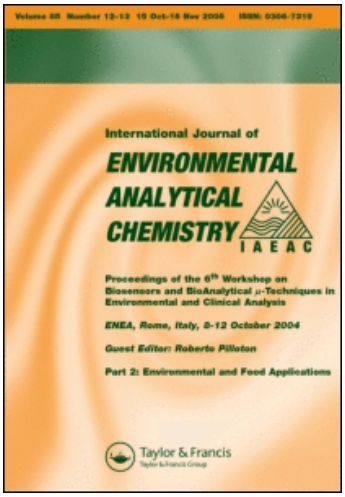

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Separation of Mutagens from Drinking Water using Coupled Bioassay/Analytical Fractionation

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A general preparative procedure has been developed for the isolation of mutagenic components from organic residues of drinking water. This procedure features the *Salmonella*-microsome mutagenicity assay coupled with an analytical fractionation method which progressively focuses to the bioactive constituents of the complex mixture. The three-step method consists of: 1) semi-solid/liquid extraction; 2) radially compressed column octadecyl-silane reverse phase HPLC; 3) SEP-PAK^R concentration and solvent exchange of HPLC subfractions for chemical and biological characterization. Using as a model a carbon chloroform extracted organic residue mixture prepared from drinking water several years ago, three mutagenic HPLC subfractions were isolated, accounting for the bulk of the mutagenic activity of this complex mixture. Preliminary GC/MS results indicate the mutagenic activity is due to isomeric chlorinated aliphatic ethers. The biological characterization suggests the mutagenic specific activity of these compounds exceeds that of several known chemical carcinogens, e.g. β-naphthylamine. This data supports the model that drinking water contains a vast number of non-mutagenic compounds, possibly some of low activity, and may contain a small number of highly mutagenic compounds. We propose this coupled bioassay/chemical fractionation method for use in testing this model for organic residues of current environmental waters.

KEY WORDS: Bioassay, drinking water, mutagens, reversed phase HPLC, organic residues.

INTRODUCTION

The search for mutagens in the organics of drinking water is conducted among chemicals of two categories: those of low solubility and with a volatility sufficient for rapid separation and identification by GC and GC/MS; and relatively non-volatile compounds which are not amenable to purging techniques but are collected by extraction or concentration.

Members of this second category of compounds comprise 90–95% of the organic material in drinking water,[1] and constitute complex mixtures of thousands of chemicals, the majority of which are unidentified. For toxicological assessment of such mixtures, initial characterization has relied upon short term bioassays of concentrated residues. The *Salmonella*/microsome mutagenicity assay has been used extensively for this purpose with positive responses being reported for organics extracted by a variety of methods.[2] Many of these extracts show toxic as well as mutagenic effects, and an assessment of their risk to human health will require fractionation into less complex mixtures for definitive biological testing.

The traditional approach to fractionation of complex mixtures has been to develop a separation scheme, identify the isolated constituents, and then test these components for bioactivity.[3,4] However, the 1200 compounds thus far identified by this approach are believed to represent only a small fraction of the total drinking water constituents.[5] It is apparent that there would be advantages to a coupled approach in which the fractionation procedure is based directly upon biological activity. Such a fractionation scheme is proposed in this paper.

EXPERIMENTAL

Equipment

High performance liquid chromatography (HPLC) separations were performed on a Waters Associates (Milford, Massachusetts) Model ALC/GPC 204 equipped with two 6000 A pumps, a U6K injector, a solvent programer and a 254 nm absorbance detector. The instrument was fitted with a 3.9 mm by 30 cm prepacked analytical column of 10 μm particles bonded with octadecylsilane (μBONDAPAK-C_{18}[R]) for analytical scale (μg level) separations. For preparative scale (mg level) separations, the HPLC was fitted with a Waters radial compression module (RCM) unit containing an 8 mm by 10 cm column packed with 10 μm silica particles bonded with octadecylsilane. A guard column (3.9 mm by 2.5 cm) packed with pellicular particles coated with octadecylsilane (BONDAPAK C_{18} CORASIL[R]) was used for all HPLC separations.

Gas chromatography (GC) analyses were performed on a Perkin-Elmer (Norwich, Connecticut) Model 900 flame ionization unit fitted with a 2 mm by 1 m stainless steel column containing 10% SE30 on Chromsorb WHP 80/100 mesh (Applied Science, State College, Pennysylvania) and a 2 mm by 2 m stainless steel column containing 10% silar 10C on Chromsorb Q 80/100 mesh (Applied Science). Data were continuously collected and analyzed using a Spectra Physics Autolab System I

Computing Integrator (Santa Clara, California); chromatograms were displayed on a 10 mV recorder.

Gas chromatograph/mass spectral (GC/MS) analyses were performed on a Finnigin (Sunnyvale, California) Model 4021 unit. Gas chromatographic separations were performed on a 2 mm by 6 ft 3% OVl on Chromosorb W, 80/100 mesh (Applied Science) prior to mass spectral analyses. Data were continuously collected then analyzed using a Finnigin Model-INSCO data system containing a 25, 413 compound NBS library.

Drinking water residue organics and other chemicals

A 125 g archival sample of carbon/chloroform extracted organics (CCEO), prepared from 50,000 gal of finished Cincinnati drinking water in 1962 by Middleton *et al.*,[6] was generously provided by F. M. Middleton. The residual chloroform solvent was removed by gentle evaporation under a stream of dry nitrogen while warming the sample at 60°C. Water for HPLC and for the preparation of other solutions was purified using a Continental Water Conditioning System (El Paso, Texas) consisting of a bulk deionizer, a polishing deionizer, an organic filter, and a 0.22 micron filter. Other HPLC solvents were obtained as distilled-in-glass solvents from Burdick and Jackson Laboratories (Muskegon, Michigan). All HPLC solvents were degassed immediately prior to use by 15 min of sonication while under vacuum. Other chemicals were reagent grade and were used as obtained.

Extraction and preparation of samples for analysis

A portion of the solvent-free CCEO sample was weighed then extracted with hexane at 3 ml/gm of sample. This semi-solid/liquid extraction was accomplished by alternating 5 min of vigorous vortexing with 3 min of sonication. The hexane layer containing the neutral extract was removed for chemical separation and bioassay following phase separation. The residue was acidified by repeated additions of small amounts of 6N HCl, interspersed with vortexing and sonification, until a pH of 2.0–1.5 was achieved. This sample was extracted as described with a 3X volume of hexane, and the hexane layer containing the acid extract was removed for bioassay. The pH of the residue was then adjusted to pH 12 by the addition of saturated aqueous sodium carbonate in a manner similar to acidification. The basic sample was extracted as described with a 3X volume of hexane, and the hexane layer containing the base extract was removed for bioassay. The remaining residue was neutralized by the addition of 6N HCl using the same manipulations described. This sample

was extracted with a 3X volume of n-butanol, and the butanol extract was tested for mutagenic activity. A diagram of this extraction scheme is presented in Figure 1.

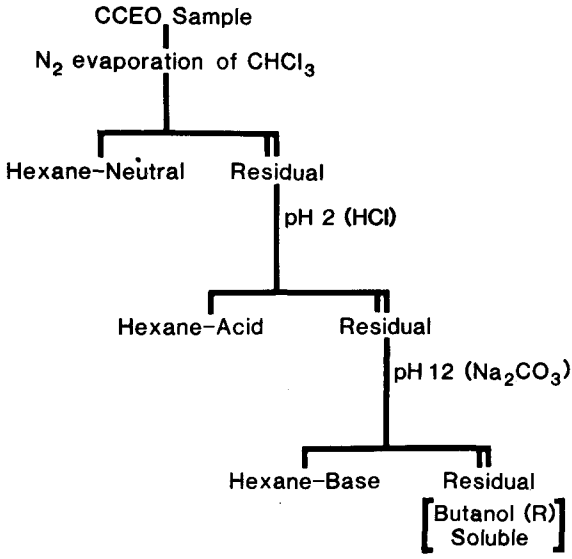

FIGURE 1 Semi-solid/Liquid Extraction Scheme.

High performance liquid chromatography

Samples for HPLC were injected as 100 μg to 100 mg per ml solutions in acetonitrile. Following injection, the column was flushed with water at 2.0 ml/min until no more 254 nm absorbing material eluted. The column was then eluted via gradients from water to acetonitrile as described in the text.

Gas chromatography and gas chromatography/mass spectroscopy

GC analyses of the HPLC subfractions and SEP-PAK^R concentrates were performed by the slow injection of 7.5 μl of the appropriate acetonitrile/water or methylene chloride solution. The nitrogen carrier gas was flowing at 18 ml/min, and the temperatures of injector and detector were 280°C and 350°C respectively. A linear temperature program from 120°C to 220°C at 4°C/min was initiated at the time of injection. Approximate weight values for sample constituents were calculated based on peak areas

compared to peak areas obtained from chromatography of 1 μl of a 1 mg/ml chloroform solution of AOCS Oil Reference Mixture No. 6, run under conditions identical with those for the unknown samples.

GC/MS analyses of the HPLC subfractions and SEP/PAK^{R} concentrates were performed by the injection of 2 to 8 μl of a solution of the sample. The helium carrier gas was flowing at 30 ml/min, and the temperature of the injector and separator were 260°C and 280°C respectively. Following an initial solvent divert and temperature hold of 0.5 min at 100°, a linear temperature program was run to 200°C at 10°C/min. Mass spectrometry conditions were as follows: a 250°C ionizer temperature; an electron multiplier voltage of -1700; an emission current of 0.350 ma. Electron impact spectra were run at 40 or 70 electron volts, as indicated.

Biological analysis

Bioassays were performed using the *Salmonella* microsome mutagenicity assay described by Ames,[7] who provided strains TA1535, TA1537, TA1538, TA98 and TA100. Strain TA1535 is an indicator of base substitution mutagenesis; strains TA1537 and TA1538, and TA98 which is TA1538 with the plasmid pKM101, are detectors of frameshift mutagens. Similarly TA100 is TA1535 containing pKM101. Presence of pKM101 increases the sensitivity of these strains to many known mutagens, and in TA100 its presence may alter specificity to include detection of frameshift as well as base substitution mutagens. Bacterial stocks were prepared weekly and their characteristic mutagenesis[7,8] was verified for each fresh stock and again as part of each experiment. Liver homogenate 9000 g supernatant fraction (S9) from rats induced with a polychlorinated biphenyl mixture Aroclor 1254 was prepared locally as described[8] or was used as obtained from Litton Bionetics, Inc., (Kensington, MD); the concentration of S9 was fixed at 20 μl/plate. Tests were conducted by standard soft agar platings from DMSO according to Ames *et al*[7] using triplicate plates for spontaneous mutagenesis. Assays of initial samples utilized duplicate plates at 5 or more dose levels selected to yield some plates whose colony count values were at least twice the spontaneous count. Where the amount of material was limiting as with subfractions from re-chromatography (HPLC), single platings at 4 dose levels were employed; again the dose range was selected to yield at least a doubling of the background spontaneous revertant colony count. For the organic extract described here the distribution of mutagenic activity among subfractions could be monitored using one strain, TA100, in the presence of microsomal activation.

RESULTS

CCEO residues

The parent CCEO sample, following chloroform removal, was tested using strains TA98 and TA100 and showed mutagenicity only to TA100 in the presence of microsomal activation (Fig. 2A). An apparent toxicity of this sample is indicated by the decreased rate of response at higher doses. From repeated assays involving low dose levels initial rate values indicate a minimum mutagenic activity of 700 net revertant colonies per mg. Such a specific activity is comparable to that typically seen with residues isolated more recently by other methods; the activity differs from that of most other drinking water residues thus far examined in the requirement for microsomal activation.[2]

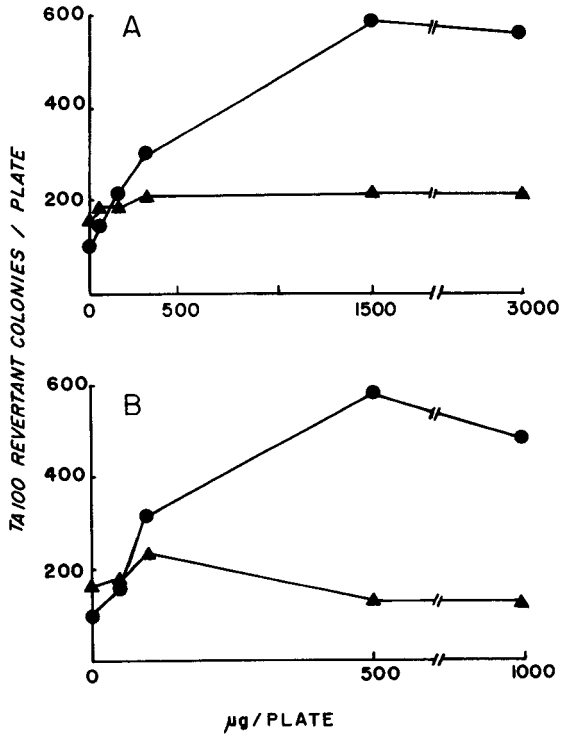

FIGURE 2 Microsomal dependent activity of parent CCEO sample (A) and neutral fraction (B) with threefold higher mutagenesis per microgram. Single experiment using duplicate plates: absence (▲) and presence (●) of microsomal activation.

Liquid/liquid extraction

The approach to fractionation of the organic residues was to separate the organic constituents according to acid, base, and neutral components. A conventional liquid/liquid procedure was attempted,[3,9] by which CCEO in methylene chloride was extracted with aqueous acid, pH 2.0, followed by aqueous base pH 12.0. However this extraction procedure was beset with problems: 1) formation of emulsions, which could not be dispelled by conventional methods; 2) excessive time requirement, greater than four fold that of our semi-solid/liquid procedure; and 3) inconsistencies in reproducibility from extraction to extraction.

Semi-solid/liquid extraction and properties of the neutral fraction

Applying the semi-solid/liquid procedure described in Figure 1 to the CCEO sample yielded reproducible distributions of weight and mutagenic activity. The neutral fraction contained one-third of the total material (Table I) and nearly all (>80%) of the mutagenic activity, as is illustrated

TABLE I

CCEO sample: Distribution of fractions by weight

Starting material and fractions	%
CCEO	100
Neutral	32
Acid	5
Base	1
Residual	62

by comparison of Figure 2A and 2B. As with the parent CCEO sample, the decreasing rate with increasing dose suggests that for this fraction the apparent initial rate mutagenic specific activity, 2000 revertant TA100 colonies/mg, is an approximate but minimum value. The neutral fraction was more extensively characterized using the *Salmonella* mutagenicity assay prior to further chemical separation. Results of tests at low dose levels using all 5 strains are presented in Figure 3. The property of microsome activation-dependent mutagenesis for strain TA100 is seen also to characterize the response of TA1535. As described in the Experimental

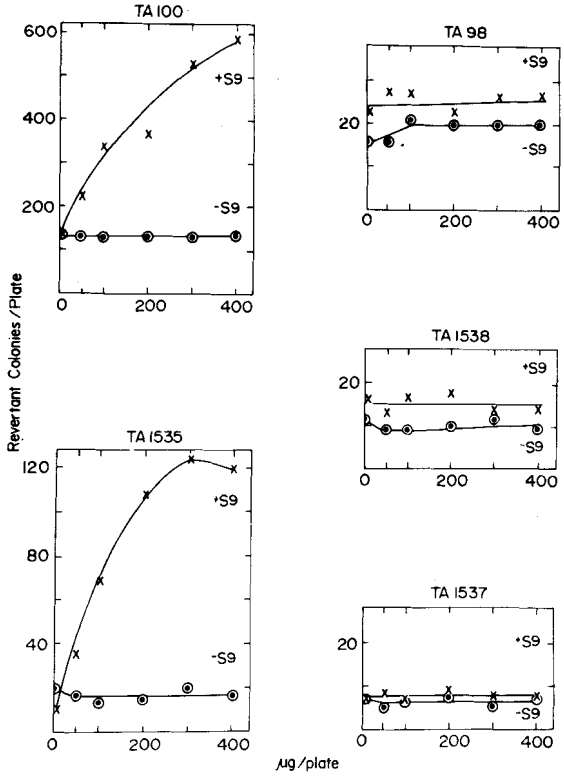

FIGURE 3 Dose response curves of the mutagenic effects of the CCEO-neutral organics mixture. Assays were conducted in the absence (−S9) and presence of microsomal activation (+S9). Data points are the means of duplicate plate counts obtained in a single experiment using TA98 and in replicate experiments using the other 4 strains.

section, both these strains are known to detect base substitution mutations. The other three strains, which are particularly sensitive to frameshift mutagens, failed to show mutagenesis over the dose range tested. Because of its high sensitivity and its other potential advantages over TA1535,[10] TA100 was chosen for use in monitoring the distribution of mutagenic activity in subsequent studies.

HPLC separation of the CCEO neutral fraction

Further separation of the neutral fraction was conducted by reverse phase octadecylsilane HPLC. Twenty micrograms were chromatographed on an analytical column using a linear gradient from water to acetonitrile. The

chemical complexity of this sample is depicted by the HPLC profile in Figure 4 which shows an array of components separated according to decreasing polarity. Resolution of three related technical problems was necessary before such isolated subfractions could be assayed for mutagenic activity. The first requirement was a great increase in sample size. Theoretically components of only one, or a few, or many of these subfractions could contribute to the mutagenic activity. Assuming the simplest case in which all the activity added to the column was fully recovered in one subfraction, and applying the specific activity value of 2000 revertant colonies/mg, a sample of 150 μg would be required to allow

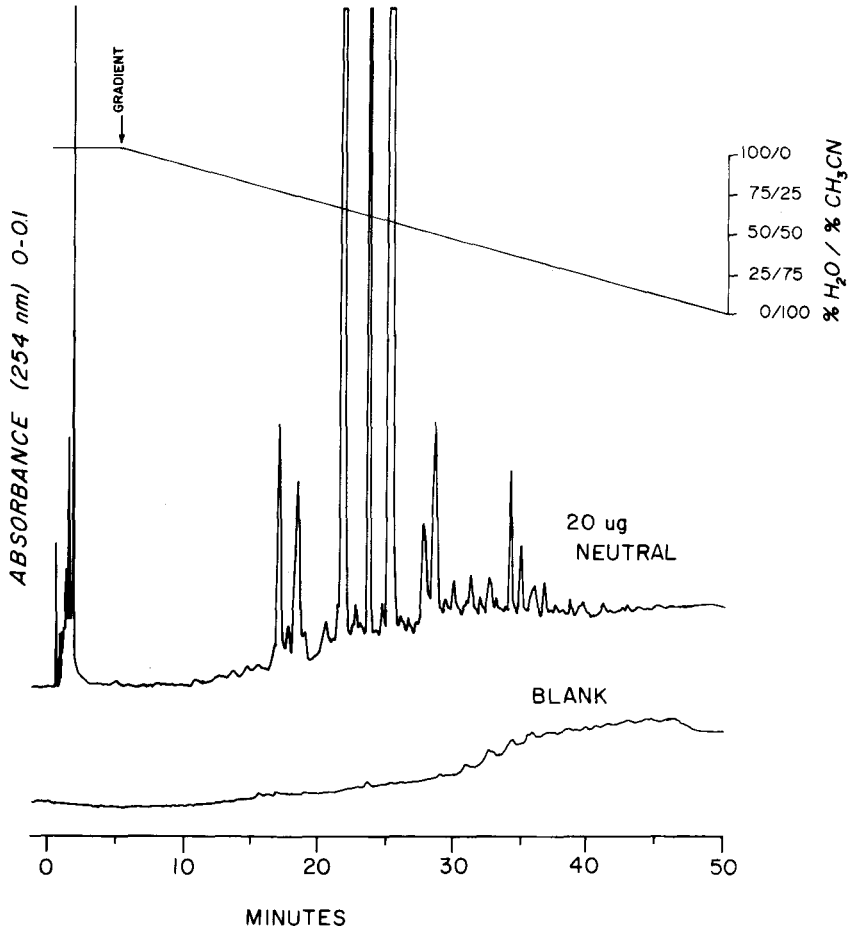

FIGURE 4 Absorbance (254 nm) profile of analytical reverse phase HPLC of neutral fraction.

for an acceptable dose response assay. And at this level all the subfraction would be consumed in the bioassay.

The second problem was that subfractions would be generated in dilute solution and would require reconcentration for assay. The third complication was that these components would be in a water/acetonitrile solvent which was itself toxic to the *Salmonella* tester strains. These latter two problems were considered first. A solution of CCEO neutral sample in water/acetonitrile, 50/50 V/V, was tested using a series of solvent concentration/exchange methods. Gentle evaporation under a stream of nitrogen resulted in the total loss of mutagenesis, even when the glassware had been silanized by a variety of methods. Gentle concentration with a Kuderna Danish evaporator resulted in a recovery of approximately 20% of the mutagenic activity and required excessive time. Since the CCEO neutral could be chromatographed on a reverse phase support, small disposable columns containing octadecylsilane (SEP-PAK^R C_{18} catridges) were examined as a means for concentration. The sample was diluted with water until the solvent polarity was sufficiently high for the components of interest to adsorb to the packing material, usually a 3 fold dilution. The dilute sample was loaded and concentrated on the cartridge, and was eluted with a small volume of methylene chloride. To avoid taking the solutes to dryness, this volatile solvent was partially removed by evaporation and a small amount of DMSO was added. The remaining methylene chloride was then evaporated and portions of the resultant solution were plated in the mutagenicity assay. Recovery of bioactivity by this procedure typically exceeded 80%.

To achieve separation of adequate sample levels, a variety of gradient elution conditions were investigated using the analytical column as quantities were scaled up through μg to mg levels. Using the RCM unit containing a higher capacity octadecylsilane, 20 mg samples were routinely chromatographed by a modified elution procedure as depicted in Figure 5. Fractions containing compounds of similar polarities were collected and processed via the SEP-PAK^R procedure for mutagenic testing. We observed that if narrow fraction cuts were taken the bioactive components could not be reproducibly collected in the same subfractions, in replicate HPLC runs. The broader fractional cuts shown at the bottom of Figure 5 were much more reproducible, with all the mutagenic activity being eluted in the subfraction indicated in the figure by a check. This bioactive fraction was diluted with water and reloaded onto the reverse phase RCM HPLC unit. The sample was eluted by a shallow gradient from 45% water/55% acetonitrile to 30% water/70% acetonitrile, and fractions were collected as shown in Figure 6A. Mutagenic activity was found in fractions 5 and 6; these too were diluted with water and each was further separated by reverse phase RCM HPLC using a shallow gradient as

shown in Figure 6B, (water/acetonitrile, 50/50 to 45·55 (%)). Fractions from this third HPLC run were collected and assayed for bioactivity, and were assessed on GC using polar and non-polar stationary phases. Mutagenic activity recovered was all contained in the 3 subfractions indicated by checks in Figure 6B, and totaled about 50% of that subjected to the initial HPLC. Typical GC results from a nonpolar phase column, Fig. 7, indicated these HPLC subfractions are nearly pure.

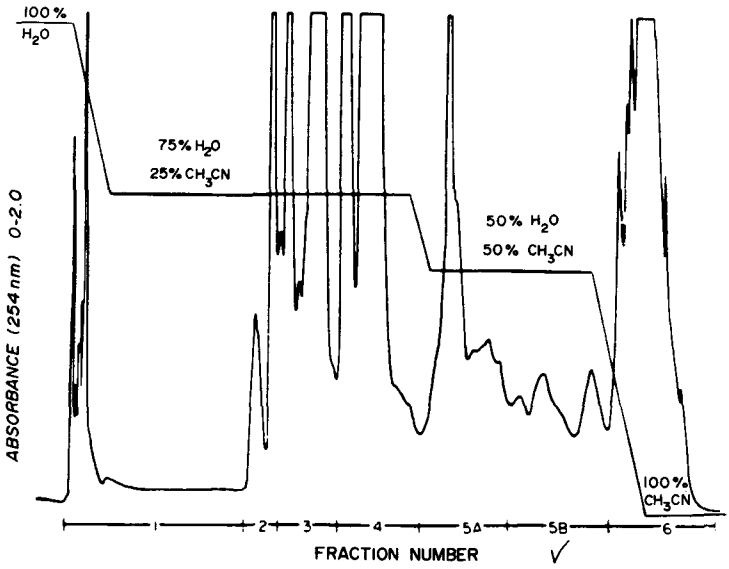

FIGURE 5 RCM/HPLC reverse phase HPLC of 20 mg sample of neutral fraction for mutagen isolation. Subfraction 5B containing activity for TA100 is indicated by a check, √.

The five major components of these three bioactive HPLC subfractions have undergone a preliminary examination using low resolution GC/MS. The electron impact mass spectrum at 40 and 70 EV for the single component of fraction 5B/6/3 (Figure 7) is shown in Figure 8A and 8B. The base peak at m/e 75 and isotope patterns at m/e 111, 141, and 189 suggest the compound is polychlorinated; however a molecular ion was not observed. The fragmentation pattern additionally suggests the compound to be an aliphatic ether.

Although the same overall mass spectral features were observed for the first GC component in each of the other two bioactive fractions (5B/5/2 and 5B/6/2, Figure 6), the intensities of the major MS peaks at 40 EV, and the patterns of the minor MS peaks at 70 EV indicate an isomeric

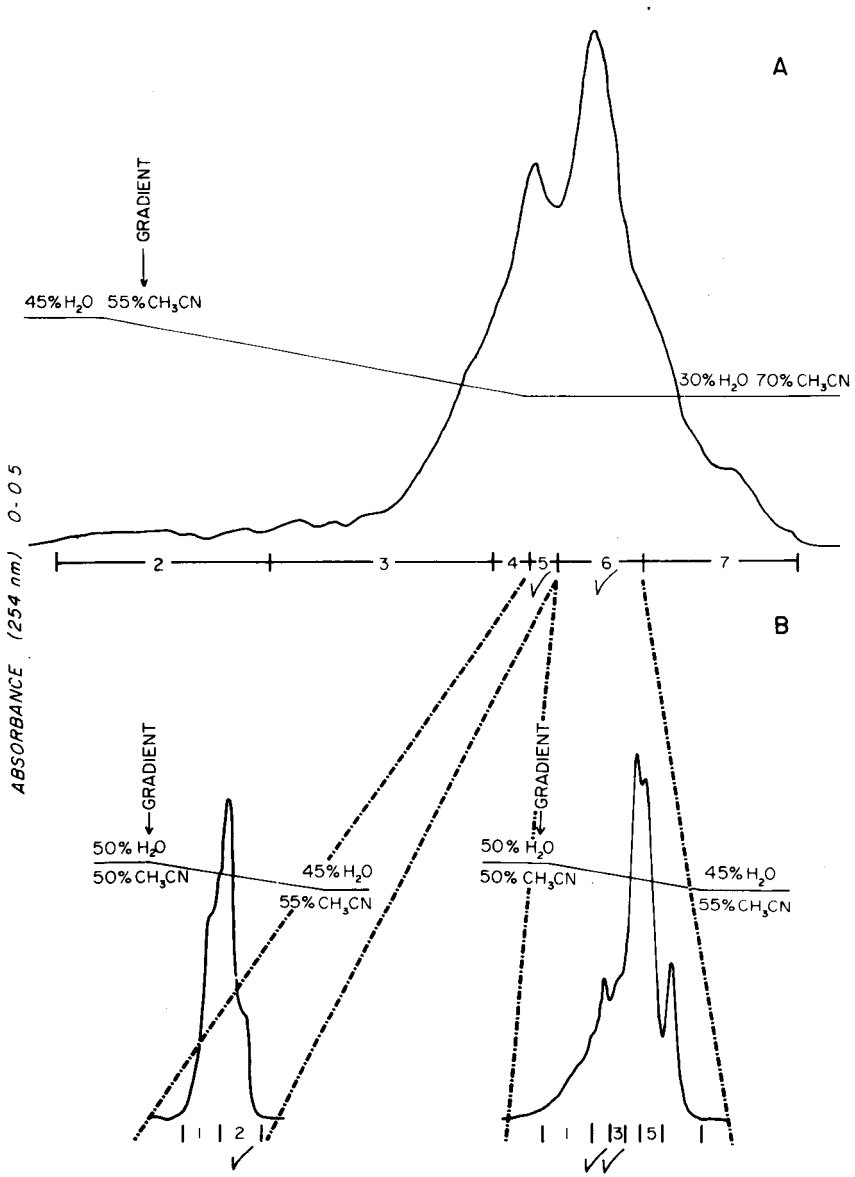

FIGURE 6 Isolation of mutagenic subfractions by RCM/HPLC. A: Absorbance profile of fraction 5B of Fig. 5, with the resultant mutagenic subfractions indicated by a check, √. B: Fractions 5B/5 and 5B/6 were rechromatographed separately to yield the mutagenic subfractions indicated by a check, √.

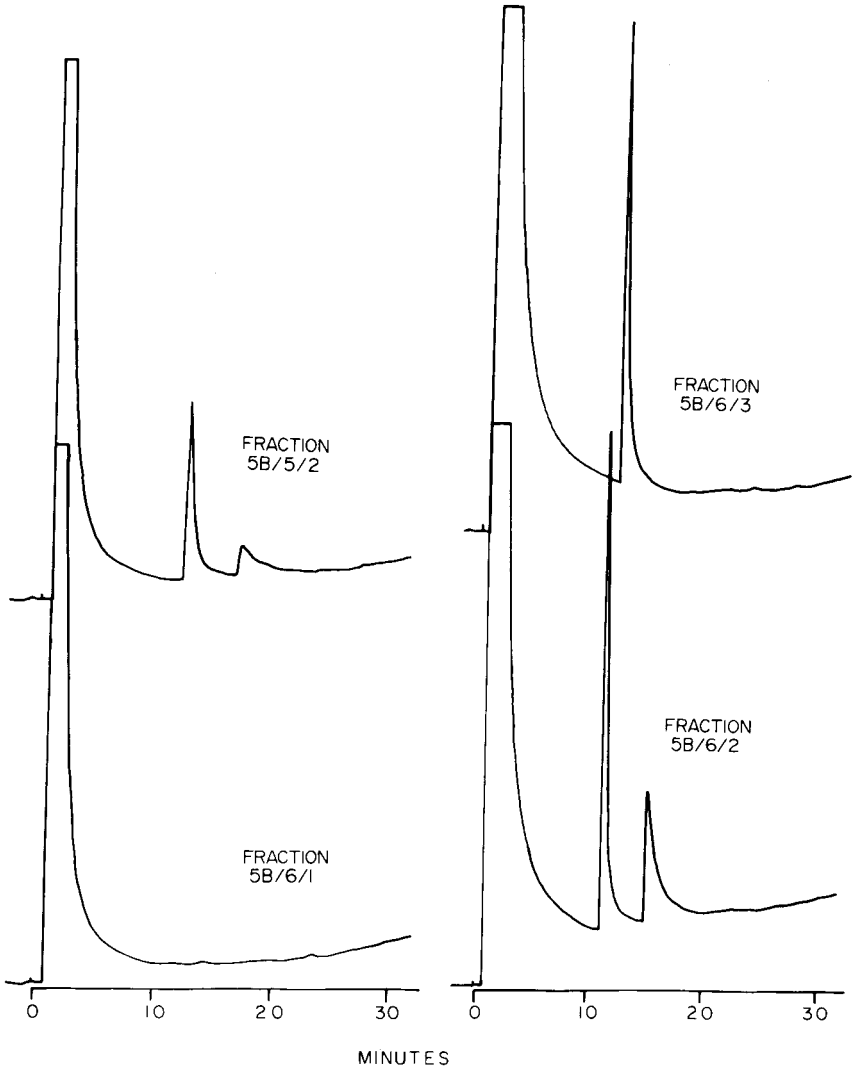

FIGURE 7 Flame ionization gas chromatograms of the contiguous fractions in the mutagenic regions depicted in Fig. 6B. GC analysis employed a 10% SE30 column as described in EXPERIMENTAL.

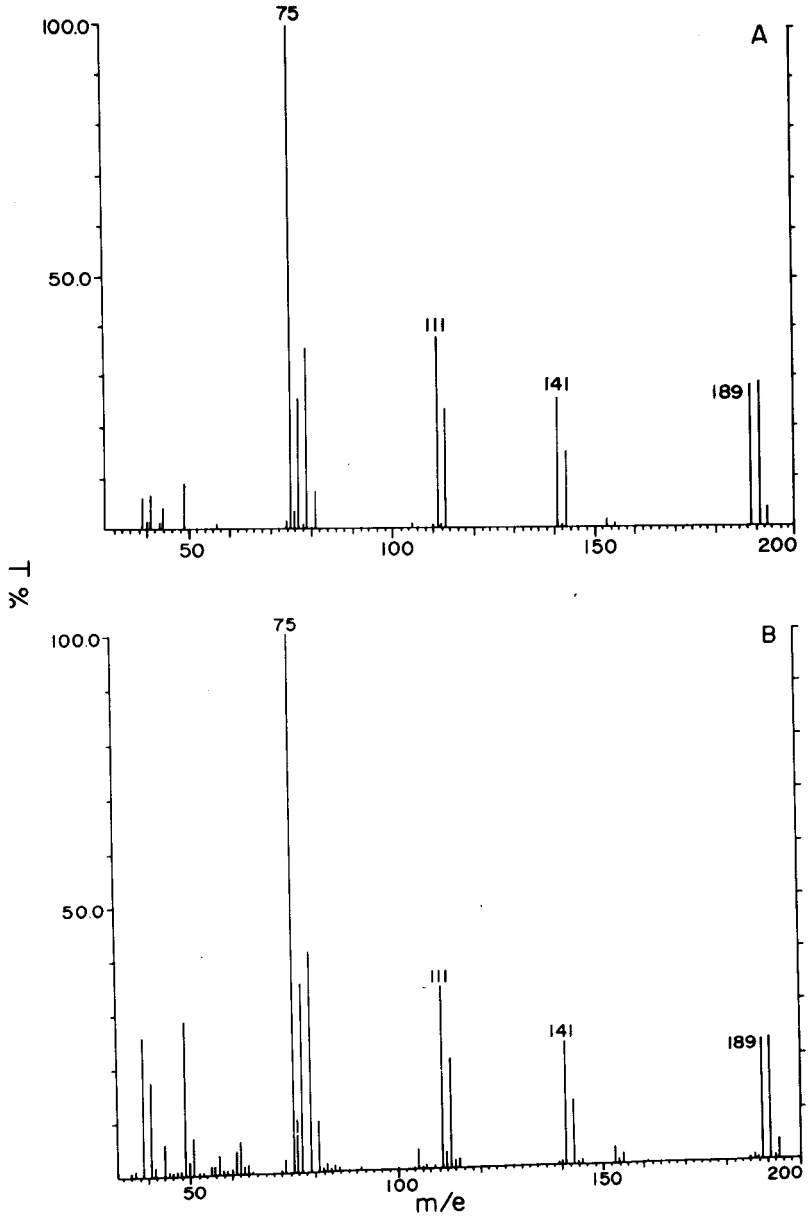

FIGURE 8 Electron impact mass spectra of fraction 5B/6/3 (see Fig. 6B, Fig. 7) A: 40 EV; B: 70 EV.

relationship for these three chlorinated compounds. The second GC component of each of these two HPLC subfractions (Figure 6) gave similar electron impact mass spectra with a base peak at m/e 127, suggesting a monosubstituted naphthylene. Other features of the spectra support this suggestion; again a molecular ion was not observed. Chemical ionization and high resolution mass spectra have not been obtained for any of the components at this time.

A few additional features of these compounds can be noted: 1) the two components of fractions 5B/5/2 and 5B/6/2 reverse their order of elution in a GC run using polar support Silar 10C; and 2) GC results using SE30 suggest the isomeric aliphatic compounds in the fractions elute in the 175°–185° range, and the naphthyl compounds in the 185°–195°C range; 3) these fractions exhibit little UV adsorbance above 220 nm.

We conclude that there were only 4 or 5 major components among the contents of the 3 tubes and that one mutagenic fraction contained only one component. Approximate weight values for the components in those 3 mutagenic subfractions were obtained as described in EXPERIMENTAL. The mutagenesis recovered from 40 mg of CCEO neutral sample was contained in a combined weight of 96 μg. For the one isolated mutagenic component the mutagenic specific activity for TA100 assayed in the presence of microsome activation was 4×10^5.

Thus this coupled bioassay/HPLC fractionation method has proved effective for the isolation of a few components as the agents of the mutagenic activity of this particular complex mixture. A summary diagram of the overall method appears in Figure 9.

DISCUSSION

Analytical methods that are consistent with theoretical principles and that work well with select compounds in model systems may present difficulties when applied to actual environmental mixtures. The sample used in this study was a carbon-chloroform extract (CCEO) prepared years earlier from drinking water. It has a grease-like consistency similar so that seen with residues isolated recently from XAD resins, and doubtless contains thousands of nonvolatile compounds of diverse structure and molecular weight. Alternative liquid/liquid extractions with the CCEO as a true solution were beset with problems due to emulsion formation. Although Webb[9] successfully resolved such difficulties in his model system containing known organic pollutants of water, the complexity of this sample led to essentially intractable emulsions which rendered these methods inadequate or ineffective.

The semi-solid/liquid extraction scheme we developed using hexane is similar to techniques used by medicinal and natural products chemists in the extraction of biochemicals from native flora. It effected a reproducible 3 fold enrichment of the mutagenic activity of this CCEO sample by its extraction into the neutral fraction, and provided the starting material used in our development of the coupled *Salmonella* mutagenicity assay/HPLC fractionation system.

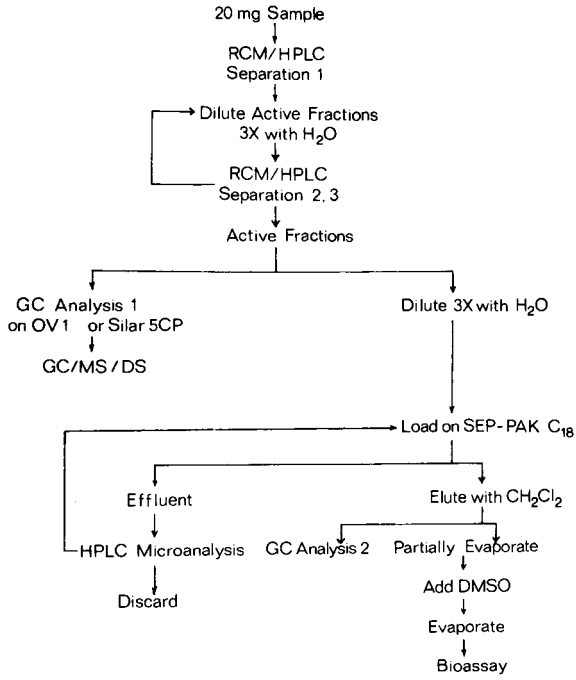

FIGURE 9 Flow diagram of coupled bioassay/chemical fraction method for the isolation of mutagens from residue organics of environmental waters.

The major features of this coupled system are:

1) High resolution and high reproducibility of separation profiles over a wide range of decreasing polarity for sample sizes from a few μg to 20 mg. This permits the monitoring of column retention and solvent exchange steps by HPLC microanalysis while retaining the major portion of the sample for separation and bioassay.

2) Use of forms of the same nonpolar stationary phase for all re-chromatography and solvent exchange steps adds predictability to the system.

3) Gentle solvent exchange from the bacteriotoxic CH_3CN, to volatile solvents such as CH_2Cl_2 and final concentration into DMSO minimizes loss of bioactivity.

4) Provision of a high recovery system for the wide range of chemical subfractions suitable for bioanalysis in alternate in vitro and in vivo tests of mutagenicity, carcinogenicity, and teratogenicity.

In the fractionation of this CCEO neutral mixture, nearly all the mutagenic activity was found to be separable into a minor portion of relatively low polarity and was brought to near purity by repeated rechromatography. Theoretically of course the mutagenic activity of the parent CCEO could have derived from the additive effects of diverse weak mutagens. This clearly was not the case for this sample. Studies reported earlier by Loper *et al.*[8] suggest a relatively few compounds account for the mutagenesis in residues recently extracted from drinking water as well. In that report hexane extraction of XAD-2 desorbed residue organics effected mutagenic specific activity enrichments of greater than 20 fold. For one such subfraction the specific activity was 10^4 revertant TA100 colonies/mg assayed in the absence of microsome activation. We have recently examined this subfraction by reverse phase HPLC.[11] Twenty major peaks were observed so the specific activity of the active mutagen(s) is likely to exceed 10^4 and may approximate that of the activation dependent CCEO mutagen described in this paper. Although final isolation, chemical characterization, and extensive biological assessment of such compounds remain for the future, it is interesting to note that their mutagenic specific activity exceeds that of several known chemical carcinogens. These relationships are presented in Table II. The carcinogens β-naphthylamine and 3-methylcholanthrene are listed for comparative purposes only; the promutagens isolated from the CCEO sample are not aromatic based upon UV absorption and preliminary data of mass spectrometry, but have properties of polychlorinated aliphatic ethers.

On the basis of these values presented in Table II, from our additional earlier observations,[8] and from those of other workers[2] we conclude that drinking water may contain a vast number of non-mutagenic compounds, and possibly some of low activity, together with a small number of compounds which are highly mutagenic. We have described a coupled bioassay/HPLC fraction procedure for the isolation of such latter mutagenic compounds based upon our experience with a CCEO residue. The procedure will be tested further in our laboratory for the separation of mutagens in residues of recent drinking water samples which have been isolated using XAD-2 and possibly other resins. By focusing upon the bioactive components during fractionation we anticipate the procedure will provide a general method of fractionating the total sample of residue organics suitable for subsequent compound identification.

TABLE II

Mutagenesis for TA100 of drinking water residue fractions in comparison to that of known carcinogens.

Mutagen[a]	Net revertant colonies per plate per milligram
Known compounds	
Direct acting:	
4-Nitrobiphenyl	5×10^4
1,2:7,8-Diepoxyoctane	7×10^2
Microsomal activation dependent:	
3-Methylcholanthrene	2×10^5
β-Naphthylamine	6×10^4
Residue fractions	
Direct acting:[b]	
XAD eluate of city 5	4×10^2
Hexane extract of XAD eluate	10^4
Microsomal activation dependent:	
Isolated HPLC component of CCEO neutral fraction	4×10^5

[a]Data for the known compounds is taken from McCann *et al.* (12).
[b]Origin of the XAD eluate and its hexane extract and specific activity values are described in Loper *et al.* (8).

Acknowledgements

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