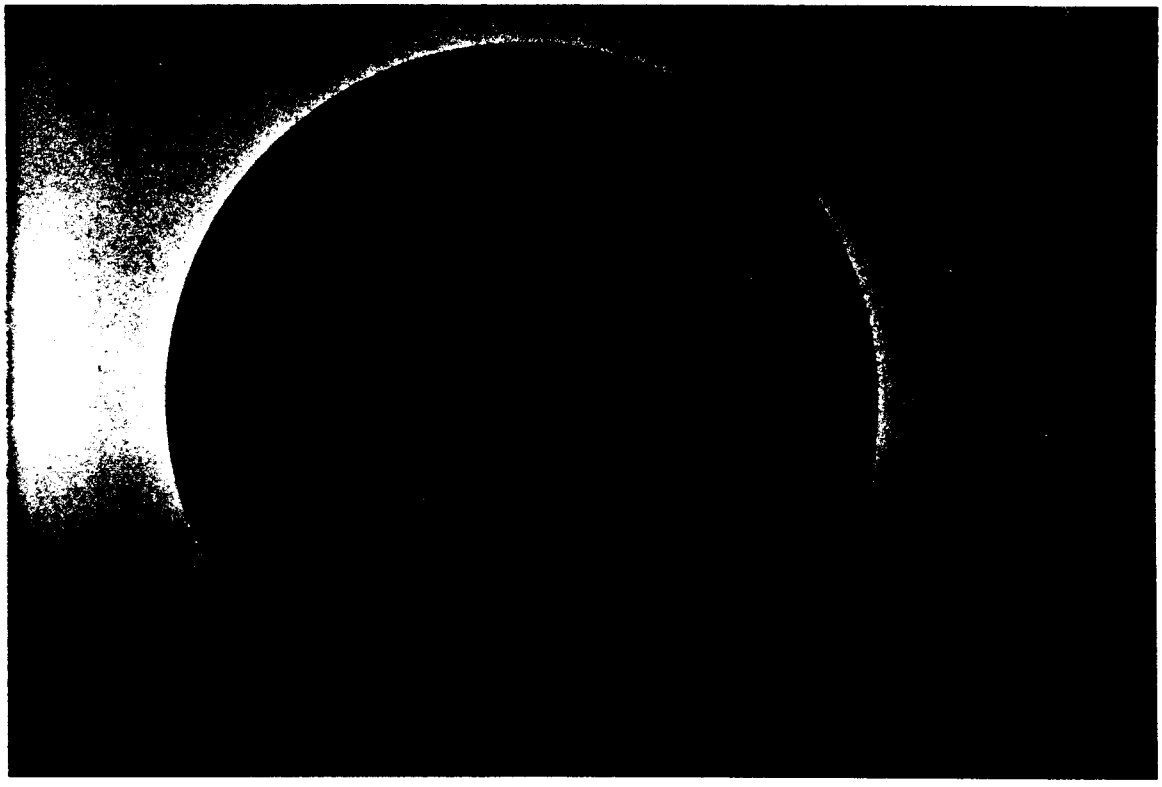
Under this program, a subcontract was awarded to Dr. Holgar Jannasch at the Woods Hole Oceanographic Institute for the purpose of screening water and sediments from deep sea sites for the presence of microorganisms which could oxidize the model compounds DBT and/or EPS. As previously reported (Second Quarterly Report, Holometrix No. 2461), nine samples from the Guaymas Basin hydrothermal vents were screened. No microorganisms grew in the presence of EPS. Two promising cultures which survived in the presence of DBT were selected for further work.

Portions from the core 1969 enrichment culture, which had a red appearance, were streaked onto 0.05% yeast extract-DBT agar plates. Two coloring types grew in approximately three weeks on plates incubated at 25°C. The first type cleared the DBT precipitate surrounding it and produced a water-soluble red-orange compound. Morphologically, the organism was a gram-negative, motile rod. The second organism did not clear the DBT nor did it produce colored products. It is a curved rod, vibrioid-to-helical in morphology, and stains gram negative. Colonies of both types were inoculated back into yeast-extract broth with DBT. They grew to visible turbidity in 72 to 96 hours and were once again streaked onto yeast-extract-DBT plates. Colonies were apparent in about three weeks and were similar to that described above.

From the yeast extract-DBT plates, the cultures were streaked onto Marine agar 2216 (a high nutrient medium). Both cultures grew and looked pure based on colony types. However, they were restreaked back onto yeast extract-DBT plates once more. Colonies again had the same appearance as above. The culture that produced the red soluble pigment is now referred to as strain GB1DBT and four subcultures are available. A photograph of GB1DBT is included as Figure 2.1. The second culture is GB2DBT and two subcultures are available. Although it is clear that strain GB1DBT oxidizes DBT, strain GB2DBT may simply tolerate large amounts of the compound.

Figure 2.1

A PLATE INOCULATED WITH GB1DBT AND COATED WITH DBT



2.2 Enzyme Kinetic Studies

2.2.1 Introduction

Subsequent to a more detailed evaluation of the data developed during the second quarter of work on this project, it became evident that UV spectrophotometric analyses could only provide qualitative information in regard to chemical alterations of the organic sulfur "model" compounds. We then turned our attention to gas chromatographic and mass spectroscopic techniques to obtain more quantitative analyses and identification of the reaction products. Furthermore, although we had identified a range of reaction conditions that favor enzymatic alteration of DBT and related compounds, it became increasingly evident that additional enzyme kinetic information would be required to develop reaction systems with the appropriate stoichiometry for specific enzymes and substrates. During this third quarter of work, Dr. Marquis' laboratory carried out an extensive series of rate studies designed to provide this information. The results are summarized below, and a more comprehensive summary of the earlier spectrophotometric work is also provided.

2.2.2 Summary of Key Enzyme Processing Experiments With Model Organic Sulfur Compounds

Over 100 enzyme reaction experiments were run during the second quarter of work. From the results, entirely UV spectrophotometric analyses, we were able to determine reaction conditions that facilitate degradation of DBT. The key studies (i.e., those that produced marked change in the DBT spectrum and provided evidence of a structural alteration in the DBT molecule) are summarized in the accompanying table (Table 2.1). While these experiments were discussed in our previous progress report, the observations made then bear some reconsideration in light of more recent findings.

These experiments showed us that relatively small amounts of HRP (on the order of less than 1 g/l of reaction mixture) can produce substantial alterations in the UV spectrum for millimolar concentrations of DBT in dioxane (DX). In these studies, it was also evident that proportionately large amounts (95%) of organic solvent (DX) favor the enzymatic

Table 2.1

SUMMARY OF KEY ENZYME PROCESSING EXPERIMENTS
WITH MODEL ORGANIC SULFUR COMPOUNDS
(PRELIMINARY DATA)

Date	Enzyme	Substrate	Results
10/27/88	HRP 250µg/ml in Tris buffer	29mM DBT in DMF or DX	•T = 2 days, increased resolution of 255nM peak DX •T = 5 days, increased height and resolution of 255nM peak in DX •T = 7 days, 95:5 ratio of DX: Tris produces significant spectral shift
11/14/88	HRP 250µg/ml in Tris buffer	1.7mM DBT in DX	•70:30 DX: Tris only ratio tested •T = 4 days, marked shift of UV spectra with decrease of peaks >300nM
11/17/88	HRP 250µg/ml in Tris buffer	2.9mM DBT in DX	•Clearly demonstrates that 95:5 DX: Tris is optimal for HRP degradation of DBT •Controls exhibit no non-enzymatic effects of the magnitude seen with HRP •T = 5 days, marked decrease and loss of peaks >300nM and small increase of peaks <300nM
11/23/88	HRP 100µg/ml in Tris buffer	2.9mM DBT in varying organic solvents	•T = 5 days •Isopropyl ether and ethyl acetate best solvents as evaluated by UV spectra

Samples were dilutes 1:10 to read UV spectra.

alteration of DBT. A survey of a wide range of organic solvents demonstrated that more hydrophilic solvents such as isopropyl ether and ethyl acetate provide a more favorable environment for HRP reactivity. Also, controls were run to demonstrate the enzymatic nature of the effects on DBT (i.e., the marked loss of peaks in 300+ nm range are not seen with hydrogen peroxide alone).

2.2.3 Summary of Enzyme Kinetic Data for the Interaction of Model Organic Sulfur Compounds With Oxidative and Hydrolytic Enzymes

The data presented in Tables 2.2 and 2.3 summarize the inhibition of three different enzymes by organic sulfur compounds and the development of kinetic constants to describe the quantitative interaction between enzyme(s) and inhibitor(s). Initially, we measured the relative potency of the organic sulfur compounds as inhibitors of horseradish peroxidase (HRP), laccase (LAC), and sulfatase (SULF) in standard conditions of assay. The assay procedures for each enzyme (i.e., the defined substrates, buffers, and reagents that are conventionally used for determining specific activity of each enzyme) are described below.

Horseradish Peroxidase Assay: In both the I-50 and kinetic determinations, HRP was assayed in the presence of hydrogen peroxide as co-factor and 4-aminoantipyrine in phenol as substrate/indicator in 25 mM Tris buffer, pH 6.0. The total reaction volume was 1.5 ml including organic solvent. Blanks were run without H_2O_2 and without enzyme. Activity was measured at 510 nm. Enzyme was added to a complete reaction mixture at t = 0 and at t = 5 minutes, the activity was read over a period of three minutes reaction time.

Laccase Assay: For both the I-50 and kinetic determinations, LAC activity was assayed with the substrate syringaldazine made up in organic solvent and the enzyme in a buffer of 0.1 M Na_2PO_4, pH 6.5. A blank was run without enzyme. Activity was measured at 530 nm. Enzyme was added at t = 0 and t = 8 minutes, the activity was read over a period of three minutes reaction time.

Sulfatase Assay: Sulfatase activity was found to be especially sensitive to temperature, so all assays were run at a constant temperature

Table 2.2

DETERMINATIONS OF I-50 FOR ORGANIC SULFUR COMPOUNDS AND RELATED DERIVATIVES: EFFECTS ON OXIDATIVE AND HYDROLYTIC ENZYMES (PRELIMINARY DATA)

	I-50 (mM)*		
	HRP	LACCASE	SULFATASE
DBT	2.8 ± 1.2[1]	1.5 ± .47	.80 ± .30
DBT-Sulfoxide	8.1 ± 0.2	8.6 ± 2.6	.32 ± .03
DBT-Sulfone	>30mM	>20mM	.54 ± .03
0,0-Biphenol	>40mM	>20mM	>30mM
EPS	4.2 ± 0.2	3.9 ± .43	.34 ± .09
EPSulfoxide	8.8 ± 1.5	8.8 ± 3.5	.31 ± .05
EPSulfone	>30mM	>20mM	.64 ± .05

*The I-50 is the concentration of inhibitor that reduces enzyme activity by 50% under standard conditions of assay (described in the text) and optimal ratios of organic solvent (dimethylformamide) to aqueous buffer.

[1]Data are presented as the mean ± standard deviation of 4 experiments.

Table 2.3A

KINETIC CONSTANTS FOR ORGANIC SULFUR COMPOUNDS:
INHIBITION OF HORSERADISH PEROXIDASE*
(PRELIMINARY DATA)

	HRP[1]	
	Km (mM)	Vmax (ΔO.D./min)
Control	1.9 ± .25[2]	.08 ± .009
DBT	0.8 ± .21	.06 ± .04
EPS	0.8 ± .26	.03 ± .009 (NC)
Control[3]	6.1 ± 2.0	.15 ± .04
DBT-Sulfox[3]	6.6 ± 1.0	.31 ± .16
EPSulfox[3]	7.9 ± .59	.18 ± .06

*Km and Vmax were determined by double-reciprocal evaluation of enzyme activity data measured under standard conditions of assay (described in the text) and optimal ratios of organic solvent (dimethylformamide) to aqueous buffer.

[1]Horseradish peroxidase was purchased from Sigma Chemical Co. (P-3912) as a suspension in 2.0 M $(NH_4)_2SO_4$, pH 7.0.

[2]Data are presented as the mean ± standard deviation of 4 experiments.

[3]These experiments were run on a second batch of HRP.

Table 2.3B

KINETIC CONSTANTS FOR ORGANIC SULFUR COMPOUNDS:
INHIBITION OF LACCASE*
(PRELIMINARY DATA)

	LACCASE[1]	
	Km (μM)	Vmax (ΔO.D./min)
Control	.05 ± .03[2]	.12 ± .05
DBT	.35 ± .001	.52 ± .04
EPS	.22 ± .13	.33 ± .15
DBT-Sulfox	.08 ± .07	.14 ± .08
EPSulfox	.45 ± .38	.53 ± .38

* Km and Vmax were determined by double-reciprocal evaluation of enzyme activity data measured under standard conditions of assay (described in the text) and optimal ratios of organic solvent (dimethylformamide) to aqueous buffer.

[1]Laccase was purchased from Sigma Chemical Co. (L-5510) from *Pyricularia oryzae*.

Table 2.3C

KINETIC CONSTANTS FOR ORGANIC SULFUR COMPOUNDS:
INHIBITION OF SULFATASE*
(PRELIMINARY DATA)

	SULFATASE[1]	
	Km (mM)	Vmax (ΔO.D./min)
Control	.28 ± .03[2]	.48 ± .02
DBT	.83 ± .44	.49 ± .08 (C)
EPS	1.1 ± .55	.59 ± .19 (C)
DBT-Sulfox	.48 ± .22	.29 ± .07 (NC)
EPSulfox	.34 ± .09	.25 ± .04 (NC)
DBT-Sulfone	.27 ± .08	.34 ± .04 (NC)
EPSulfone	.62 ± .20	.55 ± .11 (C)

*Km and Vmax were determined by double-reciprocal evaluation of enzyme activity data measured under standard conditions of assay (described in the text) and optimal ratios of organic solvent (dimethylformamide) to aqueous buffer.

[1]Sulfatase was purchase from Sigma Chemical Co. (S-9751), Type H-2 from Helix pomatia.

[2]Data are presented as the mean ± standard deviation of 4 experiments.

of 37°C. For both the I-50 and kinetic determinations, SULF activity was assayed with p-nitrocatechol sulfate as substrate in a buffer of 0.2 M NaAc, pH 5.0. Parallel assays were set up to contain substrate, buffer, organic solvent, inhibitor, and enzyme or an equal volume of buffer. In the assays without enzyme (blanks), 5 ml of 1 N NaOH were added at t = 0 to stop the reaction. Blanks and enzyme assays were incubated in a water bath for 30 minutes. At t = 30 minutes, the enzyme assays were stopped with 5 ml of 1 N NaOH. The t = 0 blanks were zeroed at 515 nm, and the activity of the enzyme assays were read as single points.

For all three enzymes, dose-response curves were developed with at least five different substrate concentrations, and the data were evaluated by linear regression analysis of double-reciprocal (Lineweaver-Burk) plots of activity vs. substrate concentration. The following organic sulfur compounds were examined: DBT (Aldrich), DBT-sulfoxide (K&K Biochemicals), DBT-sulfone (Lancaster Biochemicals), EPS (Aldrich), ethylphenylsulfoxide (Lancaster Biochemicals), ethylphenylsulfone (Lancaster Biochemicals), and o,o-biphenol (Aldrich).

As indicated in the legends to the tables, these kinetic measurements were carried out in ratios of organic solvent (dimethylformamide; DMF) to aqueous buffer that provided maximal solubility of both the enzyme and the organic sulfur compound (i.e., inhibitor) while retaining the expected enzyme activity against the standard substrates. While that is generally in the range of 85:15 (DMF:I) for LAC and SULF, it was found that HRP required ratios closer to 50:50 for measurable activity.

2.2.4 Relative I-50 Data

The data in Table 2.2 demonstrate that for each series of organic sulfur compounds (i.e., DBT and EPS and their respective oxidized products, DBT-sulfoxide, DBT-sulfone, o,o-biphenol, EPS sulfoxide, and EPS sulfone) there are some significant differences in their ability to inhibit enzyme activity against conventional substrates. If one accepts the I-50 as a reasonable indication of binding of organic sulfur compound to the enzyme, one can draw certain conclusions that are applicable to the proposed use of the enzymes for oxidation or desulfurization of the sulfur compounds.

First of all, it is clear that none of the enzymes studied are readily inhibited by the biphenol compound, the end-product of DBT desulfurization. This is at least a first-pass indication that the industrial process we are proposing may not exhibit the problems associated with end-product inhibition. Furthermore, both the sulfones are seen to react poorly with HRP and LAC, but they do react with SULF. It is, thus, suggested that a sulfatase or related enzyme may be required in addition to HRP and LAC for complete desulfurization of either of the parent sulfur compounds, DBT and EPS. It is also significant to note that the I-50 for each compound and its respective sulfoxide is very similar (differences are only two or three fold), so it is suggested that these enzymes may carry out at least two steps in the desulfurization pathway, namely oxidation to the sulfoxide and to the corresponding sulfone.

The decision to include a sulfatase in the present experimental design was based on our earlier suggestion, in fact, in our original proposed Statement of Work, that a sulfatase may be of value in catalyzing the complete desulfurization of coal. It was, therefore, of particular interest to us to find the substantial reactivity that is evident in Table 2.2.

2.2.5 Apparent Kinetic Constants

We have begun to examine the thermodynamic nature of the interaction between the organic sulfur compounds and each enzyme by running experiments with a single concentration of inhibitor (close to the I-50) over a range of varying substrate concentrations. This permitted us to plot conventional double-reciprocal plots and to measure the Km (affinity) and Vmax (activity) for each inhibitor-enzyme pair. Clearly, a major pitfall exits, namely, whether each enzyme follows Michaelis-Menten kinetics, and whether the inhibitors are truly reversible. Enzymes such as HRP which require a co-factor may not be adequately evaluated by simple double-reciprocal plots. Studies are underway at this time to address these concerns.

The data presented in Tables 2.3A-C are preliminary data and additional experiments are in progress to fill in the gaps. For example, we are currently repeating the HRP assays with higher concentrations of inhibi-

tors, and evaluating the effects, if any, of incubation time and H_2O_2 concentrations on the kinetic parameters. However, there are at least some indications that binding of the organic sulfur compounds may occur outside the catalytic site for the conventional substrates (i.e., noncompetitive (NC) inhibition was evident with some of the compounds). Also, it is likely that the modulation of these enzymes by the organic sulfur compounds may be significantly affected by the enzymatic action on the "inhibitor" itself. This is a process that is probably occurring, since we have already seen effects of HRP and LAC on the structure of DBT and EPS, and it would certainly account for the apparently mixed nature of the effects on kinetic parameters.

2.3 Laccase Assays - Evaluation of Reaction Conditions

2.3.1 Overview

At Holometrix, work with laccase and DBT has continued. Three series of assays, each requiring extensive analytical GC, were completed. In the first series, the effect of pH on the laccase activity in DMF and dioxane was examined with the use of an acetate buffer, pH 5.2. In the second series, distilled water at pH 2.5 and water buffered to pH 7 with dibasic phosphate were compared. The concentration of DBT was lowered and the concentrations of laccase was raised. The amount of water present was greatly reduced. The third series was a repeat of the second experiment.

For gas chromatography (GC) analysis, samples were filtered through Rainin nylon 0.45 μ filters. The gas chromatographic conditions were altered slightly from the prior report. A Varian 3700 gas chromatograph with a FID detector was fitted with a 3 percent SP2250, 100/120 Supelcoport column. The carrier gas was N_2 at 30 ml/minute. The column conditions were: detector = 260°C, injector = 260°C. The program was: initial temperature of 150°C for 5 minutes; rise = 10°C/minute to 260°C; hold at 260°C for 5 minutes. The attenuation was 1 and the range was 10^{-11}. A Varian 4270 integrator was used to record and report the gas chromatograph detector output. UV spectra were obtained on a Hewlett Packard 8451A diode array spectrophotometer.

2.3.2 Acetate Buffer Experiment (20 mM DBT, 0.2 mg/ml Laccase)

In these experiments, the initial conditions were: organic solvent (95%), 0.01 M sodium acetate (5%), laccase 0.05 mg/ml, DBT 20 mM. The total volume per tube was 10 ml. The temperature was 30°C. Two organic solvents were used: DMF and dioxane. Four sets of experiments were run, each in duplicate: DMF-enzyme added dry; DMF-enzyme added in buffer solution; dioxane-enzyme added dry; dioxane-enzyme added in buffer solution. Samples were taken at T=0 and T=24 hours and analyzed by GC. As can be seen in the summaries of average areas under curves or peaks at various retention times, shown in Tables 2.4A-D, the only set of conditions which produced any drop in DBT was the DMF with wet enzyme addition.

2.3.3 Phosphate Buffer Experiment (2.0 mM DBT, 0.2 mg/ml Laccase)

Distilled water was brought up to pH 7 with dibasic phosphate. Laccase (19.4 mg) was added to 1 ml of the buffer. Six screw cap test tubes were prepared as follows:

- Three tubes (1-3) each received 9 ml of acetonitrile and 1 ml of 21 mM DBT in DMF.
- Three tubes (4-6) each received 9 ml of ethylacetate and 1 ml of 21 mM DBT in DMF.

A magnetic stir bar was added to each tube and also 100 µl of the enzyme solution. Final concentration of laccase was 0.2 mg/ml. Some precipitate was observed. Samples were taken for GC at T = 0, 24 hrs, 2 days, 3 days, 7 days, 8 days, and 16 days.

For each GC run, the areas of peaks or curves were converted to percents of total area (excluding solvent). All good runs (at least two for each sample) were averaged. The results, shown in Table 2.5A-B, indicate that there was reactivity against the DBT; however, it is not yet clear what the products were, as they did not necessarily fall only where known products were expected. After two days, the percentage of total area attributed to observed products began to decrease. By the 16th day, only DBT was

Table 2.4A

RESULTS OF ASSAYS IN DMF
(ENZYME PREDISSOLVED IN ACETATE BUFFER)

RETENTION TIME	PERCENT OF TOTAL	
	T=0 (n=1) (%)	T=24 (n=2) (%)
5.8- 7	0.00	1.59
8.0- 9	0.00	0.00
10.0-10.9	0.00	0.00
11.0-11.9	98.20	97.83
12.0-12.9	0.00	0.00
13.0-13.9	0.01	0.00
14.0-14.9	0.00	0.00
15.0-15.9	0.36	0.15
16.0-16.5	0.48	0.04
16.5-16.9	0.00	0.00
17.0-17.5	0.82	0.00
17.5-17.9	0.00	0.02
18.0-18.5	0.00	0.00
18.5-18.9	0.10	0.00
19.0-19.9	0.00	0.15
20.0-20.9	0.02	0.00
21.0-21.9	0.00	0.00
22.0-22.9	0.00	0.00
23.0-23.9	0.00	0.00
24.0-24.9	0.00	0.22
25.0-25.9	0.00	0.00

Table 2.4B

RESULTS OF ASSAYS IN DMF/ACETATE BUFFER
(ENZYME ADDED DRY)

RETENTION TIME	PERCENT OF TOTAL	
	T=0 (n=1) (%)	T=24 (n=2) (%)
5.8- 7	0.00	0.00
8.0- 9.9	0.00	0.00
10.0-10.9	0.00	0.00
11.0-11.9	99.09	99.78
12.0-12.9	0.00	0.00
13.0-13.9	0.00	0.01
14.0-14.9	0.00	0.00
15.0-15.9	0.00	0.00
16.0-16.5	0.00	0.02
16.5-16.9	0.85	0.03
17.0-17.5	0.00	0.00
17.5-17.9	0.01	0.00
18.0-18.5	0.00	0.05
18.5-18.9	0.00	0.00
19.0-19.9	0.03	0.00
20.0-20.9	0.01	0.02
21.0-21.9	0.00	0.00
22.0-22.9	0.00	0.00
23.0-23.9	0.00	0.00
24.0-24.9	0.00	0.08
25.0-25.9	0.00	0.00

Table 2.4C

RESULTS OF ASSAYS IN DIOXANE
(ENZYME PREDISSOLVED IN ACETATE BUFFER)

RETENTION TIME	PERCENT OF TOTAL	
	T=0 (n=1) (%)	T=24 (n=2) (%)
5.8- 7	0.00	0.00
8.0- 9	0.00	0.00
10.0-10.9	0.00	0.00
11.0-11.9	99.97	99.48
12.0-12.9	0.00	0.00
13.0-13.9	0.00	0.00
14.0-14.9	0.00	0.00
15.0-15.9	0.00	0.04
16.0-16.5	0.00	0.11
16.5-16.9	0.00	0.03
17.0-17.5	0.00	0.17
17.5-17.9	0.01	0.11
18.0-18.5	0.00	0.00
18.5-18.9	0.02	0.05
19.0-19.9	0.00	0.00
20.0-20.9	0.00	0.01
21.0-21.9	0.00	0.00
22.0-22.9	0.00	0.01
23.0-23.9	0.00	0.00
24.0-24.9	0.00	0.00
25.0-25.9	0.00	0.00

Table 2.4D

RESULTS OF ASSAYS IN DIOXANE/ACETATE BUFFER
(ENZYME ADDED DRY)

RETENTION TIME	PERCENT OF TOTAL	
	T=0 (n=1) (%)	T=24 (n=2) (%)
5.8- 7	0.00	0.00
8.0- 9	0.00	0.00
10.0-10.9	0.01	0.00
11.0-11.9	99.81	99.58
12.0-12.9	0.00	0.00
13.0-13.9	0.00	0.00
14.0-14.9	0.00	0.00
15.0-15.9	0.01	0.00
16.0-16.5	0.12	0.07
16.5-16.9	0.00	0.00
17.0-17.5	0.00	0.29
17.5-17.9	0.00	0.00
18.0-18.5	0.00	0.06
18.5-18.9	0.05	0.00
19.0-19.9	0.00	0.00
20.0-20.9	0.00	0.00
21.0-21.9	0.00	0.00
22.0-22.9	0.01	0.00
23.0-23.9	0.00	0.00
24.0-24.9	0.00	0.00
25.0-25.9	0.00	0.00

Table 2.5A

RESULTS OF ASSAYS IN ACETONITRILE
(ENZYME PREDISSOLVED IN PHOSPHATE BUFFER)

RETENTION TIME	AVERAGE AREA (PERCENT OF TOTAL)						
	T=0 (%)	T=1 (%)	T=24 (%)	T=48 (%)	T=144 (%)	T=168 (%)	T=192 (%)
5.8- 7	0.00	0.10	0.08	0.03	1.15	0.02	0.00
8.0- 8.9	0.86	3.18	2.79	1.52	0.00	0.62	0.00
9.0- 9.9	0.00	0.00	1.50	4.26	0.00	0.00	0.00
10.0-10.9	0.00	0.00	0.17	0.00	0.00	0.02	0.00
11.0-11.9	98.98	96.50	94.49	90.17	94.68	97.34	98.72
12.0-12.9	0.00	0.02	0.00	0.00	0.00	0.04	0.00
13.0-13.9	0.00	0.00	0.04	0.03	0.00	0.08	0.00
14.0-14.5	0.01	0.01	0.01	0.00	0.00	0.00	0.00
14.0-14.9	0.00	0.00	0.00	0.01	0.00	0.12	0.00
15.0-15.9	0.00	0.00	0.00	0.00	0.00	0.00	0.00
16.0-16.5	0.03	0.02	0.52	2.40	2.69	0.60	0.00
16.5-16.9	0.05	0.07	0.28	1.57	1.45	0.08	1.28
17.0-17.5	0.06	0.02	0.05	0.00	0.00	0.27	0.00
17.5-17.9	0.02	0.03	0.00	0.00	0.00	0.00	0.00
18.0-18.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
18.5-18.9	0.00	0.02	0.03	0.00	0.03	0.24	0.00
19.0-19.9	0.00	0.00	0.00	0.00	0.00	0.11	0.00
20.0-20.9	0.00	0.02	0.03	0.00	0.00	0.25	0.00
21.0-21.9	0.00	0.00	0.00	0.00	0.00	0.00	0.00
22.0-22.9	0.00	0.01	0.00	0.00	0.00	0.22	0.00
23.0-23.9	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24.0-24.9	0.00	0.00	0.00	0.00	0.00	0.00	0.00
25.0-25.9	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 2.5B

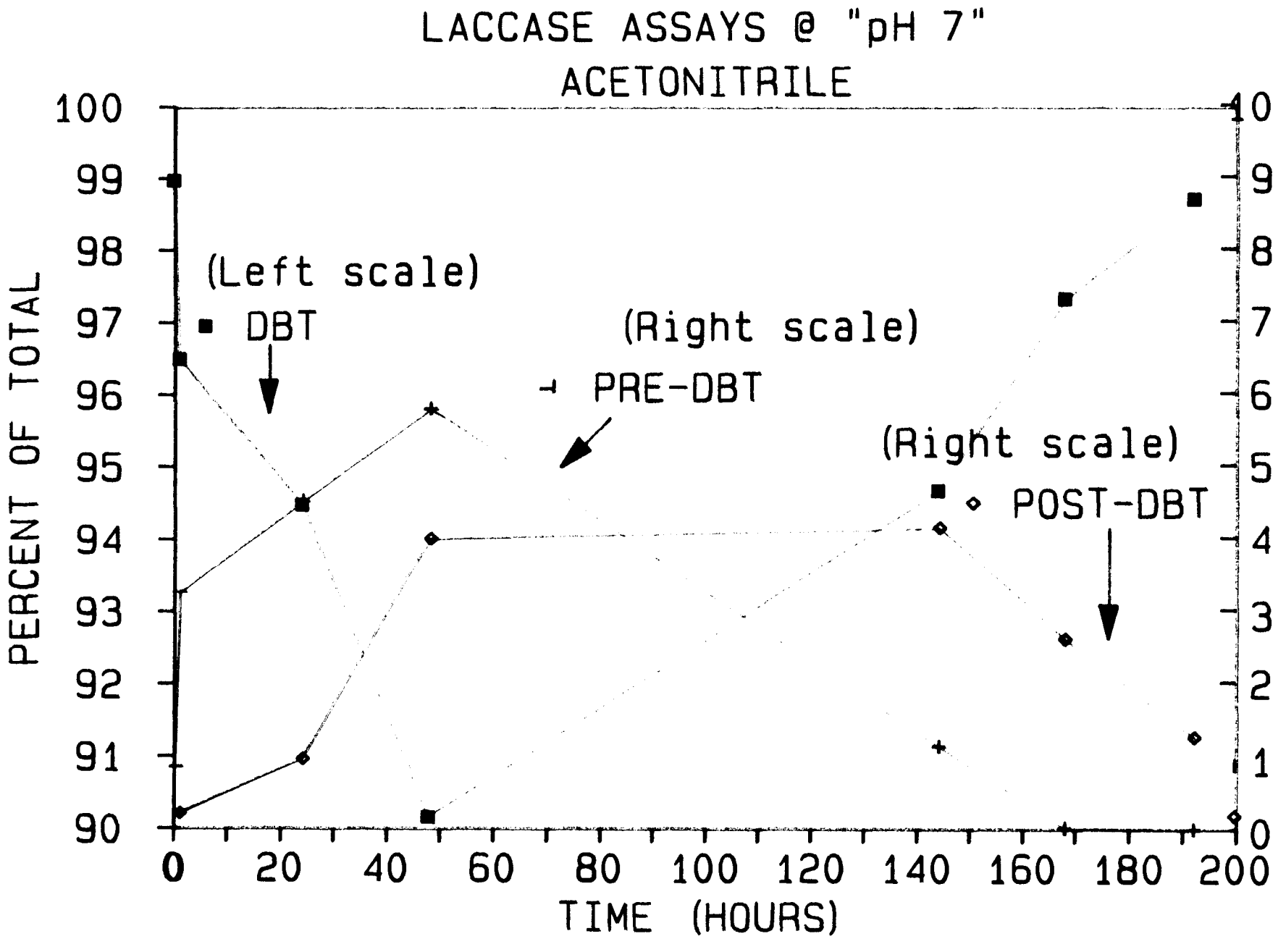
RESULTS OF ASSAYS IN ETHYLACETATE
(ENZYME PREDISSOLVED IN PHOSPHATE BUFFER)

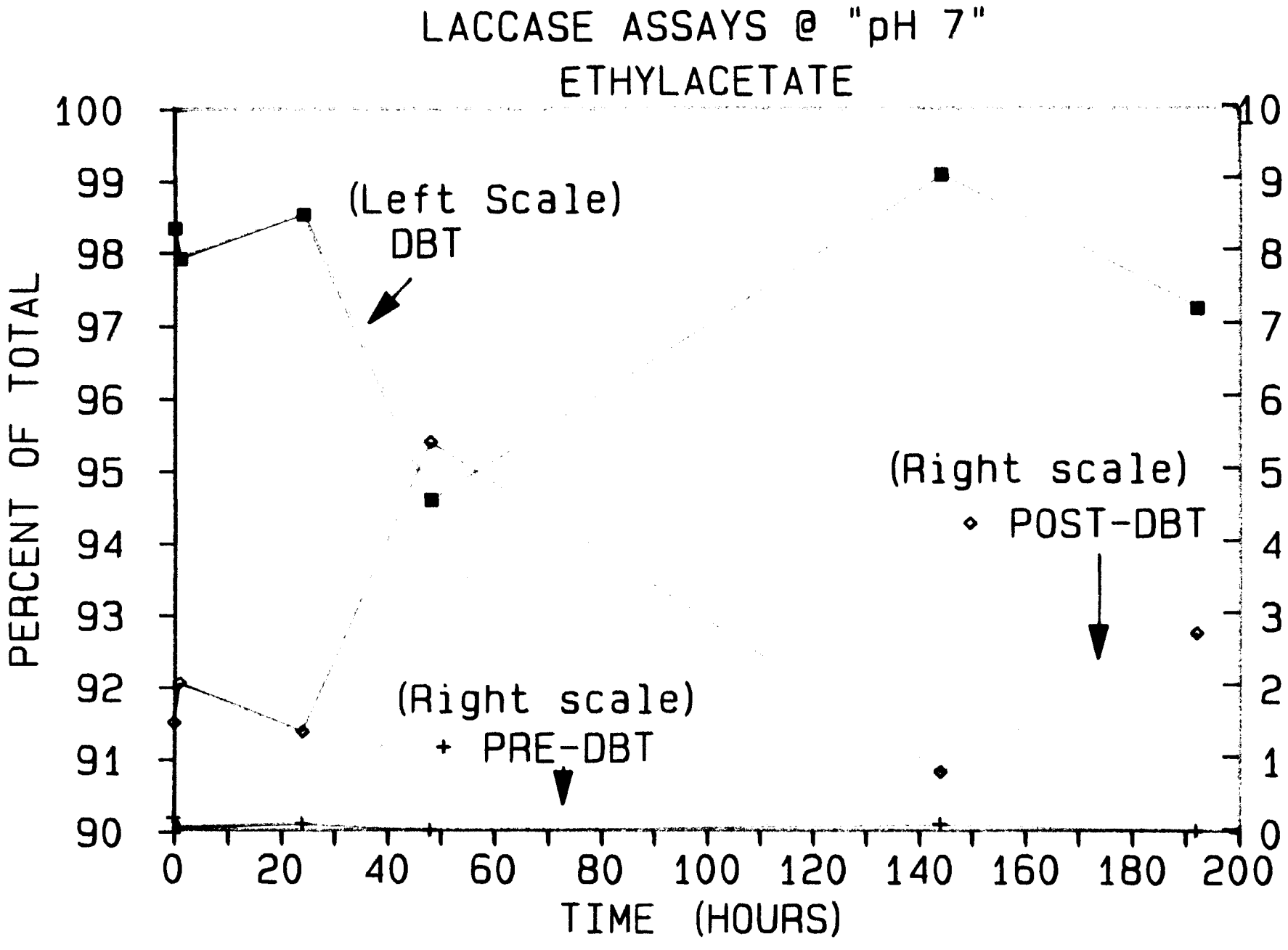
RETENTION TIME	AVERAGE AREA (PERCENT OF TOTAL)					
	T=0 (%)	T=1 (%)	T=24 (%)	T=48 (%)	T=144 (%)	T=192 (%)
5.8- 7	0.17	0.03	0.09	0.00	0.09	0.00
8.0- 8.9	0.00	0.00	0.00	0.00	0.00	0.00
9.0- 9.9	0.00	0.00	0.00	0.00	0.00	0.00
10.0-10.9	0.00	0.11	0.07	0.04	0.00	0.00
11.0-11.9	98.35	97.93	98.55	94.62	99.10	90.00
12.0-12.9	0.00	0.01	0.00	0.00	0.00	0.00
13.0-13.9	0.01	0.03	0.02	0.17	0.03	0.00
14.0-14.5	0.00	0.10	0.17	0.00	0.00	0.00
14.0-14.9	0.00	0.02	0.00	0.65	0.01	0.00
15.0-15.9	0.09	0.18	0.00	0.00	0.00	0.00
16.0-16.5	0.51	0.29	0.83	1.31	0.04	0.00
16.5-16.9	0.44	0.03	0.25	1.61	0.66	0.00
17.0-17.5	0.22	0.35	0.02	1.33	0.02	0.00
17.5-17.9	0.12	0.30	0.00	0.00	0.00	0.00
18.0-18.5	0.00	0.22	0.00	0.00	0.02	0.00
18.5-18.9	0.05	0.02	0.00	0.16	0.00	0.00
19.0-19.9	0.00	0.16	0.00	0.00	0.00	0.00
20.0-20.9	0.02	0.07	0.00	0.10	0.03	0.00
21.0-21.9	0.00	0.11	0.00	0.00	0.00	0.00
22.0-22.9	0.02	0.00	0.00	0.00	0.00	0.00
23.0-23.9	0.00	0.03	0.00	0.00	0.00	0.00
24.0-24.9	0.00	0.00	0.00	0.00	0.00	0.00
25.0-25.9	0.00	0.00	0.00	0.00	0.00	0.00

seen in any of the samples. To simplify examination of these experiments, the areas of all peaks emerging "pre-DBT" on the GC were summed, as were the areas of all peaks emerging "post-DBT". As is shown in Figure 2.2A, for the acetonitrile assay, through Day 2 there was a drop in %DBT explained by a rise in both "pre-DBT" and "post-DBT" peaks. Subsequently, the % content of DBT returned to 100%. The ethylacetate samples (Figure 2.2b) showed a similar but less dramatic pattern of change in %DBT. The difference was almost entirely due to "post-DBT" material. We thought that this pattern could be due to conversion of intermediates from compounds separated on the GC to those that were not seen, either because they did not come off the column during the period of observation (e.g., too early or too late) or they had precipitated in solution and were removed by filtration before GC analysis.

On Day 17, experiments were made to see if the products had precipitated. For samples 1-3, 2 ml were taken from each, and 1 ml of EtOAc and 1 ml of .1 M $NaHCO_3$ were added to each. No phase separation occurred, but the solution was clear. A sample was filtered and run on the GC. Only DBT was seen, confirming the results reported in the paragraph above. Additionally, 1 ml of the reaction mixture was mixed with 1 ml of 1% AcOH and a filtered aliquot was run on the GC. Again, nothing but DBT was found. For samples 4-6, 2 ml was taken from each, and 1 ml of EtOAc and 1 ml of .1 M $NaHCO_3$ were added to each. Phase separation occurred and the solutions were clear. The organic layers were removed by pipette. Samples were filtered and run on the GC. In the aqueous phase, a peak at the position assigned to the sulfone/sulfoxide accounted for 11.3% of the area, with DBT being the remainder. In the organic phase, most of the material seen was DBT, although a trace amount of material appeared at later retention times.

On Day 15, samples were taken for UV examination. From each sample tube, an aliquot was removed and an equal volume of water was added. Samples 1-3 cleared but did not phase separate. Samples 4-6 did phase separate (both phases were clear) and the upper, organic phase was removed by pipette. UV scans from 200 to 500 nm were run first vs. distilled water. Subsequently, new scans were made in which 1-3 were diluted with acetonitrile 10 times and scanned vs. acetonitrile. Samples 4b-6b were diluted 10 times with ethylacetate and scanned vs. ethylacetate, and 4a-4b were rescanned vs. ethylacetate with an absorbance range limited to 0 to 1.


LACCASE ASSAYS @ "pH 7"
ACETONITRILE
PERCENT OF TOTAL
100
99
98
97
96
95
94
93
92
91
90
(Left scale)
DBT
(Right scale)
PRE-DBT
(Right scale)
POST-DBT
10
9
8
7
6
5
4
3
2
1
0
0
20
40
60
80
100
120
140
160
180
200
TIME (HOURS)


LACCASE ASSAYS @ "pH 7"
ETHYLACETATE
PERCENT OF TOTAL
100
99
98
97
96
95
94
93
92
91
90
10
9
8
7
6
5
4
3
2
1
0
(Left Scale)
DBT
(Right scale)
POST-DBT
(Right scale)
PRE-DBT
0
20
40
60
80
100
120
140
160
180
200
TIME (HOURS)

While DBT-like absorbance is seen in all the samples, it appears that the aqueous phases of 4-6 may contain biphenol-like material.

2.3.4 A Repeat of the Experiment in Section 2.3.3

We wished to confirm the observations reported in Section 2.3.3. In the repeat series of assays, each tube was initially cloudy with flocculant material. It seemed that the amount of this material increased with time. Although the insoluble material was seen in the enzyme-only tubes as well, we were anxious to insure that reaction products were not being filtered out prior to analysis. Thus, at each measurement time, two aliquots were taken and while one was simply filtered as before, the other was mixed with an equal volume of distilled water. In both the ethylacetate and the acetonitrile aliquots, at each time immediate clearing occurred. The ethylacetate samples phase separated and the upper phase was removed to a separate container prior to filtering (two filtered samples were thus obtained for each of the diluted aliquots).

Each sample was analyzed by GC with two or more injections. In looking at the data from the undiluted samples, no significant change in DBT concentration was observed and no significant appearance of new compounds was seen in filtered assay aliquots taken at Day 1, and at Days 15 or 16. In the aliquots which were diluted or extracted with water, many new peaks were observed in the GC analyses, but these often appeared in the enzyme-only controls as well. The results were erratic and not reproducible. One sample was also run on the GC-MS (it is discussed in Section 2.4) and there it did not show the same peaks. At this time we are not sure if the water in the samples was causing erratic results or if material dissolved by the water in the assay samples has contaminated the GC system. We have just changed the GC column and will be looking for more reliable results in the next quarter. We have confirmed that the enzyme used had the normal level of activity vs. syringaldazine.

2.4 Progress With the GC-Mass Spectrometer

2.4.1 The System

Considerable progress has been made in the use of the gas chromatograph-spectrometer (GC-MS) on this project. At the time of the

Second Quarterly Report the instrument had been installed and the designated operator had just begun to become familiarized with the basics of operation. The instrument is a versatile computer driven machine and just learning the "lingo" of the computer menus is not trivial. The manufacturer provides a large file of reference spectra (a library) for use in compound identification. When a sample is run, the software is designed to choose representative scans from the major peaks and to choose the compounds from the library which best match the same scans. Scans from a sample may not exactly resemble the scans from the library, because the sample collection conditions are not necessarily the same as those used in the library data base. For this reason, the matching parameters are not too rigorous and many potential matches are made. If no spectrum in the library resembles the sample spectrum, no identification is made. A very useful feature of the software is that the operator can interactively review the individual column peaks and select other scans in each peak for analysis. In this way, if the column peak contains more than one compound, multiple identifications can be made.

The compounds in the reference library include DBT, DBT-sulfoxide, and 2,2-biphenol. The DBT-sulfone is not present and will be added by the operator from an in-house standard. When a sample containing DBT-sulfone is processed, the identification given from the library is thioanthrene, which has the correct molecular weight, but the wrong molecular formula and structure.

The system presently utilized with our standards and assay samples is as follows:

Hewlett Packard 5890A gas chromatograph with a 5988a mass spectrometer column, Hewlett Packard HP1 cross-linked methylsilicone gum (12m x 0.22 mm, 0.33 μ film thickness)

Source Temperature	:	200°C
Analyzer Temperature	:	280°C
Initial Column Temperature	:	35°C
Final Column Temperature	:	300°C
Hold at Ti for 10 minutes		
Temperature Rise Rate	:	7°C/minute
Hold at Tf for 5 minutes		

2.4.2 Results

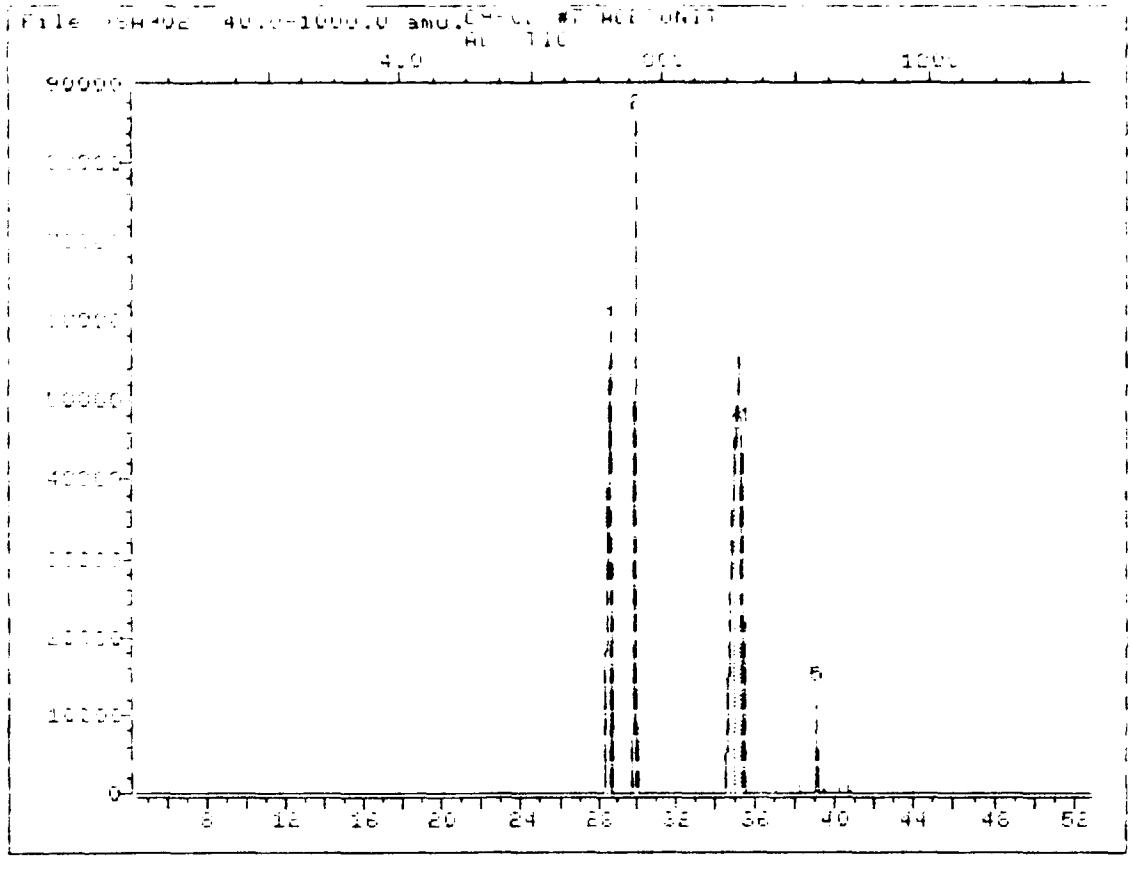
Two examples of GC-MS runs are described and illustrated here as examples of the utility of the method in our program. The first example is a "4S" standard run (solvent was acetonitrile, contents were equimolar amounts of [1,1'-biphenyl]-2,2'-diol, DBT, DBT sulfoxide, and DBT sulfone). In this standard run, five major peaks were found above the set threshold. The fifth peak was less than two percent of the total area and is an unidentified contaminate in the standard. The column scan is shown in Figure 2.3

Figures 2.4A through 2.4D are copies of the computer printouts of the program identifications of the four peaks. The first peak was clearly and correctly identified as the biphenol. The second peak was correctly identified as the DBT. The third and fourth peaks (expected to be the DBT sulfoxide and DBT sulfone) ran very close and were only partially resolved, as on the Holometrix GC program; and the distribution of areas was not equal. Peak number three was apparently difficult to identify from the sample scan, but the correct compound, the DBT-sulfoxide, was one of the tentative identifications made from the computer library. Peak number four was not correctly identified by the computer, as the DBT-sulfone was not in the compound library, however the correct mass ion of 216 was found in scan. Once the DBT-sulfone is placed in the library, correct identification should be obtained.

The second GC-MS analysis shown is from an actual laccase assay. The column scan is shown in Figure 2.5. Two column peaks were of interest, although because we set low threshold limits for this run seventeen peaks were selected. Peaks 5 and 10 were examined in detail as shown in Figures 2.6A through 2.6B. The major peak, consistant with our expectations, was identified as DBT. The much smaller earlier peak came out near the retention time of biphenol, however, it was identified from the library as a reduced form of DBT in which the bond directly between the two benzyl rings has been reduced (benzene, 1,1'-thiobis). The probability assigned to this identification is low and we are planning to work on the identification of this material as it appears consistantly in our reaction mixtures.

Figure 2.3

COLUMN CHROMATOGRAM - STANDARD RUN (GC-MS)

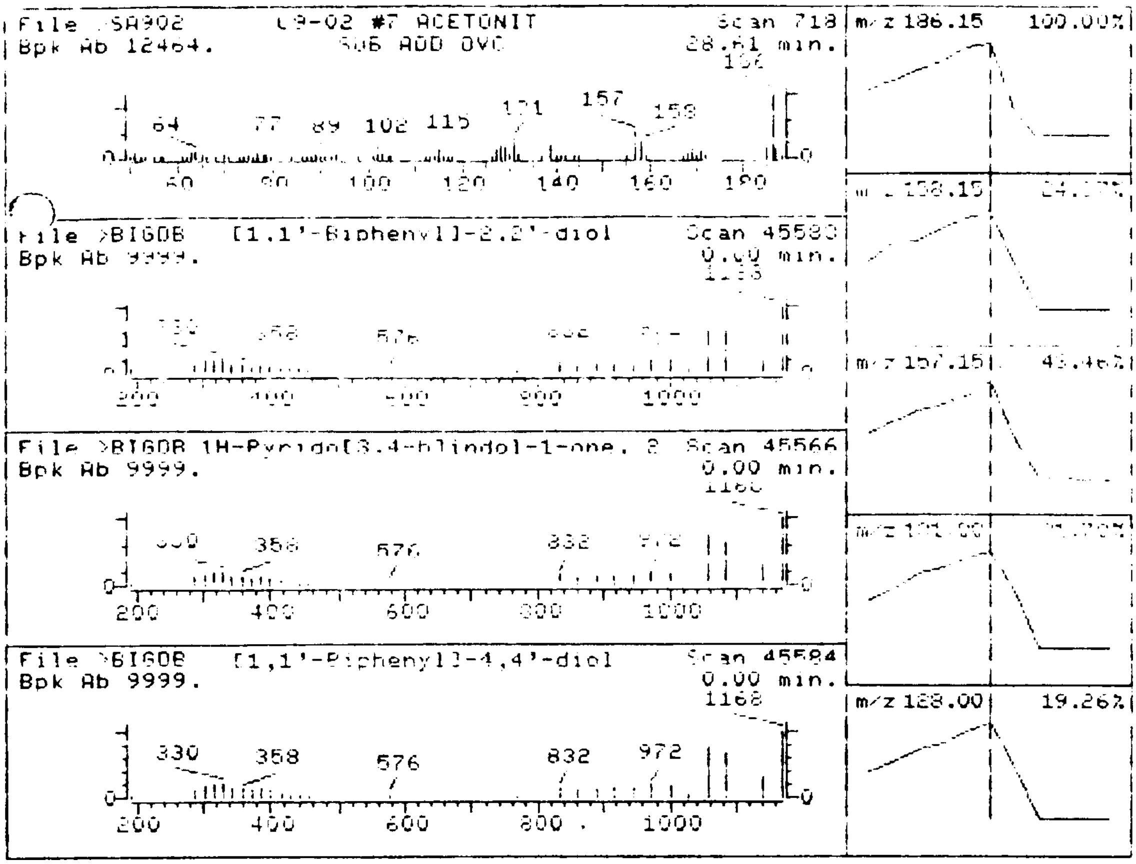


Peak #	R.T. min.	first scan	max scan	last scan	peak height	raw area	corr. area	corr. % max.	% of tot.
1	26.01	708	718	721	58040	671785	671785	98.01	[illegible]
2	27.98	752	759	762	85648	647012	647012	87.80	28.[illegible]
3	35.02	894	909	910	44869	724181	720044	100.00	31.[illegible]
4	35.39	918	920	922	45807	177745	176282	24.46	[illegible]
5	39.02	1026	1028	1030	11243	38595	38595	5.36	1.[illegible]

Sum of corrected areas: 2[illegible]

Figure 2.4A

IDENTIFICATION OF STANDARD PEAK 1 (GC-MS)



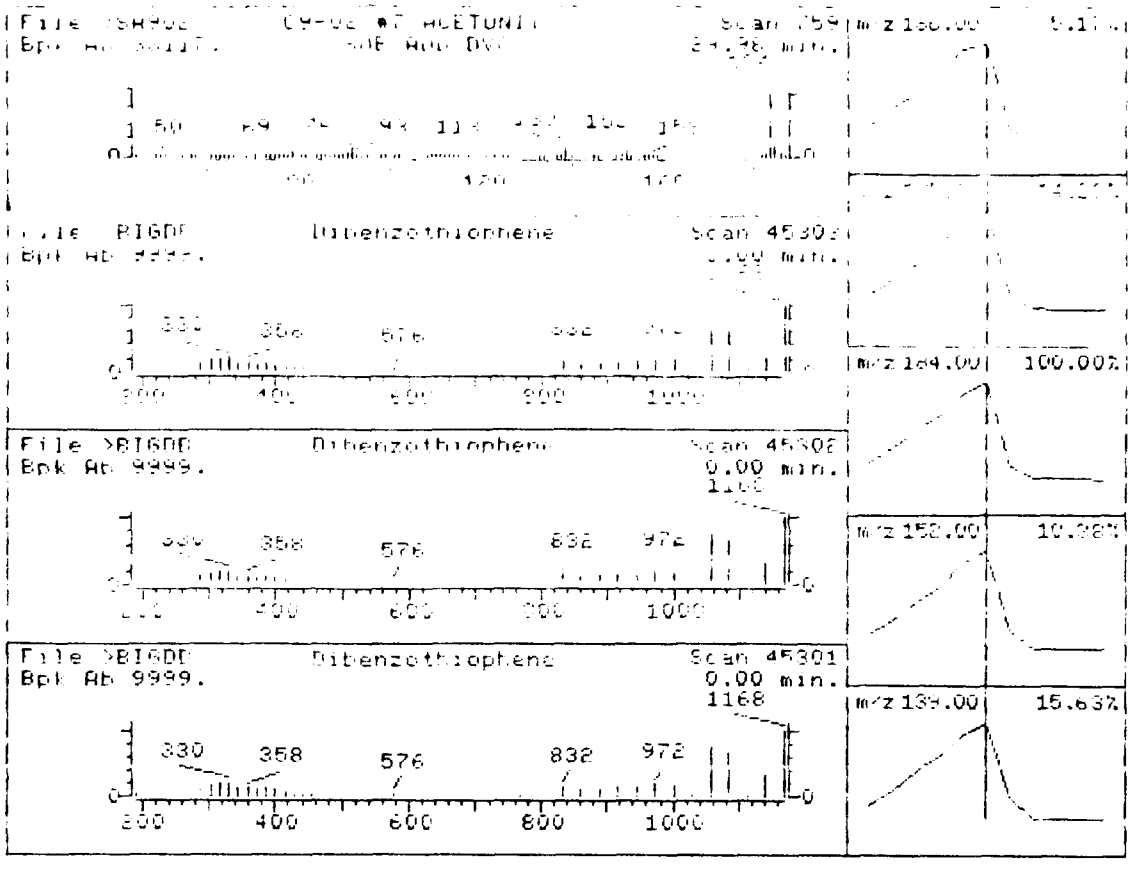
1. [1,1'-Biphenyl]-2,2'-diol 186 $C_{12}H_{10}O_2$
2. 1H-Pyrido[3,4-b]indol-1-one, 2,3,4,9-tetrahydro- 186 $C_{11}H_{10}N_2O$
3. [1,1'-Biphenyl]-4,4'-diol 186 $C_{12}H_{10}O_2$
4. Phenanthrene, octahydro- 186 $C_{14}H_{18}$
5. 1,4-Naphthalenedione, 2,3-dimethyl- 186 $C_{12}H_{10}O_2$

Sample file: SA902 Spectrum #: 718
Search speed: 1 Tilting option: 3 No. of ion ranges searched: [illegible]

	Prob.	CAS #	CON #	ROOT	K	DK	#FLG	TILT	%	CON	R_IIK
1.	92*	1806297	45583	^BIGDB	[illegible]	48	0	0	[illegible]	21	[illegible]
2.	35*	17952620	45566	^BIGDB	22	92	5	0	151	50	14
3.	31*	92886	45584	^BIGDB	[illegible]	61	2	0	100	[illegible]	12
4.	27*	29966049	45614	^BIGDB	25	119	5	0	100	54	12
5.	25*	2197501	45579	^BIGDB	42	77	5	0	100	45	8

Figure 2.4B

IDENTIFICATION OF STANDARD PEAK 2 (GC-MS)



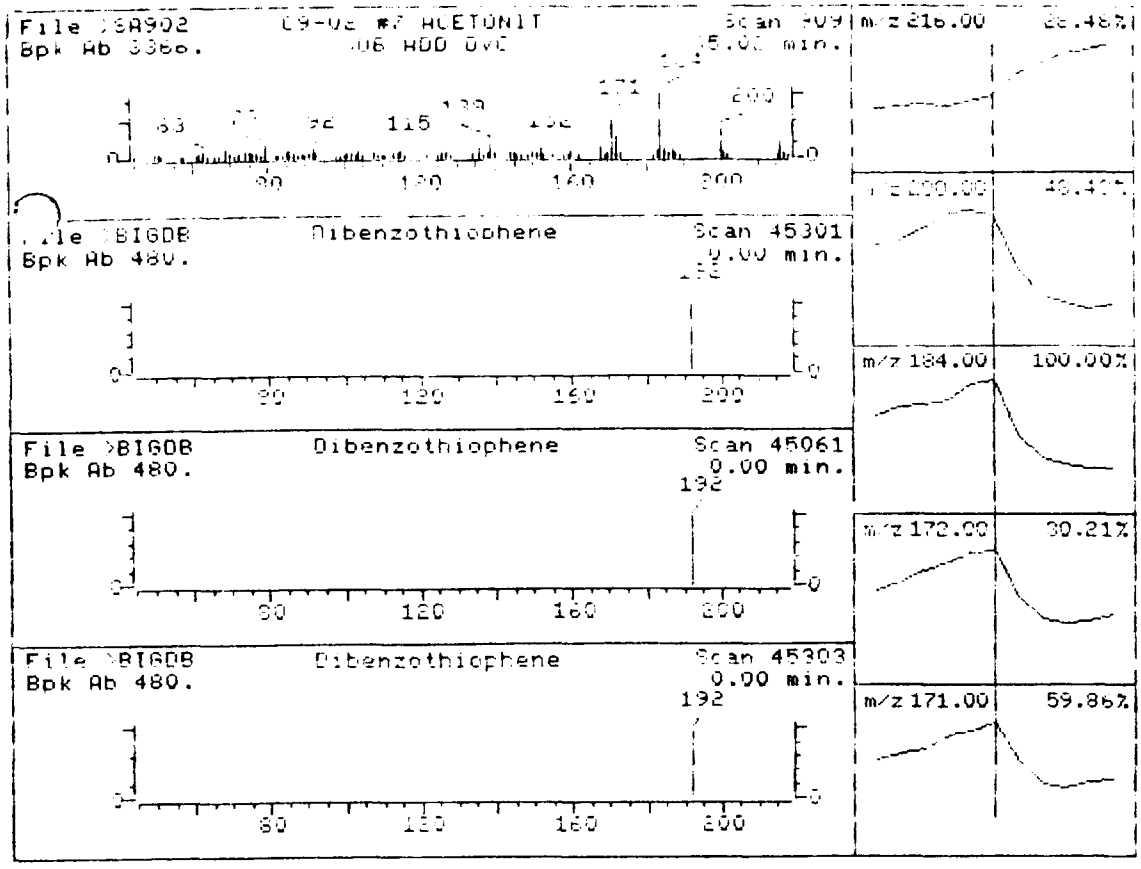
1. Dibenzothiophene 184 C12H8S
2. Dibenzothiophene 184 C12H8S
3. Dibenzothiophene 184 C12H8S
4. Dibenzothiophene 184 C12H8S
5. Dibenzothiophene 184 C12H8S

Sample file: >SA902 Spectrum #: 759
Search speed: 1 Fitting option: 3 No. of ion ranges searched: 4

	Prob.	CAS #	CON #	Root	K	DK	#FLG	TILT	%	CON	C_1	r_1
1.	96*	132650	45303	"BIGDB	98	3	1	0	93	3		
2.	96*	132650	45302	"BIGDB	82	17	0	0	82	5	32	
3.	95*	132650	45301	"BIGDB	87	14	0	0	70	5	72	
4.	88*	132650	45061	"BIGDB	70	43	2	0	80	1	60	
5.	86*	132650	45304	"BIGDB	66	40	2	-1	89	3	80	

Figure 2.4C

IDENTIFICATION OF STANDARD PEAK 3 (GC-MS)



1. Dibenzothiophene
2. Dibenzothiophene
3. Dibenzothiophene
4. Dibenzothiophene, S-oxide
5. Phenoxathiin

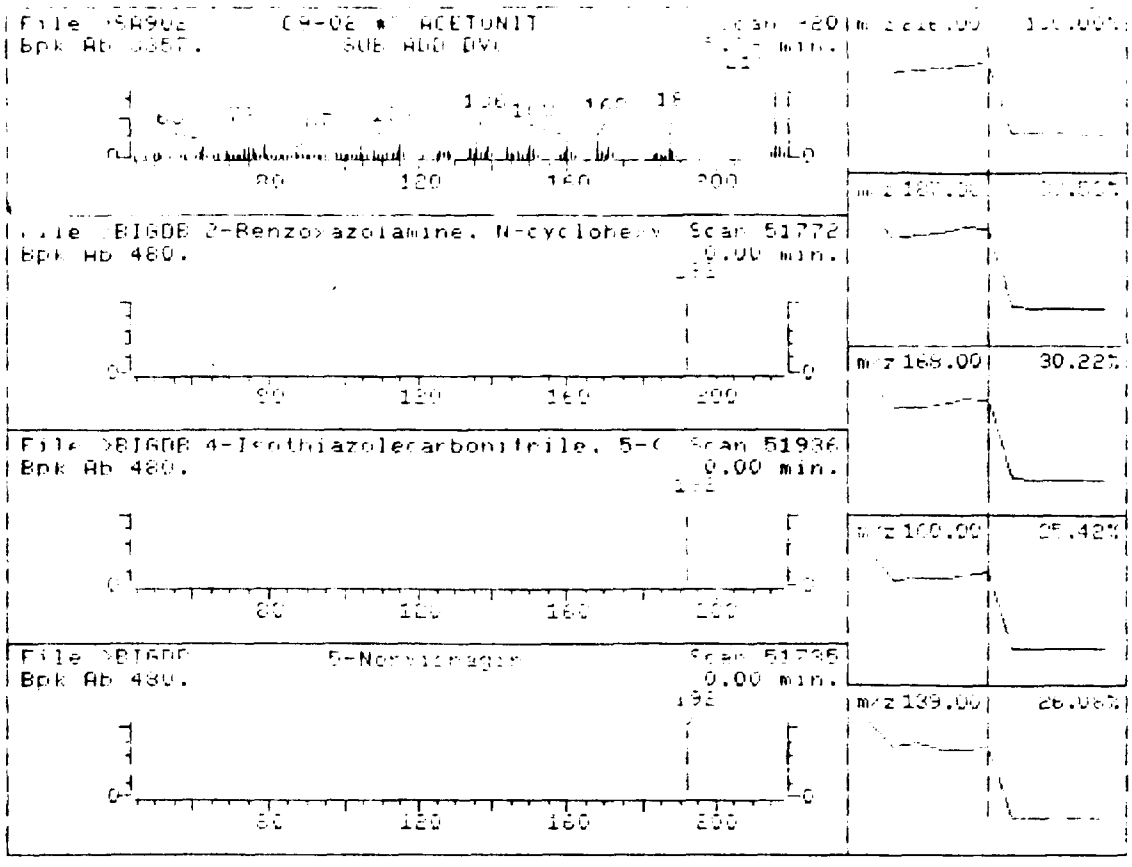
184 C12H8S
184 C12H8S
184 C12H8S
200 C12H8OS
200 C12H8OS

Sample file: SH902 Spectrum #: 909
Search speed: 1 Tilting option: 5 No. of ion ranges searched: 4

	Prob.	CAS #	CON #	ROOT	K	DK	#FLG	TILT	%	CON	C_1	R_
1.	33*	132650	45301	"BIGDB	61	40	0	0	88	60	12	
2.	21*	132650	45061	"BIGDB	78	55	2	0	87	60	8	
3.	20*	132650	45303	"BIGDB	48	55	0	0	85	60	7	
4.	24*	1013256	45123	"BIGDB	52	72	0	0	100	55	7	
5.	20*	262204	48901	"BIGDB	43	60	2	1	91	55	5	

Figure 2.4D

IDENTIFICATION OF STANDARD PEAK 4 (GC-MS)



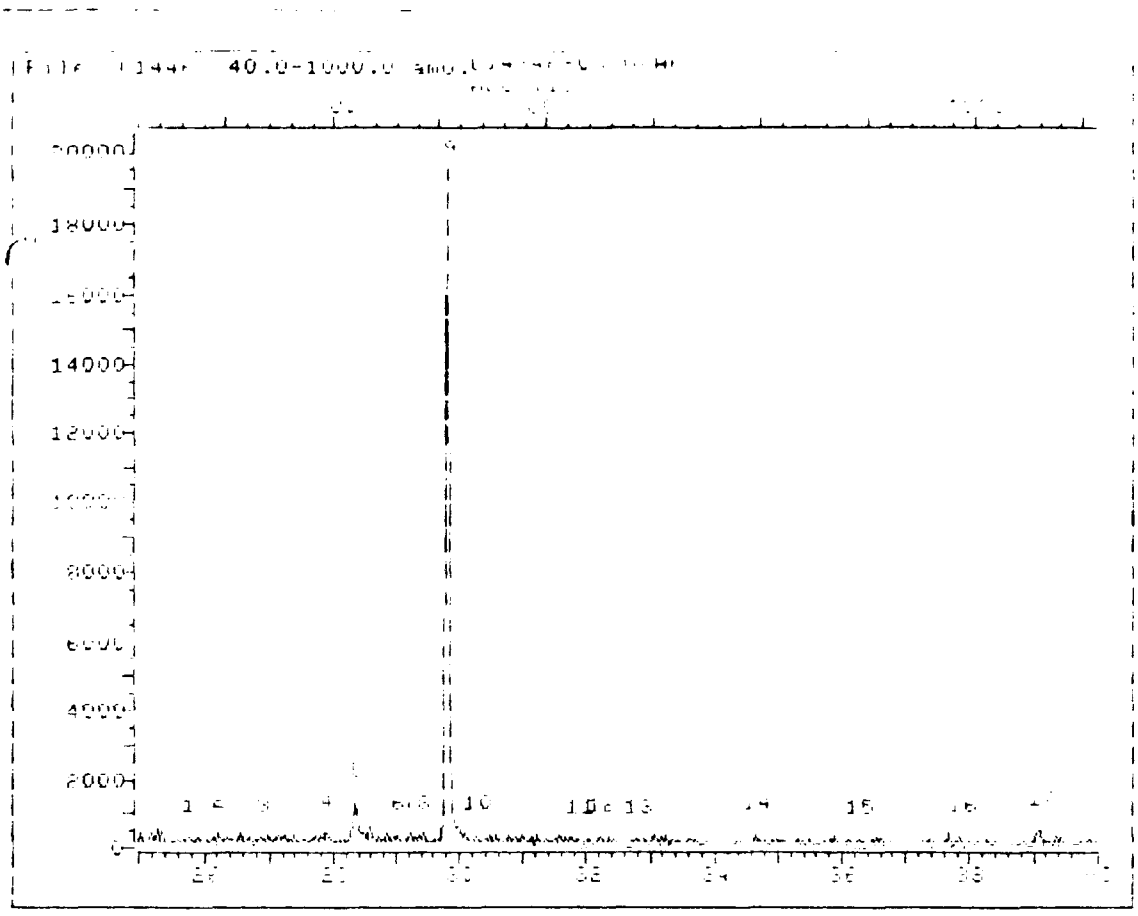
1. 2-Benzoxazolamine, N-cyclohexyl-
2. 4-Isothiazolecarbonitrile, 5-(ethylthio)-3-methyl-th
   io)-
3. 5-Norolshagin
4. DI-(4-AMINOPHENYL)-SULFIDE
5. N-ACETYL,N-PROPYL LEUCINE

Sample file: >SA902 Spectrum #: 920
Search speed: 1 Tilting option: S No. of ion ranges searched: 49

	Prob.	CAS #	CON #	ROOT	K	DK	#FLG	TILT	%	CON	L_1	R_1
1.	25*	10450110	51772	"BIGDB	23	100	3	0	19*	47	7	1
2.	25*	21882506	51930	"BIGDB	31	125	3	0	100	46	.	1
3.	20*	4481601	51735	"BIGDB	28	87	3	0	100	53	5	1
4.	20*	0	51755	"BIGDB	29	90	2	0	22	35	5	1
5.	15*	0	51699	"BIGDB	27	34	3	0	100	60	.	1

Figure 2.5

COLUMN CHROMATOGRAM - LACCASE ASSAY SAMPLE (GC-MS)

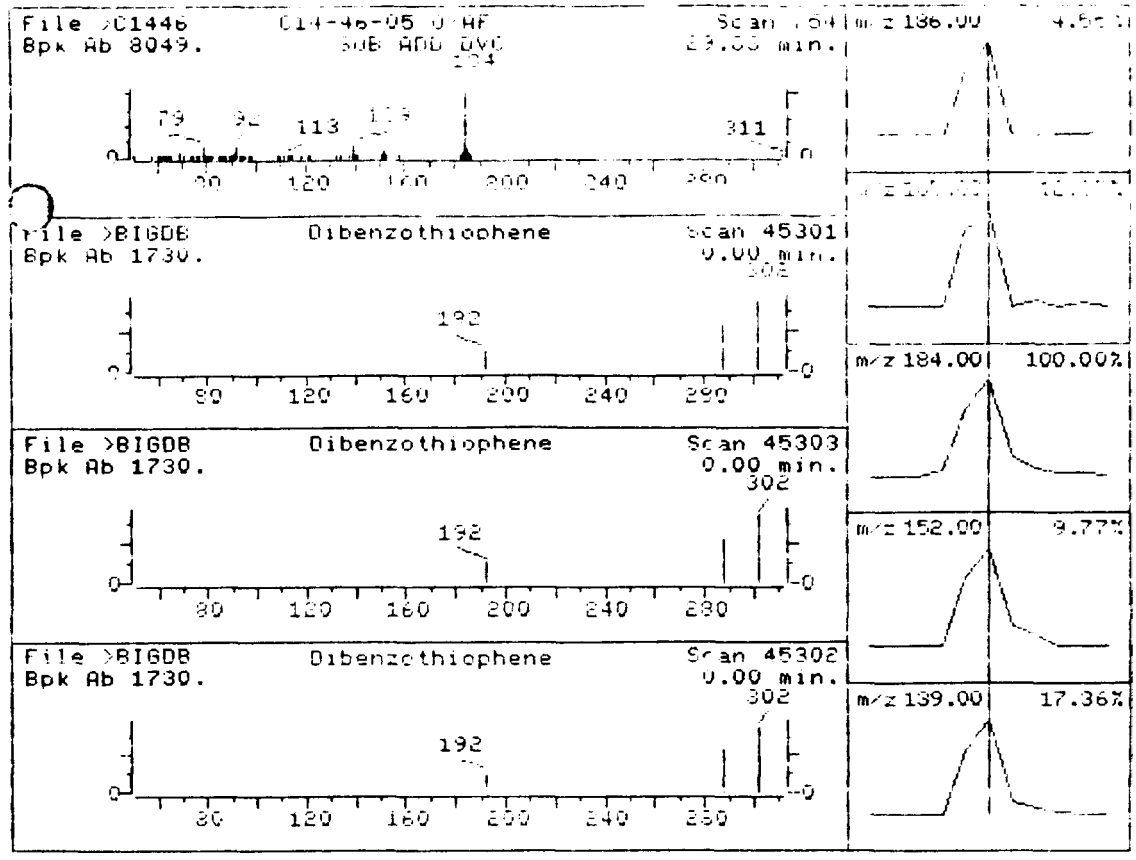


Peak #	R.T. min.	first scan	max scan	last scan	peak height	raw area	corr. area	corr. % max.	% total corr.
1	[illegible]	630	633	635	[illegible]	[illegible]	1744.	2.11	1.[illegible]
2	26.20	645	648	649	390	2709	2296	2.78	1.8
3	26.68	662	666	666	270	3074	1973	2.39	1.6
4	27.88	695	696	700	355	3315	2075	2.51	1.7
5	28.35	707	710	716	1205	11000	8158	9.87	6.7
6	29.03	727	730	735	323	2814	2016	2.44	1.6
7	29.23	733	736	737	329	2415	1883	2.28	1.5
8	29.43	739	742	745	326	3216	1969	2.38	1.6
9	29.83	751	754	765	19570	84607	82658	100.00	68.3
10	30.27	765	767	770	324	2366	1693	2.05	1.3
[illegible]	31.88	813	815	818	269	2046	1764	2.13	1.4
12	32.22	821	825	827	261	2516	1693	2.05	1.3
13	32.79	837	842	845	217	2457	1790	2.17	1.4
14	34.67	895	898	901	344	2370	2008	2.43	1.6
15	36.29	943	946	949	225	2026	1669	2.02	1.3
16	37.86	988	993	995	349	2201	2205	2.67	1.8
17	39.11	1024	1030	1031	478	4331	3413	4.13	2.8

Sum of corrected areas: 121014.

Figure 2.6A

IDENTIFICATION OF PEAK FIVE - ASSAY SAMPLE (GC-MS)



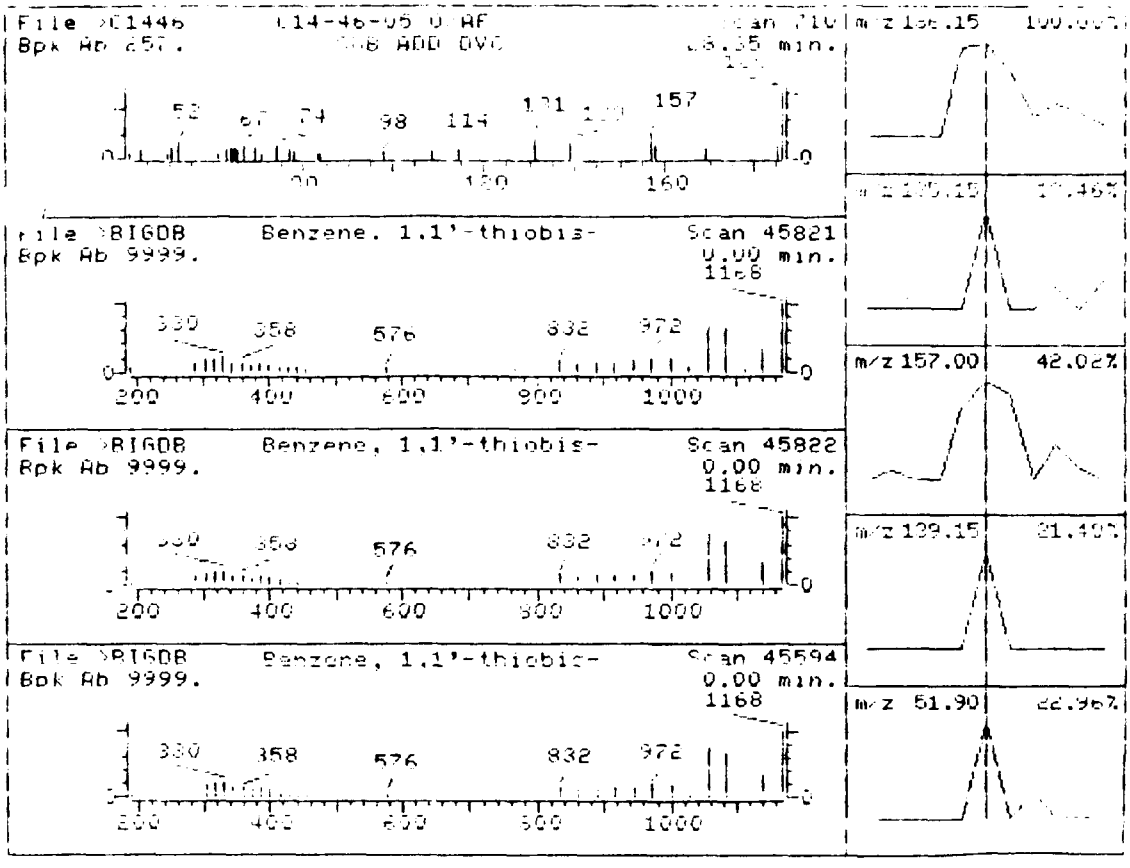
1. Dibenzothiophene
2. Dibenzothiophene
3. Dibenzothiophene
4. Dibenzothiophene
5. Dibenzothiophene

Sample File: >C1446 Spectrum #: 754
Search speed: 1 Fitting option: 5 No. of ion ranges searched: 55

	Prob.	CAS #	CON #	NOUT	K	DK	#FLG	TILT	%	CON	C_1	K_1
1.	96*	132650	45301	"BIGDB	90	11	1	0	88	5	72	9
2.	95*	132650	45303	"BIGDB	98	5	1	1	86	1	72	9
3.	95*	132650	45302	"BIGDB	91	8	0	2	82	1	72	9
4.	87*	132650	45304	"BIGDB	73	33	2	1	71	5	65	4
5.	87*	132650	45061	"BIGDB	73	40	2	1	67	1	65	4

Figure 2.6B

IDENTIFICATION OF PEAK TEN - ASSAY SAMPLE (GC-MS)



1. Benzene, 1,1'-thiobis- 186 C12H10S
2. Benzene, 1,1'-thiobis- 186 C12H10S
3. Benzene, 1,1'-thiobis- 186 C12H10S
4. Psoralene 186 C11H6O3

Sample file: >C1446 Spectrum #: 710
Search speed: 1 Fitting option: S No. of ion ranges searched: 4

	Prob.	CAS #	CON #	ROOT	K	DK	#FLG	TILT	%	CON	C_1	R_
1.	15*	139662	45821	"BIGDB	29	89	3	0	100	98	3	
2.	15*	139662	45822	"BIGDB	22	92	3	0	100	98	3	
3.	15*	139662	45594	"BIGDB	22	96	3	0	100	98	3	
4.	11*	66777	45818	"BIGDB	22	106	2	0	100	82	2	

Section 3

RESULTS

3.1 Evaluation of Progress

We are very pleased with the overall technical progress on this project. The enzyme work planned was ambitious and novel in many ways and, though there have been many problems associated with intermediate and product identification (as would be expected with enzymes which were chosen because they catalyzed a broad range of oxidations), we have been able to persevere. It is especially satisfying to be able to report the inhibition studies described in Section 2.2 because we believe that this information, when published, will be of great interest to many researchers in the field of biodesulfurization. Plainly, a great deal of coal work remains and we have much territory to cover before we see definitive results with coal as substrate.

Additionally, our task targeting microbial isolation has been successfully carried out. Two cultures of potential interest have been obtained from work at Dr. Jannasch's laboratory. It will be necessary to characterize the products in the bacterial growth media (when DBT is present) to assess the future commercial value of the organisms. We would add that our progress has been aided by the comments and questions from our project monitor at DOE. It is gratifying to receive thoughtful responses to our reports and to look forward to review meetings as events of technical relevance.

3.2 Plans for the Fourth Quarter

Work planned for the fourth quarter will focus on the selection and testing of series desulfurization reactions. When the kinetic studies are completed, we will be able to select multiple enzyme processes with proportions of starting materials chosen to avoid a build-up of inhibitory intermediates. We will utilize the GC-MS more fully in the identification of reaction products and intermediates.

While we had planned to begin work directly on coal particles at this time in the program, we believe that the change in enzyme substrate should be delayed until we have greater confidence in the kinetics of reactions with DBT and EPS. The addition of the complexities of surface chemistry to the multi-step reaction will be more easily handled within a few months.