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Supercritical Fluid Extraction of Polar Analytes Using in Situ Chemical Derivatization

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Analytical-scale supercritical fluid extraction (SFE) of polar organics from solid samples was enhanced by derivatizing the analytes to less polar species under static SFE conditions using reagents including trimethylphenylammonium hydroxide and boron trifluoride in methanol. After derivatization, the analytes were extracted with CO_2 using standard SFE techniques and analyzed without additional treatment using conventional capillary GC. Total time for derivatization and extraction can be <30 min; however some samples which contained high concentrations of reactive matrix components required multiple derivatization/extraction steps to achieve quantitative recoveries. The development and application of derivatization/SFE methods for the quantitative (>90%) derivatization and extraction recoveries of native and spiked acid herbicides ((2,4-dichlorophenoxy)acetic acid and Dicamba) from soil and sediment, microbial phospholipid fatty acids (as their methyl esters) from whole cells, and wastewater phenolics (as their methyl ethers) from water and from C_{18} sorbent disks is presented.

INTRODUCTION

Analytical-scale supercritical fluid extraction (SFE) has recently become an attractive alternative to conventional liquid solvent extraction for the recovery of organic analytes from solids and sorbent resins, both because of the drastic reduction in liquid solvents required and its potential to yield good recoveries with short (<1 h) extraction times. Several applications of qualitative and quantitative SFE have been reported and have been the subject of recent reviews.[1,2] However, the majority of reports to date have focused on relatively nonpolar analytes (e.g., analytes which are amenable to conventional GC analysis), while quantitative SFE of polar and ionic analytes has required the addition of organic polarity modifiers to CO_2 or the use of fluids (e.g., $CHClF_2$) which are less acceptable for routine applications.[3-6] In addition to the difficulty in extracting polar analytes from solid samples, many analysis schemes require that the analytes are derivatized prior to chromatographic analysis (particularly for capillary GC). As a result, a wide variety of postextraction derivatizing methods have been reported that serve to make the analytes easier to separate and/or detect.[7-15]

In situ chemical derivatization under SFE conditions has the potential to simultaneously solve the two related problems encountered during the determination of polar analytes, i.e., poor extraction efficiencies and the frequent need to derivatize polar analytes prior to chromatographic analysis. Although examples of enhanced chemical reactions under supercritical conditions have been reported, the potential for utilizing chemical derivatizations during analytical-scale SFE to increase extraction rates and recoveries has received only preliminary attention.[16-19] The ion-pairing methylating reagent trimethylphenylammonium hydroxide (TMPA) was chosen for the present study because of its solubility in methanol (a good SFE polarity modifier for polar organics, refs 3 and 5) and several previous applications of liquid-phase ion-pair derivatizations to a variety of polar analytes ranging from bentazone, phenylurea, and chlorinated acid herbicides to pharmaceuticals and biological acids.[7,8,12-14,20] This paper describes the development and use of chemical derivatization under SFE conditions for quantitative derivatization and extraction of polar organics from real-world samples including soil and sediment, wastewaters, "Empore" C_{18} sorbent disks, and microbes.

EXPERIMENTAL SECTION

Derivatization/SFE. Chemical derivatization/SFE was performed in a two-step manner by first derivatizing the analytes under static (no outlet flow from the sample cell) SFE conditions, then by recovering the derivatized analytes using dynamic SFE. The individual sequential steps in the derivatization/SFE procedure are (i) the sample is weighed into the extraction cell, and the derivatizing reagent (50–1000 μL) is added, (ii) the sample cell is placed into the heater (which was preheated to the extraction temperature) and immediately pressurized with 400 atm of CO_2, (iii) the derivatization and extraction step was allowed to occur under static SFE conditions for 5–45 min, and (iv) the outlet of the extraction cell was opened and the sample was extracted under dynamic SFE conditions for 5–15 min.

All extractions were performed using ISCO Model 260D syringe pumps (Lincoln, NE) to supply a constant pressure of SFC grade CO_2 (Scott Specialty Gases, Plumsteadville, PA) and either an ISCO Model SFX extraction unit or a simple extraction system fabricated in our laboratories. Dynamic SFE flow rates were controlled using 15-cm lengths of 25–30 μm i.d. fused-silica tubing (Polymicro Technologies, Phoenix, AZ) which resulted in flow rates of ca. 0.5–1 mL/min of pressurized CO_2 (measured as liquid CO_2 at the pump). Extracted analytes were collected by inserting the restrictor outlet into a vial containing ca. 3 mL of methanol or methylene chloride. Unless otherwise noted, temperatures for the derivatization and extraction steps were held at 100 °C (for the microbial phospholipids) or 80 °C (for the remaining samples). For wet soil samples, restrictor plugging from water freezing at the outlet of the restrictor during the dynamic extraction step was avoided by inserting the collection vial into a small beaker of room-temperature water.

The ISCO unit comes equipped with shutoff valves at the inlet and the outlet of the extraction cell (2.5 mL), allowing the static and dynamic extraction steps to be controlled by simply closing the outlet valve during the static step and opening the outlet valve for the dynamic step. The lab-built extractor consisted of an inlet shut-off valve (SSI Model 02-0120, Supelco, Bellefonte, PA) connected to a commercial extraction cell (Keystone Scientific, Bellefonte, PA) having a volume of either 0.5 mL (for the bacterial

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phospholipids) or 2.2 mL which was placed in a tube heater for temperature control. Individual components of the lab-built system were connected with $^{1}/_{16}$ in. o.d. (0.030 in. i.d.) stainless steel tubing. Rapid connection of the extraction cell to the supply line and to the outlet restrictor coupler was accomplished using fingertight fittings ("Slip-Free") from Keystone Scientific. The fused-silica restrictor was connected to the $^{1}/_{16}$ in. o.d. tubing of the Slip-Free connector with a $^{1}/_{16}$ in. tubing union and a Supelco M-2A ferrule (Bellefonte, PA). To perform the static derivatization/SFE step, the outlet restrictor of the extraction cell was flamed shut prior to assembling the cell so that no flow would result upon pressurization. After the static step was completed, the end of the restrictor (which was inserted into the collection solvent) was easily broken off with a small spatula to initiate the dynamic SFE flow. Since the outlet restrictors were inexpensive and simple to make, a new restrictor was used for each extraction. (As is the case for any high-pressure experiments, appropriate care must be taken to ensure safety of the operator should any failure in the extraction unit occur, particularly for the lab-built system. For the lab-built system used in these experiments, the tube heater, a piece of steel tubing wrapped evenly with heat tape under thermocouple control, included a shoulder on the inside to contain the cell components should failure occur. We have conducted several thousand SFE experiments with the cells provided by Keystone Scientific without any failures other than occasional minor leaks.)

Preliminary experiments with several of the samples discussed below showed no differences in the results from the commercial and the lab-built systems, and both systems were utilized throughout the study. Since the inlet valve of both the ISCO and the lab-built system was left open during the static step, there was initially concern that extracted species could diffuse into the $^{1}/_{16}$ in. tubing between the inlet valve and the extraction cell, thus causing carryover from sample to sample. However, no such carryover could be detected.

Derivatizing reagents were purchased as solutions in methanol and were used as received (except that methanol was evaporated under a gentle stream of nitrogen to prepare TMPA concentrations that were not commercially available). Trimethylphenylammonium hydroxide (TMPA) was obtained from three suppliers (Eastman Kodak Company, Rochester, NY; TCI-America, Portland, OR; and Sachem, Austin, TX) in concentrations ranging from 1.5 to 20% wt/vol. (Note: It is important to test the activity of this reagent, since two of the six lots of TMPA obtained during this study were inactive. The activity of individual lots was tested by applying the derivatization/SFE procedure with known spikes of the target analytes on sand. Fortunately, no loss of activity was observed during prolonged storage at ca. 4 °C for the active lots.) Boron trifluoride in methanol (12% w/w) was purchased from Supelco.

Extract Analysis. All extracts were analyzed by capillary GC without any additional sample preparation (except for a brief standing period at room temperature to allow the CO_2-saturated collection solvent to degas). Analyses were performed using Hewlett-Packard Model 5890 chromatographs equipped with either flame ionization (FID) or electron capture (ECD) detectors. GC/MS analyses were performed using a Hewlett-Packard Model 5985 or 5988 mass spectrometer. Chromatographic columns were either a 60-m dimethylpolysiloxane stationary-phase column (0.25 mm i.d., 0.1 μm film thickness, Restek, Bellefonte, PA) for the phospholipid-derived fatty acid methyl esters or a 25-m HP-5 column (0.25 mm i.d., 0.17 μm film thickness) for the remaining extracts. For quantitative studies, internal standards (methyl nonadecanoate for the microbial phospholipids and 1-chloronaphthalene for the 2,4-D and Dicamba analyses) were added to the extracts after the SFE step. All quantitations were based on gravimetrically prepared standard solutions of the methylated products.

Conventional analysis of the agricultural soil which contained native 2,4-D and Dicamba was performed by a laboratory which specializes in pesticide determinations. A rigorous extraction method was used which was based on two sequential extractions of 20-g soil samples with 80 mL of 0.5 N KOH in 10% KCl/water with the extraction vessel placed in a boiling water bath for 15 min and shaken mechanically for an additional 15 min (this procedure was repeated twice to hydrolyze any esters of the acid herbicides to their free acids for extraction into the basic water). The combined supernatants were washed with two 50-mL chloroform portions (to remove interferences), acidified to pH < 1.5 with H_2SO_4, and extracted three times with 70 mL of chloroform to recover the extracted pesticides (acid forms). The chloroform extracts were dried with 150 g of sodium sulfate, the solvent was evaporated, and the extracts were derivatized using diazomethane to form the methyl esters of the acid herbicides.

Table I. Recovery of 2,4-D Acid Spikes Using Derivatization/SFE

matrix	reagent	deriv time	% recovery ± SD[a]
sand	1.5% TMPA	5 min	98 ± 6
river sediment	1.5% TMPA	5 min	23 ± 2
river sediment	20% TMPA	5 min	63 ± 15
river sediment	20% TMPA	15 min	92 ± 4

[a] Each value represents three replicate extractions.

Conventional microbial lipid analysis was performed using an extraction procedure[21] involving the addition of approximately 25 mg of lyophilized microbial cells to 142.5 mL of a single phase solvent system consisting of chloroform:methanol:phosphate buffer (50 mM at pH 7.4), 1:2:0.8 (volume:volume:volume). After 3 h, the solvent system was partitioned into aqueous and organic fractions by the addition of 37.5 mL of chloroform and 37.5 mL of water. After the organic phase was concentrated and dried under N_2, the resultant lipid samples were derivatized by a mild alkaline methanolysis to produce fatty acid methyl esters.[15]

Samples. Lyophilized whole cells of the bacteria *Escherichia coli* were purchased from Sigma Chemical Co. (St. Louis, MO). River sediment was collected from the Red River in Grand Forks, ND, and air-dried before use. Analysis of the unspiked sediment showed no detectable levels of 2,4-D. Samples (2 g) were spiked at the approximate center of the sediment with 10 μL of 4 mg/mL 2,4-D in methanol, and the solvent was evaporated before extraction. Pesticide-contaminated agricultural soil (silty clay) was collected in Montana and used as received (ca. 7% water). Leachate from wood soot was prepared by stirring 50 g of soot from a residential wood stove chimney with 1 L of tap water for 18 h. Aliquots (50 mL) were then acidified to pH < 2, 3 mL of methanol was added, and the sample was filtered through Empore C_{18} sorbent disks (Analytichem International, Harbor City, CA). Prior to filtering the sample, the Empore disks were prewashed with 20 mL of methanol and 20 mL of water as per manufacturer's instructions.[22] The coal gasification wastewater was generated during the gasification of lignite coal and stored at 4 °C until used. The pH of the coal gasification wastewater was ca. 9.5 and was not adjusted before extraction.

RESULTS AND DISCUSSION

(2,4-Dichlorophenoxy)acetic Acid (2,4-D) and Dicamba from Soil and Sediment. Initial development of the derivatization/SFE procedure was performed using 20 ppm 2,4-D spikes into the approximate center of 2-g samples of clean sand (2.5-mL cell) with 0.5 mL of 1.5% TMPA reagent. Interestingly, varying the derivatization time from 5 to 30 min (at 100 °C) or the derivatization temperature from 60 to 140 °C (for 5 min) had no significant effect on the recovery of the spiked 2,4-D, and all conditions yielded essentially quantitative recovery of the spike as the methyl ester. For experimental convenience, a static derivatization time of 5 min at 80 °C followed by dynamic SFE for 15 min was chosen for subsequent recovery studies. Replicate extractions were performed, and the extracted species were collected in ca. 3 mL of methanol. The extracts were analyzed without additional treatment using GC/ECD with 1-chloronaphthalene as the internal standard. Quantitation was based on standard solutions of 2,4-D methyl ester.

As shown in Table I, good recoveries of spiked 2,4-D (recovered as the methyl ester) were obtained from the sand with only a 5-min derivatization time followed by 15 min of dy-

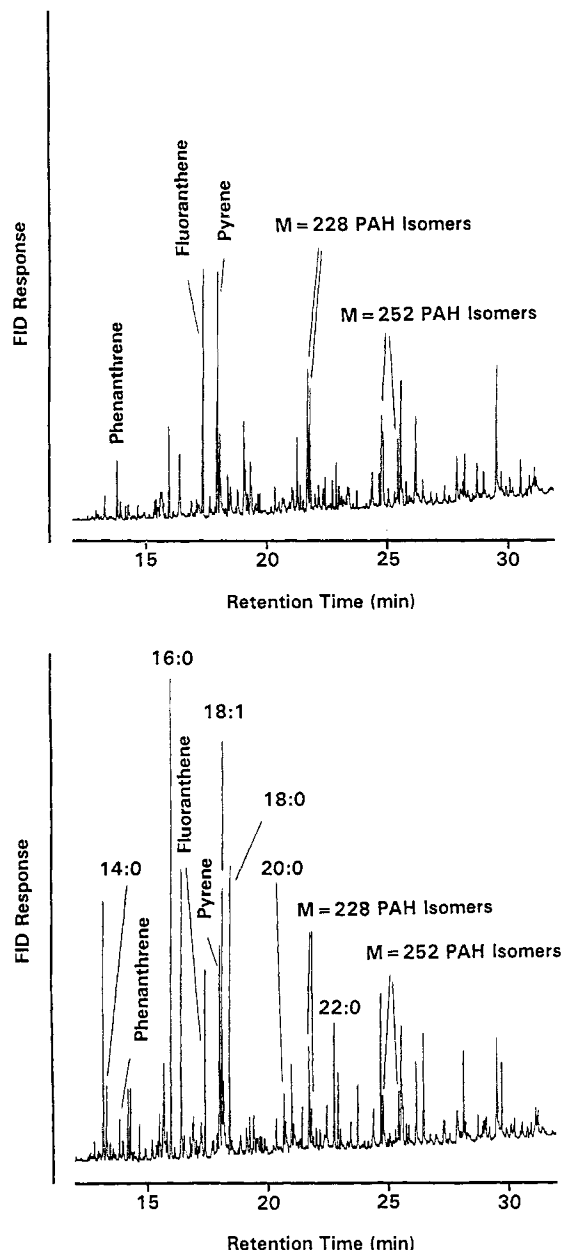

Figure 1. GC/FID chromatograms of river sediment extracts from supercritical methanol-modified CO_2 (top) and TMPA derivatization/SFE (bottom). Identifications are based on GC/MS analysis. The samples were injected splitless (0.5 min) at an oven temperature of 60 °C, followed by a temperature ramp to 330 °C at 8 °C/min. Shorthand notations for the fatty acid methyl esters indicate the parent fatty acid (e.g., 18:0 designates the methyl ester of octadecanoic acid) as defined in Table III.

namic extraction. While these results demonstrated that the derivatization, extraction, and collection system were quantitatively efficient, sand is likely an "easy" matrix from which to obtain good recoveries. Therefore, additional spiking experiments were performed in an identical manner with river sediment. Unfortunately, the recoveries dropped dramatically, and only 23% of the herbicide was recovered with the 5-min derivatization. A second sequential derivatization/SFE extraction performed on the spiked river sediment yielded ca. 35% more recovered 2,4-D methyl ester, and a third sequential step finally yielded quantitative (>90%) recovery of the 2,4-D as the methyl ester. The extracts from the sediment were dark yellow (compared to colorless extracts for the sand), indicating that several other reactive species (e.g., humic material) could be present on the sediment which could reduce the effectiveness of the TMPA reagent. This explanation is supported

Table II. Recovery of Native 2,4-D and Dicamba from Agricultural Soil

method	deriv time,[b] min	pesticide concentration, μg/g of soil ± SD[a]	
		2,4-D	Dicamba
conventional lab		0.95	0.40
SFE, 20% TMPA	15	0.53 ± 0.03	0.31 ± 0.02
	45	0.60 ± 0.10	0.29 ± 0.01
	3 × 15 (op 1)[c]	1.1 ± 0.1	0.47 ± 0.06
	3 × 15 (op 2)	1.3 ± 0.1	0.51 ± 0.05
SFE, 12% BF_3		1.1 ± 0.2	ND[a]

[a] Quantities reported for the derivatization/SFE extractions are the results of three replicate extractions. [b] Time used for the static derivatization step. The dynamic SFE step was 15 min in each case. [c] Three sequential static derivatization steps were used for each replicate sample. Operator 1 used the commercial extractor, and operator 2 used the laboratory-built unit.

by the fact that the sediment contains ca. 4% organic carbon (determined by thermal analysis). Additionally, as shown in Figure 1, the GC/FID chromatogram for the TMPA extract of the sediment contains a more complex mixture of "GC-able" organics than nonderivatized extracts prepared using CO_2 with methanol modifier (0.5 mL methanol added to the cell in the same manner as that used for the TMPA reagent). GC/MS analysis showed that both extracts contained polycyclic aromatic hydrocarbons (PAHs) and elemental sulfur (S_8), while the majority of additional species in the TMPA extract were methyl esters of carboxylic acids, probably of biological origin as discussed below.[15,23]

A higher concentration of TMPA (20%) was then used in an effort to increase the 2,4-D extraction efficiencies from the river sediment. As shown in Table I, 20% TMPA increased the recovery to ca. 60% using a 5-min derivatization step; however, a 15-min derivatization step was required to increase the recoveries to >90%. These results clearly demonstrate that the presence of reactive matrix components must be considered in selecting reagent concentrations and derivatization times with the in situ derivatization/SFE method.

Additional validation of the derivatization/SFE method was performed by comparing recoveries of native (not spiked) 2,4-D and Dicamba (3,6-dichloro-2-methoxybenzoic acid) from an agricultural soil with those obtained from a laboratory that specializes in pesticide analysis using conventional liquid solvent extraction techniques followed by diazomethane derivatization as discussed above. Derivatization/SFE was performed on the sample as received (ca. 7% water content) using 15-min static derivatization with TMPA (20% wt/vol) followed by 15-min dynamic SFE. Extracts were collected in methanol and analyzed using GC/ECD.

As shown in Table II, the derivatization/SFE procedure using one 15-min derivatization step yielded 56% of the 2,4-D and 77% of the Dicamba compared to the conventional laboratory extractions. These somewhat low recoveries obtained with one 15-min derivatization step might be expected since the soil contained ca. 5% organic matter which could react with the TMPA, as was observed for the derivatization of the 2,4-D spikes on the river sediment discussed above. To further determine whether the reagent was being exhausted or whether the derivatization/extraction procedure was simply slower for the native than the spiked herbicides, the static derivatization/SFE step was increased to 45 min (followed by 15 min of dynamic extraction). No significant increase in the amounts of the extracted herbicides resulted from the longer derivatization time (Table II). Increasing the dynamic extraction time from 15 to 45 min also failed to increase the recoveries. Preliminary experiments showed that increasing the reagent volume from 0.5 to 1.0 mL did yield higher ex-

traction efficiencies, but this approach was not satisfactory because very low flows often resulted during the dynamic extraction step. Although it was initially thought that the restrictors were plugging with matrix material, careful observation demonstrated that a slow stream of liquid methanol was flowing out of the restrictor outlets. Apparently, the use of 1.0 mL of reagent in the 2.5 mL cell resulted in a two-phase system (liquid methanol and the supercritical fluid), which, when liquid methanol entered the restrictor, resulted in near zero flows.

Since these results indicated that the derivatization step was reagent limited, four sequential derivatization/SFE steps (15-min static followed by 15-min dynamic SFE) were performed on a single sample and demonstrated that ca. 90–95% of the total extractable 2,4-D and Dicamba were found in the first two extracts, while no detectable herbicides were found in the fourth extract. On the basis of these multiple extraction results, the soil sample was again extracted using three sequential derivatization/SFE steps (15-min static followed by 15-min dynamic SFE) while the extract was collected from each step in a single vial containing 3 mL of methanol. Two sets of triplicate extractions were performed using these conditions by two different operators: one operator using the ISCO system, and one operator using the lab-built system. As shown in Table II, both operators obtained results which agreed well with each other and were slightly higher than the values obtained by the conventional extraction/derivatization methods. Note also that for all of the SFE results reported in Table II, the standard deviations of triplicate extractions were acceptable (generally <10% RSD), which demonstrates that the derivatization/SFE procedure was reproducible and that the 1-g samples were representative of the bulk sample matrix.

As shown in Figure 2 (top), the unfractionated TMPA/SFE extract from the agricultural soil yielded a fairly complex chromatogram with several ECD-sensitive organics which were not present in the TMPA/SFE reagent blank (middle chromatogram) in addition to the 2,4-D and Dicamba methyl esters (identifications of the 2,4-D and Dicamba methyl esters were confirmed by GC/MS analyses of the extracts). One advantage of chemical derivatization techniques is their potential to selectively derivatize and extract target compounds. For example, BF_3/methanol has been reported to efficiently methylate 2,4-D, but not Dicamba.[24] To investigate the potential for selectively derivatizing and extracting 2,4-D using SFE conditions, the same soil sample was derivatized using BF_3 in methanol (12% w/w) using a single 15-min derivatization step (80 °C, 400 atm) followed by a 15-min dynamic extraction step in a manner identical to that used for the TMPA derivatizations. As shown in Figure 2 (bottom), the SFE extract using BF_3 contained the methyl ester of 2,4-D, but no peak for Dicamba could be detected. In addition, the BF_3 extract contained fewer ECD-responsive organics than the TMPA extract. The BF_3 extract was also light yellow while the TMPA extract was dark brown. Derivatization/SFE with a single 15-min derivatization step also yielded quantitative recoveries for 2,4-D, as shown in Table II. These results demonstrate that in situ derivatization under SFE conditions does not necessarily require an ion-pairing reagent such as TMPA. It is also interesting to note that, while the BF_3/methanol SFE did not yield derivatized Dicamba, derivatization of the BF_3/methanol extract with TMPA (by adding 0.5 mL of 20% TMPA to the extract and heating for 2 h at 80 °C) did show the methylated Dicamba peak, presumably because underivatized Dicamba extracted in the methanol-modified CO_2 during the SFE step.

It should be noted that, in addition to causing methylation of the target analytes during the SFE step, TMPA may aid

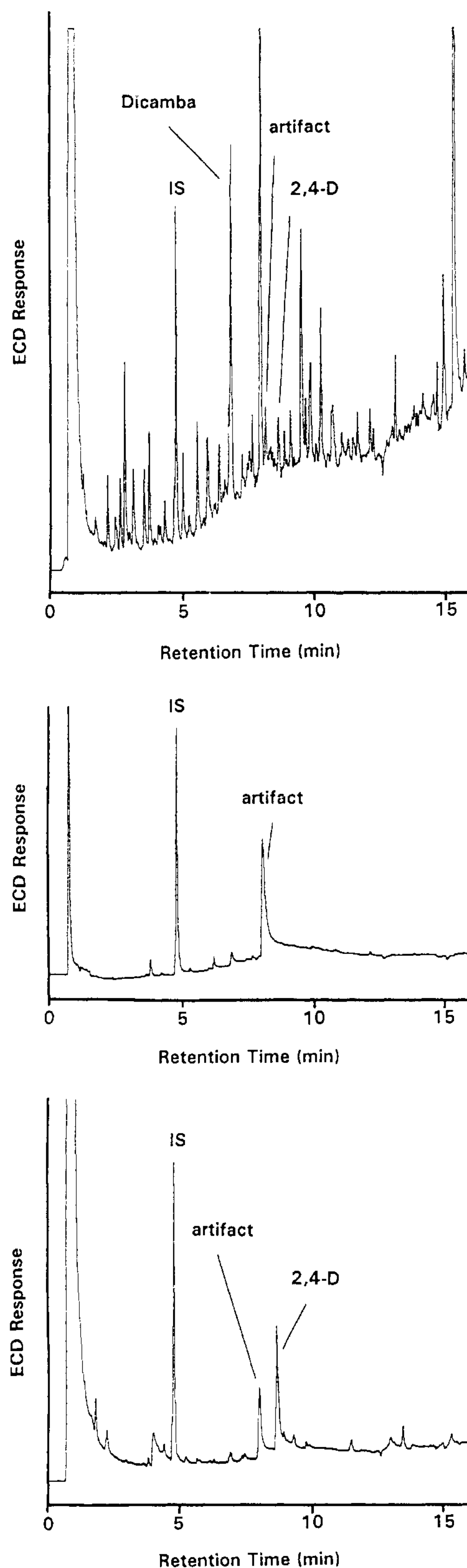

Figure 2. GC/ECD chromatograms of derivatization/SFE extracts of agricultural soil using TMPA/methanol (top chromatogram) and BF_3/methanol (bottom chromatogram) reagents. Injections were performed in the split mode (ca. 1:20) at an oven temperature of 100 °C. After a 2-min isothermal step, the oven was programmed to 330 °C at 8 °C/min. IS denotes the 1-chloronaphthalene internal standard. The derivatization blank (middle chromatogram) was generated in an identical manner to the TMPA-derivatized sample except that no sample was added to the cell. All background peaks were chromatographically resolved from the Dicamba and 2,4-D methyl ester peaks. The peak marked "artifact" was found in all extracts.

the extraction and derivatization by ion-pairing interactions and TMPA has also been previously used as a methylating reagent in heated GC injection ports.[7,8,12-14,20] Thus, it is possible that some derivatization of the extracted analytes may occur in the GC injection port after the SFE step. For example, when a solution of 2,4-D acid in methanol is injected with TMPA into a conventional split/splitless injector heated to 300 °C, the methyl ester of 2,4-D is detected. Since increasing the concentration of TMPA yielded better recoveries of 2,4-D from the sediment samples, while adding additional TMPA to the extracts after SFE did not, reaction of 2,4-D with TMPA must have occurred during the SFE extraction step. The use of on-column injection (i.e., an unheated injection port) also indicates that the methylation occurs in the SFE cell. For example, analysis of the microbial phospholipid extracts (discussed later) using cooled on-column injection shows the same fatty acid methyl esters as the analysis using injection into a heated split/splitless injection port. Derivatization with BF_3/methanol reagent is less ambiguous, since it does not cause methylation in the GC injection port (e.g., coinjection of 2,4-D with BF_3/methanol does not yield any detectable methyl ester of 2,4-D), yet SFE/derivatization using BF_3/methanol yields good recoveries of 2,4-D (Table II), clearly demonstrating that the derivatization occurred under the SFE conditions.

Microbial Phospholipids. The presence of fatty acid methyl esters in the river sediment extracts indicated that the derivatization/SFE approach might be useful for the rapid derivatization and extraction of fatty acid esters from biological lipids. Since cell membrane phospholipid-derived fatty acids are frequently used to identify bacteria and other microbes by capillary GC following liquid solvent extraction, concentration, lipid class fractionation (if required), and derivatization to their methyl esters,[15,23] whole bacteria were chosen as a test sample for the derivatization/SFE procedure. Whole lyophilized *E. coli* cells were used since phospholipids are the predominant source of fatty acids in their extracts. While no detectable recovery of the underivatized phospholipids using supercritical CO_2 was obtained, derivatization/SFE using the TMPA reagent yielded good recoveries of the phospholipid-derived fatty acids. As was the case for the 2,4-D spikes on sand, preliminary investigations demonstrated that static derivatization times longer than 15 min yielded no increase in the amounts of fatty acid methyl esters in the extracts. Therefore, each extraction was performed with a 15-min static and a 15-min dynamic step in a 0.5-mL extraction cell. Sequential derivatization/extractions on single samples using 50-μL aliquots of 1.5% and 15% TMPA indicated that either concentration was sufficient (based on the lack of significant fatty acid methyl ester peaks in the second extract) to perform the extraction on 10- to 20-mg samples. However, to avoid any possible matrix effects on the activity of the reagent, 15% TMPA was used for subsequent extractions.

Figure 3 shows the phospholipid-derived fatty acid methyl esters resulting from the derivatization/SFE of a 10-mg sample of *E. coli.* A second derivatization/SFE extraction of the same sample was performed in an identical manner and resulted in no detectable peaks, indicating that the first 15-min derivatization and 15-min extraction procedure was sufficient to quantitatively derivatize and extract the phospholipid fatty acids as their methyl esters. Note that a large chromatographic peak which elutes near the solvent peak is seen in the chromatogram. This peak has been identified by GC/MS as *N,N*-dimethylaniline which results from the TMPA reagent. The presence of this peak presents no problem for the fatty acid methyl ester analysis, and even though several hundred injections of TMPA extracts have been performed, no degradation in chromatographic performance has been observed.

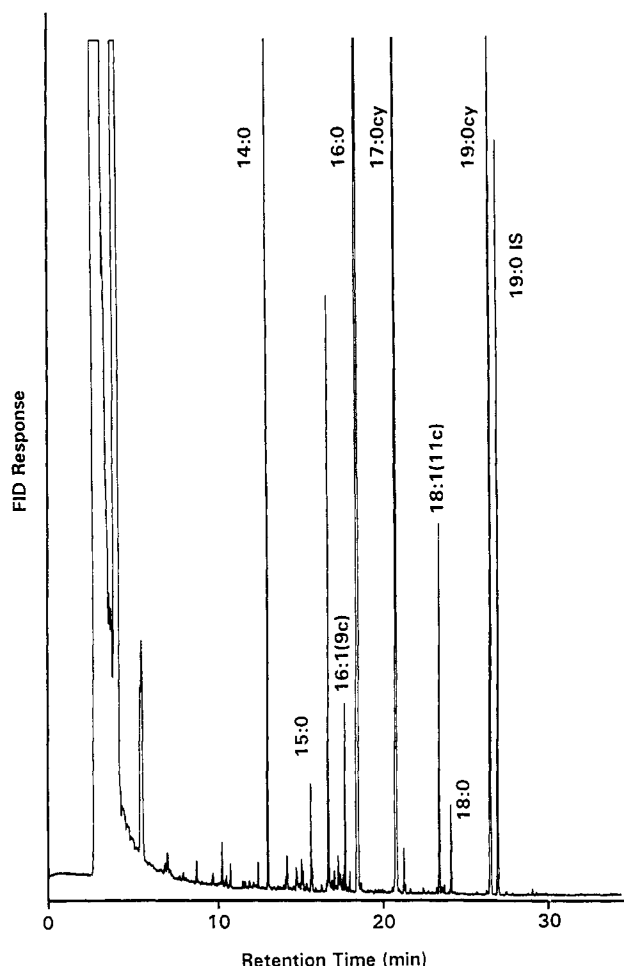

Figure 3. GC/FID chromatogram of the fatty acid methyl esters from the derivatization/SFE of a 10-mg sample of *E. coli.* The extract was injected split at an oven temperature of 100 °C followed by temperature ramps at 10 °C/min to 150 °C and then 3 °C/min to 280 °C. Shorthand notations for the fatty acid methyl esters indicate the parent fatty acid (e.g., 18:0 designates the methyl ester of octadecanoic acid) as defined in Table III.

Since TMPA was introduced into the cell as a methanol solution, it is possible that a portion of the phospholipids could be extracted without being derivatized to their methyl esters, since methanol would act as a polarity modifier during the CO_2 extraction. If this were to occur, GC analysis would detect artificially low concentrations of the fatty acid methyl esters, even though a second derivatization/SFE extraction would show no detectable species. To further investigate whether the derivatization/SFE procedure yielded quantitative derivatization and recovery of the fatty acid methyl esters, a quantitative comparison of the conventional liquid solvent extraction method[21] with the derivatization/SFE procedure for the determination of phospholipid fatty acids of *E. coli* was performed.[21] Triplicate chloroform/methanol/water extractions (followed by mild alkaline methanolysis derivatization, ref 21) and triplicate derivatization/SFE extractions using 50 μL of the 10% TMPA reagent under the conditions described above were performed. After the extractions and derivatizations were completed, 50 μmol of the internal standard (methyl nonadecanoate, 19:0) was added as an internal standard, and each extract was analyzed using GC/FID.

The quantitative comparisons of the conventional and derivatization/SFE methods are shown in Table III. The derivatization/SFE extracts showed good quantitative agreement with the conventional liquid solvent method (albeit with somewhat poorer reproducibilities), demonstrating that

Table III. Quantitative Comparison of Conventional Liquid Extraction and in Situ Derivatization/SFE for Phospholipid Fatty Acids from *E. coli*

	fatty acid concentration, μg/g dry wt								% of total[b]	
	conventional				derivatization/SFE					
fatty acid[a]	trial 1	trial 2	trial 3	X ± SD	trial 1	trial 2	trial 3	X ± SD	conventional	SFE
14:0	5.3	5.1	5.2	5.2 ± 0.1	6.0	5.2	6.4	5.9 ± 0.6	5.9	6.2
15:0	0.6	0.6	0.6	0.6 ± 0.0	0.6	0.5	0.7	0.6 ± 0.1	0.7	0.6
16:1(9c)	0.8	0.7	0.7	0.7 ± 0.1	1.1	1.0	1.2	1.1 ± 0.1	0.8	1.2
16:0	41.5	39.9	39.6	40.3 ± 1.0	47.8	39.9	47.5	45.1 ± 4.4	46.0	47.5
17:cy	24.2	23.3	22.9	23.5 ± 0.7	26.7	22.7	25.7	25.0 ± 2.1	26.8	26.3
18:1(11c)	1.9	1.8	1.8	1.9 ± 0.1	2.4	2.1	2.3	2.3 ± 0.1	2.2	2.4
19:0cy	0.6	0.5	0.5	0.5 ± 0.1	0.6	0.5	0.6	0.6 ± 0.1	0.6	0.6
18:0	15.4	14.9	14.6	15.0 ± 0.4	15.6	13.5	14.2	14.4 ± 1.0	17.0	15.1

[a] Shorthand designations of the fatty acid methyl esters refer to tetradecanoic acid (14:0), pentadecanoic acid (15:0), *cis*-hexadec-9-enoic acid (16:1(9c)), hexadecanoic acid (16:0), 2-hexylcyclopropaneoctanoic acid (17:0cy), *cis*-octadec-11-enoic acid (18:1(11c)), octadecanoic acid (18:0), and 2-hexylcyclopropanedecanoic acid (19:0cy). [b] Average percent of the total extracted phospholipid-derived fatty acids for each extraction method.

the 30-min derivatization/SFE method is capable of yielding reasonable quantitative results. The SFE method yielded somewhat higher concentrations than the conventional liquid solvent method, but the proportion of the individual fatty acids (as a percent of the total) was nearly identical for the two methods.

Phenol-Contaminated Waters. The potential for applying derivatization/SFE to polar analytes in water samples was investigated using two phenol-contaminated waters, a coal gasification wastewater, and a wood soot leachate. Since an earlier report demonstrated that extraction of phenols directly from water using supercritical CO_2 was possible,[25] direct derivatization of the coal gasification wastewater was attempted by placing 1 g of clean sand into a 3.5-mL cell (to increase surface area), adding 1.0 mL of the wastewater and 1.0 mL of 20% TMPA reagent, and performing a 15-min derivatization followed by a 15-min dynamic extraction. (The cell was inverted so that the flow of the supercritical fluid was from bottom to top, as opposed to the normal top to bottom configuration used for the solid samples, to avoid the water sample being flushed out of the cell during the dynamic SFE step.) As shown in Figure 4, phenol (which accounted for ca. 90% of the organic content in the water) was efficiently derivatized to anisole, although traces (<2%) of the phenol were not derivatized. The other significant phenolics in the water, cresol isomers, were also found as their methyl ethers. (Previous analysis of methylene chloride extracts of the acidified wastewater showed only the phenols and no detectable anisole or cresol methyl ethers).

The feasibility of forming derivatives with ECD-sensitive functional groups was also investigated using the direct derivatization of the coal gasification wastewater under the conditions described above, except that 20% TMPA reagent was mixed in a 1:4 ratio with 2,2,2-trifluoroethanol. As shown in Figure 5 (bottom), the GC/ECD chromatogram showed no significant peaks when the derivatization was performed with pure TMPA reagent; however, a peak for the ether formed from phenol and trifluoroethanol was found when the derivatization/SFE was performed using the mixed reagent (top chromatogram). GC/MS analysis verified the identity of the peak to be (2,2,2-trifluoroethyl)phenyl ether. While no attempt has been made to optimize the trifluoroethylation method, these results do demonstrate the possibility of enhancing selectivity and sensitivity of derivatization/SFE extractions by adding detector-specific functional groups.

The direct derivatization/SFE of the coal gasification wastewater successfully formed and extracted the methyl ethers of the phenols, but such an approach has inherently limited sensitivities since direct derivatization/SFE of water samples larger than a few milliliters is experimentally difficult.

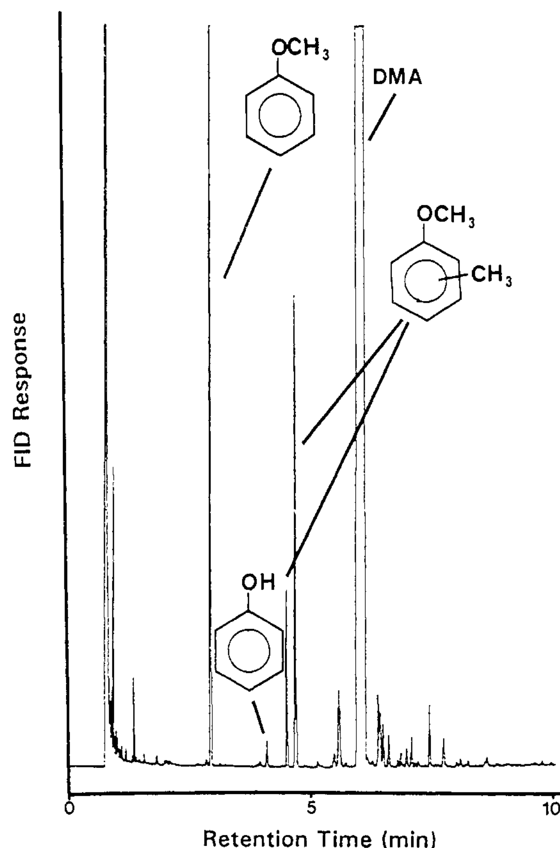

Figure 4. GC/FID chromatogram of the derivatization/SFE extract from phenol-contaminated coal gasification wastewater using the TMPA reagent. Identifications were by GC/MS. The peak marked DMA was identified as *N*,*N*-dimethylaniline, a degradation product of the TMPA reagent. The temperature program was 50 °C (2-min hold) followed by a temperature ramp at 8 °C/min to 220 °C.

Although the majority of the sample water remained in the extraction cell, it is also likely that significant water is extracted with the supercritical CO_2/methanol and thus is present in the collection solvent after the derivatization/SFE procedure is completed. An alternate approach, preconcentration of water samples on solid sorbents (e.g., Empore C_{18} sorbent disks) followed by derivatization/SFE, may be a more appropriate approach for many water samples since the only limitation on the size of sample that can be extracted is the amount of water that can be filtered through the sorbent without breakthrough losses of the target analytes.

To investigate this approach, 50-mL samples of the wood soot leachate were acidified and filtered through Empore C_{18}

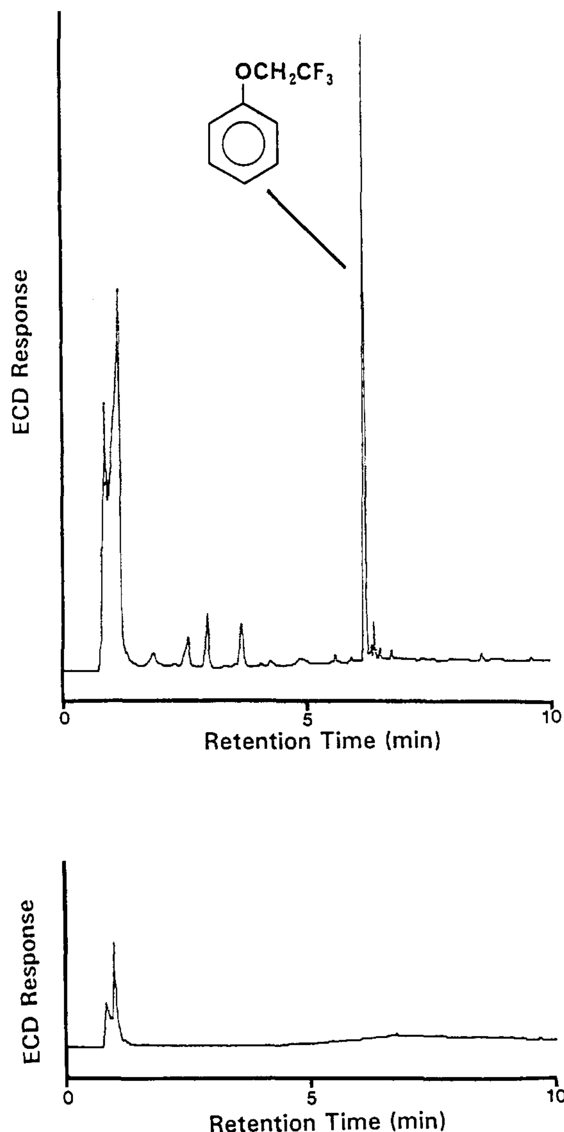

Figure 5. GC/ECD chromatograms of the derivatization/SFE extract from phenol-contaminated coal gasification wastewater using a 1:4 mixture of the TMPA reagent with 2,2,2-trifluoroethanol (top) and pure TMPA reagent (bottom). The temperature program was 50 °C (2-min hold) followed by a temperature ramp at 8 °C/min to 220 °C.

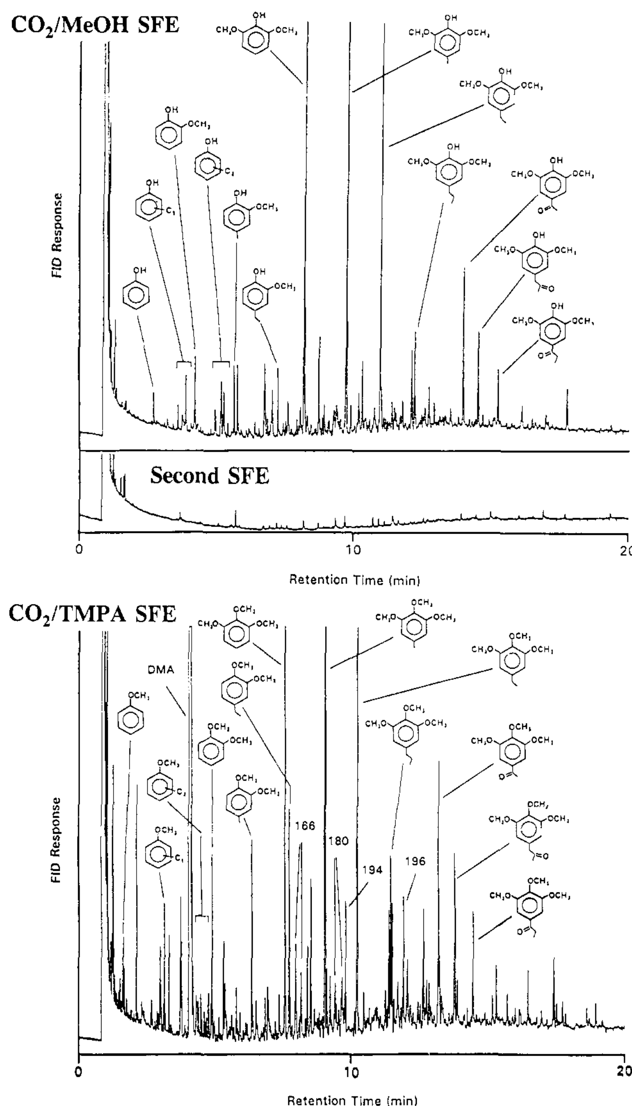

Figure 6. GC/FID chromatograms of wood soot leachate collected on "Empore" C_{18} sorbent disks and extracted using methanol-modified CO_2 (top) and derivatization/SFE (bottom). The peak marked DMA was identified as *N*,*N*-dimethylaniline, a degradation product of the TMPA reagent. The middle chromatogram shows a second extraction of a disk. The temperature program was 60 °C (2-min hold) followed by a temperature ramp at 8 °C/min to 330 °C.

sorbent disks according to the manufacturer's instructions as described above. The disks were then rolled into a cylinder and placed into the 2.5-mL (ISCO) extraction cell (no effort was made to dry the disks). Either 1 mL of pure methanol or 1 mL of 10% TMPA/methanol reagent was added, and the sample was extracted at 500 atm using a 5-min static step followed by a 10-min dynamic extraction. As shown in Figure 6 (top), the CO_2/methanol extract contained a fairly complex mixture of phenol, guaiacol (2-methoxyphenol), syringol (2,6-dimethoxyphenol), and their substituted analogues similar to the species previously identified in atmospheric wood smoke.[26] (Interestingly, the chromatogram of a pure CO_2 extract of the disks was virtually identical to that of the methanol-modified CO_2, demonstrating that the methanol modifier is not needed to extract these phenolics from the C_{18} stationary phase.) The TMPA extract (Figure 6, bottom) showed only the methyl ether derivatives of all of the phenolic species, and in general, the TMPA extract chromatogram was similar to that of the CO_2/methanol extract except that the major chromatographic peaks were shifted to earlier retention times (as would be expected for the methyl ethers).

The TMPA extract also contained several additional peaks that did not correspond to phenolics in the methanol-modified CO_2 (and that were not in a TMPA blank performed on a sorbent disk). These peaks, indicated on Figure 6 by molecular weights 166, 180, 194, and 196 all show a base peak in their mass spectra at M − 31, corresponding to the loss of a methoxy radical. All spectra are consistent with those expected for methyl esters of methoxylated benzoic acid congeners which correspond to the major methoxylated phenols (i.e., guaiacol and syringol and their alkyl derivatives). On the basis of their mass spectra, these species have tentatively been identified as the methyl esters of methoxybenzoic acid, methoxymethylbenzoic acid, and dimethylmethoxybenzoic acid (for M = 166, 180, and 194) and the methyl ester of dimethoxybenzoic acid (M = 196). Since the analogous underivatized benzoic acids were not found in the methanol-modified CO_2 extract, it is speculated that the derivatization procedure was necessary for their extraction from the sorbent disk, or it is also possible that their poor chromatographic properties prevented the underivatized acids from being detected.

GC/MS analysis of the TMPA-derivatized extract failed to find any of the original phenols, which indicates that the 5-min derivatization step was at or near 100% efficient at converting the phenols to their methyl ethers. The extracted

disks were also extracted a second time in a manner identical to the first extraction using pure CO_2 (15-min dynamic extraction), and the TMPA/methanol reagent (5-min static and 5-min dynamic SFE). As shown in Figure 6 (middle) the second extracts of the sorbent disks with CO_2 showed no significant (<1% of the amount found in the first extract based on integrated peak area) additional extracted peaks. The second TMPA derivatization/SFE also showed no significant peaks (except for the *N,N*-dimethylaniline product from the TMPA reagent). On the basis of the results of these multiple extraction studies, the 15-min SFE with CO_2 was sufficient to quantitatively remove the wood soot phenolics from the sorbent disk, while the 5-min TMPA derivatization followed by 10 min of dynamic SFE was sufficient to quantitatively methylate and extract the wood soot phenolics.

CONCLUSIONS

In situ chemical derivatization under supercritical fluid extraction (SFE) conditions can be used to reduce the polarity of target analytes during a static SFE step. This process makes polar species easier to extract and can yield extracts that are ready for capillary GC analysis without additional sample preparation. Quantitative derivatization and extraction of phospholipid fatty acids from whole bacteria cells, acid herbicides from soil and sediment, and phenols from wastewater and sorbent disks has been achieved with total derivatization and extraction times ranging from 15 to 90 min. For samples with high concentrations of reactive matrix components (e.g., organic-laden soils and sediments), the derivatization/extraction efficiencies can be dependent on the concentration of the derivatization reagent, and multiple derivatization steps may be required. Selectivity can be achieved by the proper choice of derivatization reagent, as demonstrated by the selective derivatization/extraction of 2,4-D from agricultural soil in the presence of Dicamba using BF_3/methanol. While this report focused on the use of the ion-pairing methylating reagent TMPA, a very wide range of derivatizing reagents is available which should be useful for chemical derivatization/SFE. The proper choice of derivatizing reagent should allow selective and quantitative derivatization and extraction of target compound classes, as well as increase overall sensitivity by forming derivatives that have high response in selective detectors (e.g., halogenation reactions followed by GC analysis with an electron capture detector).

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