DOE/PC/88855--T9

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DE92 006539

ENZYMATIC DESULFURIZATION OF COAL

Seventh Quarterly Report

DynaGen, Inc. Report No. 2481
DynaGen, Inc. 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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Pittsburgh, Pennsylvania 15236-0940

Prepared by:

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March 23, 1990

Submitted by:

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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 center around solving processing problems through the use of enzymes rather than live organisms for chemical catalysis. Enzymes will not accumulate sulfur; they will not add significant bulk in processing; they will be selective in their activity; and they will be functional in organic solvents, alleviating the need for the addition of water to coal.

The question of catalyst specificity has not previously been sufficiently addressed in considering pre-combustion coal desulfurization. The energy of coal is held primarily in carbon-carbon bonds; the organic matrix also contains oxygen, sulfur, and nitrogen. When coal is used as a fuel, thermal energy is gained through the addition of oxygen to the organic molecules. Complete oxidation breaks all bonds to carbon, adding oxygens to produce carbon dioxide and inorganic oxides and acids such as H_2SO_4. The aim of pre-combustion desulfurization is to achieve selective oxidative cleavage of sulfur-carbon bonds, releasing sulfuric acid. Ideally, this is accomplished without effecting the stability of the carbon-carbon bonds, thus preserving most of the energy content of the coal. A secondary issue is the disruption or "solubilization" of the coal matrix, deemed necessary by some to provide access of the catalyst to the sulfur sites. Little is actually known about the need for "solubilization" in coal treatment with various catalysts.

Our experimental approach focuses on the use of enzymes which catalyze the addition of oxygen to organic compounds. In tailoring the application of these enzymes to coal processing, we are particularly interested in ensuring that oxidation occurs at sulfur

and not at carbon-carbon bonds. For example, in the model coal compound DBT which we use to evaluate processes, there are three specific types of oxidation sites: carbon-carbon bonds in the phenyl-rings; carbon-sulfur bonds in the thiophene ring; and sulfur itself. Previous studies with DBT have shown that the reaction most frequently observed in microbial oxidative pathways is one in which DBT is oxidized at ring carbons. These reactions, as we have said, are accompanied by a considerable decrease in the energy content of the compound.

In addition, microbial pathways have been identified in which the sulfur atom is sequentially oxidized to sulfoxide, to sulfone, to sulfonate, and finally to sulfuric acid. This "4S" pathway of DBT oxidation, shown in Figure 1.1, adds one or two hydroxyls to the phenyl rings, but leaves them otherwise intact. In this case, the fuel value of the desulfurized compounds is largely retained. Identification of the multi-step ("4S") microbial reaction pathway has encouraged us to examine enzyme processes which will selectively catalyze oxidation at sulfur. We are evaluating the potential of commercially available enzymes to perform this function, and also seeking to isolate "4S" pathway enzymes from a microbial source which produces "4S" products.

1.2 Review of Earlier Results

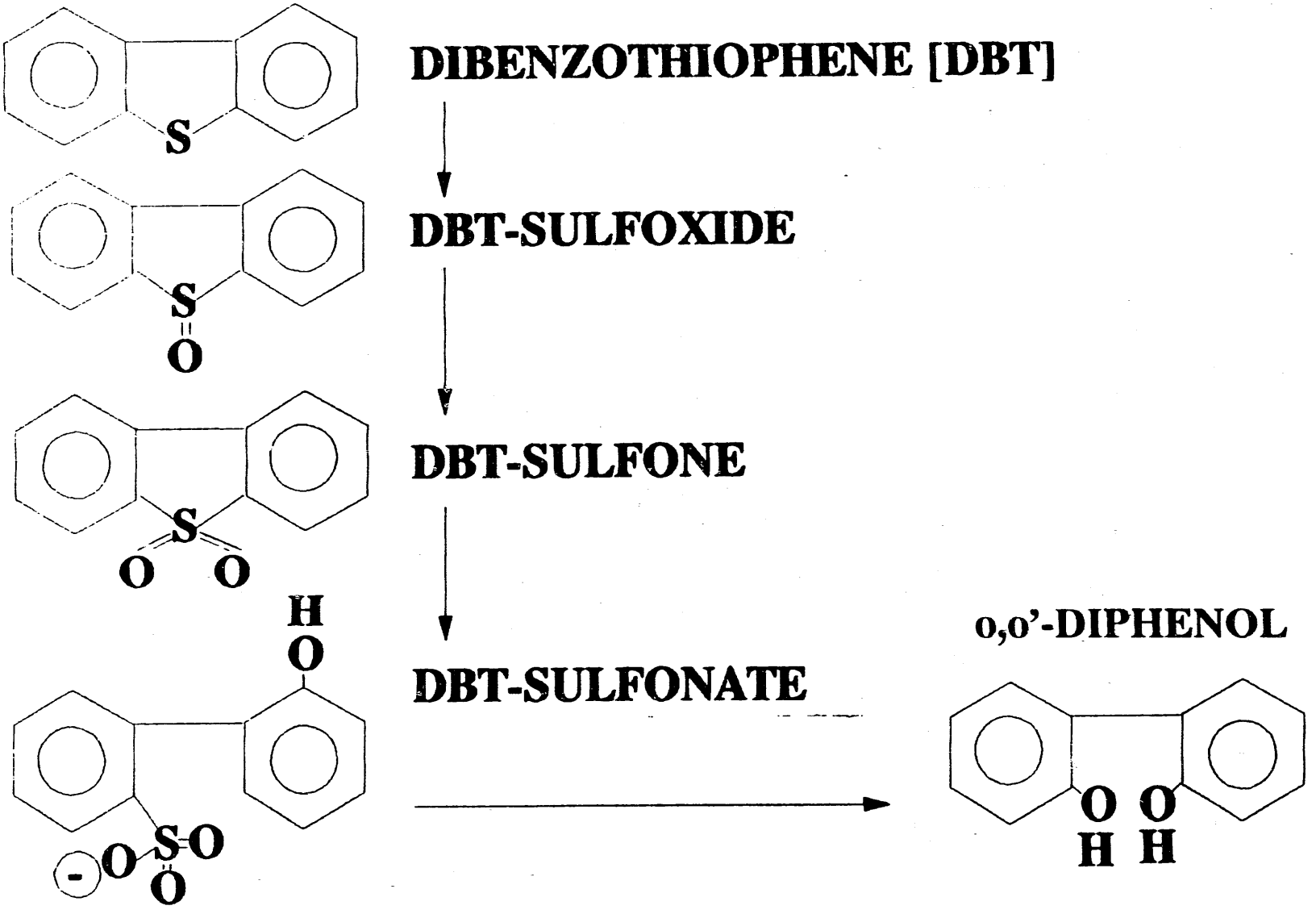
Our technical progress in the first quarter can be summarized as follows. We worked with laccase and horseradish peroxidase in buffer and in aqueous organic solvents. After establishing the activity of our enzymes in buffer, many tests of activity against standard substrates in hydrated dioxane and hydrated DMF media were made. In both solvents, some evidence of activity against dibenzothiophene [DBT] was observed. 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 "4S" 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.

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 received 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. The final report from Woods Hole is contained in Appendix A.

Figure 1.1

DBT AND ITS SULFUR OXIDATION PRODUCTS



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.

In the fourth quarter, we reported evidence of stability of laccase in hydrated ethyl acetate and hydrated acetonitrile for at least five days. Our attempts to identify reaction products in the reaction of laccase with DBT were unsuccessful. We had tested laccase in these media with DBT sulfone and no sulfur oxidation was observed. The reversibility of binding of DBT, EPS, and their sulfur oxidation products to horseradish peroxidase, laccase, and sulfatase was shown. The work with the microorganisms (GB-1 and GB-2) from the hydrothermal vents was shown in the fourth quarter to be quite fruitful. We were able to show production of DBT sulfoxide and DBT sulfone when GB-1 was grown in the presence of DBT.

During the fifth quarter, we completed more extensive testing of laccase, horseradish peroxidase, and sulfatase in hydrated organic solvents. We found that under the conditions used, the conversion of DBT to "4S" oxidation products was seen, although the yield was low. Other, as yet unidentified, oxidation products are also observed; these are most likely molecules in which carbon-carbon bond cleavage has occurred. We also began work on the isolation of the microbial enzymes from GB-1 shown to produce "4S" products.

The greatest success in the sixth quarter was the fractionation of GB-1, with the isolation of intracellular and membrane fractions in addition to the extracellular fraction previously utilized. We saw apparent production of DBT sulfoxide and DBT-sulfone from DBT with extracellular fraction in organic solvents and the intracellular fraction seemed to be similarly active. The assays with laccase and horseradish peroxidase gave ambiguous results because of the very low levels of products observed.

1.3 Summary of Seventh Quarter Results

This report covers the period of December 16, 1989 through March 15, 1990. During this time, a second lot of GB-1 has been fractionated as before, resulting in isolation of the intracellular and membrane fractions. Assays were performed with both the first and second lots of GB-1 ICF with DBT sulfone. GC/MS analysis of one assay sample indicated the presence of o,o'-diphenol and o-hydroxydiphenyl. Assays with concentrated (by ultrafiltration) GB-1 ICF were run with DBT and DBT-sulfone. From the latter, GC analysis indicated the formation of o,o'-diphenol. The discovery that significant amounts of material were lost during sample concentration impaired our analysis. We have recently found a method for reducing this loss, but no data using the new method is reported.

Section 2

EXPERIMENTAL RESULTS AND DISCUSSION

2.1 GB-1 ICF/Dibenzothiophene Sulfone Assays

Assay sample tubes were prepared as described in Section 2.3 and aliquots were removed and filtered at the indicated times. There were two tubes for each sample set: GB-1 ICF with 0.05 mM DBT-sulfone, GB-1 ICF only, and 0.05 mM DBT-sulfone only. The solvent was 90% CH_3CN with Tris buffer in the case of samples containing ICF, and distilled water in the DBT sulfone controls. Day 4 aliquot filtrates were analyzed by HPLC as obtained and also by evaporation to dryness and resuspension in a smaller amount of solvent to effect a 20-fold concentration. These aliquots were also concentrated 20-fold by evaporation to dryness and then derivatized prior to GC analysis.

HPLC analysis of neat reaction media indicated the presence of a very slight amount of o,o'-diphenol in the ICF/sulfone samples, whereas none was present in either set of controls. However, the HPLC analysis of the concentrated sample did not detect any o,o'-diphenol while it did detect small amounts of o-hydroxydiphenyl which had not been seen in the neat samples. The GC analysis of concentrated aliquots from this sample set indicated the presence of o,o'-diphenol in one sample but not the other and also showed peaks that possibly indicated the presence of o-hydroxydiphenyl. GC analysis of a concentrated DBT sulfone control also indicated the possible presence of o,o'-diphenol, but was not conclusive.

Based on the HPLC analysis which suggested the presence of o,o'-diphenol and o-hydroxydiphenyl in the Day 4 aliquot filtrates from the ICF with DBT-sulfone assays, coupled with the lack of any indication of these compounds in either control set, a portion of one of the filtrates from this sample set was taken to Boston University Medical School for GC/MS analysis. The sample was concentrated 15-fold by evaporation to dryness and derivatized with Tri-Sil/BSA prior to analysis. The GC/MS analysis confirmed the presence of o,o'-diphenol and o-hydroxydiphenyl in this sample. A copy of this data is included in Appendix B. No controls were analyzed by GC/MS.

The uncertainty raised by the loss of material during evaporation as described in Section 2.5.2 precludes any discussion as to the quantity of materials present in these assay samples. Other than the qualitative indication of the presence of o,o'-diphenol and o-hydroxydiphenyl in the sample analyzed by GC/MS, the question of whether material is truly not present in the control samples or has been lost during evaporation prevents any meaningful conclusion as to qualitative or quantitative results. Further refinements in the

concentration procedure are needed prior to further GC/MS confirmation before any conclusions can be drawn.

2.2 Preparation of a Second Lot of Intracellular Fraction (ICF) from GB-1

As described previously, 2 liters of a 3 day old GB-1 culture was centrifuged, washed with 10 mm Tris buffer, pH 7.0, and since growth was less dense than the previous time, the pellet was resuspended in 32 ml (instead of 50 ml) of the same buffer. The cells were then disrupted by sonication (3.5 minutes at an average of 60 watts using 15 second bursts with 1 minute intervals) in an ice bath. The ICF was obtained by ultracentrifugation at 35,000 rpm for 1 hour. The ICF was divided into 2 ml aliquots and kept frozen at -20°C until used. The membrane fraction was discarded.

2.3 Repeat of GB-1 ICF/Dibenzothiophene Sulfone Assays and Comparison of Two Lots of GB-1 ICF

This experiment was multipurpose. First, we wanted to repeat the assays of the GB-1 ICF against DBT sulfone in 90% CH_3CN with a larger number of samples. The second purpose was to compare the effects of different lots of GB-1 ICF. Table 2.1 summarizes the sample sets.

In general, the procedure for the assays in all sample sets was that the 0.99 mM DBT-sulfone solution (in CH_3CN) and CH_3CN were added to screw cap test tubes (Table 2.1). The indicated ICF solution (or distilled water in the case of Sample Set No. 3) was then added to each tube. The tubes were mixed by gentle inversion and a 1 ml aliquot was removed and filtered through a 0.22 μ Nylon-66 (Rainin No. 38-159) syringe filter unit. All filtrates were refrigerated until analysis. After the initial aliquots were removed, the assay tubes were agitated by shaking in a temperature controlled room (24-25°C). Mechanical failure of a shaker support rack interrupted the agitation during some of the first 24 hours of the assay. Another 1 ml aliquot was removed and filtered after this first 24 hour period, and the remainder of the assay sample was filtered after 4 days. These aliquot filtrates were similarly refrigerated until analysis.

Initial analysis of some Day 4 aliquot filtrates by GC and HPLC yielded inconclusive and confusing results (data not shown). Some species detected by GC analysis were not detected by HPLC analysis and vice versa. There was not good agreement between methods as to concentrations of the same materials found to be present in the same samples. Difficulties in analysis due to losses during drying (as will be mentioned in Section 2.5.2) may have contributed to this inconclusion. This analysis will be repeated with better concentration procedures and, if necessary, GC/MS confirmation will be obtained.

Table 2.1

SUMMARY OF 0.05 mM DBT SULFONE ASSAYS WITH GB-1 ICF

Sample Set No.	No. of Tubes in Set	DBT-Sulfone Added (ml, 0.99 mM)	ICF Added/ Date Prepared (ml)	CH_3CN (mls)	Distilled H_2O Added (mls)
1	6	0.252	0.500 10/19/89	4.248	---
2	6	---	0.500 10/19/89	4.500	---
3	6	0.252	---	4.248	0.500
4	3	0.252	0.500 01/16/90	4.248	---
5	3	---	0.500 01/16/90	4.500	---

It should be noted that assay tubes utilizing ICF (Sample Set Nos. 1 and 2) had turned a slightly cloudy pink upon the addition of the ICF. When ICF prepared on January 16, 1990 was added to tubes in Sets 4 and 5, the tube contents turned decidedly cloudy and a white flocculent precipitate settled out while the tube contents had stayed relatively colorless.

2.4 Results of Incubation of Concentrated ICF With DBT and DBT-Sulfone

Previous experiments with GB-1 ICF were assayed after 4 days of incubation with substrate. We decided to perform a 1 day incubation experiment using concentrated ICF.

The ICF was concentrated with a 43 mm Amicon ultrafiltration membrane (Diaflo No. 5YC05) with a molecular weight cut-off of 500. This low cut-off was used assuming that the enzyme(s) that brings about DBT or DBT-sulfone oxidation might need a co-factor(s) of low molecular weight. The ICF was kept cold during the concentration step by pumping ice-cold water through tubing surrounding the filtration chamber. Thus, 26 ml of ICF was concentrated to 3.4 ml.

This concentrated ICF was incubated separately with 0.1 mM DBT and 0.1 mM DBT-sulfone in 90% acetonitrile. Controls included ICF incubated without substrate and each of the substrates incubated without ICF. After 24 hours of incubation at 24°C with shaking, the samples were filtered through 0.2 μ Nylon filters and refrigerated in vials until tested. One hundred microliters (100 μl) of each sample was concentrated by drying in reactor vials at room temperature without letting the sample go to dryness. Samples were analyzed by HPLC and GC.

The results of these analyses are shown in Table 2.2.

From the GC, there was evidence of formation of o,o'-diphenol from DBT-sulfone. No products in these samples were observed on the HPLC, although the sulfone controls had a peak close to the retention time of o,o'-diphenol. In ICF incubated with DBT, DBT-sulfone, o,o'-diphenol, as well as DBT-sulfoxide, were detected by HPLC while DBT-sulfone/sulfoxide and a peak close to the retention time of o-hydroxydiphenyl were detected by the GC. While the DBT controls showed some DBT-sulfone, the samples with ICF and DBT had larger DBT-sulfone peaks. These results should be confirmed by GC-mass spectrometry.

2.5 Methods of Sample Concentrations

2.5.1 Extraction of Phenols from Assay Samples

When GB-1 ICF was incubated with 0.05 mM sulfone for 4 days at 25°C, we detected the formation of o,o'-diphenol and o-hydroxydiphenyl. In order to confirm o-hydroxydiphenyl, we developed a scheme to separate the phenols from the ICF-sulfone mixture. This was based on the principle that the phenols will have greater solubility in basic solutions. The procedure was initially done with a standard mix containing o-hydroxydiphenyl, o,o'-diphenol, and sulfone in 90% acetonitrile and 10% Tris buffer. To 2.5 ml of this solution was added 2.5 ml of 0.1 N NaOH and 2.5 ml methylene chloride.

Table 2.2

ANALYSIS OF ASSAYS OF CONCENTRATED GB-1 ICF WITH DBT-SULFONE OR DBT

	Method of Analysis								
	HPLC					GC			
Sample	DBT Sulf-oxide (mM)	DBT Sulfone (mM)	o,o'-Diphenol (mM)	o-Hydroxy-Diphenyl (mM)	DBT (mM)	DBT-Sulfoxide/ DBT-Sulfone (mM)*	o,o'-Diphenol (mM)	o-Hydroxy-Diphenyl (mM)	DBT (mM)
Conc. ICF and	±	0.055	ND	ND	0.006	0.08	0.0015	ND	0.05
DBT-Sulfone	±	0.057	ND	ND	0.007	0.05	0.0003	0.012	0.006
in 90% CH_3CN									
Conc. ICF	ND	±	ND	ND	0.005	0.012	ND	0.002	0.013
in 90% CH_3CN									
DBT-Sulfone	±	0.047	X	ND	0.0001	0.129	ND	ND	0.003
in 90% CH_3CN	±	0.047	X	ND	ND	0.137	ND	ND	ND
Conc. ICF	0.003	0.002	0.0002	ND	0.067	0.007	ND	?	0.09
and DBT in	±	0.0004	0.0002	ND	0.07	0.005	ND	?	0.10
90% CH_3CN									
DBT in	ND	0.0003	ND	ND	0.057	0.001	ND	ND	0.07
90% CH_3CN	ND	0.00005	ND	ND	0.035	0.0004	ND	ND	0.09

* Since the GC program does not separate DBT-sulfoxide from DBT-sulfone, the reported concentrations for the combined peaks were calculated based on a standard containing only DBT sulfone.
Samples were collected after 24 hours of shaking at 24°C.

ND = none detected.

± = Small peaks incorrectly integrated on the HPLC.

X = A peak with retention time close to that of o,o'-diphenol was detected by HPLC analysis.

? = A peak with retention time close to that of o-OH-diphenyl was detected by GC analysis.

This was mixed thoroughly and the layers were allowed to separate. The top basic layer was removed. To the CH_2Cl_2 layer was added 2 ml of base and after vortexing and separation of the layers, the CH_2Cl_2 layer was extracted twice more with base. All the base layers were pooled, acidified by the addition of concentrated HCl, and extracted twice in 2 ml of CH_2Cl_2. At each extraction, the layers were analyzed by HPLC to make sure that no more phenols were left behind in the CH_2Cl_2/base layers. The CH_2Cl_2 layers were pooled, dried to concentrate the phenols, and resuspended in a small volume of CH_2Cl_2. This was then analyzed by HPLC. By this procedure, about 50% of the o,o'-diphenol and only 10-20% of the o-hydroxydiphenyl could be recovered. It became apparent that the loss was occurring during the concentration/drying step. The concentration of the phenols by evaporation of CH_2Cl_2 was a required step since the amount of phenol in the samples was not high enough to be detectable without concentration.

2.5.2 Loss of Materials During Drying

In order to confirm the loss of phenols due to drying, a standard solution of o-hydroxydiphenyl and one of a mixed "4S" standard containing DBT-sulfoxide, DBT-sulfone, o,o'-diphenol, o-hydroxydiphenyl, and DBT was prepared in 90% acetonitrile, 10% Tris buffer. A small volume of each of these standards was dried in a heat block at 40°C and analyzed either: (1) immediately after drying; (2) on keeping for 1 hour at 40°C after drying; and (3) on keeping for 2 hours at 40°C after drying.

The results are presented in Figs. 2.1 and 2.2. It is clear that a substantial loss of DBT and o-hydroxydiphenyl occurs during such a procedure. This loss may be due to adherence of these materials to the glass vessel rather than to volatilization. We are currently attempting to eliminate this possibility by coating the glassware with DriCote (Fisher No. D140-50). An initial experiment was performed to determine any differences in levels of recovered materials from samples evaporated in silane coated glassware. Equal volumes, 300 μl, of 0.1 mM "4S" standards were placed in two silane coated vials. One sample was allowed to evaporate until the volume had been reduced to 13.5 μl. The second sample was allowed to evaporate to complete dryness and then was resuspended to a volume of 13.5 μl. Both samples were analyzed by HPLC. Figure 2.3 shows the results. In this case, the quantities of phenolic compounds were better conserved, however, losses of DBT-sulfoxide and DBT-sulfone were larger than in unsilanized vials.

Disposable Sep-Pak cartridges (Water Associates) were tested as a means of separating and concentrating all the reaction products or the phenols selectively. The C18 and CN Sep-Paks tried were not effective in binding either the phenols or the sulfone preferentially and pretreatment of the sample by making it acidic did not improve the procedure. Because the initial samples are in acetonitrile, they do not readily partition into the Sep Pak.

Figure 2.1

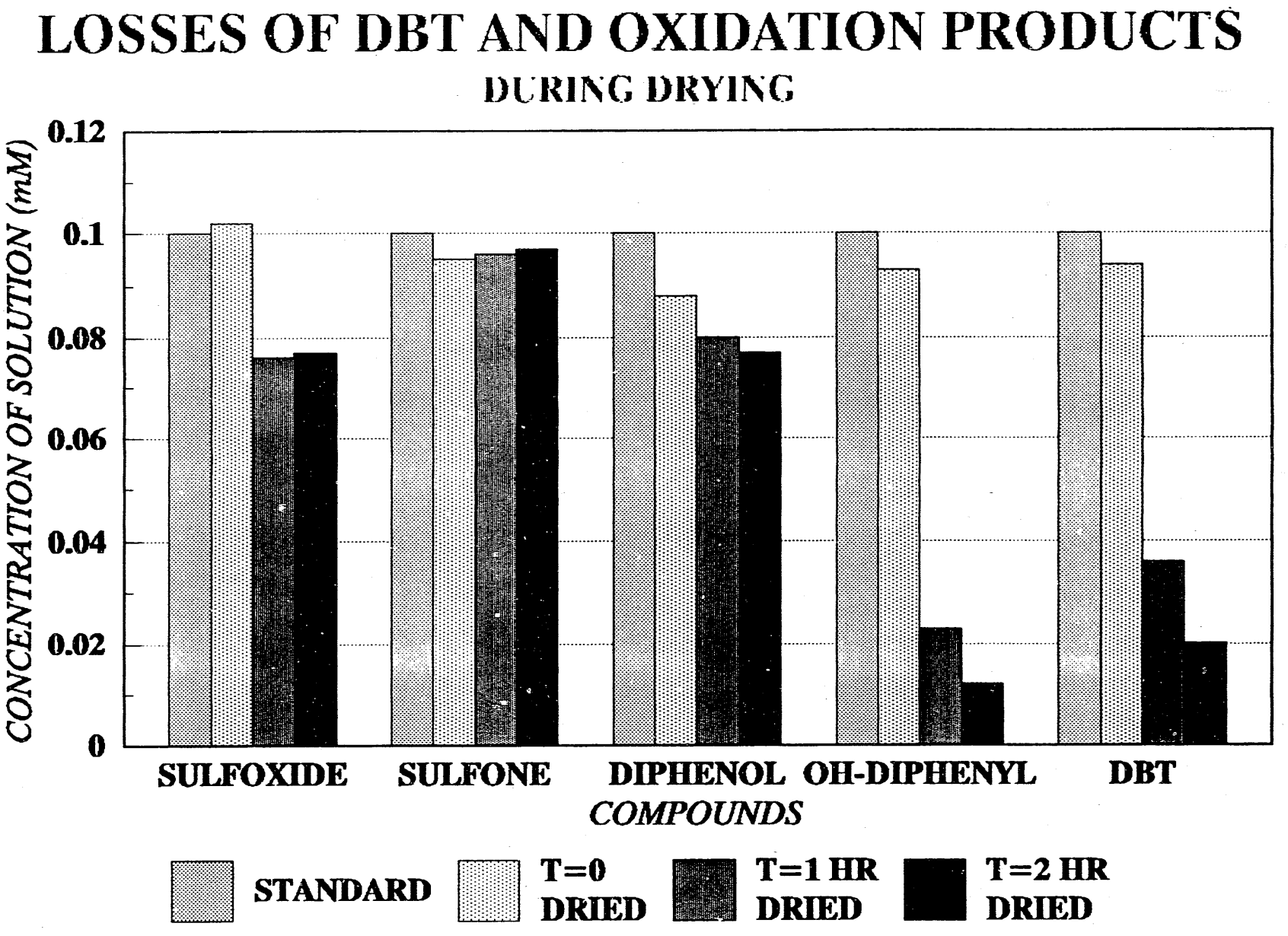

LOSSES OF DBT AND OXIDATION PRODUCTS
DURING DRYING
CONCENTRATION OF SOLUTION (mM)
0.12
0.1
0.08
0.06
0.04
0.02
0
SULFOXIDE
SULFONE
DIPHENOL
OH-DIPHENYL
DBT
COMPOUNDS
STANDARD
T=0 DRIED
T=1 HR DRIED
T=2 HR DRIED

Figure 2.2

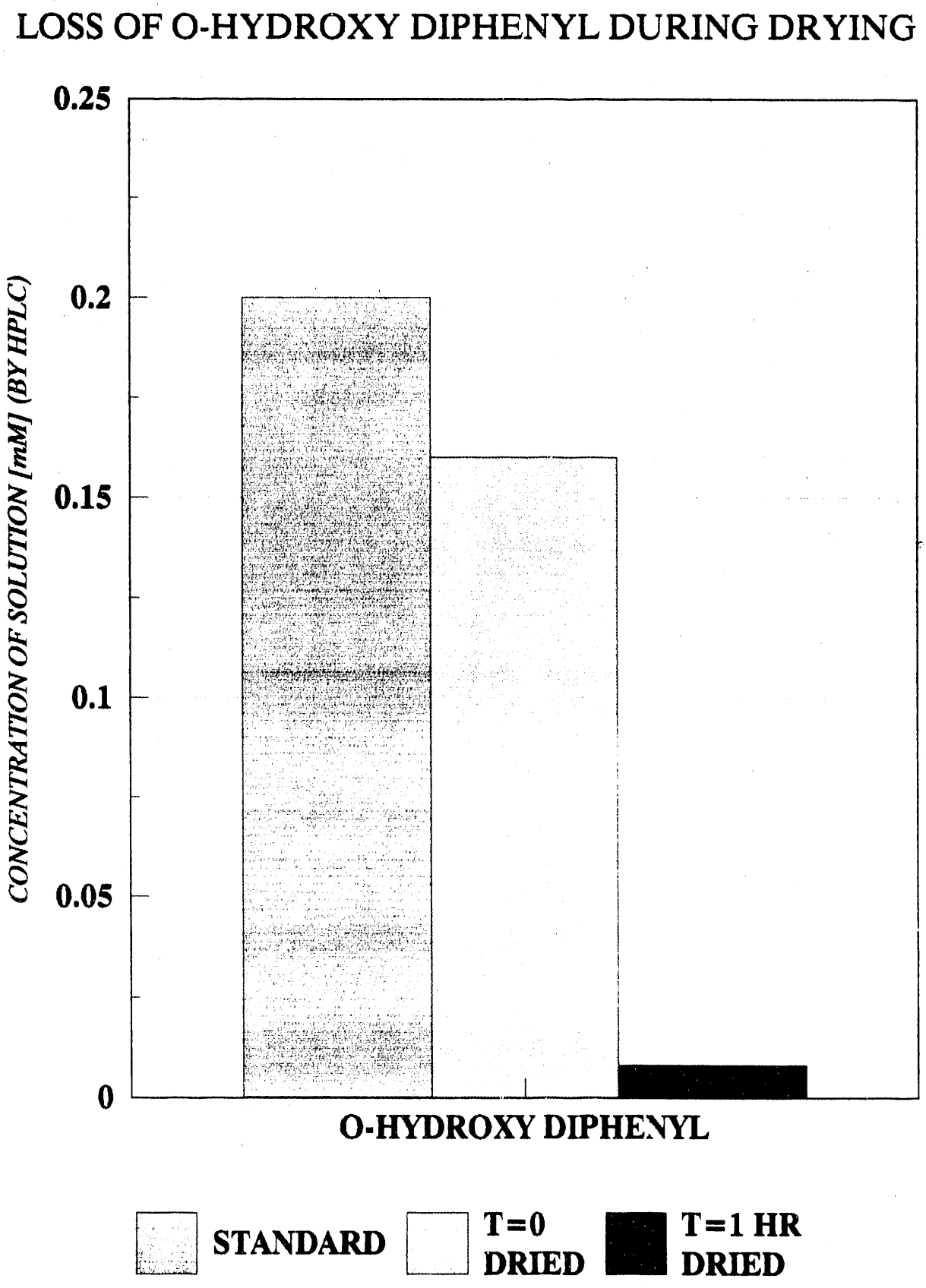
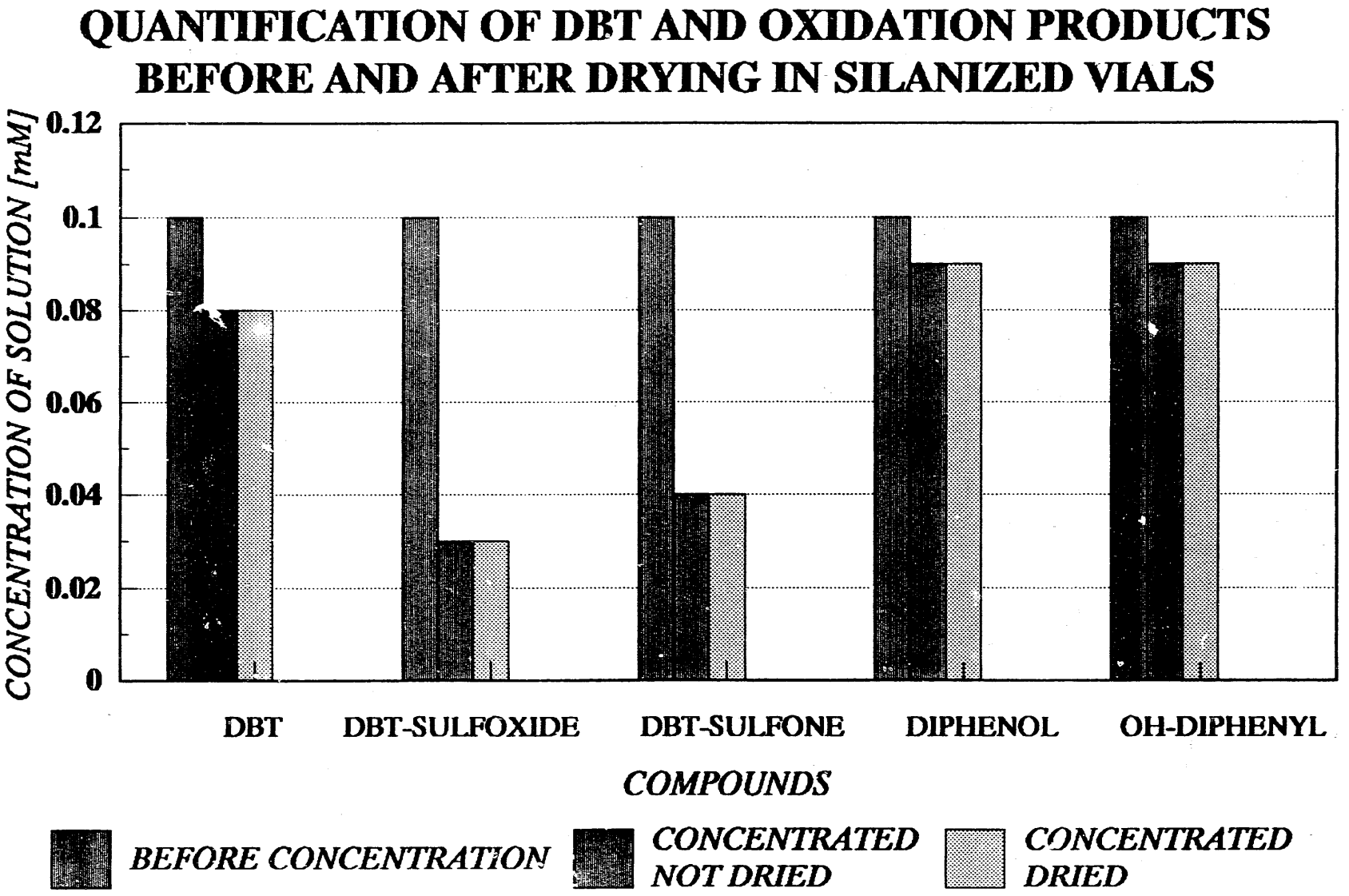

LOSS OF O-HYDROXY DIPHENYL DURING DRYING
0.25
0.2
0.15
0.1
0.05
0
CONCENTRATION OF SOLUTION [mM] (BY HPLC)
O-HYDROXY DIPHENYL
STANDARD
T=0
DRIED
T=1 HR
DRIED

Figure 2.3


QUANTIFICATION OF DBT AND OXIDATION PRODUCTS
BEFORE AND AFTER DRYING IN SILANIZED VIALS
CONCENTRATION OF SOLUTION [mM]
0.12
0.1
0.08
0.06
0.04
0.02
0
DBT
DBT-SULFOXIDE
DBT-SULFONE
DIPHENOL
OH-DIPHENYL
COMPOUNDS
BEFORE CONCENTRATION
CONCENTRATED NOT DRIED
CONCENTRATED DRIED

Section 3

PLANS FOR FUTURE WORK

3.1 Current and Future Tasks

In the eighth quarter, we plan to continue development of our analytical methods for assay samples. We are currently performing a study to determine whether coating glassware with silane will eliminate the observed uncertainties in our analysis. Another lot of GB-1 is being prepared. This lot will be fractionated to isolate the intercellular fraction (ICF) which will be concentrated by ultrafiltration. Both GB-1 whole cells and the concentrated ICF will be used in assays utilizing coal depyritized by Thiobacillus ferrooxidans as the substrate. These assay samples will be returned to DOE for analysis. A sample of a new, more potent type of laccase will be used for assays using model compounds as substrates to compare with our earlier work with laccase.

APPENDIX A

FINAL REPORT SUBMITTED TO HOLOMETRIX, INC.
Cambridge, Massachusetts 02139

by

Dennis A. Bazylinski

Biology Department
Woods Hole Oceanographic Institution
Woods Hole, Massachusetts 02543

"ISOLATION OF MARINE DIBENZOTHIOPHENE- AND ETHYLPHENYLSULFIDE-TRANSFORMING MICROORGANISMS"

I. Sources of Inocula

Sources of inocula for the isolation of marine dibenzothiophene (DBT) and ethylphenylsulfide (EPS) came from a number of different sources including sediment and water from: 1) Eel Pond, Woods Hole, MA; 2) the Black Sea; and 3) the Guaymas Basin. Enrichments using material from the first two sites were negative for these types of organisms. Because of this and other reasons (see below), we concentrated our efforts on samples from the Guaymas Basin.

The Guaymas Basin is one of a series of deep semi-closed basins in the Gulf of California (Byrne and Emery, 1960) which is tectonically active (Curray et al., 1979). It is separated by a 20 km transform fault into two rift valleys, the Northern and Southern Troughs (Simoneit and Lonsdale, 1982). Hydrothermal vents are numerous in the Southern Trough which is the area we sampled with the use of the DSV *ALVIN* during February 1987. Exuding hydrothermal fluids discharge through chimneys at between 270°-325°C and through sediment at about 50°C (Lonsdale et al., 1980; Lonsdale and Becker, 1985; personal observations).

The Guaymas Basin is unique in that rapidly accumulating hemipelagic sediment which amasses at a rate of 1.0-1.2 m per 1000 yr (Curray et al., 1979) is pyrolyzed under high temperature conditions to petroleum-like products (Simoneit and Lonsdale, 1982; Simoneit, 1984, 1985). These products consist of gasoline-range aliphatic and aromatic hydrocarbons and predominantly residual polar asphaltic material (Simoneit and Lonsdale, 1982). The sediments also contain large amounts of sulfur (Bazylinski et al., 1988) some of which is found in organic compounds particularly in the form of cyclic polysulfides (Kawka and Simoneit, 1987).

Geochemical analyses of extracted petroleums from sediments of the Southern Trough of the Guaymas Basin and microbiological studies of the area suggest that microbial degradation of hydrocarbons is a significant process at the site (Simoneit, 1985; Bazylinski et al., 1988, 1989). Because of the presence of organic sulfur compounds and hydrocarbon-utilizing bacteria as well, we thought this area to be a likely source of material from which to isolate DBT- and/or EPS-utilizing microorganisms.

II. Experimental Procedures

Approximately 1-2 g of sediment and water were used as inocula and added to flasks containing the following basal artificial seawater medium (g L^{-1} unless otherwise noted): NaCl, 23.477; $MgCl_2 \cdot 6H_2O$, 4.981; Na_2SO_4, 3.917; $CaCl_2 \cdot 2H_2O$, 1.102; KCl, 0.664; $NaHCO_3$, 0.192; KBr, 0.096; H_3BO_3, 0.026; $SrCl_2 \cdot 6H_2O$, 0.024; NH_4Cl, 0.5; and 5 ml of Wolfe's mineral elixir (Wolin et al., 1963) modified by the addition of 0.4 g L^{-1} of $Na_2MoO_4 \cdot 2H_2O$ rather than 0.01 g L^{-1}. The medium was autoclaved after which 2.7 ml L^{-1} of sterile potassium phosphate buffer (750 mM, pH 7.2) and 5 ml L^{-1} of a filter sterilized vitamin solution (Bazylinski et al., 1989) were added. The pH of the medium was adjusted to 7.2 with sterile 0.1 M NaOH or HCl. Sterile DBT or EPS was added to the medium at a concentration of 0.1% (w/v) as the sole carbon source. DBT was dissolved in acetone or ether and filter-sterilized and EPS was filter-sterilized directly (solvent resistant FP Vericel; porosity, 0.2 μm; Gelman Sciences, Inc., Ann Arbor, MI). The acetone or ether was evaporated under a stream of sterile nitrogen gas.

Previous results with hydrocarbon-utilizing bacteria isolated from oily Guaymas Basin sediments showed that most if not all of these bacteria are mesophilic (growth optima >20°C and <45°C) (Bazylinski et al., 1989). For this reason, all enrichment cultures were incubated at 25°C.

Enrichment cultures were checked daily for: 1) turbidity, 2) appearance of colored compounds, 3) change in the appearance of DBT and EPS, and 4) fluorescence under short wave ultraviolet light (indicating the presence of certain intermediates in the desulfurization of DBT - J. Marquise, personal communication). Cultures were considered positive if any of the above criteria were met and were then used for subsequent steps in isolation of DBT- and EPS-utilizing microorganisms.

Material from positive enrichment cultures were streaked onto similar medium solidified with 1.5% Purified Agar (Difco). Some agar plates also contained various amounts (0.01-0.05%, w/v) of Yeast Extract (Difco). DBT was dissolved in acetone and ether (2%, w/v) and

sprayed onto the surface of the agar plates using a nebulizer. Approximately 1.0-1.5 ml was sprayed onto each agar plate. Once separated colonies were apparent, material from these colonies were streaked onto new plates. This was repeated at least three times to ensure the use of a pure culture.

III. Results

EPS: - All enrichment cultures containing EPS using inocula from all sampled sites were negative for turbidity (growth). In addition, there was never any change in the appearance of the EPS and microscopic examination of the culture showed no increase in bacterial numbers.

DBT: - All enrichment cultures containing DBT with inocula from Eel Pond and the Black Sea were negative based on the previously mentioned criteria.

Although most enrichment cultures inoculated with Guaymas Basin sediment were also negative, one culture containing sediment from an oily core obtained on *ALVIN* dive number 1967 was positive. After two weeks of incubation, the culture fluid was red and some of the DBT disappeared. The culture was slightly turbid and microscopic examination of the culture indicated large numbers of a motile, rod-shaped bacterium. There was no fluorescence under short wave ultraviolet light. A sample of red culture fluid was filtered and analyzed spectrophotometrically. The pigment had a visible absorbance maximum at about 392 nm.

The enrichment procedure was repeated using more sediment from the same source and material from the original positive enrichment was streaked onto DBT agar plates as described earlier. As in the first enrichment culture, culture fluid from the second also became reddish and slightly turbid after two weeks. Microscopic examination of the culture showed organisms of similar morphology to those observed in the first enrichment culture. This result suggests that these organisms are distributed throughout the sediment of the sample. Red areas became apparent on the DBT-containing agar plates (with and without yeast extract) in about 11-12 days. However, single colonies grew much more quickly when yeast extract was included. Several colonies were restreaked onto DBT agar plates containing 0.05% (w/v) yeast extract. In two weeks colonies formed with reddish zones surrounding them. This red pigment eventually diffused throughout the medium indicating that the pigment was water soluble. In addition, sprayed water-insoluble DBT particles disappeared around the colonies. This and the fact that no reddish color appeared on control agar plates lacking DBT indicates that the red material results from a bacterial transformation of DBT. This restreaking was repeated once more.

Once returned to liquid culture, the strain also produced red pigment from DBT which occasionally precipitated out of the growth medium late in the growth cycle. Several strains were purified and kept. These were designated GB1DBTa, GB1DBTb, GB1DBTc, and GB1DBTd. The organism was rod-shaped, motile, and stained variably with the Gram stain. The KOH test for the determination of the Gram reaction (Buck, 1982) was also equivocable.

Smaller colonies which did have clear zones on DBT agar plates and did not produce any pigment were also restreaked to obtain a pure culture. Two strains were obtained and designated GB2DBTa and GB2DBTb. The organism was vibrioid-to-helical in morphology, motile, and Gram-negative. There was no indication that this organism could transform DBT in any way but more specific tests should be done to determine this.

IV. Final Considerations

This study has resulted in the isolation of at least one organism (strain GB1DBT) which can transform DBT to a red water soluble compound(s). It is unclear whether this organism can remove sulfur during this transformation. In addition, the red compound has not been identified. It is interesting that a strain of *Pseudomonas putida* has been reported to convert DBT to DBT sulfone via DBT-5-oxide and to 3-hydroxy-2-formyl benzothiophene via a previously described pathway (Kodama et al., 1970, 1972) while also producing an unidentified red-colored product (Mormile and Atlas, 1989). Further studies may show that strain GB1DBT and the strain of *P. putida* utilize similar pathways of DBT oxidation.

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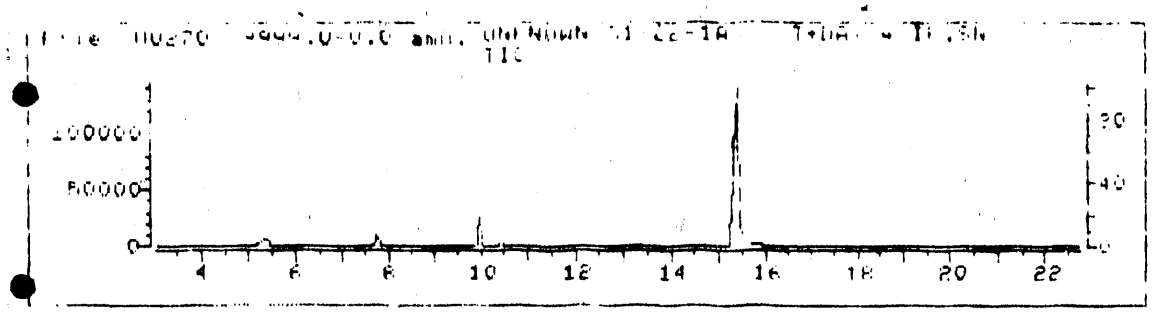
APPENDIX B



UNKNOWN

FOUND:

1) Hydroxybiphenyl
2) DBT
3) 0-0' Diphenol Derriv
4) DBT sulfoxide
5) DBT sulfone

```
*      ID File: [illegible]                    *
* Output File: [illegible]0270::D4             *
*----------------------------------------------*

*-------------------------*
* Start QUANT Test Mode  *
*-------------------------*

*---------------------------------------------------------------*
* Comp#   1   O-HYDROXYBIPHENYL                         (ISTD)  *
*---------------------------------------------------------------*

    Entering 3 ions over range:  6.50:  [illegible] minutes.
    Integrating mass 211.0.... slope:    .20  min area:      [illegible]

    Peaks in user window ( [illegible] +/-  1.00 minutes): 1

      RT    Start   Max     Stop    Raw Area CorrArea
    ------ ------ ------ ------ -------- --------
      7.74    154    158    167    13212    13046

     *--------------* Peak at RT:   7.74 minutes *--------------*

     Rel abund error allowed:   50.00% (by AREA)
                Raw        Corr      Rel    Target
     Mass       Area       Area       %       %
    ------   --------  --------  ------  ------
    211.0 Q    13212     13046  100.00  100.00
    227.2      12045     11879   91.05   98.00
    242.2       9080      8985   68.87   65.00

     All ions pass maximization check.

     Peak PASSES qual criteria.
     Q-value:  93

  1 [redacted]YL                 RT: 7.74 Area:   13046 1000.0          [redacted]

*---------------------------------------------------------------*
* Comp#   2   DBT                                               *
*---------------------------------------------------------------*

    Entering 3 ions over range:  8.43: 11.43 minutes.
    Integrating mass 184.0.... slope:    .20  min area:     1500

    Peaks in user window (  9.93 +/-   1.00 minutes):  1

      RT    Start   Max     Stop    Raw Area CorrArea
    ------ ------ ------ ------ -------- --------
      9.96    226    231    245    92610    91813

     *--------------* Peak at RT:   9.96 minutes *--------------*

     Rel abund error allowed:   50.00% (by AREA)
                Raw        Corr      Rel    Target
     Mass       Area       Area       %       %
    ------   --------  --------  ------  ------
    184.0 Q    92610     91813  100.00  100.00
    152.0       8310      7930    8.64    9.00
    139.8        592       384     .42   14.00

     All ions pass maximization check.

     Peak PASSES qual criteria.
     Q-value:  79
```

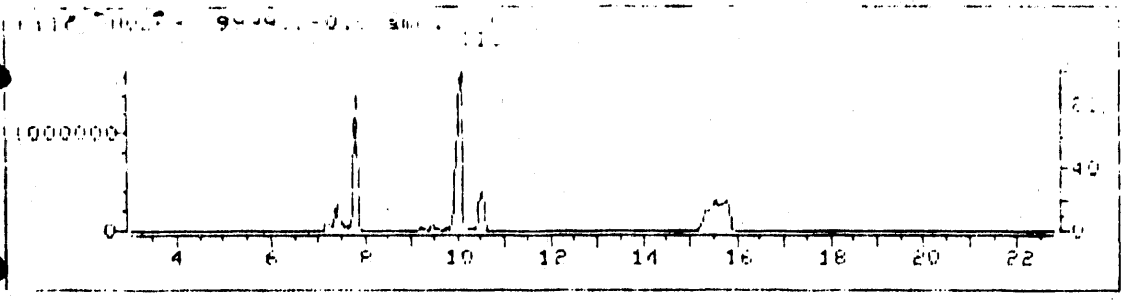
2) [redacted] RT: 9.96 Area: 91813 91813. NO CALIBq [redacted]

```
*-------------------------------------------------*
* Comp#  3  O-O'DIPHENOL DERRIV                   *
*-------------------------------------------------*

 Entering 2 ions over range:  8.92: 11.92 minutes.
 Integrating mass 330.2.... slope:    .20  min area:      800

 Peaks in user window ( 10.42 +/-   1.00 minutes):  1

   RT    Start  Max    Stop   Raw Area CorrArea
------ ------ ------ ------ -------- --------
 10.45    244    247    251    11227    11061

  *-------------* Peak at RT: 10.45 minutes *-------------*

  Rel abund error allowed:  50.00% (by AREA)
           Raw      Corr    Rel   Target
 Mass      Area     Area     %       %
------  -------- -------- ------ ------
330.2 Q    11227    11061 100.00 100.00
331.1       2916     2814  25.44  28.00

  All ions pass maximization check.

  Peak PASSES qual criteria.
  Q-value:  95
```

3) [redacted] RT:10.45 Area: 11061 11061. NO CALIBq [redacted]

```
*-------------------------------------------------*
* Comp#  4  DBT SULFOXIDE                         *
*-------------------------------------------------*

 Entering 2 ions over range: 13.67: 16.67 minutes.
 Integrating mass 184.0.... slope:    .20  min area:      800

 Peaks in user window ( 15.17 +/-   1.00 minutes):  1

   RT    Start  Max    Stop   Raw Area CorrArea
------ ------ ------ ------ -------- --------
 15.43    403    411    431    42725    41602

  *-------------* Peak at RT: 15.43 minutes *-------------*

  Rel abund error allowed:  50.00% (by AREA)
           Raw      Corr    Rel   Target
 Mass      Area     Area     %       %
------  -------- -------- ------ ------
184.0 Q    42725    41602 100.00 100.00
200.0       1919     1459   3.51  33.00

  All ions pass maximization check.

  Peak PASSES qual criteria.
  Q-value:  47
```

```
*-------------------------------------------------*
* Comp#  5  DBT SULFONE                           *
*-------------------------------------------------*

Entering 3 ions over range: 13.74: 16.74 minutes.
Integrating mass 216.0.... slope:   .20  min area:     800

Peaks in user window ( 15.24 +/-   1.00 minutes):  2

  RT    Start  Max    Stop   Raw Area CorrArea
------ ------ ------ ------ -------- --------
 15.40    403    410    413   656692   655781
 15.49    413    413    451    56323    47838

 *-------------* Peak at RT: 15.40 minutes *-------------*

 Rel abund error allowed:  50.00% (by AREA)
          Raw      Corr    Rel   Target
 Mass     Area     Area     %       %
------  -------- -------- ------ ------
216.0 Q   656692   655781 100.00 100.00
136.0     220220   219746  33.51  35.00
160.0     137945   137544  20.97  20.00

 All ions pass maximization check.

 Peak PASSES qual criteria.
 Q-value:  97

 *-------------* Peak at RT: 15.49 minutes *-------------*

 Rel abund error allowed:  50.00% (by AREA)
          Raw      Corr    Rel   Target
 Mass     Area     Area     %       %
------  -------- -------- ------ ------
216.0 Q    56323    47838 100.00 100.00
136.0      22134    18636  38.96  35.00
160.0      13128    10704  22.38  20.00

 Maximization check failed for mass: 136.0
 Maximization check failed for mass: 160.0

 Peak FAILS qual criteria.

5) [redacted]                    RT:15.40 Area: 655781 655781 NO CALIBq[redacted]

*---------------------*
* End QUANT Test Mode *
*---------------------*
```



← STD

Start QUANT Test Mode

Comp# 1 O-HYDROXYBIPHENYL (Istd)

Entering 5 ions over range: 6.50 9.30 minutes.
Integrating mass 211.0.... slope: .20 min area: 800

Peaks in user window (7.80 +/- 1.00 minutes): 5

RT	Start	Max	Stop	Raw Area	CorrArea
7.08	132	134	134	878	849
7.17	134	137	141	238978	237236
7.39	141	144	153	764253	760787
7.90	161	161	188	35114	26847

Peak at RT: 7.08 minutes

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
211.0 Q	878	849	100.00	100.00
227.2	953	906	106.71	98.00
242.2	791	754	88.81	65.00

Maximization check failed for mass: 227.2
Maximization check failed for mass: 242.2

Peak FAILS qual criteria.

Peak at RT: 7.17 minutes

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
211.0 Q	238978	237236	100.00	100.00
227.2	229215	227524	95.91	98.00
242.2	162908	161561	68.10	65.00

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value: 97

Peak at RT: 7.39 minutes

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
211.0 Q	764253	760787	100.00	100.00
227.2	722073	718727	94.47	98.00
242.2	513377	510709	67.13	65.00

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value: 98

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
211.0 Q	3209422	3206658	100.00	100.00
227.2	2867819	2865172	[redacted]	
242.2	1934217	1932103		

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value: 92

------------- Peak at RT: 7.90 minutes *-------------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
211.0 Q	35114	26847	100.00	100.00
227.2	33082	25134	93.62	98.00
242.2	23814	17466	65.06	65.00

Maximization check failed for mass: 227.2
Maximization check failed for mass: 242.2

Peak FAILS qual criteria.

Auto Qdel: Largest area is hit number 3.
Istd: Removing all but best hit.

1) *O-HYDROXYBIPHENYL RT: 7.84 Area:3206658 1000.0 q: 92

---
* Comp# 2 DBT *

Entering 3 ions over range: 8.55: 11.55 minutes.
Integrating mass 184.0.... slope: .20 min area: 1500

Peaks in user window (10.05 +/- 1.00 minutes): 6

RT	Start	Max	Stop	Raw Area	CorrArea
9.12	198	201	201	3983	3917
9.21	201	204	206	233570	219416
9.44	210	213	215	384687	301804
[redacted]					
10.16	235	235	244	111763	61102
10.58	244	249	251	60179	28922

------------- Peak at RT: 9.12 minutes *-------------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
184.0 Q	3983	3917	100.00	100.00
152.0	532	318	8.12	9.00
139.0	40	12	.31	14.00

Maximization check failed for mass: 152.0

---------- Peak at RT: 9.21 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
184.0 Q	233570	219416	100.00	100.00
152.0	19935	18422	8.40	9.00
139.8	552	470	.21	14.00

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value: 78

---------- Peak at RT: 9.48 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
184.0 Q	384687	301804	100.00	100.00
152.0	33043	26628	8.82	9.00
139.8	902	665	.22	14.00

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value: 79

---------- Peak at RT: 10.09 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
184.0 Q	11766366	11688732	100.00	100.00
152.0	1039826	1028727	8.80	9.00
139.8	24942	24450	.21	14.00

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value: 79

---------- Peak at RT: 10.15 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
184.0 Q	111763	61102	100.00	100.00
152.0	20543	13374	21.89	9.00
139.8	672	326	.54	14.00

Maximization check failed for mass: 152.0
Maximization check failed for mass: 139.8

Peak FAILS qual criteria.

---------- Peak at RT: 10.56 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %	
184.0 Q	60179	28922	100.00	100.00	
152.0	117626	113263	391.62	9.00	Fails
139.8	3174	2932	10.14	14.00	

All ions pass maximization check.

Peak FAILS qual criteria.

2) DBT RT: 9.21 Area: 219416 219416 NO CALIBq: 78
2) DBT RT: 9.48 Area: 301804 301804 NO CALIBq: 79
(2) DBT RT: 10.09 Area: ******* ****** NO CALIBq: 79

----
* Comp# 3 O-O'DIPHENOL DERRIV *

Entering 2 ions over range: 9.06: 12.06 minutes.
Integrating mass 330.2.... slope: .20 min area: 800

Peaks in user window (10.56 +/- 1.00 minutes): 6

RT	Start	Max	Stop	Raw Area	CorrArea
9.76	219	222	223	27796	27570
9.85	223	225	227	42329	11572
10.03	227	231	232	143933	100240
10.12	232	234	236	149516	50543
[redacted]					
10.64	251	251	268	18301	9222

---------- Peak at RT: 9.76 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %	
330.2 Q	27796	27570	100.00	100.00	
331.1	7883	7759	28.14	95.00	Fails

All ions pass maximization check.

Peak FAILS qual criteria.

---------- Peak at RT: 9.85 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %	
330.2 Q	42329	11572	100.00	100.00	
331.1	11605	3355	28.99	95.00	Fails

All ions pass maximization check.

Peak FAILS qual criteria.

---------- Peak at RT: 10.03 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %	
330.2 Q	143933	100240	100.00	100.00	
331.1	39963	28297	28.23	95.00	Fails

All ions pass maximization check.

Peak FAILS qual criteria.

---------- Peak at RT: 10.12 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %	
330.2 Q	149516	50543	100.00	100.00	
331.1	40262	13708	27.12	95.00	Fails

All ions pass maximization check.

Peak FAILS qual criteria.

---------- Peak at RT: 10.55 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %	
330.2 Q	2021697	[redacted]		0	
331.1	563867				s

All ions pass maximization check.

Peak FAILS qual criteria.

---------- Peak at RT: 10.64 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %	
330.2 Q	18301	9222	100.00	100.00	
331.1	5147	2462	26.91	95.00	Fails

Maximization check failed for mass: 331.1

Peak FAILS qual criteria.

* Comp# 4 DBT SULFOXIDE *

Entering 2 ions over range: 13.86: 16.86 minutes.
Integrating mass 184.0.... slope: .20 min area: 800

Peaks in user window (15.36 +/- 1.00 minutes): 1

RT	Start	Max	Stop	Raw Area	CorrArea
15.34	399	406	415	1389769	1340804

---------- Peak at RT: 15.34 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
184.0 Q	1389769	134080	[redacted]	
200.0	446434	445821		

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value: 32

4) DBT SULFOXIDE RT:15.34 Area:1340804 ****** NO CALIBq: 32

* Comp# 5 DBT SULFONE *

Entering 3 ions over range: 13.93: 16.93 minutes.
Integrating mass 216.0.... slope: .20 min area: 800

Peaks in user window (15.43 +/- 1.00 minutes): 2

RT	Start	Max	Stop	Raw Area	CorrArea
[redacted]					1
15.89	424	424	455	123525	89456

---------- Peak at RT: 15.56 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
216.0 Q	1915785	1915211	[redacted]	
136.0	608640	607820		
160.0	381089	380624		

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value: 96

---------- Peak at RT: 15.89 minutes *----------*

Rel abund error allowed: 50.00% (by AREA)

Mass	Raw Area	Corr Area	Rel %	Target %
216.0 Q	123525	89456	100.00	100.00
136.0	48282	34609	38.69	35.00
160.0	27557	19195	21.46	20.00

Maximization check failed for mass: 136.0
Maximization check failed for mass: 160.0

Peak FAILS qual criteria.

5) DBT SULFONE RT:15.56 Area:1915211 ****** NO CALIBq: 96

----
* End QUANT Test Mode *

END

DATE FILMED

2/27/92