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Determination of Polybrominated Biphenyls in Serum by Negative Chemical Ionization Mass Spectrometry

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Individual polybrominated biphenyls (PBBs) from Firemaster BP-6 (an environmental pollutant) were quantified in serum by selected ion monitoring of bromine anions (m/e = 79) obtained by dissociative electron capture negative chemical ionization (nitrogen reagent gas). Internal standard was decachlorobiphenyl. Serum (1 mL) was deproteinized (methanol) and extracted with hexane–diethyl ether and the extract was purified (Florisil), evaporated to dryness and reconstituted with hexane. Detection limits were 10 pg/mL for pentabromobiphenyls and 35 pg/mL for other PBBs, an approximate 20-fold improvement over electron capture gas chromatography. The technique permits determination of overall PBB burden in the general population (1 ng/mL or less of hexa isomer) and quantification of individual PBBs in serum as well as in separated blood compartments of exposed individuals.

The accidental addition of polybrominated biphenyls (PBBs) as Firemaster BP-6 (an industrial fire retardant), instead of magnesium oxide, to cattle feed resulted in the introduction of these chemicals into the food chain in Michigan in 1973. Symptoms and clinical abnormalities in humans (*1*) and in domestic and experimental animals (*2*) were reported, and it was soon realized that the excretion of PBBs was extremely slow (*3*, *4*).

Information on the body burden and absorption of PBBs has, in most studies, been obtained utilizing gas chromatography with electron capture detection. The sample preparation methodology has been discussed (*5*), the gas chromatographic properties of PBBs have been investigated (*6*, *7*), and an interlaboratory methods validation test has been conducted (*8*). Detection limits for the most abundant hexa isomer (2,2′,4,4′,5,5′ isomer), in terms of which serum and tissue levels have generally been expressed, have been quoted in the 0.2–1.4 ng of Firemaster BP-6/mL of serum range. Very few values have been reported below 1 ng/mL because of difficulties in the identification of the analytes by electron capture gas chromatography. When serum levels were used to represent the body burden of PBBs, levels of the order of 1 ng/mL were reported for a large number of subjects (*9*).

The contention that the biological effects of PBB exposure are caused by one or more of the halogenated biphenyls present (and not by contaminants) is supported by studies showing that PBB congeners produce a 3-methylcholanthrene type induction of hepatic microsomal enzymes in experimental animals (*10*, *11*) and that degradation products of PBBs caused by sunlight (identified by mass spectrometry) induced hyperkeratosis in rabbit ears (*12*). The possibility that PBB congeners are preferentially accumulated or excreted has been suggested in studies involving Michigan chemical workers and farmers (*13*, *14*) as well as in experimental animals (*15*). Following a report on altered lymphocyte behavior in a small group of Michigan residents (*16*), PBBs were identified by positive chemical ionization mass spectrometry in separated blood compartments of a few Michigan chemical workers (*17*).

These investigations suggest that after an exposure to Firemaster BP-6 one should consider the individual constituents separately because their relative amounts do not stay constant and their biological activities may differ. Furthermore, when individual PBBs are to be determined in blood compartments (*17*), very low levels are expected. Continued work in these areas thus requires lower detection limits for

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the individual PBBs and more specificity than electron capture gas chromatography can provide.

Investigations on the negative chemical ionization mass spectrometry of polychlorinated benzo-*p*-dioxins (*18*) and various polyhalogenated hydrocarbons (*19*) revealed the presence of free halogens under several experimental conditions. The present technique is based upon the selected ion monitoring of bromine anions which form in large abundance due to dissociative electron capture when PBBs are ionized by negative chemical ionization mass spectrometry (*20*).

EXPERIMENTAL SECTION

Reagents. Firemaster BP-6, consisting of a mixture of two penta-, four hexa-, four hepta-, and two octabromobiphenyls, all available individual brominated biphenyls, and decachlorobiphenyl were purchased from Ultra Scientific Inc. (Hope, RI). All solvents were of HPLC quality (Fisher Scientific Co., Springfield, NJ). Other chemicals were of the highest purity commercially available and were used without further purification. Gas chromatographic supplies were purchased from Supelco Inc. (Bellefonte, PA); gas chromatographic carrier and chemical ionization reagent gases were obtained from Union Carbide Co. (Linde Division, Somerset, NJ). ^{14}C-labeled 2,2′,4,4′,5,5′-hexabromobiphenyl was obtained from New England Nuclear Co. (Boston, MA). Specific activity was 10.2 mCi/mmol. The purity of the product was 96% as determined by high-performance liquid chromatography and by mass spectrometry. Scintillation fluid (Betafluor) was obtained from National Diagnostics Co. (Somerville, NJ).

Extraction of PBB from Serum. The extraction of the PBBs from serum was based on methods employed by several investigators (*13*, *16*). To 1 mL of serum the internal standard (dissolved in hexane) was added to give a concentration of 800 pg/mL. After the mixture was vortexed for 15 s, 2 mL of methanol was added and the sample vortexed briefly. The precipitated protein was not removed (see Results and Discussion). The PBBs were extracted by vortexing for 1 min with two successive 5-mL portions of hexane:diethyl ether mixture (1:1). The organic layers were combined, dried with anhydrous sodium sulfate, and evaporated with dry nitrogen to a volume of approximately 0.5 mL. The extract was next filtered through a bed of Florisil. The filter comprised a Pasteur pipet stoppered with glass wool and dry-packed with Florisil to a height of 5 cm. The concentrated extract was placed on the column and eluted with 6 mL of hexane. The effluent was evaporated to dryness using dry nitrogen in a 6-mL screw-cap vial. The residue was taken up with 25 μL of hexane and a 5-μL aliquot was injected into the combined gas chromatograph–mass spectrometer.

Recovery Experiments. A quantity of 20 ng of the radiolabeled hexabromobiphenyl was added to 1 mL of serum samples. Sample preparation proceeded as described above until the dry residue was obtained. To this 2 mL of Betafluor scintillation cocktail was added, and the samples were counted for 4 min in a Beckman Model 3255 scintillation counter. For checking the amount of PBB bound to proteins, the precipitated proteins were physically removed and independently extracted. The residues from these extracts were counted as above.

Gas Chromatography–Mass Spectrometry. All analytical work was carried out on a combined gas chromatograph–quadrupole mass spectrometer (Finnigan, Model 3300)–computer (Finnigan, Model 6000) system equipped with a pulsed positive ion–negative ion chemical ionization (PPINICI) accessory and software for selected ion monitoring.

A 1 m long, 2 mm i.d. glass tube filled with SP-2100 (100% methyl silicone) on Supelcoport (100/120 mesh) was used. The column was conditioned overnight at 285 °C. Injection temperature was 290 °C; the column was operated at 260 °C isothermally. Nitrogen was used as both the carrier gas and the chemical ionization reagent gas. There was no separator between the gas chromatograph and the mass spectrometer. The connecting line was maintained at 250 °C. Nitrogen flow rate was adjusted to yield maximum sensitivity for the calibration samples. After introduction of a 5-μL sample into the gas chromatograph, the effluent was vented to vacuum for about 15 s to avoid introducing large quantities of hexane into the ion source.

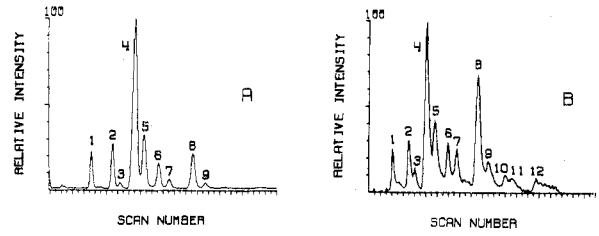

Figure 1. Total ion monitoring profiles of Firemaster BP-6: (A) positive chemical ionization (methane reagent gas); (B) negative chemical ionization (nitrogen reagent gas); mass range, 70–820; identity of peaks, (1) 2,2′,4,5,5′-pentabromobiphenyl, (2) 2,3′,4,4′,5-pentabromobiphenyl, (3) 2,2′,3,4′,5′,6-hexabromobiphenyl, (4) 2,2′,4,4′,5,5′-hexabromobiphenyl, (5) 2,2′,3,4,4′,5′-hexabromobiphenyl, (6) 2,3′,4,4′,5,5′-hexabromobiphenyl, (7) heptabromobiphenyl, (8) 2,2′,3,4,4′,5,5′-heptabromobiphenyl, (9) 2,2′,3,3′,4,4′,5′-heptabromobiphenyl, (10) heptabromobiphenyl, (11) octabromobiphenyl, (12) 2,2′,3,3′,4,4′,5,5′-octabromobiphenyl.

The mass spectrometer was operated in the negative chemical ionization mode. Resolution and focusing were adjusted daily to obtain maximum response at masses m/e = 452 and m/e = 28. Although the m/e = 452 peak of perfluorotributylamine does not arise from dissociative electron capture, tuning at this peak was selected because this compound can be introduced reproducibly through a controlled leak. Both good reproducibility and high sensitivity were concurrently obtained for the peaks resulting from the dissociative electron capture as well as resonance capture for the compounds analyzed. The m/e = 28 peak probably results from positive nitrogen ions migrating at low lens potentials when the Finnigan ion source is operated in the negative chemical ionization mode (*21*). Emission current was in the 0.4–0.8 mA range, ionization energy was 100 eV, and the electron multiplier was operated at 1.6 kV (conversion dynode voltage at 3.0 kV) with the preamplifier set at 10^{-8} A/V. For operation in the selected ion monitoring mode the masses monitored were m/e = 79 (bromine anion), m/e = 81 (bromine isotopic anion), and m/e = 498 (decachlorobiphenyl, internal standard).

RESULTS AND DISCUSSION

Mass Spectra and Profiles of Firemaster BP-6. Total ion current profiles have been obtained for Firemaster BP-6 under a number of analytical conditions. The positive chemical ionization (methane reagent gas) profile (Figure 1A) of Firemaster BP-6 resembled the chromatograms obtained by using conventional gas chromatographs with electron capture detection (*22*) or by monitoring the effluent from the gas chromatograph by electron ionization mass spectrometry (*23*). The negative chemical ionization (nitrogen reagent gas) profile, using "full scanning" (i.e., covering the mass range from m/e = 70 to m/e = 820) was still similar in general appearance (Figure 1B); however, there were apparent quantitative differences, e.g., the heptabromobiphenyl peak (peak 8) was disproportionally intense with respect to the peaks of lower bromine content. When the profile was obtained scanning only the m/e = 400 to m/e = 820 mass range, the profile was even more different in regard to the increased relative intensities of the congeners of higher bromine content. When the complete mass spectra of individual components were obtained under systematically varying conditions, it was observed that bromine anions were always present in considerable abundance in addition to the peaks in the molecular anion region. For example, in the mass spectrum of the 2,2′,4,4′,5,5′-hexabromobiphenyl (the most abundant component in Firemaster BP-6) the bromine anions accounted for approximately 90% of the total ion current with the remaining 10% being carried by isotopic ions in the molecular anion region.

A study of the negative chemical ionization mass spectrometry of PBBs revealed that both resonance electron capture and dissociative electron capture occurred (*20*). The latter resulted in the formation of bromine anions in a large

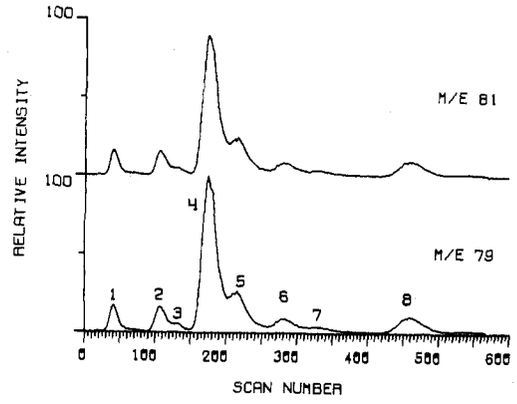

Figure 2. Selected ion monitoring of Firemaster BP-6 in the negative chemical ionization mode (nitrogen reagent gas). Masses monitored are the bromine anions at m/e = 79 and m/e = 81. Peak identities are the same as Figure 1.

excess with respect to the molecular ions. The intensities of the bromine anions were found to be related to the number and position of the bromine atoms in the molecules, and of the chemical ionization reagent gas. Extensive dissociative electron capture occurred even though the ion source was kept as cool as possible (no heater). The effects of source temperature on bromine production and on sensitivity have been investigated (*19, 20*). It is the abundant formation of bromine anions, which in fact are fragment ions, that is utilized in the present work. A selected ion monitoring profile of the Firemaster BP-6 mixture taken by monitoring only the m/e = 79 and m/e = 81 masses in the negative chemical ionization mode revealed a pattern (Figure 2) which is quite comparable to that obtained by positive chemical ionization as well as by electron capture gas chromatography. As shown by the results below, the phenomenon of bromine anion production is eminently suitable for the quantification of PBB congeners in serum at trace levels previously unattainable.

Sample Preparation. The sample preparation technique described is essentially the same as used by most workers with electron capture gas chromatography. Attention is called, however, to the fact that after proteins are precipitated, the precipitate must not be physically removed before extraction. The reason for this is our observation (unpublished, under current investigation) that PBB binds to protein in human serum in such a manner that, while precipitation of proteins with methanol does not release PBB completely, the binding becomes "loose enough" to permit subsequent complete extraction with hexane–diethyl ether. In recovery experiments, 20 ng of the ^{14}C-labeled hexabromobiphenyl (of the order of 1000 cpm) was added to serum and the samples were extracted as described. More than 95% of the added radioactivity was recovered. When the precipitated proteins were removed prior to extraction, large errors occurred because most of the PBB (order of 80%) remained bound to the proteins as evidenced by counting the extract from separated proteins. It is noted that no quenching was observed in the recovery experiments.

Internal Standard. Although the advantages of internal standards are well-known, none were used in the majority of publications on PBB determinations with electron capture gas chromatography; impressive interlaboratory methods validation was reported using an external PBB standardization (*8*). Two internal standards were investigated in the present work: decachlorobiphenyl, which had been previously used in this laboratory (*17*), and tetrabromobiphenyl (*24*). Both proved to be acceptable as far as sample preparation (extraction) is concerned.

As far as gas chromatographic properties are concerned, decachlorobiphenyl is a better choice. Its retention time is nearly the same as that of the most abundant hexa isomer of Firemaster BP-6; thus all constituents can be analyzed isothermally. When tetrabromobiphenyl was employed (the 2,2′,4,5′, isomer was found to be most useful) column temperature had to be reduced to obtain the internal standard peak and isothermal operation was no longer feasible for all constituents, thus analysis time increased considerably. From a mass spectrometric point of view, tetrabromobiphenyl is more desirable because bromine is released in this compound similarly to the constituents of Firemaster BP-6. No halogen is released from decachlorobiphenyl, thus a large jump must be made in the masses covered in selected ion monitoring. The required switching can be accomplished easily in quadrupole type mass spectrometers but is not possible in magnetic type instruments. Still other aspects to consider are the possibility that a particular batch of Firemaster might contain tetrabromobiphenyls (*24*) and also that degradation of PBBs of higher bromine content to toxic compounds of lower bromine content might be of great importance in the biological actions of PBBs (*12*).

Calibration curves were obtained for the abundant hexa isomer using both internal standards. Straight lines with r values of 0.99 were obtained with both compounds. For the reasons discussed, decachlorobiphenyl was used in the present work; however, tetrabromobiphenyl may also be used successfully.

Quantification. The quantification of PBB was accomplished with the aid of calibration curves. Computer-integrated areas were obtained for the peaks monitored. Calibration curves were obtained by plotting Br^-/internal standard area ratios against the known quantity of Firemaster BP-6 added to normal serum samples. When a series of samples with increasing Firemaster BP-6 concentrations was analyzed for the hexa isomer, the plots yielded straight lines within a concentration range varying from 100 pg/mL to 50 ng/mL Firemaster BP-6. It is noted that linearity exists up to 10 ng of injected hexabromobiphenyl. Above this level a saturation of the ionization process occurs. This means that samples with >50 ng of Firemaster BP-6/mL of serum should be appropriately diluted.

Reproducibility measurements for the major hexa isomer were made by using concentrations of 0, 200, 500, 1000, and 1500 pg of Firemaster BP-6/mL of serum sample (800 pg/mL of internal standard was added to each sample). With five independent samples analyzed at each concentration, the coefficients of variation (defined as standard deviation divided by the mean, multiplied by 100) were in the 6–13% range. Simultaneous analysis on the penta isomers from the same doped serum samples yielded comparable calibration curves.

It is emphasized that a separate set of calibration samples was extracted and analyzed together with the samples of interest every time the instrument was used for the analysis of PBBs. This was considered essential to assure that instrumentation parameters were properly adjusted to reproduce desired dissociative electron capture conditions on a day-to-day basis. In a series of five sets of runs, samples were prepared by different personnel and the mass spectrometer conditions readjusted for each set of analyses. The calibration curves gave r values in the 0.97–0.99 range.

Limit of Detection. The instrumental limit of detection for the pure compounds is illustrated by the selected ion monitoring of the most abundant hexa isomer from 2 pg of Firemaster BP-6 injected (Figure 3). This corresponds to 1.4 pg of the hexa isomer. This is the "amount" of the compound that must be present in the ion source to obtain a quantifiable signal. About half of this amount can be detected.

The PBB burden in serum (and also in tissues) has usually been expressed in terms of the amount of the most abundant hexabrominated biphenyl per milliliter of serum. The limit

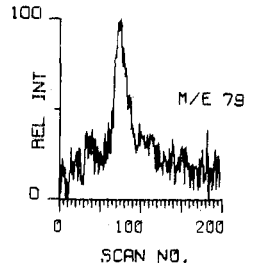

Figure 3. Detection of 2 pg of Firemaster BP-6 by monitoring the bromine anion from the most abundant hexa isomer.

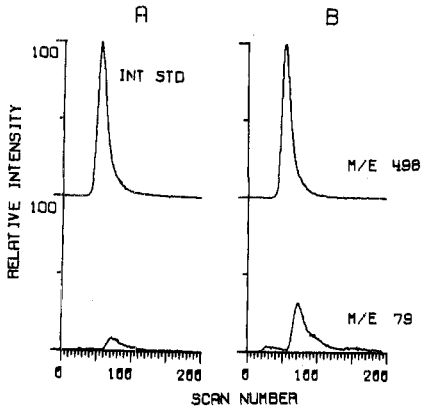

Figure 4. Determination of Firemaster BP-6 in serum by monitoring the bromine anion (m/e = 79) and decachlorobiphenyl internal standard (m/e = 498): (A) serum blank, (B) 200 pg of Firemaster BP-6/mL of serum.

of detection (detectability) is defined here as the amount of a particular constituent needed per milliliter of serum sample to produce a bromine/internal standard peak area ratio twice that of a blank sample. An amount double that of the detectable amount can be quantified with some reliability.

Low level analysis of the hexa isomer in serum is illustrated in Figure 4B which shows the signal corresponding to 200 pg of Firemaster BP-6/mL of serum. As seen in Figure 4A there was a small blank that had to be subtracted. The most likely source of the bromine anion peak in the blank is cross contamination during sample preparation. On the basis of the minimum area increment over the blank value that the computer is capable of determining, the lower limit of detection for Firemaster BP-6 in serum when the most abundant hexabromobiphenyl constituent was monitored was 50 pg/mL serum; this corresponds to 35 pg of hexabromobiphenyl/mL of serum. These detection limits represent a 20-fold increase over the 1 ng of Firemaster BP-6/mL serum (in terms of the hexa isomer) that is considered the borderline for detection by electron capture gas chromatography. The increased sensitivity allows for the more reliable quantification of the many borderline cases reported in general population studies.

There is a growing interest in the monitoring of other constituents of Firemaster BP-6 because of the possibility of different mechanisms of metabolism (*14*, *15*). Figure 5 shows the quantification of all constituents in a sample which contained 500 pg/mL Firemaster BP-6. Attention is called to the pentabromobiphenyls (peaks 1 and 2). For these compounds the present technique is more sensitive because of the increased dissociative electron capture that occurs for biphenyls with lower bromine content (*20*). An additional advantage was that the serum blank did not contain any interference (blank) for the pentabrominated biphenyls. The lower limit of detection for the two pentabromobiphenyls in serum was determined as 10 pg/mL of sample. The other hexa isomers and the hepta biphenyls can be detected to about 35 pg/mL of serum. It is noted that there was no serum blank for these compounds.

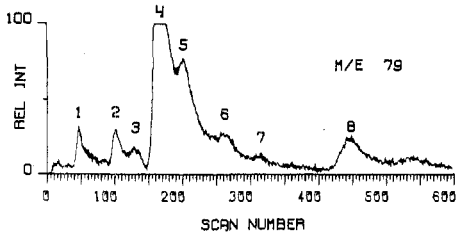

Figure 5. Selected ion monitoring profile of a serum extract containing 500 pg/mL Firemaster BP-6 for the quantification of PBBs of lower abundance. Peak identities are the same as Figure 1.

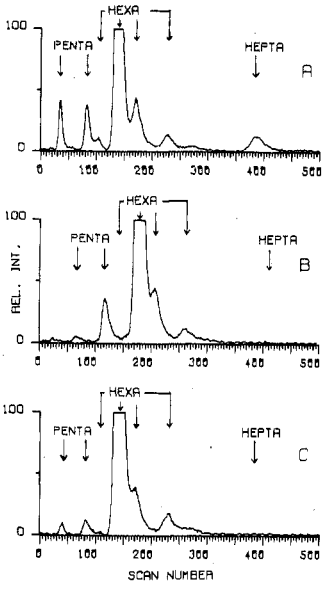

Figure 6. Selected ion monitoring of constituents in: (A) Firemaster BP-6 standard; (B) Michigan chemical worker; (C) Michigan farmer. All peaks shown were normalized with respect to the most abundant hexa isomer in the standard. Note changes in penta ratios and disappearance of hepta homologue.

The sensitivity and utility of the technique described for the assaying of PBB burden is illustrated in Figure 6. It is seen that considerable differences exist in the relative amounts of the two pentabrominated biphenyls in the samples from a Michigan chemical worker (Figure 6B) and a Michigan farmer (Figure 6C) when compared to Firemaster BP-6 (Figure 6A). It is also noted that there is an apparent absence of the major hepta homologue in both subject samples. (In Figure 6 all peaks have been normalized with respect to the major hexa isomer in Firemaster BP-6.) These data are in agreement with previous findings (*13*).

The present method requires only 1 mL of serum whereas 2–4 mL are required for all previously published methods. Another factor of 2 in detection limits may be achieved with increased sample size. More importantly, the small sample requirement of this technique conserves samples in field studies where several types of assay must be made utilizing a limited amount of blood.

Specificity. In addition to increased sensitivity, this technique increases specificity with respect to electron capture gas chromatography. Although the electron capture detector is blind to many serum constituents, halogen-, nitrogen-, and phosphorus-containing compounds present in the samples as well as in containers, reagents, and outgassing column materials, may pose a limitation to discerning sample peaks above the "chemical noise". An important advantage of mass spectrometry is to provide increased specificity. The monitoring of the molecular ion is the method of choice. When that is not possible because of either the absence or the low intensity of the molecular ion, a suitable fragment is often

selected. In the present case, the monitoring of the molecular anion was possible only when a relatively large amount of Firemaster BP-6 was present. To achieve the goal of low detection limits, we monitored the bromine fragment anions. This provided significantly lower detection limits and, at the same time, considerably increased specificity with respect to electron capture gas chromatography. In addition to the chromatographic retention times, which are common to both techniques, the present method increases specificity because only one particular mass, that of the bromine anion, is monitored. Thus, only compounds yielding a contribution to the $m/e = 79$ mass may interfere. In addition, the $m/e = 81$ peak may also be monitored for proper isotopic ratio, thus, in fact, only bromine-yielding compounds may conceivably interfere.

Areas of Application. The technique reported here provides an approximately 20-fold decrease in detection limits and increased specificity over electron capture gas chromatography. The method is thus well suited to the analysis of serum samples containing low levels of PBB such as in the borderline cases that occur in the general population, and also for the study of changes in the relative quantities of individual brominated biphenyls in samples from directly exposed individuals (such as chemical workers) and also those indirectly exposed through the food chain. In addition, the low detection limits allow for the search for PBBs in separated blood compartments and other tissues. Such studies are now in progress in this laboratory.

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Evaluation of Fast Atom Bombardment Mass Spectrometry for Identification of Nitrogen-Containing Compounds in Fossil Fuels

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The applicability of fast atom bombardment mass spectrometry (FAB/MS) to the analysis of fossil fuel materials has been explored by acquiring FAB mass spectra of 20 nitrogenous bases and a base fraction from anthracene oil. The spectra are characterized by significant $M^{+}\cdot$, $(M + H)^{+}$, and $(M - H)^{+}$ ions. Fragmentation parallels that expected for electron impact (EI) and chemical ionization and several fragmentation pathways are proposed from the observation of peaks corresponding to metastable-ion decompositions. Comparison of the FAB spectrum of the base fraction with a field ionization (FI) spectrum recorded earlier reveals that many of the molecular ions appearing in the FI spectrum are shifted to $(M + H)^{+}$ ions in the FAB spectrum. The $M^{+}\cdot$ and $(M + H)^{+}$ ions in the FAB spectrum of the base fraction are used to classify its components by nominal-mass *Z* series. This classification is in agreement with one deduced from high-resolution mass spectral data recorded earlier.

Fast atom bombardment mass spectrometry is a new technique that has been developed to provide spectra of underivatized polar molecules. To date, almost all reported applications have been made on compounds having biological significance (*1–6*). Because of the interest in the structures of polar molecules existing in coal and petroleum, we decided to apply FAB/MS to a number of nitrogenous bases representative of those found in fossil fuels and to a base fraction separated from anthracene oil to determine whether the technique has potential for analyzing nitrogenous samples of interest to the energy industry.

EXPERIMENTAL SECTION

Instrumentation. The FAB ion source was a prototype developed by Kratos Scientific Instruments, Ltd., and was fitted to an MS-80 mass spectrometer. All spectra were recorded oscillographically at low resolution with an accelerating potential of 4 kV and a scan rate of 100 s/decade. The pressure in the

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