Analysis of Biodesulfurization Products Using Solid-Phase Extraction

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Abstract

Alkylated dibenzothiophenes (DBTs) are not efficiently desulfurized by the conventional hydrodesulfurization (HDS) process, and thus they remain targets for deeper desulfurization of HDS-treated oil. Microbial degradation systems are expected to convert these heterocyclic sulfur compounds to inorganic sulfur (sulfate or sulfite). For screening and characterization of the potentially useful desulfurizing microbes, it is very important to analyze microbial metabolites of these DBTs. For this purpose, highly sensitive methods of analysis and efficient concentration are required because there would be only small amounts of the metabolites in microbial culture media.

A sample preparation technique based on solid-phase extraction (SPE) was studied to separate DBT metabolites from an organic matrix such as gas oil. Our results demonstrate that the metabolites with polar functional groups such as 2-hydroxybiphenyl or *o,o'*-biphenol produced by a desulfurizing microbe (*Rodococcus erythropolis*) can be efficiently separated from the organic matrix and concentrated quantitatively by SPE. Moreover, it has been proven that the combination of SPE with gas chromatography–mass spectrometry or gas chromatography–atomic emission detection analysis can be successfully used for detailed characterization of gas oil treated by desulfurizing biocatalysts.

Introduction

Sulfur dioxide released upon the combustion of fossil fuels such as petroleum is one of the major environmental pollutants. The conventional refining technology, hydrodesulfurization (HDS), has been used as the effective process for sulfur removal from many types of sulfur-containing petroleum components. However, some residual organosulfur compounds remain, even after HDS treatment (1,2). It is therefore important to develop a novel technology for the highly efficient removal of these organosulfur compounds because the stringency of sulfur emission standards is increasing around the world. In this respect, biological desulfurization systems have recently attracted attention for their potential advantages compared with HDS. There are several reasons for this: they work at low temperatures and under low pressure, require no addition of hydrogen gas, and possibly degrade organosulfur compounds, which are considerably resistant to HDS treatment (3–5). Dibenzothiophene (DBT) has been used as the model compound to represent the organosulfur material present in the fossil fuel matrix. In addition, it is known that substantial amounts of various alkylated derivatives of DBT are not efficiently removed by HDS. For this reason, DBT-related compounds are good targets for biodesulfurization studies.

To accomplish these studies, it is very important to establish a method for analyzing the microbial metabolites of DBT for screening and characterizing the useful desulfurizing microbes. Analyses of biodegradation products have been accomplished by combinations of liquid–liquid extraction from aqueous culture media and high-performance liquid chromatography (HPLC) or gas chromatography (GC) (6–10). On the other hand, no studies have detailed a method for determining the products of microbial degradation in petroleum, to our knowledge. In this paper, we present an efficient method based on solid-phase extraction (SPE) for separating DBT and its microbial metabolites from an organic matrix such as gas oil.

Experimental

Materials

The three aromatic compounds used in this study, 2-hydroxybiphenyl (2-HBP), *o,o'*-biphenol, and DBT, were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The purities of these compounds were greater than 98%.

The aromatic sulfur compound, dibenzothiophene sulfone (DBT-sulfone), was obtained from Aldrich Chemical (Milwaukee, WI); its purity was greater than 97%.

A mixture of these four aromatic compounds (each at approximately 2 mg/mL) was prepared in ethyl acetate.

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Two aromatic sulfur compounds, dibenz[*c,e*] [1,2]-oxathiin-6-oxide (sultine) and dibenz[*c,e*] [1,2]-oxathiin-6,6-dioxide (sultone), were obtained from the Nard Institute (Hyogo, Japan). The purities of these two compounds were greater than 99.5%. A mixture of these aromatic sulfur compounds was also prepared as described above.

All of the solvents (analytical-grade ethyl acetate and kerosene obtained from Kanto Chemical [Tokyo, Japan] and analytical-grade *n*-hexane and ethanol from Wako Pure Chemical Industries [Osaka, Japan]) were used without further purification.

Two kinds of gas oil containing 1,312 and 139 µg/mL of total sulfur, respectively, were obtained from an oil refining company in Japan. The former was a gas oil sample that is usually refined, and the latter was deeply desulfurized. These two gas oil samples were characterized by the SPE technique.

Medium and growth conditions

DBT was dissolved in ethyl alcohol (5 g/L) and added to presterilized medium A (8), a sulfur-free synthetic medium, as a sole source of sulfur.

Rhodococcus erythropolis cells were grown in 200 mL of medium A supplemented with 25 µg of DBT per milliliter by shaking (100 strokes per min) in 500-mL Erlenmyer flasks at 30°C for 2 days. The bacterial cells were resuspended in 0.1M potassium phosphate buffer (pH 7.0) after being washed twice with 0.85% NaCl and recultivated at 30°C in test tubes (125 × 14-mm i.d.) containing 4 mL of a gas oil–cell suspension (1:1 mixture) with rotary shaking (50 rpm) for 1 day.

SPE procedures

The DBT derivatives to be analyzed have polar functional groups such as S=O or OH. Therefore, a silica cartridge was selected to trap them quantitatively. The cartridges (Bond Elut) contained 500 mg silica packing with a polyethylene frit and were obtained from Varian Sample Preparation Products (Harbor City, CA). The column volume was 2.8 mL.

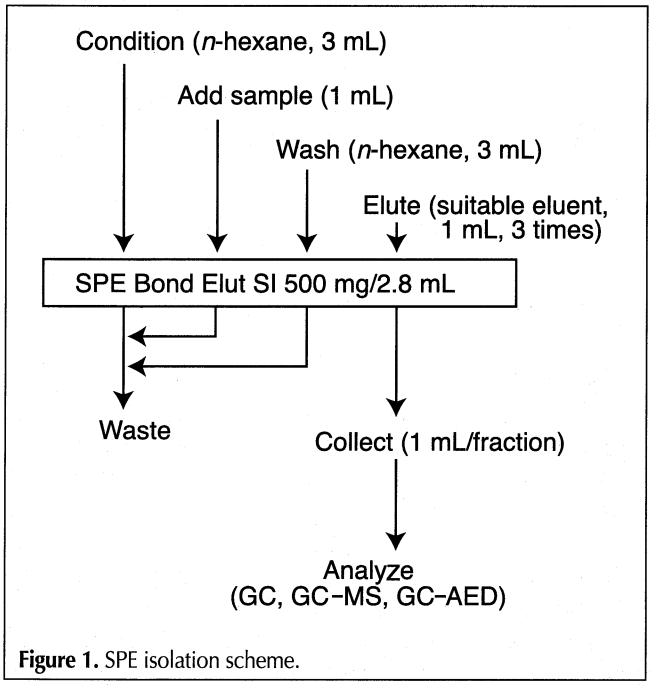

Figure 1. SPE isolation scheme.

Kerosene solutions containing the standard aromatic compound mixtures were used in all experiments for separations, recoveries, and concentration determinations. The standard mixtures were prepared by diluting mixtures containing four kinds of aromatic compounds (approximately 2 mg/mL each of 2-HBP, *o,o'*-biphenol, DBT-sulfone, and DBT) with kerosene. DBT-sulfone, 2-HBP, and *o,o'*-biphenol are the microbial metabolites of DBT. At first, the cartridge columns were conditioned by washing with 3 mL of *n*-hexane. Aliquots (1 mL) of the samples containing aromatic compounds or gas oil were pipetted into the columns. After the samples were absorbed onto the cartridge columns, they were washed with 3 mL of *n*-hexane again, and suitable eluents were added to elute the absorbed substances. The GC elution profiles were recorded for each of the components in the 1-mL fractions eluted stepwise. To accelerate the elution rate, a positive pressure was exerted on the column with a syringe. The isolation scheme is shown in Figure 1.

Analytical methods

Detection of the DBT derivatives and oil components was accomplished by using a Shimadzu GC-17A gas chromatograph with a DB-17 fused-silica capillary column (30 m × 0.25-mm i.d., 0.25-µm film) (J&W Scientific, Folsom, CA). A flame-ionization detector (FID) was used. The carrier gas was helium (1.3 mL/min), and the injector and detector temperatures were both 260°C. The column oven temperature was isothermal at 250°C. The samples (0.5-µL aliquots) were injected in the split mode (1:30).

Microbial metabolites in gas oils were identified by GC–MS and GC–AED. A DB-5 fused-silica capillary column (30 m × 0.25 mm i.d., 0.25-µm film) was used for GC–MS (Finnigan Mat, San Jose, CA). The carrier gas was helium (1.3 mL/ min). The injector and the ion source temperatures were maintained at 260°C. The initial programmed temperature was 100°C, which was increased to 250°C at 10°C/min and held 5 min at 250°C. Electron impact (approximately 70 eV) was selected as the ionization method. Aliquots (1 µL) of the samples were injected in the split mode (1:40).

An HP-1 fused-silica capillary column (25 m × 0.32-mm i.d., 0.17-µm film) (Hewlett-Packard, Wilmington, DE) was used for

Table I. Effect of Eluent Composition on Elution of DBT Metabolites (Silica Column, 500 mg/2.8 mL)

Eluent	Composition	Peak area (µV sec) 2-HBP	*o,o'*-Biphenol	DBT-sulfone
EtOAc–IPA	10:0	4127	2583	2402
EtOAc–IPA	9:1	4376	2750	3399
EtOAc–IPA	8:2	4487	2792	3264
EtOAc–IPA	7:3	4620	2859	3311
EtOAc–IPA	6:4	4606	2848	3161
EtOAc–IPA	5:5	4559	2628	2774
EtOAc–EtOH	9:1	4628	2727	3155
EtOAc–EtOH	8:2	4104	2465	3283
EtOAc–EtOH	7:3	4418	2624	3011
EtOAc–EtOH	6:4	4531	2735	3437
EtOAc–EtOH	5:5	4566	2576	3071

GC–AED. High-purity helium (greater than 99.9999%) was used as a carrier gas (2.9 mL/min). The injector, transfer line, and cavity temperatures were maintained at 250°C. The oven temperature was programmed from 60 to 250°C at 5°C/min. Aliquots (1 µL) of the samples were injected in the split mode (1:35).

Carbon and sulfur were simultaneously detected by using an AED at 193 and 181 nm, respectively. Oxygen (more than 99.995% pure) and hydrogen (more than 99.999% pure) gases were used as reagent gases.

Results and Discussion

SPE conditions

Aliquots (1 mL) of the standard mixtures (approximately 100 µg/mL of each component) were applied onto the silica cartridges to select the suitable eluent and to determine the recovery and concentration of each component. As shown in Table I, the DBT-related compounds were most efficiently eluted with ethyl acetate–isopropyl alcohol (EtOAc–IPA, 7:3 mixture) and ethyl acetate–ethanol (EtOAc–EtOH, 6:4 mixture).

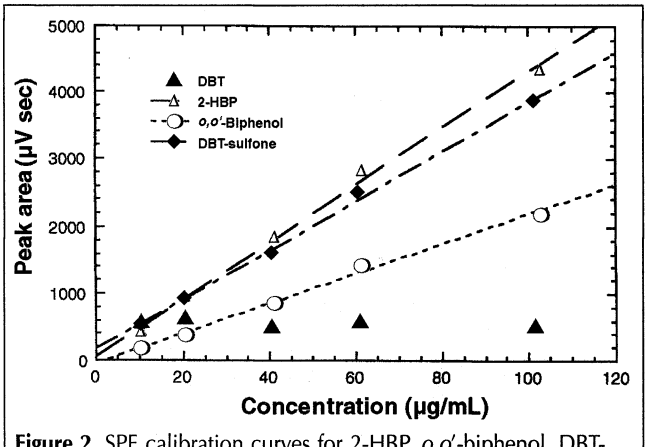

Figure 2. SPE calibration curves for 2-HBP, *o,o'*-biphenol, DBT-sulfone, and DBT (10–100 µg/mL).

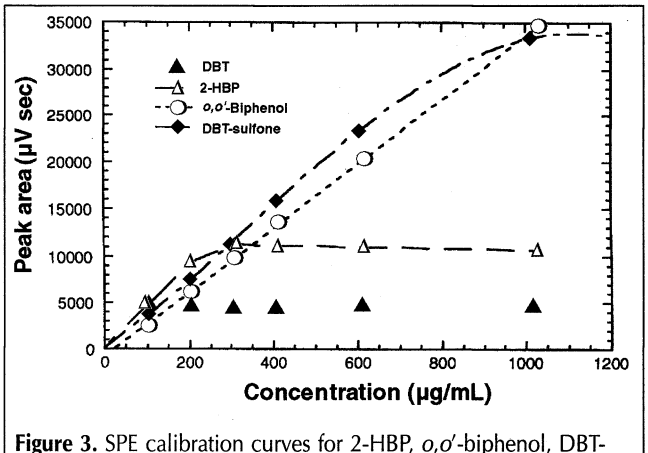

Figure 3. SPE calibration curves for 2-HBP, *o,o'*-biphenol, DBT-sulfone, and DBT (100–1000 µg/mL).

Recoveries of the eluates with the two eluents were compared. Although the average recoveries of each of the components with both eluents was greater than 90%, the coefficient of variation (CV) in the recovery with EtOAc–EtOH was less than that with EtOAc–IPA (Tables II and III). Only DBT was washed out of the silica columns with *n*-hexane because DBT did not have sufficient polarity to be adsorbed by the column packing. These data indicate that EtOAc–EtOH (6:4 mixture) is a suitable eluent for the elution of the DBT derivatives.

Concentration in the SPE method was tested with various amounts of each of the four components. As shown in Figures 2 and 3, linear relationships were observed for the amounts of three components (except DBT) applied to the columns, and GC peak areas were measured. The linear relationships were observed in the range of approximately 10–200 µg/mL for 2-HBP, 10–1,000 µg/mL for *o,o'*-biphenol, and 10–600 µg/mL for DBT-sulfone. These results imply that the DBT derivatives can be quantitatively concentrated by the SPE method. On the other hand, a linear relationship was not found with DBT, which is devoid of strong functional groups, as was expected from its behavior on the silica cartridge columns.

SPE application to sultine and sultone concentration

Application of the SPE analysis technique to sultine and sultone, intermediates produced in the course of DBT degradation by *Rhodococcus erythropolis* (11), was also examined. These two compounds were detected by GC as described above. As shown in Table IV, the recovery of sultone was high (98.5%, 1.6% CV), but the recovery of sultine was less (83.8%, 2.9% CV), and a small amount of 2-HBP (approximately 6% recovery as sultine) was detected in the same fraction (Figure 4). On the

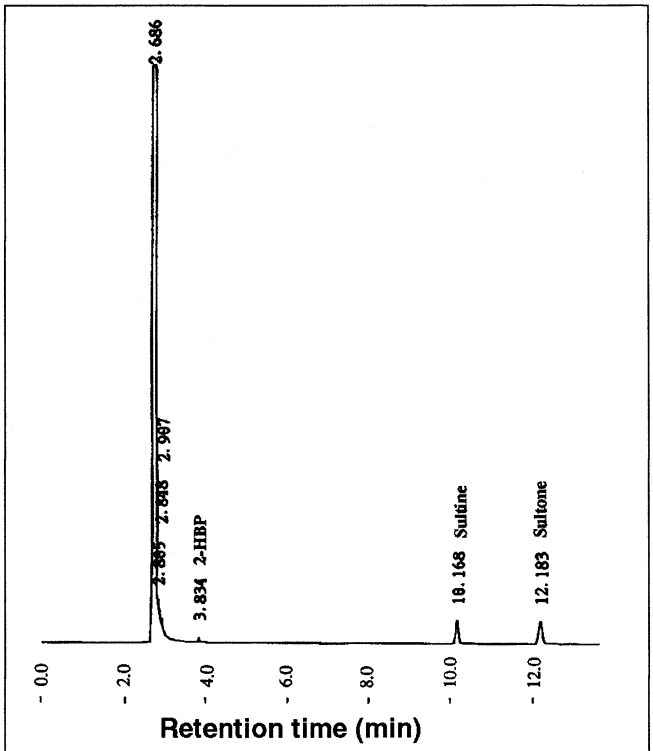

Figure 4. Gas chromatogram of sultine and sultone (100 µg/mL of each component).

Table II. Recovery of 2-HBP, *o,o'*-Biphenol, DBT-Sulfone, and DBT by SPE (EtOAc–IPA, 7:3)

Run no.	DBT (%)	2-HBP (%)	*o,o'*-Biphenol (%)	DBT-sulfone (%)
1	102.4	102.1	96.9	100.4
2	102.2	105.9	94.8	101.4
3	102.2	103.4	93.7	90.5
4	99.8	103.8	99.9	87.4
5	98.9	101.7	94.6	89.3
6	104.2	100.8	94.3	87.5
7	99.2	100.2	92.4	87.3
Mean	101.3	102.6	95.2	92.0
Standard deviation of 7 recoveries	1.986	1.959	2.463	6.217
CV (%)	2.0	1.9	2.6	6.8

Table III. Recovery of 2-HBP, *o,o'*-Biphenol, and DBT-Sulfone by SPE (EtOAc–EtOH, 6:4)

Run no.	2-HBP (%)	*o,o'*-Biphenol (%)	DBT-sulfone (%)
1	103.9	97.1	105.3
2	102.7	96.6	105.0
3	103.3	95.4	98.6
4	102.8	95.5	104.0
5	103.6	96.7	106.1
6	103.1	96.4	91.6
Mean	103.2	96.3	101.8
Standard deviation of 6 recoveries	0.4633	0.6853	5.658
CV (%)	0.45	0.71	5.6

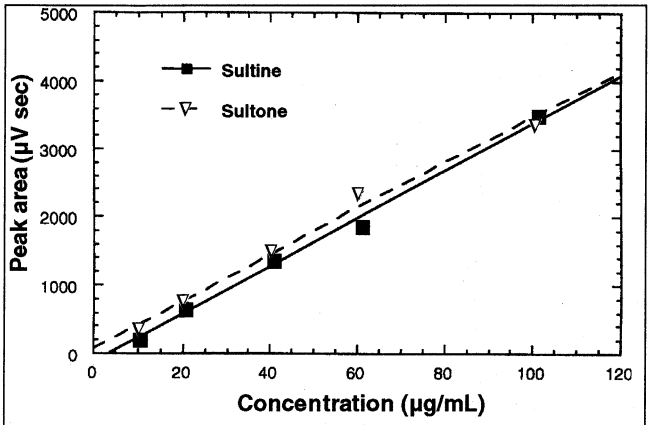

Figure 5. SPE calibration curves for sultine and sultone (10–100 µg/mL).

basis of these data, it seems likely that sultine may be converted partially to 2-HBP via a nonenzymatic process during the SPE procedure.

The concentration of sultine and sultone by the SPE technique was examined in the range of 10–1,000 µg/mL of each component (Figures 5 and 6). Linearity was observed (Figures 5 and 6) in the range of approximately 10–300 µg/mL for sultine and 10–200 µg/mL for sultone. These results suggest that sultine as well as sultone can be quantitatively concentrated despite the low recovery of the former.

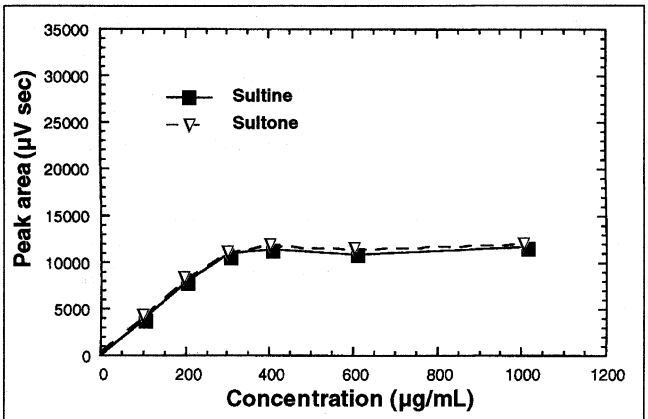

Figure 6. SPE calibration curves for sultine and sultone (100–1,000 µg/mL).

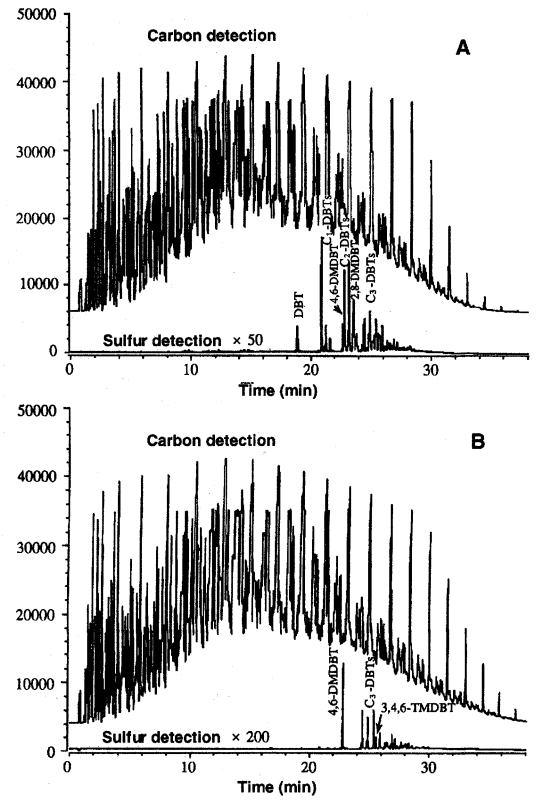

Figure 7. GC–AED chromatograms of gas oils.Total sulfur concentration: 1312 µg/mL (A) and 139 µg/mL(B). C_1-DBTs, methyl-DBTs, C_2-DBTs, dimethyl-DBTs, C_3-DBTs, trimethyl-DBTs, 4,6-DMDBT, 4,6-dimethyl-DBT, 2,8-DMDBT, 2,8-dimethyl-DBT, 3,4,6-TMDBT, and 3,4,6-trimethyl-DBT.

Characterization of biodesulfurization gas oil

Gas oils (diesel fuels) are complex mixtures of aliphatic, naphthenic, and aromatic hydrocarbons. Small amounts of

Table IV. Recovery of Sultine and Sultone

Run no.	Sultine (%)	Sultone (%)
1	81.4	97.7
2	85.1	95.2
3	86.3	99.9
4	80.0	98.2
5	84.2	98.8
6	81.8	98.4
7	86.2	99.9
8	85.5	99.8
Mean	83.8	98.5
Standard deviation of 8 recoveries	2.415	1.562
CV (%)	2.9	1.6

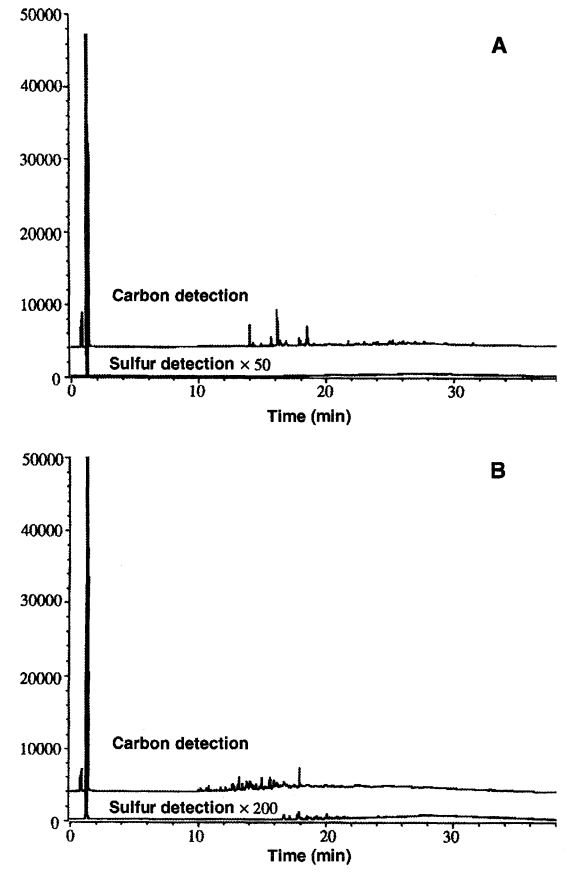

Figure 8. GC–AED chromatograms of polar components in BDS gas oils eluted from SPE. Total sulfur concentration: 1,312 µg/mL (A) and 139 µg/mL (B).

heterocyclic sulfur compounds such as DBTs are components of aromatic hydrocarbons.

Two kinds of gas oils containing 1,312 and 139 µg/mL of total sulfur, respectively, were analyzed by GC–AED (Figure 7). As illustrated in Figure 7, DBT, methyl-DBTs (C_1-DBTs), dimethyl-DBTs (C_2-DBTs), and trimethyl-DBTs (C_3-DBTs) were detected, but these two gas oils showed different GC–AED patterns.

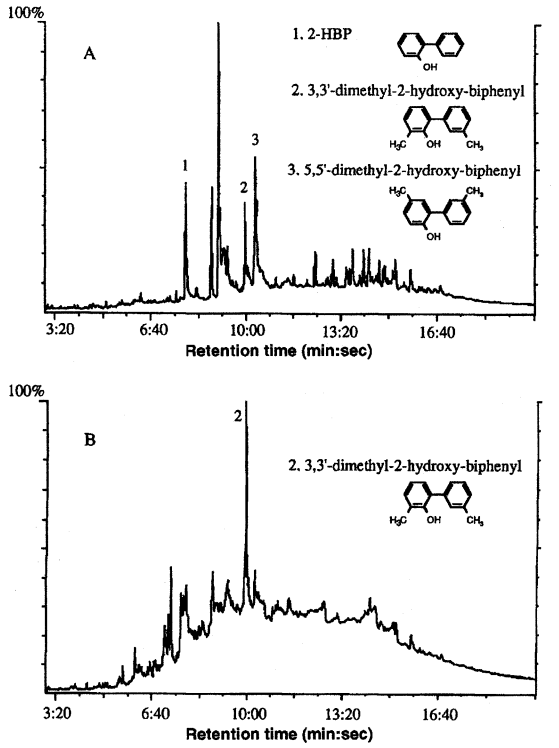

Figure 9. GC–MS chromatograms of polar components in BDS gas oils eluted from SPE. Total sulfur concentration: 1,312 µg/mL (A) and 139 µg/mL (B).

A combination of SPE with GC–AED or GC–MS analysis was used to evaluate the desulfurization of DBTs in gas oils by *Rhodococcus erythropolis* (Figures 8 and 9). As shown in Figure 8, sulfur was not clearly detected in the samples treated by SPE. Mass spectral identification of each of the peaks, which were expected to correspond to the bacterial metabolites, revealed that 2-HBP and other hydroxylated compounds such as 3,3'-dimethyl-2-hydroxy-biphenyl were produced by the microbial desulfurization (Figure 9).

Conclusion

A rapid, simple SPE procedure has been developed for the separation and concentration of some microbial metabolites in organic solvent such as kerosene. The recoveries of 2-HBP, *o,o*'-biphenol, DBT-sulfone, DBT, and sultone were in the range of 96–103%; CVs were less than 6%. However, the recovery of sultine was relatively low (84%, 3% CV) because of nonenzymatic, partial conversion, presumably to 2-HBP. All of these components, except DBT, can be quantitatively concentrated by SPE. Finally, the SPE technique, in combination with GC–MS or GC–AED, proved to be applicable to the identification and characterization of the microbial metabolites in actual gas oils.

Acknowledgment

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