HEI

COMMUNICATIONS

Theoretical Approaches to Analyzing Complex Mixtures

BIOCHEMICAL APPROACHES

Immunoaffinity Chromatography in the Analysis of Toxic Effects of Complex Mixtures
William E. Bechtold and Jon A. Hotchkiss

Stationary-Phase Programming for Liquid Chromatography: A New Concept for Separating Complex Mixtures
John G. Dorsey and H. Brian Halsall

Supercritical Separation and Molecular Bioassay Technologies Applied to Complex Mixtures
David L. Springer, James A. Campbell, and Brian D. Thrall

STATISTICAL APPROACH

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Includes HEI Comments

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Background for the Complex Mixtures Program

Introduction

It is HEI's mission ultimately to determine and provide a quantitative measure of the human health effects of exposure to vehicle emissions at concentrations relevant to actual ambient exposures. Our understanding of the toxicity of such environmental pollutants, even when they are studied in isolation, is incomplete. This problem is compounded by the fact that environmental pollutants rarely, if ever, occur as single substances, but are part of larger mixtures, which often contain thousands of compounds; these are known generally as complex mixtures. The environment, especially in heavily industrialized regions, contains a wide variety of such mixtures. Typical examples of mixtures commonly found in ambient air include cigarette smoke, toxic waste, emissions from industrial sources, and evaporative emissions or products from the combustion of fossil fuels.

Individual components of complex mixtures may be associated with toxic responses, ranging from short-term toxicity to effects with longer-term consequences including mutagenicity and carcinogenicity. However, it is not enough to simply understand the toxicity of individual components in the mixture. The toxicity of one pollutant may be affected by the presence of other pollutants (Elashoff et al. 1987; Simmons and Berman 1989). This may be attributed to a number of factors, including the effects of one substance on the pharmacokinetics or metabolic transformation of a second substance.

Within recent years, many research efforts have focused on identifying and characterizing specific complex mixtures. As analytic approaches have become more sensitive and specific, it has been possible to better identify and quantify components of individual mixtures. Less has been done to develop a systematic and more general approach that could be used to identify in a few steps the toxic components of a number of mixtures and to better understand their toxicologic interactions and effects.

The need for such a systematic approach was the basis of HEI's decision to develop RFA 90-6 "Theoretical Approaches to the Health Effects of Complex Mixtures". This RFA was intended to support several theoretical studies that might, in turn, lead to the development of better analytic approaches.

Purpose and Goals of Studies Funded Under RFA 90-6

Scientific Background To assess the toxic effects of a complex mixture, it is necessary to characterize toxicologically active components of the mixture and to understand the interaction of these components with other mixture components.

In general, two broad analytic approaches have been used. One approacb is based on partial resolution of a naturally occurring mixture into mixtures of fewer constituents (a breakdown approach). The other is based on the artificial construction of mixtures of compounds (a build-up approach). Some aspects of each approach are described in a report of the National Research Council (1988). In the first approach (Lewtas 1988, 1990; Schuetzle and Daisey 1990), various analytical techniques are used to separate complex mixtures into individual components or fractions of fewer components. During separation, the components or fractions are assayed individually for toxicity, generally using short-term tests such as those for mutagenicity. Toxic fractions are then further fractionated. This process is usually referred to as a bioassay-directed fractionation approach. In the second approach (Chapin et al. 1989; Germolec et al. 1989; Yang et al. 1989), pure compounds are combined to form defined mixtures of as few as 2 and as many as 25 to 30 components. These mixtures then are tested for toxicity, generally using short-term tests as biological end points.

Each approach has limitations. One problem of the bioassay-driven approach is that materials may be lost or chemically modified during analysis. This is of special concern if the toxic material is present in very small amounts or is not separated easily from other components. A

second problem involves the bioassay-driven strategy often used in analytic schemes. Bioassay-driven separations are specifically directed toward the toxicologically active fractions and usually do not focus on those that are toxicologically inactive. However, the inactive fractions may contain substances that modify the activity of compounds in the active fractions; if the compounds in the inactive fractions remain undetected, their toxicological role in the mixture remains unknown. Aspects of this problem have sometimes been addressed by "spiking" isolated fractions with a known toxic chemical such as benzo[*a*]pyrene (Kaden et al. 1979).

The build-up approach offers the possibility of relating interactions to a mixture of known chemical composition. However, this approach uses mixtures that are artificially constructed; thus, the specific interactions may not represent interactions found in naturally occurring mixtures, in part because the proportions of individual constituents in the constructed mixtures are unlikely to represent proportions in a "naturally occurring" complex mixtnre.

To determine chemical interactions in a complex mixture is a major challenge. One significant limitation is the large number of sample combinations that would need to be tested. In theory, if the quantitative and qualitative composition of a mixture were known, all components could be combined and all combinations tested for synergism. However, because of the amount of testing required, such an approach is not feasible. For example, to screen only one dose made up of two components of a mixture of 100 individual components, approximately 5,000 different combinations of binary mixtures would be required. This "simple" approach only would identify interactions at specified proportions; and because it would fail to identify interactions at other concentrations, it would not determine which interactions are local (occurring only at certain proportions) and which are global (occurring at all proportions). Furthermore, this "simple" approach would neither mimic all of the in vivo processes that ultimately affect toxicity nor examine all possible toxicologic effects.

Specific Goals of Funded Studies In considering how to advance the understanding of component interactions in complex mixtures, the HEI Research Committee felt that a major stumbling block was the practical impossibility of testing all component combinations to assess interaction. The Research Committee suggested that a fruitful approach might be to simplify the number of components to be tested by dividing the mixture into submixtures on the basis of functional groups that might be related to particular mechanisms of toxicity. Then it would be possible to do a preliminary assessment of interactions, without necessarily identifying the toxic components, by looking at interactions between the submixtures. An ideal analytic approach to complex mixtures wonld be adaptable to a nnmber of different mixtures, and should be designed to separate mixtures into groups according to a common characteristic in a minimum of analytical steps. Thus separated, mixtures could be tested to determine whether or not the common characteristic is a major determinant of toxicity. If the separation process yielded fewer fractions (rather than many), determination of interactions would be more feasible. It was with this in mind that proposals for theoretical approaches to the analysis of complex mixtures were solicited. These proposals were asked to respond to one or both of the following objectives:

- the development of analytic methods to separate or detect, in complex mixtures, components associated with biological activity, and
- the development of methods to determine interactive effects of toxic components in complex mixtures.

It was intended that successfully developed proposals would produce publishable theoretical papers. It was not required that papers describe an approach to a specific complex mixture; however, investigators were asked to develop a theoretical approach that could be tested experimentally using a complex mixture of known composition made up of approximately 100 different chemicals.

Thirteen applications were received. These were evaluated by an ad hoc panel of investigators with expertise in chemical analysis of complex mixtures, hioassay techniques, and statistics; and by the HEI Research Committee. Four applications were funded; three involved the

development of analytic approaches to better identify toxic components, and the fourth involved the development of methods to determine interactive effects. Although investigators were not required to include biological methods for testing, one study did address the development of such methods. Total cost for the four theoretical studies was $166,627. Periods of work ranged from three months to one year.

In addition to the funded studies, HEI held a workshop on synergy and independent action to which a number of scientists and mathematicians with expertise in statistical analysis were invited. The workshop was intended to stimulate further thinking in this area. Although the workshop identified some potentially fruitful areas, participants concluded that there was no obvious "next step" to be taken in this field and that further thought was needed. Participants agreed on the value of integrating statistical, biological, and chemical approaches in research efforts.

The Investigators' Reports were received at HEI in the winter and spring of 1993 and reviewed by outside technical reviewers and by the Review Committee at its October 1993 meeting, at which time the reports were accepted. During review of the Investigators' Reports, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the reports. Because the three analytical studies have a similar focus, and because the analytical studies and the statistical study are complementary, the Review Committee recommended publishing the set of studies together in an HEI Communication format.

There are four reports included in this publication. Three address analytical approaches to identifying toxic components:

- William E. Bechtold, Jon A. Hotchkiss, "Immunoaffinity Chromatography in the Analysis of Toxic Effects of Complex Mixtures";
- John G. Dorsey, H. Brian Halsall, "Stationary-Phase Programming for Liquid Chromatography: A New Concept for Separating Complex Mixtures";
- David L. Springer, James A. Campbell, Brian D. Thrall, "Supercritical Separation and Molecular Bioassay Technologies Applied to Complex Mixtures";

and one describes statistical approaches to analysis of interaction:

- Chris Geunings, W. Hans Carter Jr., Kathryn Dawson, "Using the Parallel Coordinate Axis System to Analyze Complex Mixtures: Determining Biological Activity and Interactions Among Components."

HEI COMMENTS

Analytical Approaches to Identifying Toxic Components

Overview of Analytical Separation Methods

The primary focus of the analytical methods developed in these studies is on identifying compounds or classes of compounds. Two approaches (Dorsey and Springer) presuppose that the specific toxic compounds or classes of compounds will not have not been identified prior to the resolution process, although knowledge of the source of the mixture might be available. Toxicity of subfractions of the original complex mixture would be assessed with a bioassay. Thus, generally these approaches are bioassay-driven (Lewtas 1988, 1990; Scheutzle and Daisey 1990). One of these two studies (Springer) describes bioassay methods that would be used for identification. A third study (Bechtold) assumes that the toxic compound or class of compounds is known. The analytical approach in this study uses a probe specific for certain identified toxic compounds to test the mixture for the presence or absence of these compounds. Thus, this approach may be said to be toxicity-driven.

Chromatography often is used to separate and identify substances in complex mixtures. Three chromatographic methods were applied to the analysis of complex mixtures in these studies: programmable size-exclusion chromatography, used by Drs. Dorsey and Halsall; affinity chromatography, used by Dr. Bechtold; and supercritical fluid extraction and chromatography, used by Drs. Springer, Thrall, and Campbell. Recent advances in each of these methods offer improved resolution and less loss of material from chemical modification or physical manipulation.

All types of chromatography involve the interaction of a sample with two phases: a mobile phase that may be a liquid or gas, and a stationary phase, generally a liquid or solid. Sample components distribute themselves between the mobile and stationary phases and move through the chromatographic medium at varying rates. Components with less affinity for the stationary phase move more rapidly through the medium; over time this results in resolution of the sample components. The efficiency of any particular chromatographic process depends on the nature of the mobile and stationary phases and their interaction with the sample. Components may be resolved at various points throughout the stationary medium (as, for example, with paper or thin layer chromatography), or they may be adsorbed onto the stationary phase and washed (eluted) from it at different times (as, for example, with column chromatography). Components of the mobile phase are designed to compete for binding sites with the bound substance(s) of the stationary phase, thus selectively removing substances from the stationary phase. There are many types of stationary-phase materials used in column chromatography. Interaction of the stationary-phase material with sample constituents may be on the basis of ionic strength, hydrogen bonding, size or shape of the molecules in the sample, or affinity between a substance (ligand) bound to the stationary phase and components of the sample to be resolved. Some types of interaction and resolution are based on more than one type of selectivity.

Specific Approaches Used in These Studies

Mobile- and Stationary-Phase Programming

In cases in which a large number of substances are to be resolved, programming the elution process to include stepwise or gradient elution generally will result in better resolution in a single chromatographic step. Better resolution also may be accomplished by multiple chromatographic steps, in which pooled fractions isolated from one chromatographic system are applied to a second system, or, alternatively, the eluent stream from the first system is applied directly to the second system. In theory, pooled fractions from a second system could be applied to a third system and so on; if the basis of each system's resolution were sufficiently different, such a multistep process might result in better resolution. However, a multistep approach can be limited in efficiency because material is lost during each resolution step. Equally important, sample components may diffuse as they are eluted, resulting in overlap

of material, especially when an eluate from one system is applied directly to a second system. An alternative strategy might be to program changes in the stationary phase within, rather than between, columns. This might be done by combining materials with different selection characteristics in a single stationary phase, or by introducing changes in the stationary phase that occur in conjunction with programmed changes in the mobile phase. To be successful, a programming strategy should be designed so that substances resolved in one way within the column would not recombine as a result of the second resolution step.

One approach to programming changes in the stationary phase takes advantage of the relationship between polypeptide conformations and such characteristics of the surrounding medium as pH, ionic strength, and solvent polarity. Variation in these characteristics will affect polypeptide conformation. This, in turn will affect the size, shape, and charge density of the polypeptide. Stationary-phase selectivity is, in part, a function of these parameters; hence, it should be possible to incorporate polypeptides into the stationary phase, and, by programming changes in solvent strength and polarity, program changes in stationary-phase selectivity.

There are a number of different polypeptide conformations; however, of particular interest are two types of repetitive conformation commonly found in a variety of both hetero- and homo-polymers of amino acids: the α-helix and the β-pleated sheet. Each of these structures requires close packing of amino acids in a repetitive pattern, and only certain amino acids, under specific conditions of pH and ionic strength, will form either type of structure. Predictive models have been developed to determine the likelihood of particular amino acids being found in either type of structure; nonetheless, whereas some agreement exists between theoretical prediction and experimental finding, the degree of predictability decreases as the amino acid composition of the polypeptide becomes more heterogeneous (Stryer 1988). Predictive models generally assume that peptides are in solution. However, the principles governing peptide folding in solution should apply to polypeptides bound to a support medium so long as the polypeptide is in contact with the solvent.

Immunoaffinity Chromatography In affinity chromatography, the stationary-phase material consists of a ligand that has biological specificity linked to a support medium. Commonly-used ligands include hormones, enzymes, and antibodies. All affinity chromatography depends upon prior identification of the material or materials to be resolved. During chromatography, sample materials interacting with the ligand are removed from the mixture as it passes through the column. The ligand-bound materials are then removed from the stationary phase by passing a solution that contains the unbound ligand through the chromatographic system. The success of affinity chromatography requires that the stationary-phase ligand bind sufficiently strongly to substances to remove them from the mixture as it passes through the chromatographic system; but binding must not be so strong that the bound material cannot be easily removed in an unmodified form during elution.

In immunoaffinity chromatography the ligand is either an antigen or an antibody. This type of affinity chromatography is both highly sensitive and selective and can be applied to a large number of different types of substances, so long as antibodies against the substances can be prepared. In some cases, it is possible to purify antibodies not only against a substance, but against a particular functional group of the substance. Antibody preparations can be very specific, as in the case of monoclonal antibodies, or more general, as in the family of immunoglobin G antibodies, specific to a number of substances with a common structural element. Antigen-antibody binding is a physical process and requires that both antigen and antibody have the "proper" molecular conformation. Antibodies assume this conformation in aqueous environments, generally at neutral pH values, thus any nonpolar substances isolated from complex mixtures also must be soluble in an aqueous neutral medium. This may require that the substances be dissolved in media such as dimethylsulfoxide. It is not clear the precise effect that this will have on the binding or recognition process.

Supercritical Fluid Extraction and Chromatography A supercritical fluid is a highly compressed gas with liquidlike properties that exists above its critical temperature. The critical temperature is the temperature

above which the substance will not exist as a liquid, irrespective of the pressure applied. However, if a gas is compressed to sufficiently high pressures at temperatures near or at its critical temperature, liquidlike interactions of the molecules become significant and the gas will exist in a single phase with liquidlike viscosity and density. Supercritical fluids are less viscous and more diffusible than liquids, although their densities are generally 100 to 1000 times those of gases. However, their properties of diffusion and density change significantly with small changes in pressure or temperature. Because of the ease with which their properties can be altered, supercritical fluids can be extraordinarily selective and capable of highly effective separation of classes of compounds. Because supercritical fluids are easily volatilized by changes in pressure, they are removed readily from the extracted compounds, leaving relatively pure compounds or classes of compounds, unaltered by extraction.

A number of factors influence the degree of a substance's solubility in a supercritical fluid. These include temperature; degree of polarity of the fluid (polar fluids are generally better solvents for polar solutes); and pressure, which is a determinant of fluid density (Gitterman and Procaccia 1983; McHugh and Krukonis 1986). The solvating or resolving capability of a supercritical fluid also can be enhanced by introduction of a small amount of a second volatile component, enabling the dissolution of a wider range of compounds or (in supercritical fluid chromatography) changing the selectivity of the mobile and stationary phases.

Supercritical fluids can be used in chromatography, as the mobile phase, as well as in solute extraction. Because the mobile phase temperature is somewhat greater than the critical temperature, mobile-phase diffusibility approximates that of a gas; however, temperatures are closer to ambient than those used in gas chromatography, making supercritical fluid chromatography useful in the separation of thermally labile compounds. Supercritical fluid chromatography may be the final step of separation, or it may be coupled with mass spectroscopy to better resolve and identify sample components.

Biological Characterization Only the study of Drs. Springer, Thrall, and Campbell describes methods to biologically characterize extracted compounds, determining cytotoxicity, mutagenicity and carcinogenicity in vitro and in vivo. Dr. Springer and colleagues propose to use two methods. Rat tracheal epithelial cells will be used to determine cytotoxicity, mutagenicity, and carcinogenic potential in cells. Cells will be exposed to vapor-phase toxicants in vitro using a hydrated collagen gel matrix system described by Zamora and associates (1983). The Big Blue Transgenic Mouse system, developed by Stratagene (Stratagene, La Jolla, CA) will be used to determine mutational frequency in the *lac*I target gene contained within a ZAP shuttle vector following in vitro exposure.

Cultured rat tracheal epithelial cells have been used to indicate the mutagenicity and carcinogenicity of a number of vapors. These cells appear to have a number of advantages in such a system (Nettesheim and Barret 1984). Mutation can be measured at the *hprt* or the *tk* loci. Furthermore, although they appear to undergo dedifferentiation when cultured, if they are reimplanted into trachea, they become reestablished as differentiated cells. Cells that have undergone changes indicating progression toward neoplasia will continue that progress when they are reimplanted into trachea. One of the first stages in this progression is enhanced cell growth, which the investigators propose to use as an end point for measuring the carcinogenic potential of extracted substances. This indicator of carcinogenic potential may be most useful for comparing different toxicants.

The transgenic mouse mammalian system that can be used to test for specific mutational events following in vivo exposure, is more representative than in vitro test methods in accounting for pharmacokinetic and metabolic processes that may occur in humans. The target *lac*I gene, contained within a shuttle vector, can be recovered easily and tested using a color detection system in *Escherichia coli*. Gene sequencing also is possible. However, some mutations may be specific to the plasmid sequence. For example, the high frequency of mutations at CpG sequences in the *lac*I gene are likely due to the presence of methylated cytosines in the gene. These bases, common to procaryotes, are unlikely to occur in mammalian systems; therefore, the mutational frequency may not be representative of true frequency in mammalian systems (Sisk et al. 1994).

Immunoaffinity Chromatography in the Analysis of Toxic Effects of Complex Mixtures

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Abstract

This report describes a new theoretical approach toward the isolation of specific components from complex chemical mixtures using immunoaffinity chromatography with monoclonal antibodies (MAbs)*. By employing an appropriate screening strategy, we can develop MAbs that recognize specific functional groups or unique structural features that impart toxicity to a class of compounds (as opposed to one specific chemical), for example, polynuclear aromatic hydrocarbons (PAHs) possessing a bay region. The MAbs can be attached covalently to a solid-phase support matrix. Complex mixtures are passed over the matrix bed, and compounds with epitopes recognized by the MAbs are retained. The high local concentration of MAbs on the immunoaffinity bed material maximizes the ability to bind and retain a low-concentration component of the complex mixture possessing the desired structural feature. This allows dilute samples to be analyzed without prior concentration. Solvent conditions then can he changed to elute the retained compounds. The two separate mixtures can be tested to determine their individual toxicities, and the contribution of the individual compounds to the toxicity of the total mixture can be assessed. A wide range of compound types can be monitored conveniently by developing a panel of class-specific MAbs. We describe the approach in detail for one group of compounds that is anticipated to contribute significantly to the toxicity of combustion mixtures, bay-region PAHs.

The specific aims of this theoretical proposal are to develop a conceptual basis for the use of monoclonal antibodies and immunoaffinity chromatography for the isolation of selected components of complex chemical mixtures, and to describe in detail one possible experimental design that could establish the utility of the approach.

Introduction

Studies of the toxic effects of complex mixtnres often center on the toxicology of a single chemical found in the mixture. Although valuable information can be gained from this approach, additional information on the toxic contribution of other compounds and on interactive effects of the mixture's components would be useful. The importance of interaction in mediating the effects of both recognized and unrecognized toxicants has spurred a report by the National Research Council (1988) which addresses methods for in vivo toxicity testing of complex mixtures.

That report identified several strategies for evaluating the toxicity of complex mixtures. One important strategy involves looking for the toxicologically important chemicals in mixtures on the basis of bioassay-directed fractionations, which are aimed at identifying the biologically active components of a mixture. Mixtures are subjected to chemical fractionation schemes that, in principle, separate them into chemically identifiable classes. The toxicities of the fractions are tested, and the most active are further fractionated. Eventually, the chemicals found in the most active fractions are identified by analytical methods. There are an extraordinary number of different fractionation schemes based on a broad range of separation phenomena.

The ideal method for isolating particular components of complex mixtures would be highly selective in separating known chemical functionalities or classes of compounds, especially those for which toxicity might be expected. It

* A list of abbreviations appears at the end of this document.

This Investigators' Report is one part of HEI Communication Number 4, which also includes three Investigators' Reports by Dorsey and Halsall, Springer and coworkers, and Gennings and coworkers, and Comments by the Health Review Committee. Correspondence concerning this Investigators' Report may be addressed to Dr. William E. Bechtold, Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, P.O. Box 5890, Albuquerque, NM 87185.

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should recover both mass and biological activities quantitatively. This might exclude harsh chemical procedures, or separation media that degrade or adsorb compounds. The method should be universal in its ability to address a variety of sample matrices, including, for instance, mainstream and sidestream cigarette smoke, both solid and liquid toxic wastes, groundwater contaminants, emissions from both gasoline and diesel engines, and emissions from coal-burning furnaces. The procedure should be fast, easy to accomplish, reproducible, and low in equipment and materials costs. Finally, the procedure should be adaptable to preparative isolations so that sufficient mass of the desired fraction can be acquired for biological testing.

Previous studies have incorporated a number of these features in biodirected fractionations of a broad range of complex mixtures (Florin et al. 1980; Guerin et al. 1980, 1983; Hanson et al. 1980, 1982, 1985; Wilson et al. 1980; Later et al. 1981, 1983; Pederson and Siak 1981; Bos et al. 1984). These studies often successfully identified several classes of compounds that significantly contribute to the toxicity of mixtures, especially those related to combustion or coal conversion processes. Important classes of toxic compounds are PAHs, particularly those containing a bay region; nitro- and amino-substituted PAHs; and azaarenes. One problem has been that although selected chemicals within biologically active fractions often can be identified, their overall contribution to the mixture's toxicity may be difficult to assess because of losses that take place during the fractionation process. In some cases no analytical standards exist for other compounds possessing similar structural features that render them toxic, making identification impossible and causing their contribution to the overall toxicity of the mixture to be ignored. Finally, interactive effects of the remaining compounds are difficult to assess owing to losses and reactions that take place during the lengthy process of fractionation.

An ideal solution to this problem would be the development of a separation medium that, when introduced into a column, selectively could recognize a chemical group or structural feature likely to be associated with the complex mixture's toxicity. Upon introduction of the complex mixture to the column, only compounds that possess the structural group of interest would be retained; all other components would pass through. Ideally, the medium could be modified selectively to retain virtually any chemical moiety, and it should be sufficiently inert to impede degradation and irreversible adsorption of applied materials. Thus the contribution of the target compound class to the total toxicity of the mixture, and the interactive effects of other components of the mixture, could be evaluated.

Chemically creating such separation media would be extraordinarily difficult; however, nature routinely achieves this level of chemical specificity with antibodies. Antibodies bind ligands ranging in size from approximately 6 Å to 34 Å with association constants in the range of 10^4 to 10^{12} L/mol (Pressman and Grossberg 1968). Antibodies have been generated selectively against biopolymers such as nucleic acids, proteins, and polysaccharides, and against smaller multifunctional molecules such as steroids and eicosanoids. More than three decades ago, immunologists generated antibodies that could selectively bind low-molecular-weight molecules such as dinitrophenol (Farah et al. 1960). The antibodies even could differentiate between the isomeric substitution patterns of the two nitro groups. Since then, interest has grown in generating antibodies to environmentally significant chemicals for trace residue analysis. Immunoassays have been developed to measure herbicides, insecticides, carcinogens, and DNA adducts (Vanderlaan et al. 1988; Bonfanti et al. 1990; Prevost et al. 1990; Rule et al. 1994).

A more recent development has been the use of MAbs in environmental toxicity testing (Galfre and Milstein 1981). Monoclonal antibodies have two great advantages over serum-derived polyclonal antibodies. First, unlimited amounts of a MAb of uniform and defined specificity can be produced, thus allowing for standardization of assay conditions and interlaboratory comparisons. Second, because each MAb recognizes only one epitope, a very high degree of chemical group specificity can be obtained.

Traditionally, MAbs used to detect specific environmental contaminants have been selected on the basis of their ability to bind to one specific compound or isomeric form of a chemical. Therefore, any MAb that binds to the compound of interest and also to other structurally related compounds is of little use and is discarded. However, a screening strategy can be

used that selects MAbs on the basis of their ability to selectively bind to a specific structural feature or functional group common to many different compounds. There is ample evidence in the literature that MAbs that recognize structural features of compounds can be developed. The concept of designing catalytic antibodies—antibodies that catalyze specific chemical reactions when they bind to substrate molecules—is rooted in the idea that the tremendous diversity and specificity inherent in the immune system can be exploited to develop antibodies that recognize almost any structural residue. MAbs have been designed that catalyze ester, carbamate, and amide hydrolysis; lactonization; aminolysis; and a Claison rearrangement (Tramontano and Schloeder 1989, and references within). All of these MAbs are remarkably selective for their predicted substrates.

A key to developing catalytic antibodies, or any antibody that must recognize a specific structural feature, is the design of an appropriate hapten and carrier system. The hapten must display the epitope to be targeted in a way that is "visible" to the immune system. The simplest method is to use a compound that contains the salient structural feature, or a fragment of that compound, as the hapten. Another possibility is to synthesize a hapten that mimics the important structural features.

Presentation of the hapten is important because the geometry of attachment of the hapten to the carrier may influence the antibodies elicited. In general, the groups farthest away from the site of attachment to the carrier are immunodominant (Pressman and Grossberg 1968). Therefore, the structural feature to be recognized by the MAb should be in the immunodominant position.

One way to favor the recognition of haptens as ligands distant from other sites on the carrier is to include a "spacer." Typically, a flexible chain of four to eight atoms is inserted (Tramontano and Schloeder 1989), which reduces steric hinderance from the carrier surface. In addition, the spacer may allow conformational variation of the epitope; increasing the number of conformational modes may produce a wider range of useful MAbs. The use of a spacer also can allow for practical methods by providing a means to efficiently couple the hapten to the carrier.

Antibodies of high selectivity typically have been used as reagents to identify and quantitate trace constituents of complex mixtures. Antibodies also have been used to isolate trace constituents by means of immunoaffinity chromatography (Tierney et al. 1986). In this process, the antibodies are linked covalently to a solid-phase support such as cyanogen bromide–activated Sepharose 4B beads. The resultant immunoaffinity bed material is introduced into a chromatography column. When mixtures are passed through the column, the desired analyte is retained; the remainder of the mixture can be collected for further analyses. The analyte retained on the column can be eluted by a different solvent regime. For example, Tierney and associates (1986) used immunoaffinity chromatography to isolate benzo[*a*]pyrene (B*a*P) diol epoxide–DNA adducts from hydrolyzates of mouse skin treated with B*a*P.

Immunoaffinity chromatography increasingly is being coupled with other analytical techniques in the analysis of complex matrices. In all cases, specificity and selectivity of the antibody interaction with the antigen are exploited to concentrate the desired analyte prior to quantitation. Immunoaffinity chromatography has been coupled with enzyme-linked immunosorbant assays (Prevost et al. 1990; Groopman et al. 1992a; Okumura et al. 1993), high-performance liquid chromatography (Groopman et al. 1992b), and gas chromatography–mass spectroscopy (Rule et al. 1994; Bonfanti et al. 1990) to measure carcinogen-DNA adducts in urine, serum, and tissue samples. These methods can be highly automated. Rule and associates (1994) recently described a fully automated system for the determination of carbofuran by on-line immunoaffinity chromatography, with coupled-column liquid chromatography–mass spectroscopy.

Theoretically, an immunoaffinity matrix can be developed to isolate and quantitate almost any constituent of a complex mixture, assuming that the ability to recognize the targeted epitope is within the genetic repertoire of the immunized host species. We describe one such approach. The structural candidate for isolation from complex mixtures is PAHs with a bay region. PAHs have been identified in a broad spectrum of complex mixtures, particularly combustion products (Zedeck 1980). We se-

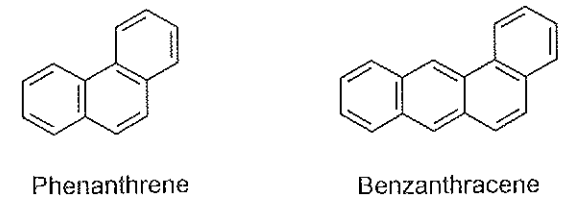
lected PAHs with a bay region because a substantial amount of research over the past 20 years has associated this structural feature with imparting carcinogenic potential to PAHs (Sayer et al. 1989). These PAHs often are metabolized to form benzo-ring diol epoxides with the epoxide group in the bay region (Dipple et al. 1985). This configuration is believed to be the consequent carcinogen for these compounds. Figure 1 shows the structures of several PAHs that form biologically active diol epoxides.

Methods and Study Design

Hapten Production Substances of molecular weight as low as 1000 generally are poorly immunogenic. Therefore, in the proposed research, B*a*P conjugated to keyhole limpet hemocyanin (KLH) will he used as the iunmunogen. Keyhole limpet hemocyanin has been shown to be superior to other proteins in eliciting specific high-affinity antibodies (Muller 1983); the antibody response of KLH was superior to that of borseshoe crab hemocyanin, which in turn was far better than ovalbumin or bovine serum albumin. Tbe strong immunogenic properties of KLH presumahly result from its molecular weight and its phylogenetic distinction from the proteins of the immunized animals.

Benzo[*a*]pyrene would be conjugated to KLH by the method of Kado and Wei (1978), as shown in Figure 2. By this procedure, an isocyanate group can be introduced to the C-6 position of B*a*P. The compound 6-nitro-B*a*P, readily synthesized by the nitration of B*a*P, will be reduced to an amino group with $SnCl_2$. The amino group will be converted by phosgene to an isocyanate group, and the B*a*P isocyanate will be coupled to KLH via a carbamido linkage. The conjugate will be purified by dialysis. The B*a*P will be spiked with a small quantity of tritiated B*a*P prior to the conjugation reactions to allow determination of the molar ratio of hapten (B*a*P) to carrier protein (KLH).

Erlanger (1980) reported that hapten densities of 8 to 25 hapten moieties per carrier molecule produce good results. For screening purposes, B*a*P and other bay-region PAHs will be conjugated to a different carrier molecule (bovine serum albumin) to select against hybrids producing antibody against KLH (see immunoassays). Other non-bay-region PAH–protein adducts, to be used as negative controls, will be synthesized in a similar fashion. Thus, 1-nitronaphthalene, 9-nitroanthracene, 1-nitropyrene, and 3-nitrofluoranthene will be converted to isocyanates and react with bovine serum albumin (instead of KLH).



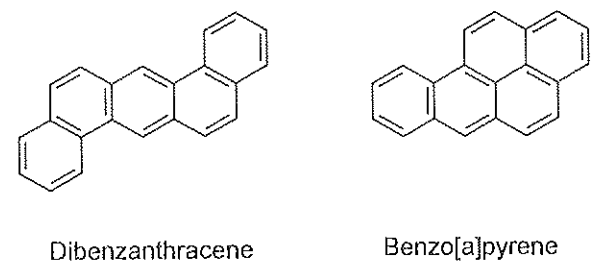


Figure 1. Structural formulas of polynuclear aromatic hydrocarbons.

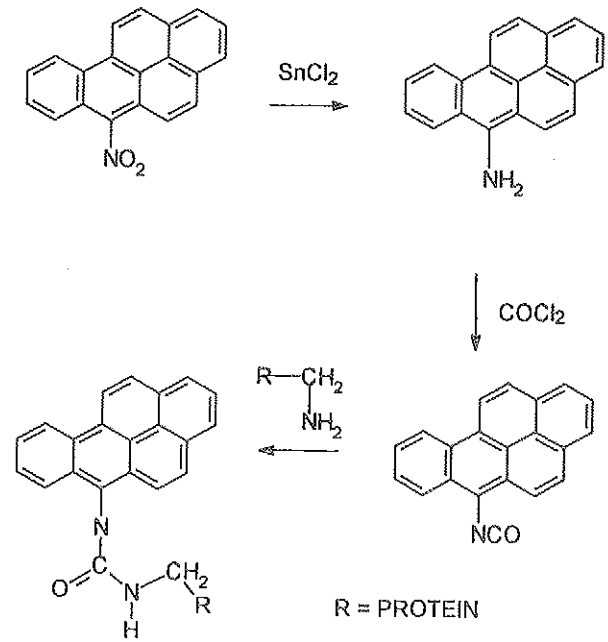


Figure 2. Scheme for synthesizing B*a*P-protein conjugates.

Immunizations Initially, female BALB/c mice will be immunized. We will inject each mouse intraperitoneally with antigen complexed with aluminum hydroxide and emulsified with Freund's complete adjuvant (Muller and Rajewsky 1981). After 14 days, the injections will be repeated, but incomplete Fruend's adjuvant will be used. On day 24, a blood sample from each mouse will be collected, diluted, and screened for immunoreactivity against the adduct with which it was immunized, using a solid-phase antibody capture assay (described in a subsequent section). The adducts will be conjugated to bovine serum albumin instead of KLH to determine the titer of hapten-specific antibodies. Serum from immunized mice will be compared to similar dilutions of normal mouse serum. On day 35, all mice will be injected intraperitoneally with antigen emulsified with incomplete Freund's adjuvant. On day 45, mice will be bled again and their serum will be tested by dot blot assay. On days 56, 57, and 58, the animals with the highest serum titers will be injected both intravenously and intraperitoneally with antigen in sterile pyrogen-free saline. All other mice will be injected intraperitoneally as before.

Hybridoma Production On day 59, splenocytes that form the best responder will be fused with the murine myeloma Sp2/0 (Shulman et al. 1978) according to the method of de St. Groth and Scheidegger (1980). Splenic lymphocytes from the immunized mouse and Sp2/0 cells in log phase growth will be fused with 50% polyethylene glycol. The heterokaryons will be plated into twelve 96-well microtiter plates, each well containing a feeder layer of murine peritoneal exudate cells. Stable hybridomas will be selected from the parental cell populations in medium containing hypoxanthine, aminopterin, and thymidine.

Ten days after the fusion, supernatants from all culture wells will be tested for antibody production. Hybridomas will be screened against the B*a*P-ovalbumin adduct described; positive cultures will be expanded and rescreened in five days. Hybridomas that are positive against B*a*P-ovalbumin and negative against non-bay-region PAH–protein adducts will be expanded and rescreened against other bay-region PAHs. Hybridomas secreting antibody that binds to bay-region PAHs but not non-bay-region PAHs will be cloned by limiting dilution; they then will be expanded, with a portion cryopreserved and frozen for future use. Monoclonal antibodies produced by the selected hybridomas will be purified from serum-free culture supernatants by protein A chromatography and stored at –80°C.

Immunoassays Several different immunoassays could be used in this project. For initial screening of immune serum and hybridoma supernatants, we will use an antibody capture assay. In this assay, B*a*P–bovine serum albumin is added to each well of a dot blot apparatus. After two hours, antigen is removed, and 5% nonfat dry milk in phosphate-buffered saline is added to block any remaining protein-binding sites on the nitrocellulose. After one hour the wells are washed by filtration, and serial dilutions of the test sera or culture supernatants are added. The primary antibody is allowed to bind, after which unbound antibody is removed. Diluted horseradish peroxidase–conjugated secondary antibody is allowed to bind and excess MAb is washed away. The entire nitrocellulose sheet then is incubated in diaminobenzidine in Tris buffer with 0.001% hydrogen peroxide to make the primary antibody visible, and the membrane is analyzed with a scanning densitometer. This assay is easily and rapidly performed, and accurate, but does not discriminate between high- and low-affinity antibodies.

It is generally believed that the affinity of a MAb for its antigen is a critical factor in determining its usefulness. For example, MAbs used to quantitate (immunoassay) or localize (immunohistochemistry) antigen require high-affinity constants ($K \approx 10^9$ to 10^{12} L/mol) so that the antigen-antibody complexes are not easily disrupted, whereas immunoaffinity chromatography is thought to require MAbs with somewhat lower-affinity constants ($K \approx 10^6$ to 10^7 L/mol) so that the antigen bound to the column can be eluted under mild enough conditions to prevent damage to either the antigen or the antibody (Van Heyningen 1986). However, there is ample evidence that little or no correlation exists between antibody affinity and ease of antigen elution (Parham 1983; Bonde et al. 1991; Pepper 1992). Therefore, we will take a more pragmatic approach and select all MAbs that bind a range of PAHs with bay regions and discriminate against those PAHs without bay re-

gions. These MAbs then will be tested directly for their ability to both bind and release a range of bay-region PAHs when coupled to a solid support.

Determination of Monoclonal Antibody Affinity and Cross Reactivity We will use a competitive antigen capture assay to determine the specificity and affinity of the anti-bay-region MAbs. The MAbs will be bound to a solid support as previously described in the dot blot assay. The amount of MAb bound per well will be adjusted so that approximately 40% to 70% of a radiolabeled tracer ([^{3}H]B*a*P; about 10^4 disintegrations per minute), in the absence of unlabeled inhibitor, is bound. The assay is performed by adding a fixed quantity of labeled B*a*P and of unlabeled B*a*P (inhibitor) to each well of the dot blot apparatus. After a two-hour incubation at room temperature to reach equilibrium (that is, no change in the inhibition values observed upon further incubation) the sample is removed and the well is washed by filtration. The nitrocellulose "dots" are removed and the bound radioactivity is determined by liquid scintillation spectrometry. The data are plotted as percent inhibition of tracer-antibody binding versus inhibitor concentration.

This assay also can be used to determine the relative affinity of the MAbs for other PAHs with and without bay regions. In this case, unlabeled PAHs with and without bay regions are substituted for the unlabeled B*a*P (inhibitor) in the previous assay. The cross-reactivity of the MAbs then can be determined by comparing the concentrations of the various PAHs required to achieve 50% inhibition of tracer binding (Muller 1983). We will select MAbs that show cross-reactivity with a range of bay-region PAHs but low affinity for non-bay-region PAHs.

Immunoaffinity Chromatography Bay-region PAHs will be isolated from complex mixtures by immunoaffinity chromatography. A basic problem will be presentation of the lipophilic PAHs to the hydrophilic antibodies. This can be accomplished by first dissolving the complex mixture in a cosolvent miscible in water. The solvents dimethylsulfoxide, dioxane, and dimethylformamide are candidates, as are detergents such as Triton X-100 and NP-40. These agents are expected to diminish the antibody affinity; therefore, the appropriate dilutions necessary to maintain sufficient antibody binding need to be determined.

Immunoaffinity matrices will be made by immobilizing the selected MAbs on an insoluble support. A wide range of matrices have been used including microporous silica (Chiong et al. 1991; Van Sommeren et al. 1993), particulate nitrocellulose (Hammerl et al. 1993), and derivatized and crosslinked agaroses such as Affigel and Sepharoses (Jack 1992). By far the most common approach is to use cyanogen bromide–activated Sepharose to couple the MAbs via exposed amino groups. Although this technique is very simple, it suffers from the fact that the MAbs are randomly oriented on the supports and therefore only a fraction of the potential antigen binding sites are available for interaction with ligand. In addition, the isourea linkage between the gel and the e-aminogroups of lysine introduces an extra positive charge at neutral pH, causing the gel to act as a weak ion exchanger at low salt concentrations (Van Sommeren et al. 1993).

We will couple the selected MAbs via the carboxyl termini as described by Yarmush and associates (1992). Briefly, all accessible carboxyl groups on the MAbs will be blocked with phenylhydrazine. The blocked MAbs will be digested with pepsin to form $F(Ab')_2$ fragments with exposed carboxyl termini, and subsequently coupled to an amine-containing solid support (AH-Sepharose 4B beads). This procedure results in the oriented coupling of the MAbs to the support matrix. Although the coupling capacity (mol protein/mL of bead) is reduced compared to other methods using random attachment, the antigen-binding capacity (mol antigen bound/mol antibody coupled) is maximized.

Validation of our procedure will begin with several bay-region PAHs. Thus, solutions of radiolabeled B*a*P will be created from the solvent systems described, added to suspensions of the MAb-conjugated resin, and incubated overnight. The samples will be poured into separate 3-mL columns and washed with five column volumes of phosphate-buffered saline; the collected eluate will be counted for radioactivity. The bound B*a*P will be recovered quantitatively by washing the columns with 100% methanol; methanol aliquots will be counted for radioactivity. The efficiency of recovery is represented by radioactivity recovered in the methanol wash divided by total applied radioactivity.

If it is successful, the same procedure will be applied to a mixture of PAHs including phenan-

threne, benz[*a*]anthracene, chrysene, B*a*P (these all have bay regions), and naphthalene, anthracene, pyrene, fluoranthene, carbazole, and dibenzo[*a*,*h*]carbazole (negative controls). Both the original eluate and the methanol extract will be analyzed for the presence of PAHs by isotope dilution gas chromatography–mass spectropscopy. This assay is chosen because most of these PAHs are not available commercially as radiolabeled. If elution with methanol is too harsh we will attempt to accomplish elution by means of pH change, chaotropic reagents, or desorption with increasing ionic strength.

The procedure will be validated further by adding radiolabeled B*a*P or pyrene to a series of complex mixtures and determining the effect of the mixture on recovery. Some mixtures might include diesel exhaust particle extracts, automobile particle extracts, solvent-refined coal, and cigarette smoke particle extracts. Again, the solvent types and dilutions may require alteration for maximum binding.

If the above approach is not successful, either in binding the bay-region PAHs or excluding the non-bay-region PAHs, we will screen other MAb clones for the appropriate affinity. The immunoaffinity approach can be applied to the above complex mixtures. If it is successful, antibodies to nitro-PAHs will be developed and the methods can be applied to diesel exhaust particle extracts.

Determination of the Total of Isolated Bay-Region Polynuclear Aromatic Hydrocarbons Total bay-region PAHs present in a complex mixture (sample) will be determined by competitive radioimmunoassay, using B*a*P as a representative PAH. A known quantity of immunoaffinity bed matrix and an aliquot containing known quantities of B*a*P or serial dilutions of column eluate will be combined and allowed to incubate overnight at 4°C. The next morning, a limiting amount of radiolabeled B*a*P will be added to all tubes and allowed to incubate for an additional two hours at 37°C. The MAb-conjugated beads will be pelleted and washed repeatedly to remove unbound labeled B*a*P; bound radioactivity will be determined by liquid scintillation spectroscopy. Unlabeled B*a*P or similar bay-region PAHs in the column eluate samples will compete for binding with the radiolabeled B*a*P. The percent inhibition of binding will be calculated and the quantity of bay-region PAHs in the column eluate will be determined from a standard curve.

Limitations The biggest limitations to this approach are the high developmental costs and time to select for the ideal antibody. Antibodies previously developed at other laboratories might be useful for initially testing the approach. Other limitations include an inability to conveniently monitor the effects of more volatile compounds. When the complex mixtures initially pass through the column, the first aqueous mixture must be lyophilized to reconcentrate the components; however, during lyophilization volatile components may be lost. One approach to recovering these components would be vacuum-line distillation of the mixture; we have successfully used this method to fractionate complex mixtures with volatile components (Hanson et al. 1985).

Discussion and Conclusions: Application to Standard Reference Material 1650 (Diesel Particulate Material)

The National Institute of Standards and Technology (NIST) has defined several Standard Reference Materials (SRMs) which are readily available to scientists and can be used both for methods development and as reference materials for interlaboratory comparisons. The SRM 1650 (diesel particulate matter) was developed to assist laboratories in validating bacterial bioassays and analytical chemistry methods for determining PAHs. The Health Effects Institute has selected SRM 1650 as one complex mixture that may be fractionated and characterized effectively by new theoretical methods for analysis. The Health Effects Institute has asked authors to address how their method might be used to characterize this SRM further.

May and associates (1992) have reviewed several studies characterizing SRM 1650 for chemical content and biological activity. Most of the chemical analyses that have been performed on SRM 1650 have been at NIST; PAHs with certified concentrations, and additional PAHs with uncertified concentrations, are reported. The certified values are established at NIST by meas-

urement using two independent and reliable methods (the likelihood of two independent methods being biased by the same amount in the same direction is presumed to be small).

Bioassays of SRM 1650 extract have been performed by several laboratories. At this writing, no bioassay-directed fractionations of SRM 1650 were available. However, Schuetzle and associates (1985) analyzed a similar diesel particulate material using solvent extraction and high-performance liquid chromatography fractionation. Both mass and direct-acting mutagenicity in *Salmonella* TA98 were measured in the fractions. Recoveries of mass and mutagenicity were 91% and 81%, respectively. Indirect activity was not measured.

Application of our methodology would be straightforward. First, the appropriate antibody would be developed as described in the experimental section. Antibodies could be raised against bay-region or nitro-substituted PAHs as desired. The SRM 1650 would need to be extracted for release, either by sonication or by soxhlet extraction, of organic compounds bound to particles. Both methods have proven successful at releasing mutagens (May et al. 1992). The material then would be concentrated by evaporation under nitrogen flow. After solubilization in dimethylsulfoxide, the material would be applied to a MAb column as described. The two resulting fractions then would be tested for PAH content and biological activity. Results could be compared to the values previously reported by May and associates.

Acknowledgments

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About the Authors

Dr. William Bechtold's principal research activities have been in biochemical toxicology and analytical chemistry. Currently at ITRI, he is exploring human exposure-dose relationships for inhaled carcinogenic volatile organic compounds. He is developing and characterizing biological markers (e.g., urinary metabolites, blood protein adducts, and DNA adducts) to assess exposures of humans in occupational and environmental settings. The goal is to identify populations that might have unique risks resulting from specific exposures.

Dr. Jon Hotchkiss is interested in the cellular and molecular mechanisms of toxicant-induced airway epithelial injury and subsequent adaptation and repair. He has conducted research in cell-specific gene expression in upper and lower airway epithelia resulting from inhaled irritants and toxins. These studies are designed to identify target-cell populations, investigate mechanisms of toxicant-induced cellular injury, secretory cell hyperplasia and metaplasia, and elucidate the role of inflammatory cells in the proliferative and adaptive responses of airway epithelia to inhaled substances. He currently works in the Experimental Pathology and Toxicology Laboratory, Michigan State University, East Lansing, MI.

Abbreviations

B*a*P	benzo[*a*]pyrene
$F(Ab')_2$	an enzymatically modified antibody
KLH	keyhole limpet hemocyanin
MAbs	monoclonal antibodies
NIST	National Institute of Standards and Technology
PAHs	polynuclear aromatic hydrocarbons
$SnCl_2$	tin dichloride
SRM	standard reference material

HEI COMMENTS

This approach seeks to isolate chemicals as a group from a complex mixture on the basis of some aspect of the mechanism of their toxicity. In order to accomplish this, Dr. Bechtold proposes to take advantage of the structure-activity relations between polynuclear aromatic compounds possessing bay regions and highly reactive, carcinogenic metabolites. Immunoaffinity chromatography would be the method used to separate such components from a sample of diesel particulate extract. The approach entails the formation of a monoclonal antibody raised against a hapten of benzo[*a*]pyrene conjugated to bovine serum albumin. Hybridomas would be screened to select those that produce antibodies selectively recognizing various polynuclear aromatic compounds containing a bay region. Subsequently, immunoaffinity chromatography columns would be prepared by coupling these monoclonal antibodies to Sepharose 4B resin.

A separation scheme predicated on the proposed mechanism of toxicity of a class of compounds is an intriguing concept. The use of immunoaffinity chromatography to separate classes of compounds from a complex mixture is a novel approach, with separation based on a toxicological rationale in addition to the physical and chemical principles which form the basis for more traditional fractionation schemes. In this respect, such an approach may provide a very useful redirection in the analysis of complex mixtures. It has, however, some potential limitations. For this approach to have widespread practical merit, the functional moieties in the chemicals responsible for the toxicity of complex mixtures must be known. Given the present gaps in knowledge of the chemical functionalities responsible for eliciting toxicity, the exact feature responsible for a chemical's toxicity is likely to be unknown. Furthermore, in any truly complex mixture, there may be several chemical classes, acting by several mechanisms, responsible for toxicity. Therefore, even with complete knowledge of the chemical features responsible for toxicity, a large number of different immunoaffinity chromatography columns would be needed to thoroughly fractionate a complex mixture of unknown chemical composition for subsequent toxicological testing. Finally, antibody recognition of an analyte is based on physical interactions, rather than chemical reactivity. Chemical reactivity is, however, a major factor involved in a compound's mode of toxicity. This fact limits the universal application of immunobased techniques to the separation and characterization of complex mixtures. Because antibody recognition is based on physical interations, some nontoxic chemicals may be retained on the column, resulting in false positives. Although this may not be a large problem, particularly if bioassay analysis follows, the possibility of false negatives is an important concern. A false negative would result when an active polynuclear aromatic compound containing a bay region does not bind the antibodies, and thus is not detected. Therefore, the use of monoclonal antibodies may not be appropriate if their specificity is too great, in which case they would produce these false negatives.

Several other practical problems should be addressed before undertaking the approach outlined in this report. Some problems are related to complex mixtures; others are related to the use of immunoaffinity chromatography. For example, there is a wide range of cross-reactivities of polynuclear aromatic compounds for a given monoclonal antibody. Levels of individual polynuclear aromatic compounds present in the complex mixture therefore may lead to unexpected selectivities of binding. The nonequilibrium nature of immunoaffinity chromatography may result in preferential selectivities and some loss.

Some technical problems also need to be addressed. For example, there will be a positive charge associated with cyanogen bromide–activated Sepharose to which the antibody conjugates. This may lead to problems related to ion exchange when the mixtures are applied to the columns. Another technical problem involves conjugating the antigen for immunization. Major chemical changes can occur within the polynuclear aromatic compound as a result of the conjugation process. Therefore, detailed antigen characterization should be performed prior to construction of the immunoaffinity chromatography columns.

An alternative strategy to consider is a two-stage separation, in which an immunoaffinity chromatography column with a general antibody could be used in an initial step to generally separate components of the mixture. The second stage of separation then could take place in an iumunoaffinity chromatography column with a monoclonal antibody. By using general antibodies with their greater yields in the first step, large amounts of materials can be screened in a "clean-up" step. Use of a second stage on the eluate of the first column then can capitalize on the specificity of monoclonal antibodies to achieve cleaner separations.

Given these limitations, it may be possible to develop a few immunoaffinity chromatography columns that would subdivide a complex mixture into fractions containing "homogeneous toxicological endpoints." This approach might provide rational experimental paradigms for testing toxicological interactions in complex mixtures. Simplification of the mixture in this manner would facilitate subsequent chemical analysis. Overall, this report outlines an interesting rationale for the separation of complex mixtures on the basis of toxicologic principles. If the method works, it could serve as an improvement over existing methods. HEI

Stationary-Phase Programming for Liquid Chromatography: A New Concept for Separating Complex Mixtures

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Abstract

We are developing theory and methodology for unique liquid chromatographic methods that combine existing technology, such as mobile-phase programming (or gradient elution techniques), with new technology developed in this laboratory predicated on the concept of stationary-phase programming. Many statistical studies have shown that for real samples containing more than a few components, there is an extremely low probability of separating all of the components in one chromatographic step. The answer to this problem is an increase in peak capacity, which is the theoretical maximum number of resolvable components in a separation. Although mobile-phase programming will accomplish this to some extent, an even more powerful approach to the problem would involve simultaneous mobile- and stationary-phase programming.

We are exploring the separational power attained by varying stationary-phase properties, such as pore diameter of silica gel, along the length of the column. This will result in a well-defined second interaction mode based on size exclusion effects, which will provide separations on the basis of both chemical class and hydrophobicity. This approach will have the benefits of a (pseudo)–two-dimensional separation on a single column, and should dramatically improve peak capacity.

A second approach to stationary-phase programming involves the utilization of chainlike molecules which will undergo changes in secondary structure with changes in mobile-phase composition. Although subtle changes in stationary-phase structure and organization occur in reversed-phase chromatography during a gradient elution process, these changes previously have neither been well understood nor deliberately induced. We are working on theory and methodology for the interaction of solutes with chainlike molecules such as polypeptides that are immobilized on a suitable support. These chainlike molecules can be induced to undergo a conformational change with changing mobile-phase conditions.

We have preliminary results for a theory of separations based on size exclusion on traditional reversed-phase stationary phases. Retention is controlled by the combination of partitioning to the hydrophobic stationary phase and a size-exclusion effect as the solute size becomes too large to access the pores of the stationary phase easily. We also have preliminary results for the bonding of poly(homo)peptides to a silica support, along with resulting chromatographies as a function of mobile-phase composition.

Introduction

The separation of complex mixtures of nn-known samples is a formidable task made even more difficult when the ultimate goal is testing for toxicity of both individual components and components in any possible combination, in which synergism may be a nonpredictable event. An obvions approach to the problem is the separation and isolation of only those compounds that exhibit the property of interest, in this case toxicity. However, when no predictive paradigms exist based on chemical structure analysis, the only remaining approach is one of brute force.

Statistical studies have shown that for multi-component samples the probability of separating all of the components in one chromatographic step is extremely low (Davis and Giddings 1983; Martin et al. 1986). The answer to this problem is an increase in peak capacity, which is the theoretical maximum number of resolvable components in a separation. Although mobile-

This Investigators' Report is one part of HEI Communication Number 4, which also includes three Investigators' Reports by Bechtold and Hotchkiss, Springer and coworkers, and Gennings and coworkers, and Comments by the Health Review Committee. Correspondence concerning this Investigators' Report may be addressed to Dr. John G. Dorsey, Florida State University, Department of Chemistry, Tallahassee, FL 32306-3006.

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phase programming will accomplish this to some extent, an even more powerful approach to the problem would involve simultaneous mobile- and stationary-phase programming.

We are developing theory and methodology showing the separational power that results when stationary-phase properties, such as the pore diameter of silica gel (either bare silica or derivatized in a reversed-phase fashion), are varied along the length of the column. This leads to a well-defined second interaction mode, predicated on size-exclusion effects, that allows separations on the hasis of both chemical class and hydrophobicity. Providing the benefits of a (pseudo)–two-dimensional separation on a single column, this method should dramatically improve peak capacity.

Using chainlike macromolecules that undergo changes in secondary structure with changes in mobile-phase composition offers a second approach to stationary-phase programming. Subtle changes in stationary-phase structure and organization occur in reversed-phase chromatography during gradient elution; however, these changes have not been well understood. The interaction of solutes with chainlike molecules, such as polypeptides immobilized on a suitable support, which can be induced to undergo a conformational change with changing mobile-phase conditions, also will result in a (pseudo)–two-dimensional separation. Both approaches should increase the peak capacity of a chromatographic separation, and improve the reliability of single peaks representing single components.

Theoretical Models for Size-Exclusion Chromatography

Size-exclusion chromatography calibration curves, simulated after taking into account the flexibility of large random-coil solute molecules, correlate very well with experimental data obtained with Hypersil. This model solves the defect of high-molecular-weight simulations attending many previous models. The simulated calibration curves of two bimodal systems show good correlation with experimental results. The effect of reversed-phase partitioning also is considered, but a full explanation of the combination of these two effects remains elusive.

In chromatography, if one component of an eluent is more strongly retained than the solute, then the solute, unable to displace eluent, will be excluded from the surface layer (Kennedy and Knox 1972); if a charged solute has the same charge as the stationary phase, it will have difficulty entering the particles due to the Donnan potential at the snrface (Knox et al. 1980); and if solute molecules have dimensions commensurate with the pore dimensions, they will be excluded sterically from part of the pore volume. When any of these phenomena occnr, the solutes travel along the column faster than the eluent (because the eluent is able to access more stationary-phase pore volume) and we say the solutes are "excluded."

The majority of chromatographers now agree (Yan et al. 1979) that size separation of molecules can be explained fully on a purely steric basis (Giddings et al. 1968; Casassa 1971) in which large molecules can permeate only partially the pore volnme of the support material. Small molecules (such as those of eluent) nearly fully permeate the pores of the support material and are eluted in the void volume, V_m*; large molecules which can not enter any of the pores are excluded totally and eluted in the extra-particle volume, V_o; molecules of intermediate size are eluted between V_o and V_m. The degree of permeation of such molecules into the pore volume of the particles, V_p, is denoted by K, and termed the "exclusion coefficient." K can be defined mathematically as: $K = V_S'/V_S$, where V_S' is the volume of pores available to a given molecular species. K is related to the elution volume, V_R, of any solute by Equation 1 (Knox and Scott 1984),

$$V_R = V_o + KV_p. \quad (1)$$

K can have any value between 0 and 1, and when $K = 1$,

$$V_m = V_o + V_p. \quad (2)$$

Theoretical Exclusion Calibration Curves

Simple Cylinder Model If we consider the solutes as "hard spheres" of radius r and the pore as an infinite cylinder of radius R, and because the center of mass of the molecule cannot approach closer than a distance r from the wall of the pore, then the part of the pore volume acces-

* A list of abbreviations appears at the end of this document.

sible to the center of mass is a cylinder of radius $(R - r)$. Thus the exclusion coefficient, which is equal to the fraction of the total pore volume accessible to the molecule (Knox and Scott 1984), is

$$K = \left(1 - \frac{r}{R}\right)^2; \left(\frac{r}{R}\right) < 1, \tag{3a}$$

and

$$K = 0; \left(\frac{r}{R}\right) > 1. \tag{3b}$$

Figures 1 and 2 show experimental and simulated exclusion curves for various stationary phases. Figure 2, curve a shows a simulated exclusion curve for a fixed pore size of 68 Å. Compared to the experimental curve in Figure 1, there are significant differences at the beginning and end of the simulated curve. Experimental curves also show much more gradual transitions to complete exclusion, a somewhat steeper gradient in the intermediate region, and a less pronounced descent at K approaching unity.

Pore Distribution Model To find the reason for deviations between the experimental and simulated curves, it is reasonable to consider the pore size as a Gaussian distribution; this allows large molecules whose size is greater than the average pore size but within the range of pore size distribution to permeate and be retained.

If we choose a support that has an average pore size R_o and a Gaussian distribution of SD δ, then according to Equation 4,

$$f(R) = \frac{1}{\delta \cdot C} \cdot e^{\frac{(R - R_o)^2}{2 \cdot \delta^2}}, \tag{4}$$

in which C is the normalization constant. This allows us to derive the expression of K,

$$K = \int_f^\infty \left(1 - \frac{r}{R}\right)^2 f(R) dR. \tag{5}$$

Figure 2, curve b is the simulated calibration curve of silica that has a pore size of 68 Å and SD of 30 Å. When we compare it to Figure 1 we see improvement in the fitting of the experimental calibration curve, but there is room for still more improvement. There are reasons other than the pore size distribution that cause the problem.

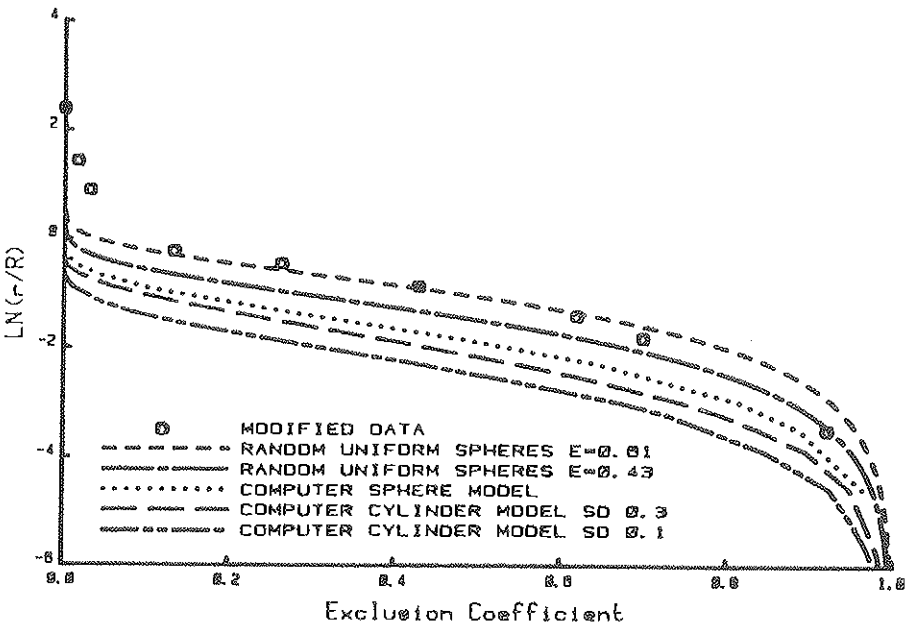


Figure 1. Experimental and simulated calibration curves (Knox and Scott 1984).

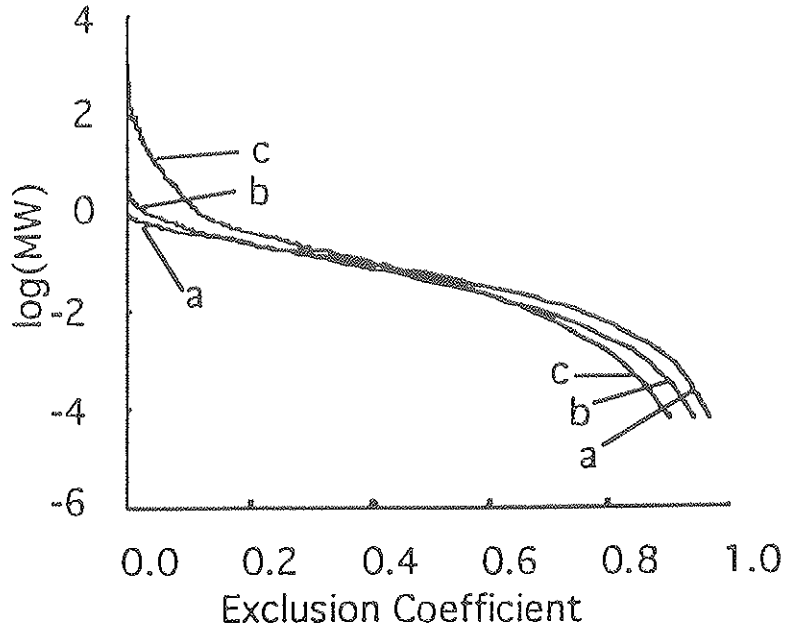


Figure 2. Simulated size-exclusion calibration curve of three models. Curve a: simple cylinder model; curve b: pore-size distribution model; and curve c: flexible molecule model.

Flexible Molecule Model: Partial Permeation Effect of the Random Chain Coil Molecules

In most cases of size-exclusion chromatography, the molecules we are separating are coiled polymers. The effective radius, r, of a sphere is related to its radius of gyration, r_g (Knox and Scott 1984),

$$r = 0.886 r_g; \tag{6}$$

and for random chain polymers r_g is produced by

$$r_g = aMW^b, \tag{7}$$

in which MW is the molecular weight and a and b are constants that are unique for every polymer.

In the previous case, the molecule radius was treated as a rigid value. If we account for the flexible coil property of the polymers, certain segments of the coiled polymer may permeate the pore and partially be retained. When the estimated molecnlar effective radius is far smaller than the pore radius, the molecule behaves as a sphere with an effective radius of r_g. As the estimated molecular effective radins approaches the pore radius, there is more and more flexible linear effect. We use an exponential function to express this tendency.

$$g(r) = e - \left(\frac{2 \cdot r}{R_o}\right)^{1/2} \tag{8}$$

Combining Equations 5 and 8, we get a new expression of the size-exclusion coefficient, K':

$$K' = 0.5 \cdot K + 0.5 \cdot g(r). \tag{9}$$

Figure 2, curve c shows the simulated curve, which is in excellent agreement with the experimental curve shown in Figure 1.

Bimodal System To demonstrate further the effectiveness of this model, we attempt to simulate the bimodal system calibration curve shown in Figure 3. This is achieved simply by Equation 10,

$$K_b = d \cdot K'_1 + (1 - d) \cdot K'_2 , \tag{10}$$

in which K_b is the exclusion coefficient of the bimodal system and d is the fraction of the pore volume of the first stationary phase to the total pore volume of the two stationary phases, and shown in Figure 4.

Using published values of the constants a and b for polystyrenes, we find the effective sphere radins r of polystyrenes:

$$r/\text{Å} = 0.121\ MW^{0.589} \tag{11}$$

Fignres 5 and 6 show simulated and experimental calibration curves of the bimodal pore sizes of 80 Å aud 500 Å, and 47 Å and 1200 Å.

How do the two simulated calibration curves compare? For the 80 Å and 500 Å bimodal system, the simulated calibration cnrve is in good agreement with the experimental results even with the simplest nodel. This is because the linear ranges of the 80 Å pore size silica and the

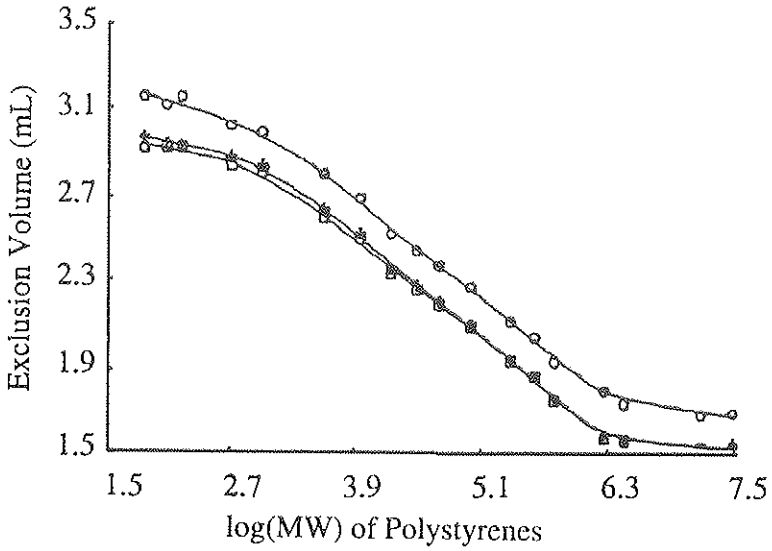


Figure 3. Experimental calibration curve of bimodal system of 80 Å and 500 Å pore size (Northrup et al. 1991). ○ = normal phase; □ = reversed phase of THF mobile phase; and + = reversed-phase mobile phase; CH_2Cl_2.

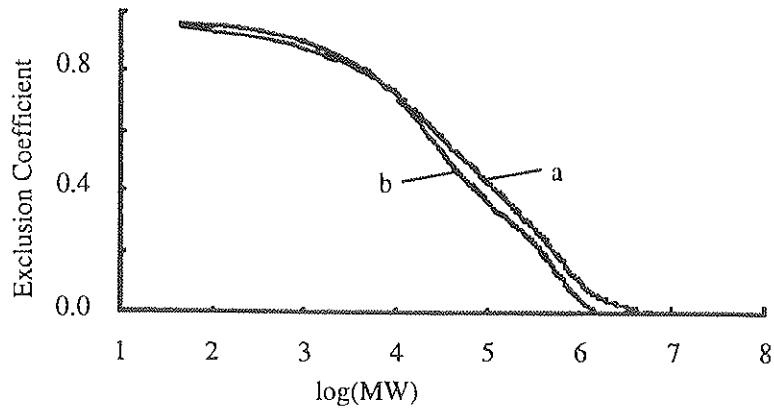


Figure 4. Simulated calibration curves of bimodal system of (a) 80 Å and (b) 500 Å pore size.

500 Å pore size silica are not overlapped but continuously connected. When we look at the 47 Å and 1200 Å bimodal system, it is obvious that the model that takes into account the effect of the flexibility of the polymer molecules produces a better simulation of the calibration curve.

Reversed-Phase Effect In a steric exclusion mechanism, it is assumed that the silica gel acts as an inert matrix that contains solvent in its pores. Interactions between the solute and the gel are assumed to be either nonexistent or the same for all solutes. However, it is not uncommon for solutes to display considerable affinity for crosslinked organic gels. For some solute-solvent gel systems K is greater than unity, which is inconsistent with a steric exclusion mechanism for which K must lie between zero and unity. If we incorporate a partitioning process in the hydrophobic stationary phase (reversed-phase partitioning), and add that to the size-exclusion effect, we can expect retention that exceeds that from the pure size-exclusion process.

In conventional liquid chromatography of small molecules, an important retention parameter is the capacity factor, k', defined by

$$k' = K_p \frac{V_i}{V_o}, \qquad (12)$$

in which K_p is the distribution coefficient for solute partition between the stationary and mobile phases; V_i is the solvent volume within the gel and in this case is identical to V_p.

For large solutes such as polymers, the fraction of V_i available will depend on solute size, so we replace Equation 12 with Equation 13.

$$k' = K_p \frac{KV_i}{V_o} \tag{13}$$

The capacity factor is related to the retention volume, V_R, by

$$V_R = V_o(1 + K'). \tag{14}$$

Therefore we get the retention volume expression of size-exclusion chromatography with the reversed-phase effect

$$V_R = V_o + KK_pV_i. \tag{15}$$

The major problem is how to define K_p, the partition coefficient. First we consider the case that the partition coefficient increases with the molecular weight:

$$K_p = (C')(MW). \tag{16}$$

C' is a weighting factor that relates the partition coefficient to the molecular weight, MW.

From Figure 7 we see that for a fixed-pore-size silica, one molecule with a specified size has the longest retention volume. The retention volume of this molecule depends on the constant C' that relates the partition coefficient to the molecular weight and the pore size.

However, the partition coefficient may be independent of molecular weight, in which case the single peaks of the size-exclusion separation may split into multiple peaks as illustrated in Figure 8.

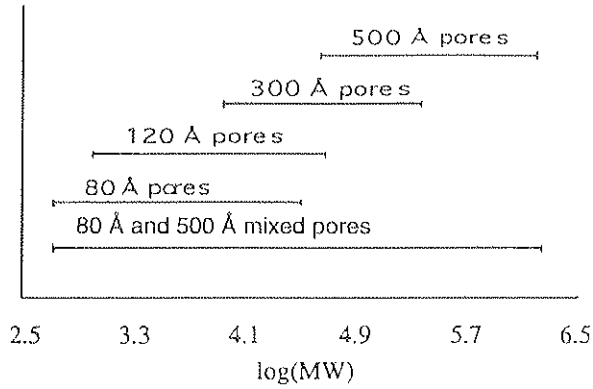


Figure 5. Linear size-exclusion chromatography molecular weight ranges (Northrup et al. 1991).

Conformational Programming of the Stationary Phase The three-dimensional structure of essentially all proteins comprises assemblies of smaller, secondary structural elements. These elements are termed α or β depending principally upon the torsion angles adopted by the polypeptide backbone and the pattern of hydrogen bonding that sustains the structure. An α-structure is helical with defined pitch and translation, and is internally hydrogen-bonded in a regular and predictable way. A β-structure is also regular and helical (Creighton 1993), but the helix of two residues per turn and a translation of 3.4 Å per residue greatly extend the

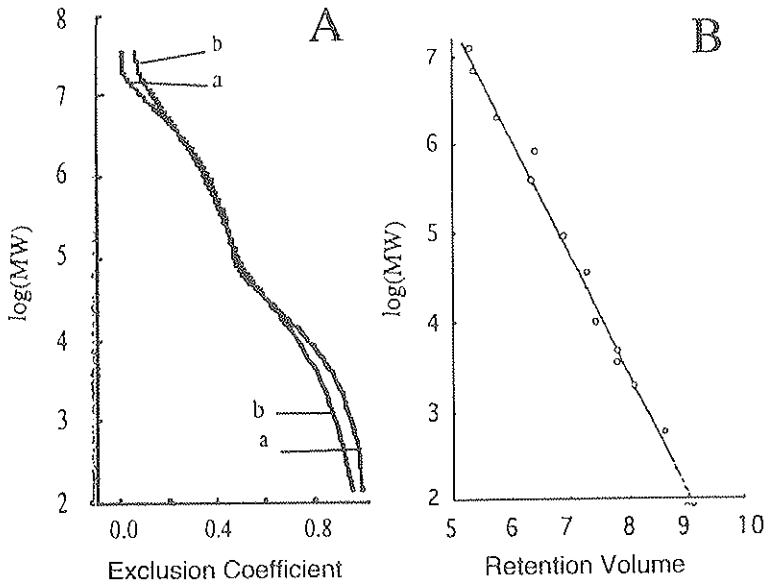


Figure 6. Panel A: Simulated bimodal system of pore size 47 Å and 1200 Å. Curve a: simple cylinder model; curve b: flexible molecule model. Panel B: Experimental result of the bimodal system of pore size 47 Å and 1200 Å (Yau et al. 1978).

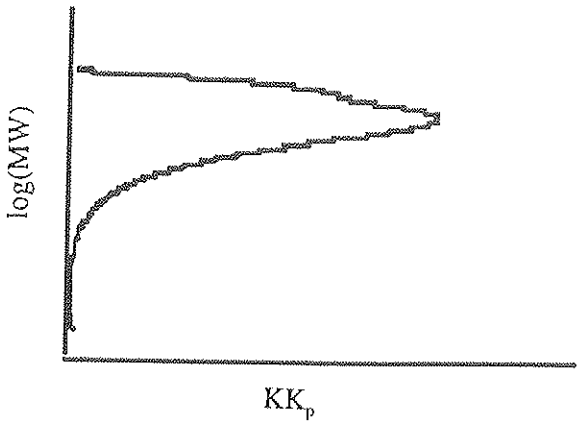


Figure 7. Simulated calibration curve of size-exclusion chromatography with reversed-phase effect.

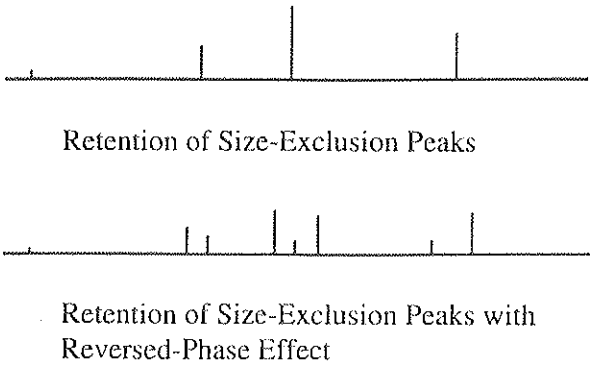


Figure 8. Illustration of the reversed-phase effect on size-exclusion chromatography.

structure such that stabilizing hydrogen bonds must form between strands, not within the helix itself, thus creating sheets. In both forms, the amino acid sidechains project into solution and define the chemical nature of the surface accessible to solvent. Regions of polypeptide not conforming to these two general classes are termed random-coil (although they are not true random coils).

The presence of the entire polypeptide chain is not necessary for the adoption of secondary structure. Short peptides can be induced to adopt secondary structure and to undergo reversible transitions between the three general forms (Mattice 1989; Scholtz and Baldwin 1992). Two principal factors appear to control these transitions: the solvent conditions and the nature of the amino acid sidechains present. In general, secondary structures are promoted by a low dielectric constant, and hindered by bulky and charged sidechains. The details of these processes are understood only poorly in heteropoly(amino acids), but are described quite well for some homopoly(amino acids) (HOAAs) (Hopfinger 1977); however, most of these latter studies have been done in aqueous solution, or in solvents not generally used in liquid chromatography.

The existence of these transitions as a combined function of solvent conditions and amino acid sidechain suggested that it might be possible to use short polypeptides to create on demand, by changing the mobile phase, a chemically well-defined surface as a chromatographic stationary phase with particular adsorptive properties.

Methods

The Stationary Phase Homopoly(amino acids) were chosen for the initial studies. The surface hydroxyls of silica, unreactive to the functional groups of the HOAAs, must be activated. Epoxide activation forms a stable silica-carbon bond and provides a group reactive to the nucleophilic amino (NH_2) terminus of the polypeptide. Such activated silica is available commercially and was chosen for initial studies to provide reproducible material. However, commercial products use proprietary bonding chemistries and polymeric epoxide phases that result in poor resistance to mass transfer and band broadening. Because we would ultimately be requiring well-characterized, monomeric derivatized silicas, we evaluated a procedure to produce them.

The silica surface initially was hydroxylated by acid washing (for 24 hours at 90°C in 0.1 M nitric acid. The product then was refluxed at 90°C under anhydrous conditions in a fourfold excess of monofunctional epoxysilane in toluene with triethylamine as a basic catalyst. This gave a monomeric epoxide coverage of 3.29 $\mu mol/m^2$ from approximately 5 $\mu mol/m^2$ of hydroxyl sites available for ligand coupling on the bare silica. This coverage is comparable to that

attained with typical reversed-phase stationary phases. Nucleophilic attack by the amino terminus opens the epoxide ring. Experiments with different ligands established the conditions for this process. Ultrasonication for five days was used as the driving force (Sentell et al. 1988), under low to moderate temperatures (5° to 40°C), to both minimize unfavorable solution reactions (oxidation, degradation) and maximize the ligand surface density by lowering the HOAA chain entropy. Hydrophilic HOAAs could be attached in 1 M phosphate buffer, whereas hydrophobic HOAAs required aprotic solvents (chloroform, tetrahydrofuran, acetonitrile).

The ligand coverage analysis procedure used differed for hydrophilic and hydrophobic HOAAs. The former were determined by acid hydrolysis of the stationary phase (50 mg derivatized silica in 1 mL 6 M hydrochloric acid for 20 hours at 150°C) followed by the ninhydrin color reaction. Hydrophobic HOAAs were determined by percent carbon analysis.

Chromatography Poly(L-lysine)$_n$ (PLL_{22}) and poly(benzylglutamic acid)$_n$ ($PBGA_{88}$) were evaluated under both normal- and reversed-phase conditions using the commercial chiral stationary phase, Chirasphere, as a control. Chromatograms with analytes and conditions are in Figures 9 through 18.

The monitoring of conformational changes is in a preliminary stage. For this initial work Fourier transform infrared spectroscopy was used to follow conformation as a function of the solution conditions by monitoring the appropriate shifts in the Amide I and II bands. By this means helix-coil shifts as a function of both temperature and pH could be seen readily for PLL_{22} and poly(glutamic acid)$_n$ (PGA_{40}). Solvent systems compatible with the Fourier transform infrared spectroscopy have yet to be found for $PBGA_{88}$. Because it is possible that interactions of the bonded phase with the silica may promote or stabilize secondary structure, it seems clear that a surface technique such as reflectance spectroscopy would be most appropriate.

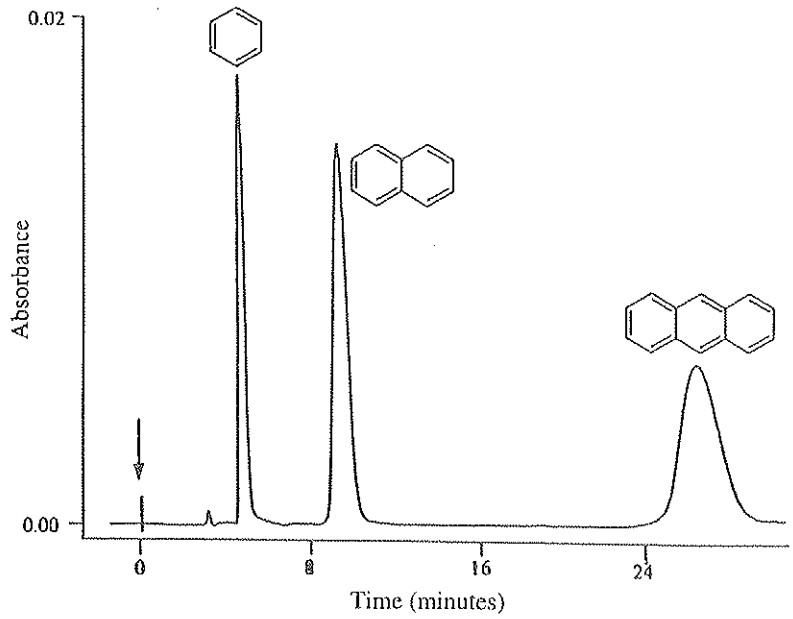


Figure 9. Reversed-phase chromatography on $PBGA_{88}$ in 70:30 water:acetonitrile at 25°C. Effluent at 1 mL/minute was monitored at 254 nm.

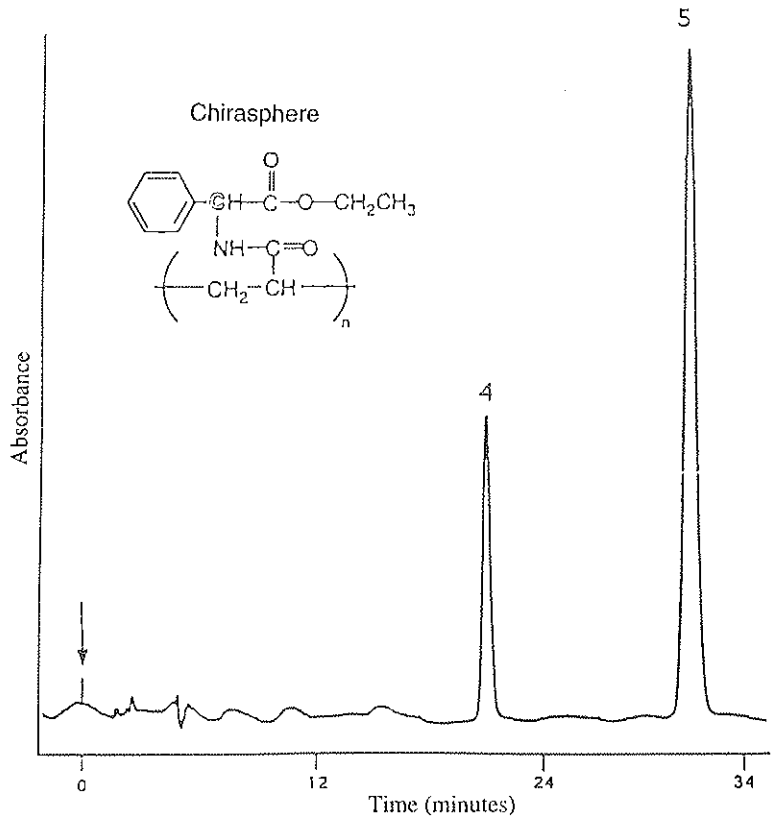
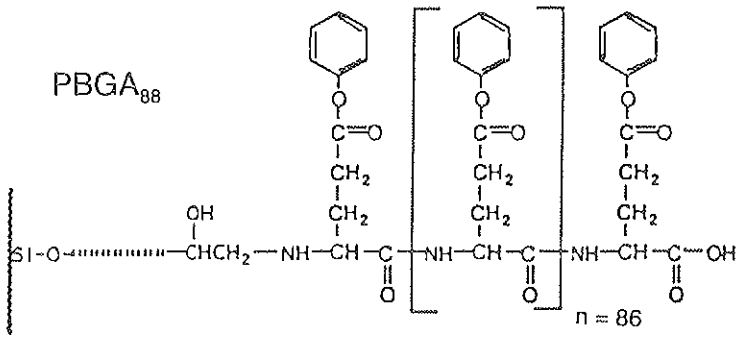


Figure 10. Reversed-phase chromatography of benzene (4) and toluene (5) on Chirasphere (250 mm × 4.6 mm, 5 µm) in 40:60 methanol:water at 25°C. Effluent at 0.5 mL/minute was monitored at 254 nm.



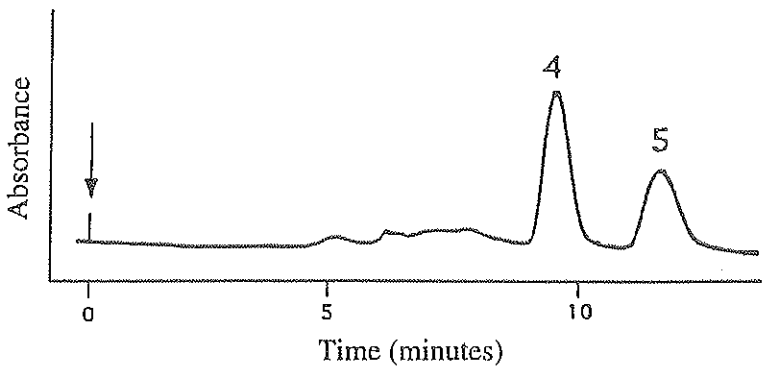


Figure 11. Reversed-phase chromatography of benzene (4) and toluene (5) on $PBGA_{88}$ (250 mm × 4.6 mm, 12 µm). Other conditions as in Figure 10.

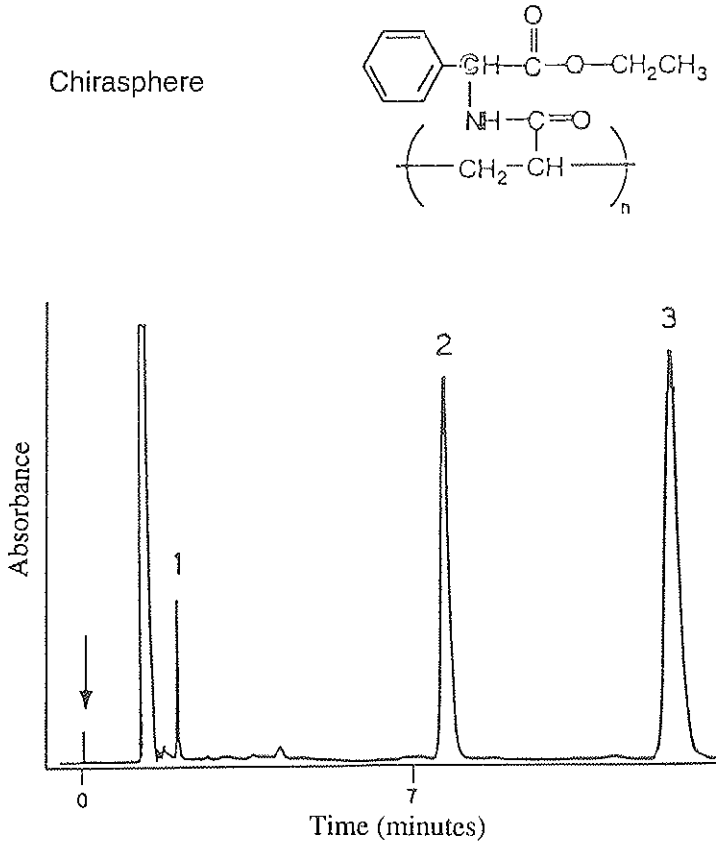


Figure 12. Normal-phase chromatography of ethylbenzene (1), phenol (2), and *p*-chlorophenol (3) on Chirasphere (250 mm × 4.6 mm, 5 µm) in 90:10 hexane:dioxane at 25°C. Effluent at 2 mL/minute was monitored at 254 nm.

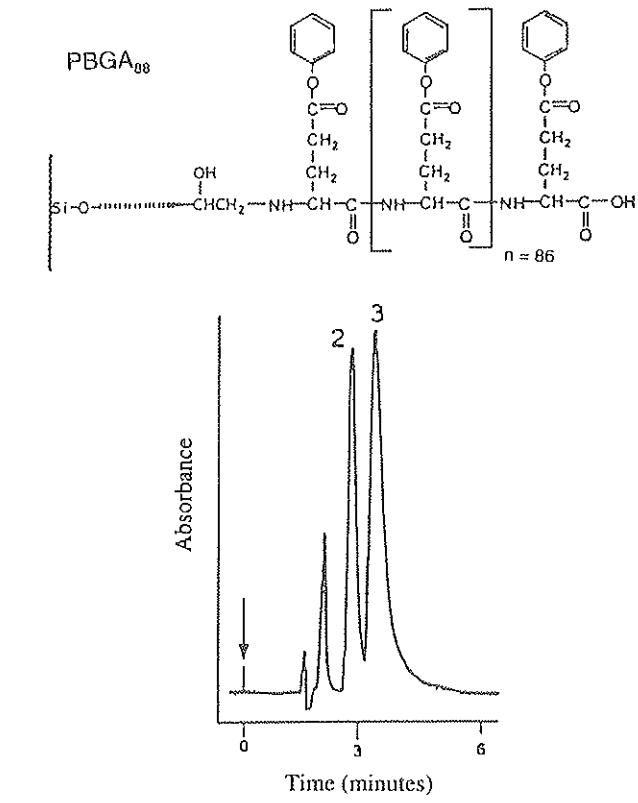


Figure 13. Normal-phase chromatography of phenol (2) and *p*-chlorophenol (3) on $PBGA_{88}$ (250 mm × 4.6 mm, 12 µm). Other conditions as in Figure 12.

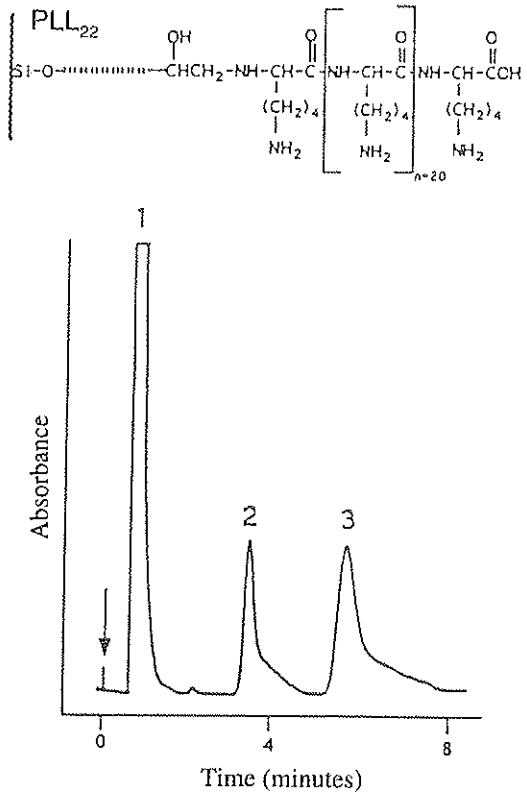
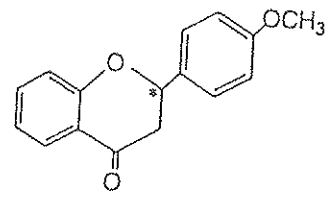
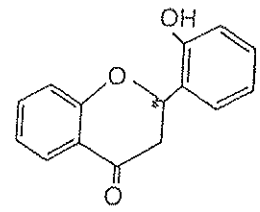


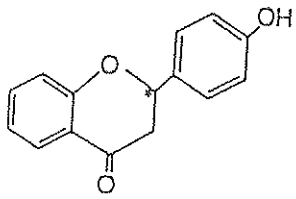
Figure 14. Normal-phase chromatography of ethylbenzene (1), phenol (2) and *p*-chlorophenol (3) on PLL_{22} (100 mm × 4.6 mm, 12 µm). Other conditions as in Figure 12.



4'-Methoxyflavanone



2'-Hydroxyflavanone



4'-Hydroxyflavanone

Figure 15. Flavanone structures.

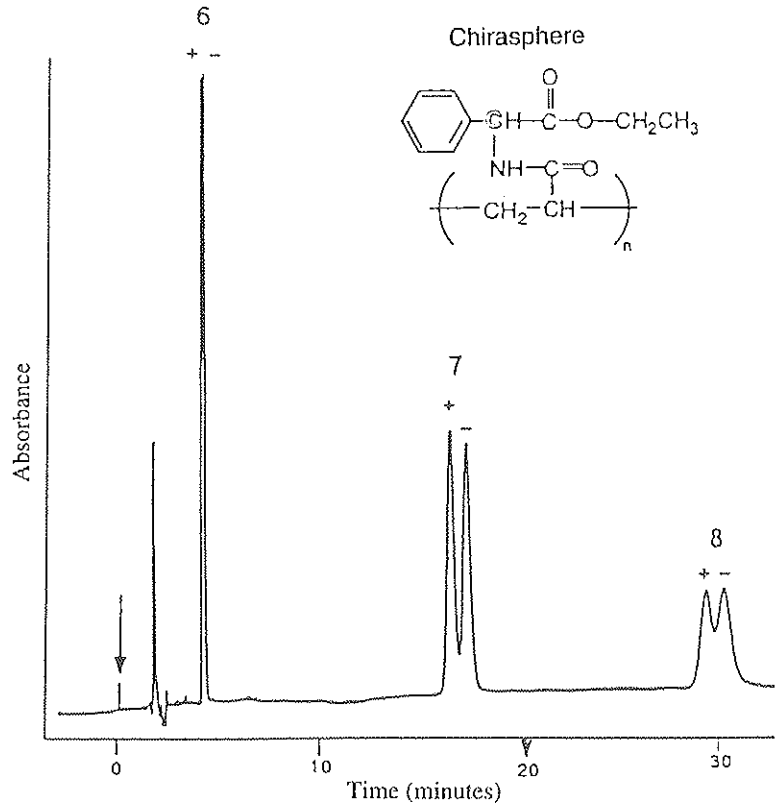


Figure 16. Normal-phase chromatography of 4′-methoxyflavanone (6), 2′-hydroxyflavanone (7), and 4′-hydroxyflavanone (8) on Chirasphere (250 mm × 4.6 mm, 5 µm) in 90:8:2 hexane:dioxane:2-propanol. Symbols + and – denote enantiomers. Other conditions as in Figure 9.

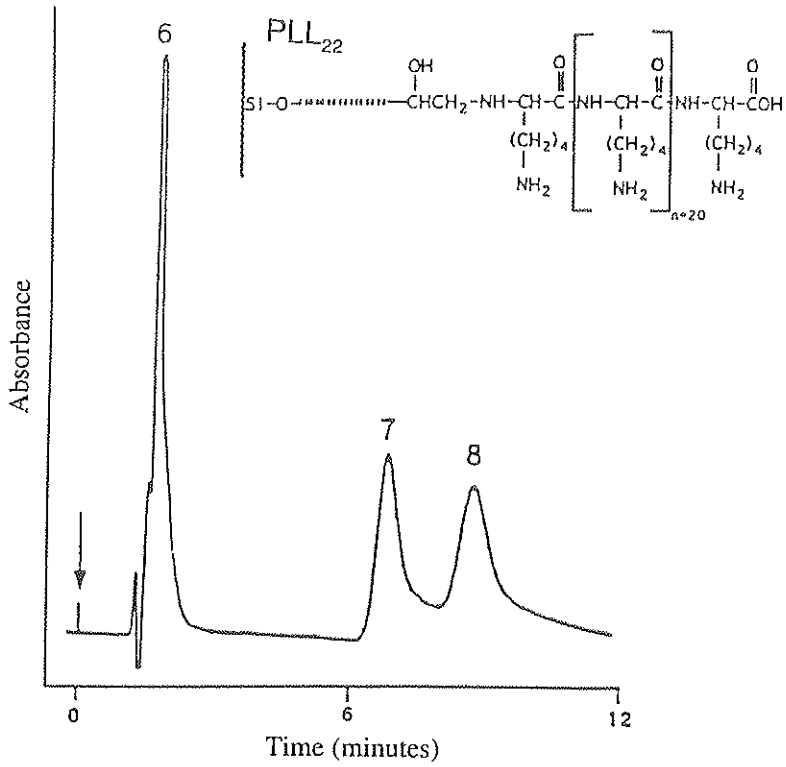
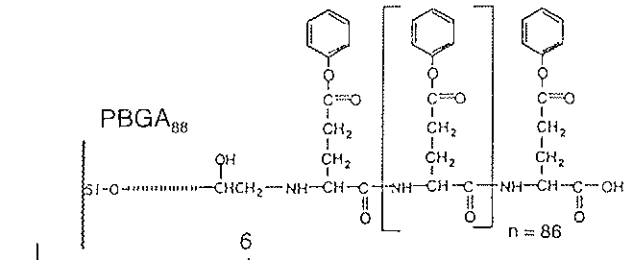


Figure 17. Normal-phase chromatography of 4′-methoxyflavanone (6), 2′-hydroxyflavanone (7), and 4′-hydroxyflavanone (8) on PLL_{22} (100 mm × 4.6 mm, 12 µm) in 90:8:2 hexane:dioxane:2-propanol. Other conditions as in Figure 9.



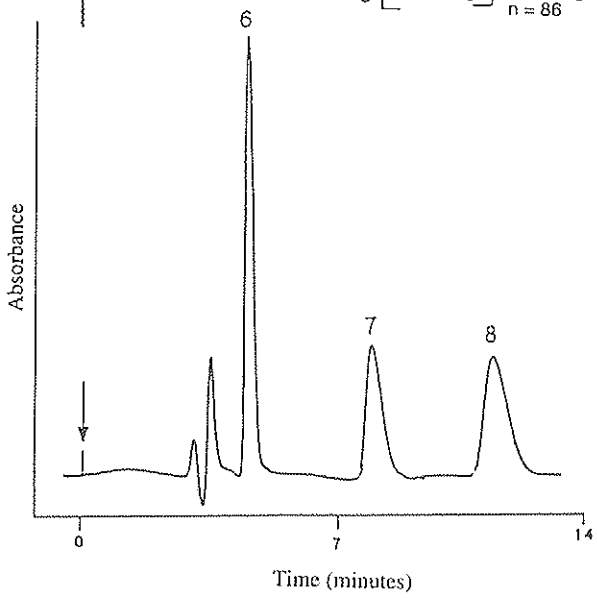


Figure 18. Normal-phase chromatography of 4′-methoxyflavanone (6), 2′-hydroxyflavanone (7), and 4′-hydroxyflavanone (8) on $PBGA_{88}$ (250 mm × 4.6 mm, 12 µm). Other conditions as in Figure 17.

Results and Consequences

The Stationary Phase Steric hindrance and conformational entropy impede the attachment of the HOAAs and limit the coverage to about 0.1 μmol/m^2 or less. Poly (L-lysine)$_n$ varied from 0.071 μmol/m^2 to 0.11 μmol/m^2 for PLL_{22} to PLL_{15}; $PBGA_{88}$ gave 0.026 μmol/m^2. There are both positive and negative features to low coverage. Capacity will be diminished (Lork and Unger 1988) and band broadening can be expected because of the presence of residual silanols (Sander 1988). However, wider spacing of chains should lessen their interactions, both steric and energetic. These effects clearly are dependent on chain length, as is the ability of the HOAAs to form secondary structure. These interrelated aspects will be important and fruitful areas of investigation in the future.

Chromatography Despite having both ionic and hydrophobic character in the sidechain, PLL_{22} did not retain any species under reversed-phase conditions. Under normal-phase conditions (90:10 hexane:dioxane), PLL_{22} resolved phenol from *p*-chlorophenol but did not retain the highly hydrophobic ethylbenzene. In contrast, under reversed-phase conditions, $PBGA_{88}$ separated benzene and toluene, and also benzene, naphthalene, and anthracene in the expected elution order. Under normal-phase conditions, $PBGA_{88}$ retained phenol, *p*-chlorophenol, and, slightly, ethylbenzene.

Chirasphere was used as a control in both reversed- and normal phase experiments. In the normal phase, PLL_{22} and Chirasphere had the same elution order with comparable retention (k') and selectivities (k'_2/k'_1); $PBGA_{88}$ had a lower retention but similar selectivities. It seems likely that the lower retention is caused by lower surface coverage, as the ratios of retention to surface coverage are similar. The similar selectivities suggest that the same simple normal-phase mechanism is operating for each.

Both Chirasphere and a Waters C_{18} μ-Bondapak reversed-phase column were used as stationary-phase controls for reversed-phase separations on $PBGA_{88}$. All three showed the same elution order for toluene and benzene. Again, the $PBGA_{88}$ and Chirasphere showed the same decrease in retention and similar selectivities. The C_{18}, however, had a twofold increase in selectivity over both of these, suggesting that a different retention mechanism may operate in this case.

Chirasphere's principal utility is as a chiral phase; to test for this property in PLL_{22} and $PBGA_{88}$, flavanones were used that exist as enantiomeric pairs (4′-methoxy, 2′-hydroxy, 4′-hydroxy) and had been separated on this commercial stationary phase. Under normal-phase conditions (90:8:2 hexane:dioxane:2-propanol), all three enantiomeric pairs were separated on Chirasphere, as expected. Under the same conditions, although neither gave chiral separations, PLL_{22} and $PBGA_{88}$ did retain and resolve the flavanones in the same elution order. The retention of flavanones by PLL_{22} and Chirasphere was not comparable, as it had been with the one-ring solutes; Chirasphere was more retentive. However, in some cases PLL_{22} and $PBGA_{88}$ had better selectivities than the Chirasphere. This may reflect only the overall polarity of the stationary-phase ligand. Most encouraging were the similar selectivities for the geometrical isomers closest in structure—the 2′- and 4′-hydroxy. The lack of ability of the two HOAAs to separate the enantiomers reflects the application of the three-point rule of chiral selectivity (Pirkle and Pochapsky 1989). Poly(L-lysine)$_{22}$ contains no benzene ring to be involved in sterically controlled π-π interactions about the chiral center, and the benzene ring in $PBGA_{88}$ is too far removed from the chiral center.

Summary

We have devised chemical procedures for attaching HOAAs to silica and have evaluated the properties of the attached substances as chromatographic stationary phases with encouraging results. This short investigation has confirmed that several challenges exist:

- The interrelations between the spacing of chains and interchain interactions, both steric and energetic, must be evaluated. These effects clearly are dependent on chain length, as is the ability of the HOAAs to form secondary structure.
- Both apolar solvents and the interaction of analytes will stabilize secondary structure. Solvent systems and peptide stationary phases have to be developed that maintain

the appropriate interaction surface and are readily conformationally reversible. Possible HOAAs are poly(benzylaspartic acid) and poly(L-tyrosine). The insertion of proline at selected positions in a polypeptide is known to modify helix formation, and this may be useful.

- Because temperature is an important parameter in the helix-coil transition, this should be investigated in concert with its role as a chromatographic variable.

Acknowledgments

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Abbreviations

Å	Angstrom units
CH_2Cl_2	dichloromethane
HOAAs	homopoly(amino acids)
k'	capacity factor
K	exclusion coefficient
K'	size-exclusion coefficient
K_b	exclusion coefficient of the bimodal system
K_p	partition coefficient
MW	molecular weight
NH_2	amino group
$PBGA_{88}$	poly(benzylglutamic acid)$_n$
PGA_{40}	poly(glutamic acid)$_n$
PLL_{22}	poly(L-lysine)$_n$
r	radius of a sphere
R	radius of a cylinder
r_g	radius of gyration
R_o	average pore size
SD	standard deviation
THF	tetrahydrofuran
V_i	solvent volume within the gel
V_m	void volume
V_o	extra-particle volume
V_p	pore volume of particles
V_R	elution volume
V_S'	volume of pores available to a molecule

RESEARCH METHODS

Many studies have shown that a single chromatographic step is unlikely to separate all of the components of a complex mixture. In response to this challenge, Drs. Dorsey and Halsall propose novel techniques for increasing the resolving power of chromatography. Their approach is to combine mobile-phase and stationary-phase programming to improve the separating power of liquid chromatography, a process described as pseudo–two-dimensional chromatography. The stationary-phase programming considers the resolving abilities of various stationary-phase properties, such as pore diameter of the silica gel, along the length of the column. Preliminary data are presented for the theory of size exclusion, based on traditional reversed-phase stationary phases, and for the bonding of poly(homo)peptides to a silica support, which imparts chromatographic properties as a function of mobile-phase composition. Theoretical

models then are presented for combined size-exclusion and reversed-phase chromatographies and stationary-phase programming.

The concept of using structural changes in the stationary phase ligand to enhance selectivity is intriguing, and may result in the development of some novel stationary phases. However, by failing to deal with how chromatographic theory is related to stationary-phase and mobile-phase programming, as well as with basic issues of diffusional processes, the investigators have not addressed some advantages and disadvantages of using pseudo–two-dimensional chromatography as opposed to true two-dimensional chromatography. For example, there may be no advantage of using a combined approach if the number of theoretical plates is not increased by combining these two processes. Statistical theory states that peak width limits the number of peaks that can occur in a sequence for a given time interval. Therefore, unless combining the two processes decreases the peak widths relative to those obtained from using each process individually, an increase in resolution will not be gained for the given time interval. Discussion with respect to diffusion and mixing (band-broadening) also would alert the reader to possible problems with this approach, as band-broadening may cause problems in resolution. The example used in the report does uot address this issue, because resolution was not a problem to begin with.

Another potential problem with the use of pseudo–two-dimensional separation is that although unresolved peaks may become resolved in the process, formerly resolved peaks may become unresolved. In Figure 1 peaks a, b, f, and g become resolved though the process, but the resolution between peaks b, c, e, and f is lost.

In summary, the idea of combining mobile- and stationary-phase programming to increase separation is a good one, but this approach may be more useful as an analytical tool for identification than as a preparative tool for separating complex mixture components for toxicity testing. Possibly, stationary phase modifications to cause preferential retention of compounds with common toxicologic properties would make this technique more useful.

Given the existing techniques applied to the analysis of complex mixtures, a discussion by the investigators of how the described approach compares to more traditional approaches, particularly in the context of assessing toxicologic endpoints, would have been appropriate.

This report offers potentially fruitful new approaches to improving selectivity in analytical chromatographic systems. However, additional research on the actual effect of stationary-phase conformational changes on chromatographic selectivity will be necessary for this approach to have a major impact on research in complex mixtures. HEI

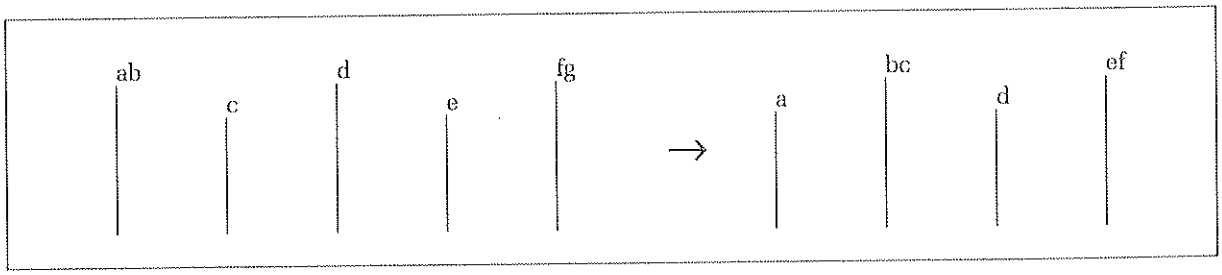

Figure 1. An illustration of how changing conditions can alter the resolution of peaks *a* through *g*.

Supercritical Separation and Molecular Bioassay Technologies Applied to Complex Mixtures

David L. Springer, James A. Campbell, and Brian D. Thrall
Pacific Northwest Laboratories, Battelle Memorial Institute, Richland, WA

Abstract

The purpose of this work is to develop an approach for the chemical and biological characterization of volatile and semivolatile compounds resulting from vehicular engine combustion of gasoline, diesel, and other types of fuel. There are two specific objectives. The first is to develop theoretical avenues for the extraction, separation, and characterization of the chemical mixtures using techniques based on the solvation properties of supercritical fluids. The second is to develop a way to evaluate these materials for potential adverse health effects using cellular, biochemical, and molecular assays. Ultimately this approach will provide information about the effects of these mixtures on the biological processes involved in tumor initiation and progression; other toxicological endpoints remain to be addressed.

Current methods of extraction and purification of volatile and semivolatile combustion products have several limitations including the incomplete extraction of certain analytes; poor resolution, leading to overlap between chemical class fractions; analyte loss during removal of the substantial quantities of solvent often required for extraction; and the creation of large volumes of waste solvents. We selected supercritical fluid chromatography (SFC)* and supercritical fluid extraction (SFE) because they offer superior separation characteristics compared to solvent extraction and other methods. Our premise for this work is that SFE and SFC are simple, gentle, and efficient methods for extraction and separation of volatile and semivolatile components from combustion products.

* A list of abbreviations appears at the end of this document.

This Investigators' Report is one part of HEI Communication Number 4, which also includes three Investigators' Reports by Bechtold and Hotchkiss, Dorsey and Halsall, and Gennings and coworkers, and Comments by the Health Review Committee. Correspondence concerning this Investigators' Report may be addressed to Dr. David L. Springer, Battelle Memorial Institute, P.O. Box 999, Battelle Boulevard, Mail Stop P7-56, Richland, WA 99352.

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Introduction

Supercritical fluids may be defined as gases near their critical temperatures and compressed to pressures (or densities) at which intermolecular distances are shorter. This results in liquidlike molecular interactions. The physical properties of a supercritical fluid can be made to vary between the limits of a normal gas and those of a liquid by controlling pressure and temperature. The combination of physical properties, such as viscosity and diffusion rates, with variable solvent properties provides the basis for the advantages of SFE and SFC. This includes enhanced solvating capabilities because of the liquidlike density of supercritical fluids.

Supercritical fluids typically are used at densities ranging from 10% to 80% of their liquid densities, which produce pressures that are typically 100 to 1000 times greater than those of the gas at ambient temperatures. The diffusion coefficients of supercritical fluids are substantially greater than those of liquids. For example, the diffusivity of supercritical carbon dioxide varies between 10^{-4} and 10^{-3} cm^2/s over the range of conditions usually utilized, whereas liquids typically have diffusivities of less than 10^{-5} cm^2/s. Similarly, the viscosity of supercritical fluids mirrors the diffusivity and is typically 10 to 100 times less than the viscosity of liquids (McHugh and Krukonis 1986). Practical pressures for applications range from less than 50 bar to more than 500 bar.

The pressure-density relationship for carbon dioxide in terms of reduced parameters (for example, pressure, temperature, or density divided by the appropriate critical parameter) is shown in Figure 1. This relationship generally is valid for most single-component systems. The isotherms at various reduced temperatures show the variations in density that can be expected with changes in pressure. Table 1 gives the critical parameters for a number of common and potential supercritical solvents (Wright and Smith 1989).

Using a binary fluid mixture can enhance solvating power by increasing the range of molecular species that: (1) are soluble in a single supercritical fluid, (2) alter the critical temperature of the mobile phase, or (3) change the chromatographic selectivity of the mobile and stationary phases. The phase behavior of binary systems is highly varied and much more complex than that of single-component systems. Examples of binary fluid systems are carbon dioxide with isopropanol or methanol, and propane with isopropanol. The phase behavior of carbon dioxide–methanol is shown in Figure 2, which depicts their pressure and composition relations at three temperatures (Brunner 1985). The single-phase supercritical regions are above the curves, and the regions beneath the curves correspond to two-phase subcritical mixtures at the respective temperatures. It is essential that the fluid mixtures used for mobile phases be selected so that they can be mixed and pumped as a single phase, preferably at ambient temperatures.

The range of solvating power of supercritical fluids essentially defines the limit of application. The solubility of analytes typically increases with supercritical fluid density or with temperature under conditions of constant density. A maximum rate of increase in solubility with pressure is seen near the critical pressure, where the rate of increase in density with pressure is greatest (Gitterman and Procaccia 1983). Often there is a linear relationship between the log (solubility) and fluid density for dilute solutions of nonvolatile compounds. Where volatility is extremely low, and at densities less than or near the critical density, an increase in temperature will typically decrease solubility (Smith and Udseth 1983a,b). However, at sufficiently high temperatures, solubility may increase and the solute vapor pressure also becomes significant. The highest supercritical fluid densities at a constant temperature are obtained near the critical temperature; the greatest solubilities often will be obtained at somewhat lower densities but higher temperatures. Simi-

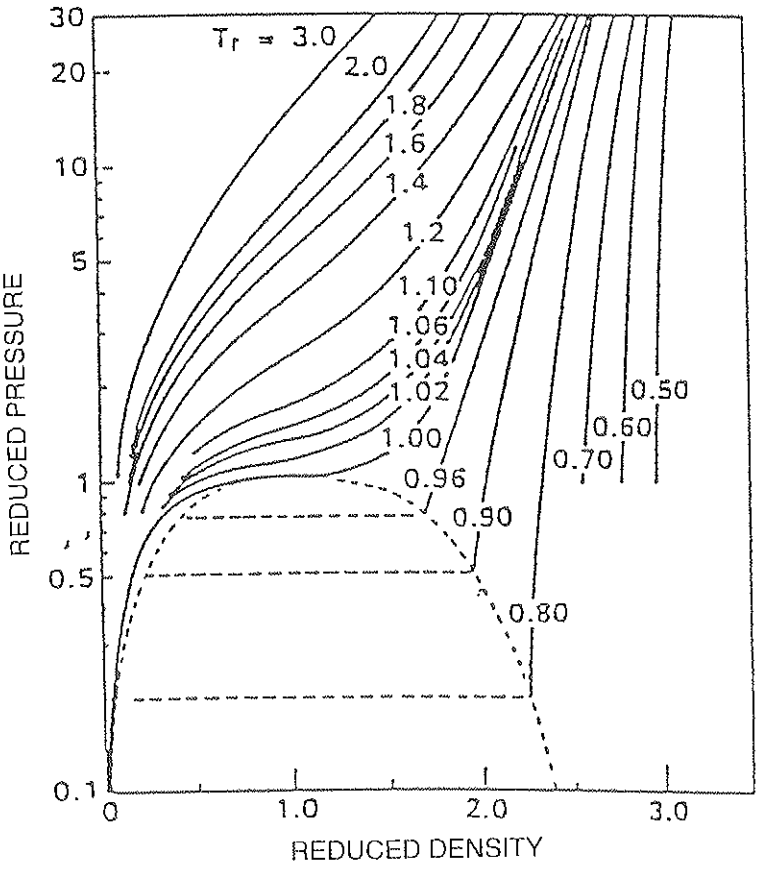

Figure 1. The pressure and density relations of carbon dioxide expressed in terms of reduced parameters. The area beneath the dotted line represents the two-phase gas-liquid–equilibrium region. T_r is the reduced temperature.

Table 1. Common Supercritical Fluid Chromatography Solvents and Properties[a]

Compound	Boiling Point (°C)	Critical Temperature (°C)	Critical Pressure (Bar)	Critical Density (g/cm^3)
CO_2	–78.5	31.3	72.9	0.448
NH_3	–33.4	132.4	112.5	0.235
H_2O	100	374.2	218.3	0.315
N_2O	–88.6	36.5	71.7	0.450
Ethane	–88.6	32.3	48.1	0.203
Propane	–42.1	96.7	41.9	0.217
Pentane	36.1	196.6	33.3	0.232
Methanol	64.7	240.5	78.9	0.276
Isopropanol	82.5	235.3	47.0	0.273

[a] Adapted from Wright and Smith 1989.

lar to liquids, the more polar solutes are most soluble in polar supercritical fluids, although nominally nonpolar fluids can be remarkably good solvents for moderately polar compounds (McHugh and Krukonis 1986).

Proposed Experimental Approach

The experimental approach will have three phases. During Phase I we will collect and analyze volatile and semivolatile compounds from various combustion sources. During Phase II, we will evaluate individual compounds and synthetic mixtures of compounds in an effort to estimate dosimetry and interaction with cells. In Phase III, we will assess the cytotoxicity of supercritical fluid extract fractions, and examine their ability to produce mutations in representative target cell lines.

Phase I Volatile combustion products will be collected on adsorbent materials and analyzed by purge and trap gas chromatography–mass spectrometry (GC–MS). Semivolatile compounds also will be collected on adsorbent materials, and particulates will be collected on filters; both will be extracted and fractionated with supercritical fluids. As a result, relatively pure fractions consisting of classes of compounds will be obtained for biological testing. Extracts will be analyzed by SFC–MS and GC–MS.

Some typical compounds detected in vehicle diesel exhaust emissions are shown in Table 2 (Westerholm et al. 1991). Similar types or classes of compounds may be expected for other combustion sources. The particulate and semivolatile phase crude extracts are fractionated according to polarity. The five fractions include light aliphatic hydrocarbons, heavy aliphatic hydrocarbons, nitro-polynuclear aromatic hydrocarbons, dinitro-polynuclear aromatic hydrocarbons, and quinones and other polar material. Fractionation will be accomplished with open column chromatography and high performance liquid chromatography (HPLC).

Proposed Approaches to Separation Characterization The most widely used procedure for sampling ambient air for volatile and semivolatile organic compounds is to pass measured volumes of air (typically 2 to 100 L for most volatile organic compounds and 2 to 500 m^3 for semivolatile organic compounds) through a solid material that sorbs the components of interest. Steel canisters, with inert interior walls electropolished to prevent decomposition of the collected organic compounds, are commonly used for collecting ambient air samples; Tedlar™/Teflon™ bags also can be used. Condensing volatile organic compounds from air into a cryogenic trap is an attractive alternative to sor-

bent sampling, particularly when it is combined with ambient air sampling in appropriate containers (Clark et al. 1982; Hutte et al. 1984). After the compounds of interest are collected, solvent extraction or thermal desorption is used to recover them.

Nonvolatile compounds, for example elemental carbon and compounds with high molecular weights, have negligible concentrations in the vapor phase; these compounds often are bound to solid particles. High-volume samplers commonly are used to collect particles and associated vapors from air. Filter packs sometimes are used as prefilters; Teflon or similar particle prefilters may be followed by a nylon filter to remove nitric acid. Impingers (bubblers)

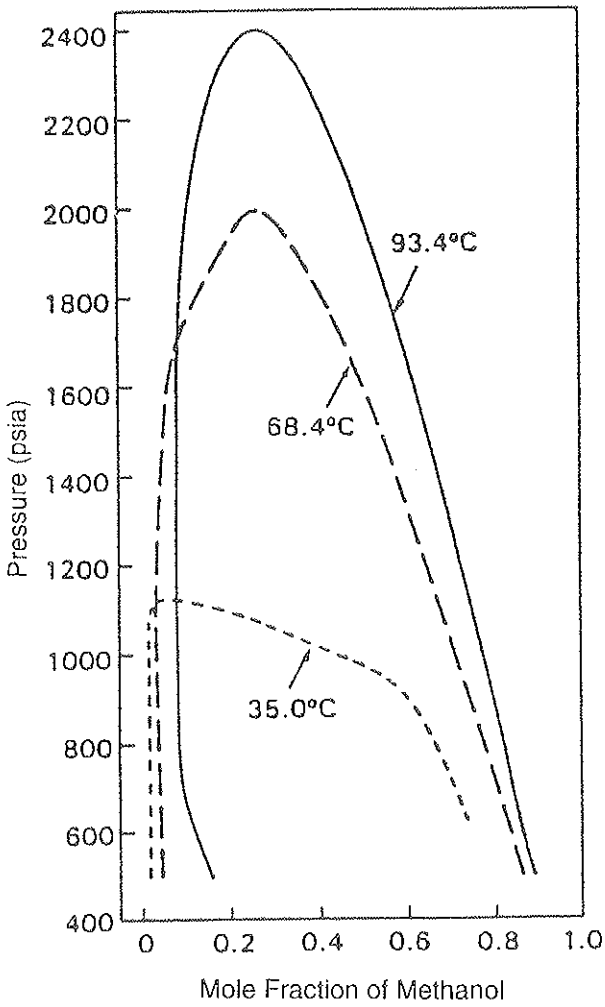

Figure 2. Pressure composition diagrams for carbon dioxide–methanol fluid mixtures at three temperatures. Two-phase gas-liquid–equilibrium regions exist inside the curves at the given temperatures.

Table 2. Examples of Oxygenated Compounds, Light Aromatic Compounds, and Particle-Associated Polynuclear Aromatic Compounds Identified in Heavy-Duty Diesel Exhaust[a]

Acrolein	2-Methylfluorene
Acetone	Phenanthrene
2-Propanol	Anthracene
Methacrolein	Pyrene
Benzene	Coronene
Toluene	Nitropyrene
Xylene	Dibenzothiophene
n-Butanal	Methyldibenzothiophene
Ethylbenzene	Perylene

[a] Adapted fromWesterholm et al. 1991.

commonly have a liquid collection medium in which chemical speciation, for example, the valence form of a metal, can be changed substantially. Impingers are not recommended for long-term, routine monitoring because evaporation of the collection fluid can be significant (Winberry et al. 1990). The Environmental Protection Agency has published sampling protocols (Winberry et al. 1990) for toxic compounds in ambient air, and these are listed in Table 3.

Air pollutant studies have emphasized the less volatile organic compounds trapped on solid adsorbents or the particulates trapped on filters (Egebaeck et al. 1981; Scheutzle and Lewtas 1986; Westerholm et al. 1991). Soxhlet extraction or sonication typically is used to recover analytes from the adsorbents. Both techniques may require long extraction periods, large volumes of solvent, and may result in incomplete recovery or sample decomposition. The resulting extracts are concentrated by rotary vacuum or by nitrogen-blowdown techniques prior to trace analysis. If bioassays are part of the experimental protocol, the solvent must be exchanged to one that is compatible with bioassays, such as dimethyl sulfoxide, prior to biological testing (Egebaeck et al. 1981).

In contrast, supercritical fluids have several properties that may make them useful for the rapid and quantitative extraction and recovery of organic pollutants from adsorbents and environmental solids. Because a supercritical fluid's solvent strength is directly related to its density, the capacity of a supercritical fluid to solvate a particular analyte species can be modified easily by changing the extraction pressure and, to a lesser extent, the temperature. Supercritical fluids with different polarities are avail-

Table 3. EPA Sampling Methods[a]

Method Number	Description	Types of Compounds Determined
TO-1	Tenax GC adsorption and GC–MS analysis	Volatile, nonpolar organics (e.g., aromatic hydrocarbons, chlorinated hydrocarbons) having boiling points in the range of 80° to 200°C.
TO-2	Carbon molecular sieve	Highly volatile, nonpolar organics (e.g., vinyl chloride, vinylidene chloride, benzene, toluene) having boiling points in the range of −15° to +120°C.
TO-3	Cryogenic trapping and CG–FID or ECD analysis	Volatile, nonpolar organics having boiling points in the range of −10° to +200°C
TO-4	High volume PUF sampling and GC–ECD analysis	Organochlorine pesticides and PCBs
TO-5	Dinitrophenylhydrazine liquid impinger sampling and HPLC–ultraviolet analysis	Aldehydes and ketones
TO-6	HPLC	Phosgene
TO-7	Thermosorb/N adsorption	*N*-Nitrosodimethylamine
TO-8	Sodium hydroxide liquid impinger with HPLC	Cresol and phenol
TO-9	High volume PUF sampling with HRGC–HRMS	Dioxin
TO-10	Low volume PUF sampling with GC–ECD	Pesticides
TO-11	Adsorbent cartridge followed by HPLC detection	Formaldehyde
TO-12	Cryogenic preconcentration and direct FID	Nonmethane organic compounds
TO-13	PUF–XAD-2 adsorption with GC and HPLC detection	PAHs
TO-14	SUMMA® passivated canister sampling with GC	Semivolatile and volatile organic compounds

[a] Adapted from Winberry and associates 1990.

able; in addition, solvent modifiers can be used to alter the polarity of a supercritical fluid. The ability to select and change the solvent strength of a supercritical fluid is important for optimizing the extraction of a particular analyte species. The use of different extraction pressures, solvent modifiers, and solvents with varying polarities may be valuable particularly in developing class-selective extraction methods. However, the use of modifiers must be evaluated carefully so that they can, if necessary, be removed before the bioassay. Many supercritical solvents are sufficiently volatile that the extracted species can be concentrated at ambient or even subambient temperatures, thus reducing the loss of volatile analytes and reducing the amount of solvent that requires disposal. The loss or transformation of compounds found in the original mixture, as well as analyte loss from the extraction procedure, is minimized.

The potential for using supercritical fluids to provide class-selective extractions has been investigated using the National Bureau of Standards diesel exhaust particulate sample (Hawthorne and Miller 1986). Class fractionation of the alkanes and polynuclear aromatic

hydrocarbons was based on the fact that changing a supercritical fluid's pressure can greatly affect its ability to solvate organic compounds (Giddings et al. 1968). The sample first was extracted with 75 atm carbon dioxide for 5 minutes at 45°C, and then was extracted for an additional 90 minutes at 300 atm and 45°C. The extracted organic compounds were collected and analyzed using selective ion monitoring GC–MS; *m/z* 57 was monitored to quantitate the alkanes. Approximately 85% of the alkanes were removed by the 5-minute, 75-atm extraction, while more than 90% of the polynuclear aromatic hydrocarbons were retained until the extraction pressure was raised to 300 atm. These results demonstrate the possibility of class fractionation, even though only one set of class-selective extraction conditions was used. Class fractionation also has been accomplished using a different series of supercritical fluids on high-molecular-weight residuals from coal liquefaction processes (Campbell et al. 1992).

In SFC, the mobile phase is maintained at a temperature somewhat above its critical point; at typical SFC pressures, its density is usually several hundred times that of the gas but less than that of the liquid. It is generally advantageous to use the highest temperature compatible with the SFC system and the material being analyzed, as the more favorable diffusion coefficients obtained with operation at higher temperatures and lower densities improve chromatographic separation efficiencies (Fields and Lee 1985). Because it is possible to use low-critical-temperature fluids and reduced temperatures, mild thermal conditions can be applied to labile compounds that can not be analyzed by GC. Nearly all current SFC techniques use either packed columns with 5- to 10-µm particles prepared for HPLC, or wall-coated open tubular fused silica capillaries of 25 to 100 µm interior diameter. Capillary columns have much greater permeabilities and have demonstrated higher numbers of total effective plates than packed columns.

Supercritical fluid chromatographic instrumentation incorporates elements of both HPLC and GC, as both high-pressure mobile phases and higher than ambient operating temperatures are used. High pressure is maintained throughout the column; detectors must work at high pressures or be interfaced to the decompressed gas flow of the column effluent. The chromatographic columns also must be stable to the mobile phase's solvating influences.

The advantages of coupling a chromatographic technique with mass spectrometry are considerable, as evidenced by the major role of GC–MS in organic analysis. The mass spectrometer is an ideal detector for SFC, providing both sensitive and selective detection with universal applicability. In contrast to many detection methods, MS is compatible with a broad range of mobile phases. The added flexibility to choose ionization modes such as electron impact or chemical ionization, independent of the mobile phase, provides the basis for obtaining either structural information or high sensitivity and selectivity.

Proposed Approaches to Sampling and Collections The sampling apparatus will comprise a series of impingers and adsorbents for collecting volatile and semivolatile components; filters will collect particulate matter. The sampling procedure would be similar to that used by Westerholm and associates (1991) for sampling diesel exhausts.

Regulated emissions will be measured as follows: carbon monoxide with a nondispersive infrared analyzer (Beckman 864), total unburned hydrocarbons with a flame ionization detector (Beckman 402), oxides of nitrogen (NO_x) with a chemiluminescence analyzer (Beckman 955), and particulate emissions by the use of Teflon-coated glass fiber filters (Pallflex T60A20; Pallflex, Inc.).

A cryogenic sampling technique (Jonsson and Berg 1983), in which a cryogradient is established over a sampling tube packed with a sorbent bed, will be used for oxygenates and light aromatic compounds. The use of two parallel cryogenic sampling devices will double the capacity to sample the semivolatile phase. The device is described elsewhere in detail (Stenberg et al. 1983), as is the adsorbent sampler, filled with XAD-2 resin (Amberlite 0.3–0.78 mm; BDH Chemicals Ltd.) (Alsberg et al. 1983). The XAD-2 samples, extracted with a series of supercritical fluids and concentrated for analysis, will be used primarily for biological testing. Semivolatile phase samples, collected on poly-

urethane foam plugs, will be extracted and treated in the same way as the particulate extract.

The adsorbent materials containing semivolatile compounds and particulates will be extracted sequentially with a series of supercritical fluids to obtain a class fractionation. Certain supercritical solvents may actually dissolve the adsorbent material; if this occurs, the adsorbent material will be removed by filtration before assay work. If the adsorbent material can not be removed, its effects on the bioassay will be evaluated. Further separation and class fractionation will be done as necessary by SFC or additional sequential SFE. The supercritical fluid with modifier will be removed by heating sufficiently to volatilize it but not the sample components. In the bioassay portion cells will be subjected to the separated class fractions.

We have not yet determined the actual sequence of supercritical fluids for extraction. However, studies have shown that model compounds, including dichloromethane, 2-nitrofluorene, and fluoranthene, could be extracted from various adsorbents using carbon dioxide for 60 minutes at 3000 psi, followed by extraction at 6000 psi with 12% hexane in carbon dioxide (Wong et al. 1991). The temperature, pressure, and type and per cent of modifier will be adjusted to obtain class fractionation. Other possible supercritical solvents, including methanol and dimethyl- or trimethylamines, will be evaluated on the basis of separatory power and ease of removal prior to the bioassay.

Once we have identified a specific class of compounds, several components indicative of that class can be spiked into the adsorbent and, with adjustment of the parameters (temperature, modifier, etc.), the desired fractionation can be obtained.

The volatile components will be identified by purge and trap GC–MS. Carbonyl compounds will be collected by bubbling a portion of the vehicle exhaust through two impingers, each of which contains 25 mL of an acetonitrile solution containing 2,4-dinitrophenylhydrazine. Subsequent analysis of the derivatives formed by carbonyl and 2,4-dinitrophenylhydrazine will be made using HPLC in a manner similar to that used in the studies by Hoekman (1992).

Semivolatile compounds and particulate extracts will be analyzed by SFC–MS and by GC–MS. No modifications of the commercially supplied GC–MS interface (Hewlett-Packard Model 5988A GC–MS) will be made. The source temperature and the interface probe temperature will be held at 270°C. A Superbond (Lee Scientific) column (SB-octyl-50, 10 m × 100 μm interior diameter, 0.25 μm film thickness) will be used in these studies.

Extracts will be fractionated further using SFC and high-resolution microcolumn HPLC. This technique uses fused silica tubing slurry-packed with 5-μm particles to provide columns that approach separation efficiencies of 100,000 theoretical plates. In addition to the enhanced resolution, microcolumn separations offer a number of inherent advantages including minute sample volumes, enhanced mass flow detection sensitivity, and the feasibility of using exotic mobile phases. Expected concentrations of classes of compounds range from microgram to milligram per gram of mobile source material.

Phase II Phase II involves the evaluation of compounds and synthetic mixtures for dosimetry and interaction with cells, because extracts from the semivolatile components and particulate fractions are expected to be much more complex, requiring further fractionation. Because we anticipate fewer compounds in the volatile fraction, it should be possible to prepare well-defined synthetic mixtures for biological studies. Radiolabeled compounds, chosen on the basis of their presence in the volatile and semivolatile fractions and on their commercial availability, will represent the major classes of compounds present in the mixtures (short-chain hydrocarbons, alcohols, ketones, aldehydes, and organic acids). Individual radiolabeled compounds will be used to establish methods for delivery of the test compounds to the cultured cells as well as to estimate recovery. This approach also will provide information on partitioning of the test compound in the vapor phase above the cells, solubility in the growth media, and amount reaching the cellular fraction. Mixtures containing one or several radiolabeled compounds will be used to evaluate toxicity to cells and to establish conditions for mutagenesis assays.

Individual compounds and synthetic mixtures representative of combustion product volatile and semivolatile components will be

evaluated for toxicity. Initial concentration-response information will establish relative cytotoxicities for individual compounds and the synthetic mixtures; these components then will be evaluated for mutagenic and transformation activities.

Phase III Phase III will involve direct evaluation of supercritical fluid extracts for toxicity. Fractions showing significant mutagenic activity will be candidates for further evaluation of mutagenicity in transgenic mice; these studies will depend on the extraction of sufficient quantities of material for whole animal evaluation. Extracts are expected to contain individual compounds or a few compounds with similar functional groups. Positive controls will ensure that the assay is working properly and control for inherent assay variability. It is anticipated that the SFC-purified fractions will comprise many different compounds, thus it will be important to determine whether interactions between compounds are synergistic or antagonistic in their biological effects. The extracts also will be evaluated for activity in the presence of ozone, which may interact with extract components, relevant to the situation in many atmospheric environments.

Bioassay Rationale Characterization of biological activity of the samples will involve separation of the volatile components into class fractions, primarily on the basis of functional groups and solubility. For example, a theoretical class fractionation of compounds identified in heavy-duty diesel exhaust by Westerholm and associates (1991) is shown in Table 2. We anticipate that concentrations of chemical class fractions recovered by SFE will be in the microgram to milligram per gram range, and that microgram amounts of each class fraction will be required for a typical experiment. If volatile components are extracted with supercritical fluids that include modifiers, the modifier probably will have to be removed because of its potential toxicity to cells. Analyte loss could occur during this removal process. Because prior analysis would have identified volatile analytes, these gases will be obtained from commercial sources and blended to reflect the composition of the starting material. Cells will be exposed to these more easily standardized and controlled gas mixtures as well as directly to the isolated fractions. This approach will be applicable as well for either the semivolatile or particulate extracts. The extracting fluid can be removed, without loss of the analytes, by heating the solution slightly.

We propose a tiered bioassay approach using the analyte fractions. Initial in vitro assays for cytotoxicity and mutagenic activity will identify potential biologically active fractions, which will be studied further for potential activity in vivo. Activity initiated by these fractions also will be compared with activity associated with the unfractionated parent mixture to determine which fraction is responsible for the major biological activity. In addition, we will blend class fractions selectively to attempt to recreate the parental mixture activity, with the objective of identifying possible interactions between class fractions.

Analysis of In Vitro Activity Because the respiratory epithelium is the target tissue most likely at risk of cancer from exposure to volatile mutagens, an ideal system for studying potential volatile mutagens would use cells that are histologically and metabolically comparable to respiratory epithelial cells. Rat tracheal epithelial (RTE) cells histologically resemble human bronchi (Kendrick et al. 1974), and are isolated and cultured easily in vitro. These cells maintain the nontransformed, differentiated phenotype in culture and, with the addition of growth factors, can be cultured under conditions permissive for growth. In addition to RTE cells, a human bronchoepithelial cell line recently has become commercially available and should be evaluated for use in these experiments.

We will expose RTE cells to vapor-phase fractions using the hydrated collagen gel matrix system described by Zamora and associates (1983a), in which cells are maintained by the medium in a collagen gel support, so that the upper cell surface can be exposed to gaseous agents. This system has been used successfully to detect the cytotoxicity and mutagenicity of agents such as ethylene oxide, propylene oxide, and 1,2-dichloromethane (Zamora et al. 1983a); nitrous oxide (Zamora et al. 1986); and phenol, formaldehyde, and diesel exhaust (Zamora et al. 1983b).

Cytotoxicity of the class fractions will be evaluated initially to determine both relative biological activity of the fractions and concen-

trations to be used in further assays. Standard assays for in vitro mutagenesis, such as the hypoxanthine-guanine phosphoribosyltransferase and thymidine kinase systems, will be used to screen for potentially active fractions.

Extracted fractions that demonstrate potentially mutagenic activity also will be studied for their ability to cause changes associated with neoplastic transformation in cells. Upon exposure to carcinogens, the first detectable stage of neoplastic transformation in RTE cells is the formation of "enhanced growth" (EG) variants which are resistant to serum-mediated differentiation (Thomassen et al. 1983; Nettesheim and Barrett 1984). Cells resembling EG variants have been noted in tracheas of animals exposed to carcinogens (Thomassen 1986) and, although nontumorigenic, they can become tumorigenic with additional time in culture. Background (spontaneous) development of EG variants has been shown to be dependent on cellular proliferation, occurring at a constant rate estimated to be 7.5×10^{-6} variants per cell generation (Thomassen 1986). The induction of EG variants of RTE cells has been observed with a number of known and suspected carcinogens, including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, dimethylbenz[*a*]anthracene, benzo[*a*]pyrene, and volatile compounds such as formaldehyde and ethyl acrylate (Steele et al. 1989; Thomassen et al. 1985; Nettesheim and Barrett 1984). Multiple exposures of RTE cells to ozone (0.7 parts per million; 9 total exposures) also induced EG variants in vitro (Thomassen et al. 1991). Most notably, this system has been used as a short-term assay to evaluate potential respiratory carcinogens (Steele et al. 1989), with an accuracy of 88% relative to in vivo results.

In Vivo Approaches to Mutagenesis Analysis In vitro approaches to mutagenesis analysis do not take into account such pharmacodynamic parameters as dose distribution, in vivo metabolism, or excretion. An advantage of the RTE cell system is that the frequency of induction of EG variants can be determined after either in vitro or in vivo exposure. This in vitro–in vivo approach provides a mechanism for calibrating the extrapolation of results observed in vitro with expected results obtained in whole animals.

The use of transgenic animals also has provided a means for genetic toxicology assays combining both long- and short-term assays in a single test system. The *lac*I transgenic mouse system (Stratagene, La Jolla, CA), a rapid and simple system for detection of mutations at the DNA level, avoids the multiple steps required with plasmid-based systems. This mutagenesis assay utilizes a *lac*I target gene contained within a λ ZAP shuttle vector (Short 1989) that has been integrated stably in 30 tandem head-to-tail copies at a single locus within the mouse genome. Mutation rates can be measured throughout the respiratory tract from trachea to deep lung, providing, in effect, a measure of target organ dose and distribution.

Transgenic mice have been exposed to a number of well-studied and representative mutagens (Kohler et al. 1990, 1991), including *N*-ethyl-*N*-nitrosourea, benzo[*a*]pyrene, and cyclophosphamide. With the two former mutagens, a mutation frequency above the background was detected. The spontaneous mutations analyzed in the mice produced spectra consistent with the types of mutations observed in other systems. It should be noted that the proposed use of transgenic mice does not preclude more standard bioassay approaches, as this system does not appear responsive to nongenotoxic carcinogens (Gunz et al. 1993). However, the *lac*I mouse system provides the unique advantage of a short-term in vivo assay for comparison of mutation rates in both target and nontarget organs.

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Abbreviations

atm	atmospheres
ECD	electron capture detection
EG	enhanced growth
EPA	Environmental Protection Agency
GC	gas chromatography
GC–ECD	gas chromatography–electron capture detection
GC–FID	gas chromatography–flame ionization detection
GC–MS	gas chromatography–mass spectrometry
HPLC	high performance liquid chromatography
HRGC–HRMS	high resolution gas chromatography–high resolution mass spectrometry
m/z	mass-to-charge ratio

NO_x	oxides of nitrogen
PAHs	polynuclear aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PUF	polyurethane foam
RTE cells	rat tracheal epithelial cells
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
MS	mass spectrometry
SFC–MS	supercritical fluid chromatography–mass spectrometry

HEI COMMENTS

This report uses the solvation properties of supercritical fluids as an approach for characterizing volatile and semivolatile compounds in a complex mixture. Drs. Springer, Thrall and Campbell propose a theoretical framework for chemical separation followed by biological testing using cellular, genetic, and molecular toxicity assays.

Supercritical fluids retain both the high diffusibility of gases and the high solvency properties of liquids. They can be used for chromatographic separation as well as for extracting chemicals from a matrix. Supercritical fluid chromatography is well developed and documented as a separation tool, and can be interfaced with mass spectrometry. In contrast, the theory and application of supercritical fluid extraction is much less developed. The proposed method could extend the use of supercritical fluid extraction techniques and perhaps provide a more rigorous foundation or theoretical basis for the technique than currently exists. The use of supercritical fluid extraction and chromatography for analyzing complex mixtures can serve two purposes: developing supercritical fluid extraction technology, and advancing the analysis and characterization of complex mixtures.

Supercritical fluid extraction has the advantage of reducing artifacts and improving solvent disposal problems, compared to conventional solvent extraction methods. The material remaining after supercritical fluid extraction is pure and easy to remove. Although there are limited analytical advantages to the use of supercritical fluids, with the possible exception of speed, there are considerable practical advantages; the ease of removing the extracting medium leaves more concentrated analytes for delivery to instrumental analysis or bioassay.

As is the case with conventional extraction techniques, chemical artifacts and incomplete recovery of chemicals can occur with the use of pure carbon dioxide as a supercritical fluid to extract polar chemicals from matrices. However, carbon dioxide can be used at room temperature and has solvent properties intermediate between those of pentane and methylene chloride. Dr. Springer and his colleagues recognize these limitations and propose the use of binary systems to increase the range of solvating power, allowing the recovery of both polar and nonpolar chemicals.

The proposed use of supercritical fluid chromatography for resolving semivolatile and polar chemicals in mixtures is potentially a significant improvement over high performance liquid chromatography, because theoretically higher resolution can be achieved. However, as it is presented in this report, the approach lacks a toxicological rationale for separation. The use of supercritical fluid technology in the field of complex mixtures would be more applicable if some characteristic of the toxic properties could be used as the basis for separating them from the mixtures. This may be possible with the use of extraction modifiers, in which case supercritical fluid extraction could be used to extract preferentially chemical classes that are most related to the toxicity of the complex mixture. However, the use of such modifiers should be considered carefully, because if a supercritical fluid such as carbon dioxide spiked with a polar solvent (for example, methanol, isopropanol, etc.) is used, the modifiers must be removed prior to bioassay. This may be difficult and the original, primary advantage of a pure solvent system may be lost. HEI

HEI COMMENTS

Statistical Approaches to Interactive Effects

How chemical effects interact, whether additively or synergistically, is an important question in the study of the health effects of mixtures. Statisticians and toxicologists have proposed many different statistical and graphical strategies for evaluating the relations among several chemicals and their effects in biological systems. One aim of the HEI complex mixtures program was to develop approaches that would determine interactive effects without the need for a prohibitive number of bioassays. The study by Drs. Gennings, Carter, and Dawson involved the development of methodology for the statistical analysis of data from complex mixtures to detect departures from additivity.

Whether a biological response is additive in terms of exposnre to two or more chemicals depends on how we choose to quantify both responses and exposures.[1] The use of "additive" both in the report of Gennings and colleagues and in tbe HEI comments assumes a particular quantitation of exposnre, namely linear in concentration, and would be more precisely worded "additive and linear in concentration." This should be kept carefully in mind in assessing the implications of the work, especially because it is not uncommon for biological responses to be additive and linear in other measures of exposure, such as, for instance, the logarithm of their concentrations (or doses).

Gennings and colleagues took two approaches to evaluating interactive effects:

(1) The extension of relatively new but established regression methods, based on generalized linear models, a random-effects model, and generalized estimating equations, to test for departures from additivity using published experimental data; and

(2) the use of a parallel axis coordinate system to depict graphically the planes of additivity.

[1] If any one or more exposures and responses vary over a narrow range, how each one is quantified, although making a difference in principle, may not make a difference large enough to be detected in practice. This is, however, not the most common situation.

In the first approach, Gennings and colleagues developed statistical tests for departures from additivity using 3 types of models: generalized linear models, generalized estimating equations, and random-effects models. These techniques were applied to data sets comprising a collection of concentration-response experiments on each of a mixture's chemicals studied alone. Under the assumption of additivity, the model-based predicted response can be compared to the observed response to a specific mixture. In the second approach, departures from additivity were examined through the use of isobolograms. An isobol is a line (for a two-component system) or plane (in a three-component system) of constant response. A parallel axis coordinate system must be used to represent constant response graphically in systems of more than three components.

The first method investigated by Gennings and colleagues, the Estimation of Additivity Model (Method I), is designed to be applied to data sets comprising concentration-response experiments on each of the chemicals in a mixture. The authors describe methods for combining the results from these experiments to estimate, under the assumption of additivity, the predicted response at any mixture of concentrations of these chemicals, including a point estimate and a prediction interval. An observed response to a specific mixture then can be compared to this interval to evaluate the appropriateness of the additivity assumption at that mixture.

Gennings and colleagues assume that the additivity of the effects of c compounds are under investigation, that data from single chemical concentration-response studies are available for each of these chemicals, and that each of these experiments has a concurrent control group. If the response also is available for a particular multichemical exposure, additivity of effects at that exposure can be assessed by comparing the observed response to that predicted from the concentration-response curves of the c single-chemical experiments and the assumption of additivity.

The test for additivity requires specification of a concentration-response model for each chemical. For the example discussed in the re-

port, the mutagenic activity of kerosene soot samples, the authors assume that the mean number of mutant colonies increases exponentially with dose. If x_{ij} and μ_{ij} are the jth dose of chemical i and the mean number of mutant colonies at that dose, respectively, the mean number of colonies is modeled as $\mu_{ij} = \exp(\beta_{0i} + \beta_i x_{ij})$.

Three methods are considered for estimating the parameters of this model. In the fixed-effects model technique, standard statistical methods are used to estimate (β_{0i}, β_i), $i = 1, \ldots, c$. The observed counts are assumed to be independent within and between chemicals. The method allows a test of the hypothesis that the intercepts, representing the background mutation rates, are constant across chemicals. A prediction interval is constructed to compare the observed (mean) response of the combination of interest to that predicted under the assumption of additivity.

In the random-effects model, the background mean responses are assumed to be realizations from some underlying probability distribution. This corresponds to the idea that the c experiments represent replications of a common experimental situation, but that control rates vary randomly from replication to replication. The authors propose that this underlying distribution is a Gamma distribution, and then apply established methods for random-effects models of this type to estimate the slopes, β_i, of the c concentration-response curves.

Finally, the generalized estimating equations method is closely related to the fixed-effects method. In the generalized estimating equations method, the experiments are assumed to be independent and to conform to the model used in the fixed-effects model, but observations (at different concentrations) in the same experiment are assumed to be correlated. The generalized estimating equations method permits estimation of this correlation and adjustment for it in the analysis.

Because the model for the background rates and regression coefficients is identical for the fixed-effects and generalized estimating equations methods, one would expect these two methods to yield similar parameter estimates. The random-effects method produces estimates of the background rates that are more similar to exponential data than those obtained with the other methods, reflecting this method's assumption that these rates are realizations from a single underlying distribution, and that this will affect the estimates of the regression coefficients.

In the c-Dimensional Isobologram Model (Method II), Gennings and colleagues investigate methods for assessing the additivity of effects of c chemicals by plotting points from isobolograms of constant response in the parallel axis coordinate system. To appreciate this work, the reader must become familiar with several geometric ideas. Every mixture of c chemicals can be represented as a point in c-dimensional space whose coordinates are the c concentrations. Because c-dimensional surfaces are difficult to visualize for $c > 3$, toxicologists have sometimes studied the geometric surfaces defined by the sets of mixtures which give the same biological response. These surfaces are called isobolograms. When c chemicals are under investigation, isobolograms will have the dimension $c - 1$, making them just as difficult to visualize. In this report, Gennings and colleagues propose plotting the points on the isobologram in the two-dimensional parallel axis coordinate system. We illustrate the concepts underlying this work with a simple example.

If two chemicals have additive effects, then the set of pairs of concentrations of the two chemicals that give a constant biological response will lie along a straight line in the plane. For example, for any biological response E, the sets of concentrations of the two chemicals (x_1, x_2) that give the response E can be represented by a linear equation,

$$a_1x_1 + a_2x_2 = E.$$

A hypothetical case corresponding to the linear equation

$$2x_1 + x_2 = 10$$

is given in Figure 1 in Gennings' report. This straight line is an isobologram (a set of mixtures giving the specified response).

Gennings and colleagues investigate the utility of plotting points from isobolograms in the parallel axis coordinate system. The two-dimensional parallel axis coordinate system is constructed by representing the c axes as c vertical lines parallel to what is ordinarily called the Y-axis, one unit apart horizontally. A specific mixture is plotted by placing a point on each axis at a distance from the origin equal to the concen-

tration of that chemical in the mixture, and then connecting those points (Gennings' Figure 2). When the effects of the two chemicals are additive, points on the isobologram will produce line segments in the parallel axis system that intersect at a unique set of $c - 1$ points that define the isohologram (Gennings' Figure 3).

These ideas can be extended to studies of the effects of any number of chemicals. If the concentrations of c chemicals are represented by the symbols x_1 to x_c, any mixture can be plotted as a point in c-dimensional space whose coordinates correspond to the concentrations of the c chemicals. The set of points corresponding to the set of concentrations giving any specified biological response defines an isobologram. Gennings and colleagues investigate the utility of plotting points from isobolograms in the parallel axis coordinate system. Each point on the isobologram generates a line segment in the parallel axis coordinate system of dimension c (Gennings' Figure 4).

The c chemicals are said to have additive effects in a biological system when any portion of one chemical in the mixture can be exchanged for a portion of another chemical, determined by the relative potency of the two chemicals, with no change in biological response. When c chemicals have additive effects, the sets of mixtures producing a specified biological response, E, will lie on a planar surface of dimension $c - 1$ in $(c - 1)$-dimensional space.

Inselberg (1985) showed that all sets of points lying on a plane of dimension $c - 1$ will, when plotted as line segments in the parallel axis coordinate system, pass through a common set of c points. These points uniquely characterize the plane. Thus, if one could select different points producing the same biological response, one could plot these points in the parallel axis system. If the effects of the chemicals were additive, each of the sets of line segments would pass through the c points that characterized the plane of constant effect.

Using the Parallel Coordinate Axis System to Analyze Complex Mixtures: Determining Biological Activity and Interactions Among Components

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Abstract

The classical approach for detecting interactions in a combination of drugs or chemicals is that of the isobologram, quantified and generalized by Berenbaum (1981), which states that contours of constant response of the concentration-response surface are planar if the drugs do not interact. This report describes the use of a parallel axis coordinate system for depicting a *c*-dimensional isobologram in the analysis of the combination of *c* chemicals. We analyze the effect of three chemicals in combination on liver function as an example. The advantage of such a technique is that in theory the methodology is not affected by the number of chemicals in the mixture. However, the approach is limited in practice by the size of the experiment required to find data on an isobol (the contour of constant response).

A more economical approach describes departure from additivity by comparing observed responses to those predicted under additivity using an additivity model. The estimation of the additivity model requires concentration-response data for only the individual components and the specific combinations of interest that reflect the local rather than global nature of departure from additivity, in contrast to the larger experiments required to estimate the multidimensional concentration-response surface for the combination. Procedures to incorporate historical data are developed for a fixed-effects model, for a random-effects model, and through the use of a generalized estimating equations approach. An example illustrates the application of these techniques to the analysis of a mixture of polynuclear aromatic hydrocarbons found in kerosene soot.

Introduction

In the study of drug or chemical combinations, many authors indicate that a planar contour of constant response (isobol) of the concentration-response surface is synonymous witb additivity (Berenbaum 1981). Isobolograms have been used with two-compound combinations to detect departure from additivity through evidence of a nonlinear isobol. The statistical properties of these procedures are unknown. Some authors have developed statistical tests for additivity, yet have based these tests on smoothed data representations (MacCarthy 1987; Gennings et al. 1988; Kapetanovic et al. 1990).

Other authors (Finney 1964; Kelly and Rice 1990) have looked for interactions by comparing predictions under an additivity model to the observed responses at combination groups. This approach requires concentration-response data for only the individual components and the specific combinations of interest that reflect the local rather than global nature of departure from additivity. Finney's assumption of similar joint action is equivalent to assuming parallel concentration-response curves for the mixture's components. Kelly and Rice's approach is more flexible in that they use monotone splines for estimating concentration-response curves.

One objective for this research was to extend the work of these authors to the case in which the number of components in the mixture is large. Concentration-response information from each component (or class of components) is all that is required to estimate an additivity model. From these concentration-response data, multiple control groups may be available and useful in describing the background response rate.

We develop and present methodology to determine departures from additivity by comparing observed responses for combination groups to those predicted from an additivity model.

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We have developed three techniques on the basis of variability across the control groups. Procedures are developed for a fixed-effects model, for a random-effects model, and through the use of a generalized estimating equations approach. These techniques are illustrated using a mixture of polynuclear aromatic hydrocarbons (PAHs)* found in kerosene soot.

The isobologram associated with a mixture having c components is c-dimensional. When $c > 3$, such a graphical approach to detect and characterize departure from additivity is cumbersome if not impossible. Inselberg (1985) developed procedures for visualizing c-dimensional relations using a coordinate system whose axes are parallel. Eickemeyer (1992) has developed algorithms for representing planarity of c-dimensional figures by utilizing the parallel coordinate system of Inselberg; however, his procedures have been developed in the absence of random variation in the data. Another goal for this research was to develop statistical methodology useful for detecting departure from additivity (departure from planarity) based on plots of c-dimensional isobols produced from "raw" data. We demonstrate our methodology to date using a parallel axis coordinate system. In general, the rationale of this approach is to plot data from a specified c-dimensional isobol. If this multidimensional isobol is different from the plane of additivity, departure from additivity is suggested. Unfortunately, although it is theoretically possible to find points on a c-dimensional isobol, the practical consequences when c is large make this approach costly.

Specific Aims

The stated objectives of RFA 90-6, "Theoretical Approaches to the Analysis of Health Effects of Complex Mixtures", were (1) to support the theoretical development of methods for identifying those components of complex mixtures that may produce adverse health effects; and (2) to support the theoretical development of methods for determining interactive effects among components. Our research has been in this latter area. Our initial direction was to extend the logic of an isobologram to the case of c components in a mixture, for large c, by using the parallel axis coordinate system. Although theoretically possible, practical concerns of estimating concentrations on a specified contour of constant response (isobol) in multidimensions suggested the approach was infeasible. Therefore, we modified our research direction and considered the more economical logic of estimating the plane of additivity, which reqnires only single component concentration-response information. This methodology permits us to determine departures from additivity by comparing observed responses for mixtures of interest to predictions from an additivity model. If we recognize the local rather than global nature of the interaction of components in a specified mixture of interest, it is not necessary to explore the entire multidimensional relationship of the components.

Both phases of the research are described and illustrated with examples. The method for using an additivity model to predict the response for a specified mixture is illustrated with the analysis of ten components in the PAH fraction of kerosene soot. Of the two approaches, this is more practical from a design point of view when the number of chemicals or components in the mixture is large. The method for depicting a c-dimensional isobol using the parallel axis coordinate system is illustrated with the analysis of the combination of three chemicals.

Estimation of the Additivity Model Consider a combination of c compounds yielding data of the form (y, x), where y is the observed response or effect at the concentration combination x. We will distinguish between two groups in the data. The first group comprises m observations where the concentrations given are combinations of at least two chemicals. It is for these particular combinations that it is of interest to detect and characterize departures from additivity. The other group comprises data from which individual concentration-response curves can be estimated. More generally, for the ith chemical, $i = 1, \ldots, c$, there are a total of n_i observations distributed across d_i concentration levels with n_{ij} replications at each of the d_i levels. Thus,

$$n = \sum_{i=1}^{c} n_i, \quad \text{in which } n_i = \sum_{j=1}^{d_i} n_{ij}.$$

* A list of abbreviations appears at the end of this document.

This scenario allows for the case of one large experiment with a concurrent control group, but is general enough to allow for the situation of $c + q$ ($q \leq m$) separate control groups, that is, one for each of the individual c chemicals and one for each of q combination groups.

A data source for such an analysis is historical concentration-response information for each of the c chemicals and a current study that comprises the responses for combinations of interest. We assume that responses for concurrent control groups are available for each single chemical, resulting in the availability of multiple control groups.

Under the hypothesis of no interaction an additivity model can be constructed as

$$g(\mu) = \beta_0 + \sum_{i=1}^{c} \beta_i x_i \qquad (1)$$

in which $g(.)$ is a monotone differentiable function specified by the user; μ is the mean response; β_0, β_i, $i = 1, \ldots, c$ are unknown parameters; and xi is the concentration of the ith constituent in the combination. For fixed response, this model is algebraically equivalent to the interaction index form assumed by Berenbaum (1981; see Equation 2 in the next section). The basic assumption is that contours of constant response are planar if the chemicals in the combination are additive.

To estimate the unknown parameters given in Equation 1, we require single-chemical concentration-response data with control groups to estimate the slope parameters and the background response. The single parameter associated with the background response, β_0, will be incorporated into the additivity model in three ways.

The first approach uses a generalized linear model (McCullagh and Nelder 1989) framework to model the concentration-response curves of each chemical alone, while using a fixed-effect parameterization for the intercept. Let the concentration-response curve for the ith chemical in the combination be described by

$$g(\mu_{ij}) = \beta_{0i} + \beta_i x_{ij}$$

in which μ_{ij} is the mean response for the jth concentration group of the ith chemical ($i = 1, \ldots, c$; $j = 1, \ldots, d_i$); β_{0i} and β_i are unknown parameters; and x_{ij} is the jth concentration of the ith chemical. Because we allow the variance of the response to depend on the mean, the unknown parameters can be estimated using an iteratively reweighted least squares algorithm discussed in McCullagh and Nelder. This model assumes that the variability in background responses (as observed in the control groups) is a function of sampling error and the different β_{0i} assumed in the model, and is not overdispersed relative to variability in the concentration-response data. The hypothesis of a common intercept across control groups, H_0:$\beta_{0i} = \beta_0$, for all i, can be tested with the usual F test using quadratic forms with weighted least squares. With the estimated additivity model we can predict tbe response under additivity for combination concentrations of interest. A $100(1 - \alpha)\%$ prediction interval can be constructed to compare the observed (mean) response for combination concentrations of inieresl to that predicted under the assumption of additivity. A prediction interval is used instead of a confidence interval, as the former accounts for the sample variability of the observed response and the variability in the prediction under additivity.

Following the philosophy of Prentice and coworkers (1992) in dealing with historical control data, the second approach is based on a random-effects assumption. This approach assumes the responses from the different control groups are exchangeable; that is, the unobserved mean control responses across studies are independent random variables from a specified distribution, say $h(\beta_0)$. Conditional on a value for β_0, we assume the response, y, follows a distribution, say $f(y; \mu \mid \beta_0)$. Following the rules of conditional probabilities, the unconditional probability distribution of y often can be found; if it is, maximum likelihood estimates for the unknown parameters in Equation 1 can be obtained. Following tbis approach, large sample prediction intervals can be estimated around the response under additivity associated with chemical combinations of interest.

The final approach involving the use of estimating equations (Liang and Zeger 1986) assumes that observations within a study are correlated and those across studies are not related. This approach makes general assumptions about the distribution of y (a member of a family of distributions that includes the nor-

mal, binomial, Poisson, and gamma, among others). It also allows for the estimation of the correlation structure of observations within studies, implying that changing experimental conditions across studies may affect the observed responses. The paper by Liang and Zeger outlines more of the details. As in the case of the random-effects model, large sample prediction intervals can be constructed around the response under additivity for chemical combinations of interest.

Further statistical details of these three approaches are given in Gennings and Carter (1995). An example involving the PAH fraction of kerosene soot follows. Because the methodology described is new, it is difficult to find existing data suitable for thorongh analysis to illustrate these approaches. Thus the the following example is included for illustration only and not as a complete analysis of the kerosene soot mixture.

Data Analysis of the Polynuclear Aromatic Hydrocarbon Fraction of a Mixture of Kerosene Soot Kaden and colleagues (1979) investigated the mutagenic activity of the PAH-containing fraction of kerosene soot samples. From a chemical analysis of the PAH fraction (20 µg/mL), 21 compounds were identified using gas chromatography–mass spectrometry. Eighteen percent of the weight of the mixture was uncharacterized in that it either was material lost in the characterization process or could not be identified by gas chromatography–mass spectrometry. Concentration-response information for 10 of the 21 characterized compounds was made available by graphically extracting the means of two independent determinations from charts provided in Kaden (1978) and Kaden and colleagues (1979); the original data were not available. The compounds and their concentrations are listed in Table 1. Those compounds for which concentration-response information was available are underlined. Using the available data, we constructed interval estimates for the mean response of this mixture under the hypothesis of additivity using the three procedures discussed in the previous section.

Mutagenic activity was measured in *Salmonella typhimurium*, using resistance to the purine analog 8-azaguanine (8-AG) as a genetic marker. The assay used was a forward bacterial mutation assay as specified by Skopek and associates (1978). Exponentially-growing cultures of *Salmonella typhimurium* strain TM677 were exposed to several concentrations of the test agent and a microsomal fraction of a liver homogenate (S9) of Aroclor-pretreated male rats. Colonies were counted after growth for two days. The response is the fraction of viable bacteria that can form colonies in selective media because of a mutation in the *hprt* gene. The mutant (8-AG–resistant) fraction was calculated by dividing the number of colonies observed under selective conditions by the number of colonies observed under permissive conditions and multiplying by appropriate dilution factors.

Because the denominator of the mutant fraction (the number of colonies observed under permissive conditions) is ronghly five orders of magnitude larger than the numerator, it was assumed to be constant across concentration groups, making it reasonable to model the number of colonies observed under selective conditions as a count (nonnegative integer). The mean response was modeled as a function of concentration as $\mu_{ij} = \exp(\beta_{0i} + \beta_i x_{ij})$, $i = 1, \ldots, 10$; $j = 1, \ldots, d_i$. Eight of the ten compounds were observed at three concentration levels in addition to a control group; perylene was observed at six, and anthanthrene two, concentration levels other than a control level. The average number of colonies observed when cultures were exposed to the mixture described in Table 1 was 21.7 (with standard deviation of 2.2). We compared these observed responses to those predicted under the assumption of additivity.

Assume the data for each compound come from identical experimental conditions so that the control group responses are independent estimates of a common background rate. Table 2 presents the (extracted mean) responses from the concentration groups for each chemical. A fixed-effects model can be used for constructing the additivity model. The test for common intercept could not be rejected ($p = 0.18$). The estimated coefficients and associated (large sample) p values are given in Table 3. Using the mixture composition listed in Table 1 for the available data, a predicted response of 42.1 mutants per 10^5 cells was estimated. The 95% large sample prediction interval on the mean response under additivity was [35.1, 49.0]. In order to interpret this interval, it is necessary to assume that neither the uncharacterized com-

Table 1. Components of Methylene Chloride Extract from Kerosene Soot

Compound[a]	Wt (%)	Amount Present (μg/mL)
Acenaphthylene	23	4.6
Cyclopenta[*cd*]pyrene	15	3.0
Pyrene	8	1.6
Benzo[*ghi*]perylene + anthanthrene	8	1.6
Coronene	5	1.0
Fluoranthene	4	0.8
Naphthalene	3	0.6
Benzo[*ghi*]fluoranthene	3	0.6
Phenanthrene + anthracene	2	0.4
Benzacenaphthalene	2	0.4
Benzofluoranthene	2	0.4
Perylene	2	0.4
Acenaphthalene	1	0.2
Indeno[1,2,3-*cd*]pyrene	1	0.2
Benzo[*a*]pyrene + benzo[*e*]pyrene	1	0.2
4H-Cyclopenta[*def*]phenanthrene	1	0.2
Benzo[*b*]fluorene	0.4	0.008
Fluorene	0.3	0.006
Uncharacterized material[b]	18.3	3.7
Methylene chloride extract	100	20

[a] Underlining indicates a compound for which concentration-response information was unavailable.
[b] Material lost in the characterization process, plus those compounds that could not be identified by gas chromatography–mass spectrometry.

pounds in the mixture nor the compounds without available concentration-response information have an effect over background, and that they do not affect the concentration-response relation of the ten compounds considered. Under this important assumption, because the concentration-response curves are increasing, and because the observed response was less than the lower bound of the prediction interval (35.1), a less than additive (or antagonistic) relationship is suggested among the ten chemicals at the specified mixture.

For a random-effects model, we assume the experimental conditions associated with each study are such that the background mean responses are exchangeable, resulting in an overdispersed model. That is, the degree of variability within a study is less than that across control group means. Assume that the number of colonies observed within a study follows a Poisson distribution conditional on a background count, where the mean control counts (μ_{i0}) across studies, $i = 1, \ldots, 10$, are independent unobservable random variables such that $\mu_{i0} = \exp(\beta_{0i})$ is a random variable that follows a gamma ($\alpha1$, $\alpha2$) distribution. From this distribution, the mean control response is $\alpha2/\alpha1$. The slope parameters are assumed to be fixed and unknown. The method of maximum likelihood was used to estimate the model parameters using the Nelder-Mead direct search algorithm with the constraints that $\alpha1, \alpha2 > 0$; the estimates are presented in Table 3. Using the mixture composition given in Table 1 for the components underlined, the predicted number of colonies under the assumption of additivity was 67.9. A large-sample symmetric 95% prediction interval on the response for this specific

Table 2. Group Mean Responses for Each Compound

Compound	Concentration (mg/mL)	Extracted Mean Colonies
Acenaphthylene	0	9
	20	12
	100	10
	200	5
Cyclopenta[*cd*]pyrene	0	3
	1	10
	5	50
	10	138
Pyrene	0	4
	10	5
	50	21
	100	21
Anthanthrene	0	9
	2	13
	10	28
Fluoranthene	0	1
	2	3
	6	128
	12	315
Perylene	0	6
	0.1	9
	0.2	11
	0.3	16
	1	28
	3	37
	10	38
Benzo[*a*]pyrene	0	4, 11
	5	51, 52
	10	83, 84
	20	99, 189
Acenaphthalene	0	4, 6, 7
	50	5, 6, 7
	100	8, 9, 11
	200	9, 10, 14
Benzo[*e*]pyrene	0	3, 9
	50	20, 31
	100	10, 23
	201	15
Benzo[*b*]fluorene	0	12
	5	16
	25	31
	50	60

Table continues next column

combination under the hypothesis of additivity is given by [3.2, 132.5]. Under the same assumptions about the unidentified and uncharacterized components in the mixture, because the observed mean response, 21.7, is included within the prediction interval, the overall relation of the components in this mixture does not appear different from additive.

The third approach uses generalized estimating equations with an exchangeable correlation structure. Because the only data available for analysis were the means at each concentration, data were insufficient for convergence of the iterative algorithm for parameter estimation. For illustrative purposes only, we simulated replicated responses from the graphically extracted means. In order to simulate true experimental conditions with replications at each concentration of the ten compounds, four Poisson (μ_{ij}) random variables were generated for $i = 1, \ldots, 10$, and $j = 1, \ldots, g_i$, where μ_{ij} is the observed mean for the jth treatment of the ith compound. For estimation of the additive model, the observations within a study are assumed to have a common correlation, ρ, and the observations from different studies are assumed to be uncorrelated. For these data, the within-study correlation was estimated at 0.12. Model parameters were estimated by iterating between a modified Fisher scoring algorithm for estimating the intercept and slope parameters and a moment estimator for ρ; regression parameters are given in

Table 3. Estimated Regression Coefficients

	Fixed-Effects Model[a]		Random-Effects Model		Estimating Equations[b]	
Parameter	Estimate	*p*-Value	Estimate	*p*-Value	Estimate	*p*-Value
β_0	2.66	< 0.001			2.68	< 0.001
α_1			0.0379	< 0.001		
α_2			0.675	< 0.001		
β_1 (Acenaphthylene)	-3.84×10^{-3}	0.394	-1.82×10^{-3}	0.794	-1.94×10^{-3}	0.763
β_2 (Cyclopenta[*cd*]pyrene)	2.53×10^{-1}	< 0.001	2.83×10^{-1}	0.044	2.56×10^{-1}	< 0.001
β_3 (Pyrene)	4.72×10^{-3}	0.371	1.07×10^{-2}	0.462	6.23×10^{-3}	0.440
β_4 (Anthanthrene)	7.15×10^{-2}	0.181	1.74×10^{-1}	0.385	8.06×10^{-2}	0.321
β_5 (Fluoranthene)	2.67×10^{-1}	< 0.001	2.81×10^{-1}	0.006	2.68×10^{-1}	< 0.001
β_6 (Perylene)	1.15×10^{-1}	0.005	2.21×10^{-1}	0.255	1.03×10^{-1}	0.109
β_7 (Acenaphthalene)	-1.91×10^{-3}	0.403	1.10×10^{-3}	0.792	3.23×10^{-4}	0.952
β_8 (Benzo[*a*]pyrene)	1.23×10^{-1}	< 0.001	1.78×10^{-1}	0.003	1.16×10^{-1}	< 0.001
β_9 (Benzo[*e*]pyrene)	1.99×10^{-3}	0.429	5.29×10^{-3}	0.440	1.46×10^{-3}	0.761
β_{10} (Benzo[*b*]fluorene)	2.88×10^{-2}	< 0.001	4.42×10^{-2}	0.152	2.94×10^{-2}	0.009

[a] The var(Y) is assumed to be of the form $\sigma^2\mu$; MSE = 7.25.

[b] The data analyzed with estimating equations were based on simulated replications of the extracted means. The fixed-effects and random-effects analyses were based on the extracted means.

Table 3. The predicted response under the assumption of additivity for the specified mixture was 44.1. The model-based estimator of the large-sample covariance matrix of model parameters was used. As the number of studies is not large relative to the number of estimated parameters, the empirically-based estimator of the covariance matrix is unstable. A large-sample symmetric 95% prediction interval for the response under additivity for the mixture is given by [25.0, 63.3]. A similar conclusion of antagonism can be made with the same restrictions as above. Because simulated data were added for this analysis, it should be emphasized that this conclusion is made for illustration only.

These three models make different assumptions about the control group means. With knowledge of the experimental conditions, the user chooses a preferred model. All three are included here for illustration; they are not meant to be compared. We have used single-chemical concentration-response information to estimate an additivity model. From this estimation, inferences can be made about the overall effect of combinations or mixtures of interest without estimation and exploration of the entire multidimensional response surface. This strategy offers the user an economical qualitative description of the nature of the overall interaction of the components.

A *c*-Dimensional Isobologram In the study of combinations of two compounds, plots of the contours of constant response of the concentration-response surface (isobolograms) are used to characterize the interaction between the compounds. The method involves comparing the isobol to the "line of additivity," that is, the line connecting the single-compound concentrations that yield the level of response associated with the contour. The interaction is described as synergistic, additive, or antagonistic according to whether the contour is below, coincident with, or above the line of additivity, respectively; systematic deviation from an additive model is attributed to the presence of an interaction.

Berenbaum (1981) proposed the following mathematical index to detect and characterize interactions for c (≥ 2) compounds:

$$\frac{x_1}{X_{1E}} + \frac{x_2}{X_{2E}} + \ldots + \frac{x_c}{X_{cE}} \quad \left\{ \begin{array}{l} <1 \text{ for } \textit{synergy} \\ =1 \text{ for } \textit{additivity} \\ >1 \text{ for } \textit{antagonism} \end{array} \right\}$$

in which x_i is the concentration of the ith component in combination that yields response E, and X_{iE} is the concentration of the ith compound that yields response E when given alone, $i = 1, \ldots, c$. Let $A_i = \frac{1}{X_{iE}}$. Then, under additivity the interaction index is the equation of a plane,

$$A_1x_1 + A_2x_2 + \ldots + A_cx_c = 1 \Rightarrow \textit{no interaction}. \quad (2)$$

If the isobol is a hyperplane in c-dimensions [called a $(c-1)$-flat], then it can be concluded that the compounds combine additively, i.e., with no interaction. If the isohol is not a $(c-1)$-flat, then the compounds are said to interact.

We demonstrate a visual model-free procedure for detecting departure from planarity in c-dimensional isobols while accounting for data variability using a parallel coordinate axis system. Inselberg (1985) developed the parallel coordinate axis system, embedding a c-dimensional axis system in the $[X, Y]$ plane by constructing c lines perpendicular to the X-axis, each one unit apart, starting at $X = 0$. The c axes in the parallel system all have the same positive orientation as the Y-axis. In this system the point $(x_1, x_2, \ldots, x_c)$ is represented by the line formed by the connected line segments with endpoints $[0,x_1]$, $[1,x_2]$, $\ldots$, $[c-1,x_c]$ in which $[.,.]$ represents the location in the $[X, Y]$ plane. The five-dimensional point ($x_1 = 3$, $x_2 = 7$, $x_3 = 5$, $x_4 = 8$, $x_5 = 2$) is plotted in Figure 1. In such a coordinate system a $(c-1)$-dimensional surface can be visualized by plotting a set of c-dimensional points satisfying a given functional relation, as that given in Equation 2.

Detecting Planarity A duality exists between plotting in the parallel coordinate system and the usual orthogonal coordinate system. (To avoid confusion in nomenclature, we will use the words 0-flat, 1-flat, 2-flat, etc. to mean a point, line, and three-dimensional plane; the words "points" and "lines" will refer to the objects that construct the representation of these figures in a coordinate system.) From Figure 1 we see that the representation of a 0-flat in five dimensions using a parallel axis coordinate system is made up of connected line segments. In two dimensions, a 0-flat is represented by a line. Inselberg (1985) and Gennings and colleagues (1990) demonstrated that a 1-flat in two

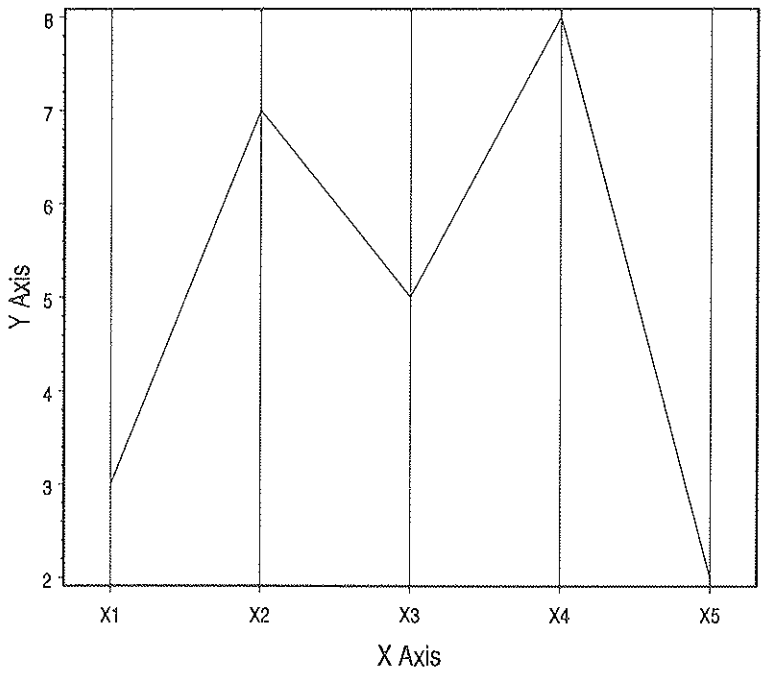

Figure 1. Representation of the 5-dimensional point (3, 7, 5, 8, 2) in the parallel coordinate axis system.

dimensions, say $y = mx + b$, can be represented by a single point, located at $\left[\frac{1}{1-m}, \frac{b}{1-m}\right]$, because the line segments that represent the 0-flats on the 1-flat all connect at this single point.

Eickemeyer (1992) has generalized this concept for the representation of a $(c-1)$-flat using $c+1$ points such that the location of the points can be used to determine the equation of the $(c-1)$-flat. His methodology augments the parallel axes by copying the axes to the right of the cth axis where the distance between the axes is still one unit. The additional axes are required in order to uniquely determine the equation of the flat. The $c+1$ points can be obtained by plotting points of intersection between 0-flats that lie on the $(c-1)$-flat; then points of intersection between these 1-flats; then points of intersection between these 2-flats; etc. The resulting $c+1$ points are located at $[x_1,y]$, $[x_2,y]$, $[x_3,y]$, . . . , $[x_{c+1},y]$ in the X-Y plane. Figure 2 shows a representation of a 2-flat in three dimensions; the four points are labeled 1, 2, 3, and 4. The coefficients of the equation of the $(c-1)$-flat in c space are given by Equation 2 in which $A_i = \frac{x_{i+1} - x_i}{y \cdot c}$, $i = 1, \ldots, c$. A one-step procedure also can be implemented to find the location of the $c+1$ points, $[x_1,y]$, $[x_2,y]$, $[x_3,y]$, . . . , $[x_{c+1},y]$, that uniquely define the $(c-1)$-flat. By selecting c 0-flats that span a $(c-1)$-flat we can find the coefficients of the equation of the flat, $A_1, A_i, \ldots, A_c$, given in Equation 2 using matrix theory. Then

$$y = \left(\sum_{i=1}^{c} A_i\right)^{-1}, \quad x_1 = \frac{\sum_{i=2}^{c} (i-1)A_i}{y},$$

$$x_j = x_1 + \frac{c \sum_{i=1}^{j-1} A_i}{y}, \quad j = 2, \ldots, c+1.$$

The theory of the plotting technique by Eickemeyer states that in order to detect a $(c-1)$-flat, $c+1$ distinct points must result. In the presence of random variation, $c+1$ clusters may be observed instead of unique points (Figure 3). The algorithm for determining these clusters is analogous to randomly selecting without replacement c data points from the isobol. These c data points uniquely determine a $(c-1)$-flat. If c more points are randomly selected from the remaining data points another $(c-1)$-flat is uniquely determined. This process continues until there are no longer c points from which to select. Each uniquely determined plane is then plotted in the parallel axis system as c unique points. These are the points that make up the observed clusters.

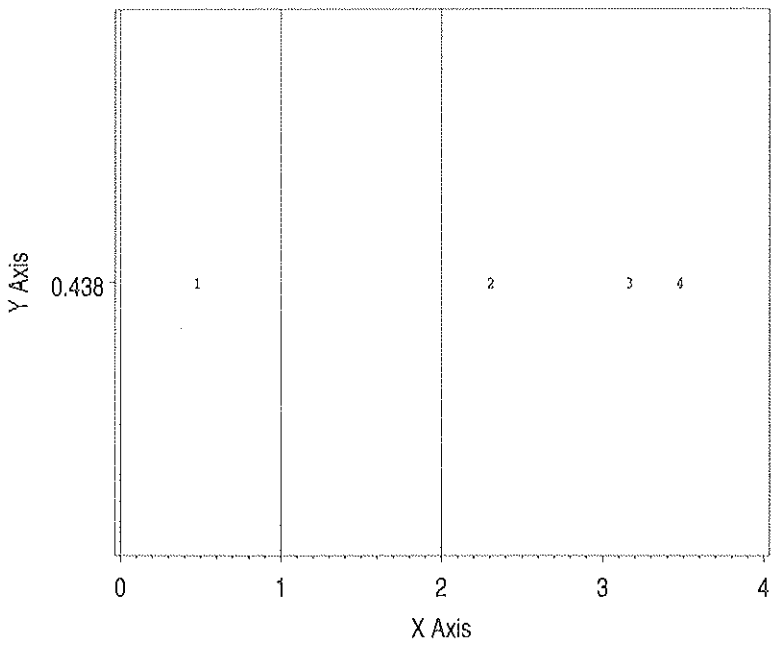

Figure 2. The plane of additivity given by $1.39x_1 + 0.655x_2 + 0.236x_3 = 1$, where x_1 is the concentration of carbon tetrachloride, x_2 is the concentration of chloroform, and x_3 is the concentration of trichloroethylene. The plane is plotted without noise. The points labeled 1, 2, 3, and 4 represent the location of intersecting line segments plotted in the parallel axis coordinate system of any 0-flat (a point) on the plane; therefore, the representation of the plane with these points is a unique representation.

It is useful to test a hypothesis about these clusters in order to determine whether the isobol is different from the "plane of additivity" or if the randomly determined c-dimensional planes are estimates of the "plane of additivity." A multivariate sign test (Hettmansperger 1984) can be used to test this hypothesis of no interaction. Although other model-free tests are available, this test makes fewer assumptions about the point distribution. Define $x_i = [x_{1i}, \ldots, x_{c+1,i}]'$, $i = 1, \ldots, n$ as the x coordinates from the ith randomly selected $(c-1)$-flat from the isobol, and $x^* = [x^*_1, \ldots, x^*_{c+1}]'$ as the x coordinates of the plane of additivity. Then the multivariate sign test is based on the statistic $S = [S_1, \ldots, S_{c+1}]'$, in which

$$S_j = \sum_{i=1}^{n} \text{sgn}\,(x_{ij} - x^*_j),$$

$j = 1, \ldots, c+1$, and is a model-free test for no interaction.

Characterizing Departure from Planarity

Carter and associates (1988) demonstrated the relation between departure from additivity as determined by Berenbaum's (1981) interaction index and a parameter in a response-surface model. For increasing concentration-response relations, if the interaction parameter (one associated with a cross-product term) is statistically significant and positive, a synergism occurs; if the interaction parameter is significant and negative, an antagonism occurs; if the interaction parameter is not different from zero, the compounds are said to have no interaction. For decreasing concentration-response relations, the characterizations are switched.

Curvature in the isobol (departure from planarity) can be identified by comparing the points from the isobol to models for the isobol with higher-degree terms. The comparison can be made visually using the parallel axis representation of the data. These higher-degree terms are analogous to those used by Carter and associates.

An important property of the representation of a $(c-1)$-flat using the parallel axis system is that the distance between adjacent points from the $c+1$ points plotted is related directly to the coefficients of the plane. Therefore, the distance and direction between clusters associated with an interaction parameter may be used to determine the presence of interaction and its characterization, respectively. A signed rank test for paired data may be useful in indicating important interaction parameters.

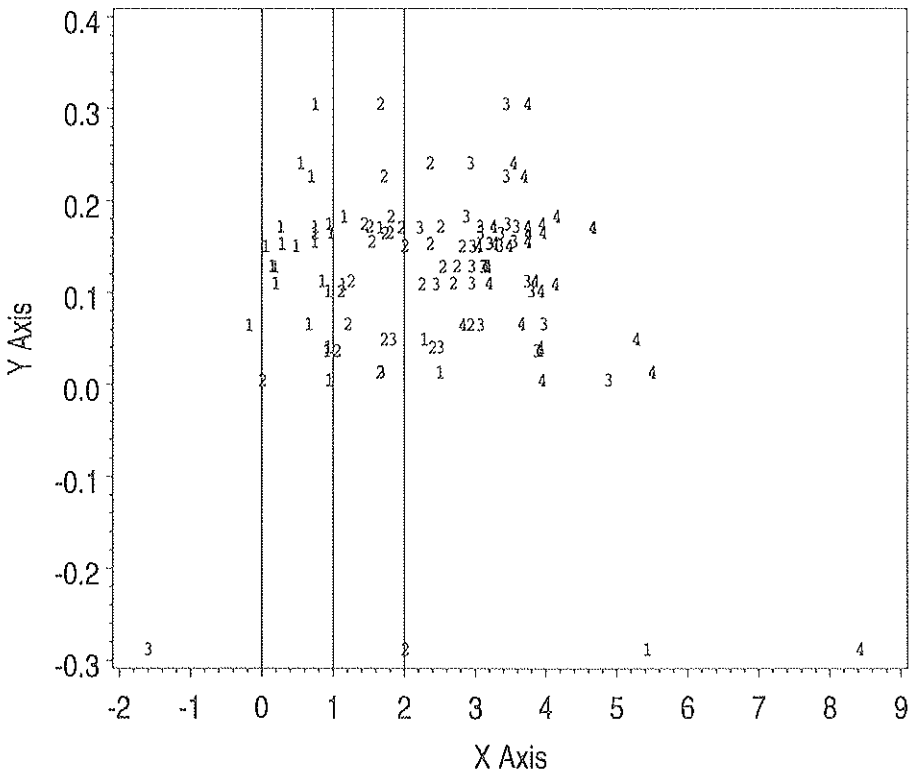

Figure 3. Random planes constructed from random data around an isobol. If the data are taken from a hyperplane without noise, the plot is equivalent to Figure 1 where the four points of intersection are coincident. When the data reflect random variation around a hyperplane, clusters of points of intersection are evident; the clusters are not present if the data follow a curvilinear relationship. Here, each set of horizontal points labeled 1, 2, 3, and 4 represent a random selection of four data points on the contour.

Data Analysis of a Three-Dimensional Isobologram Data were obtained from Dr. J. E. Simmons at the Environmental Protection Agency Health Effects Research Laboratory studying the effect of carbon tetrachloride, chloroform, and trichloroethylene in combination. Male F344 rats (Charles River Lab) 60 to 70 days old, held in metabolism cages, were used in the study. The objective was to determine how carbon tetrachloride, chloroform, and trichloroethylene combine to affect percent liver weight [percent liver weight = (100)(liver weight)/(body weight)]. The study design comprised nine rays combining the three compounds in differing proportions: {(1,0,0), (0,1,0), (0,0,1), (1/3,1/3,1/3), (2/3,1/6,1/6), (1/6,2/3,1/6), (1/6,1/6,2/3), (5/6,1/12,1/12), (1/12,5/6,1/12)}, with between two and five concentrations along each ray.

We want to estimate a contour of constant response within (or near) the experimental range of concentrations along all the rays. A response of 4.0 was selected. Estimates for concentration combinations that yielded a response of 4.0 units (ED_4) were found by fitting a nonlinear model to response and total concentration along each ray. The sigmoid-shaped nonlinear model used was $y = \alpha[\exp\{-(\beta_0 + \beta_1 x)\}]^{-1} + \varepsilon$, in which y is percent liver weight, x is the total concentration; α, β_0, and β_1 are unknown regression parameters; and ε is an unobservable random error term. This model was selected because of its flexibility in representing a concentration-dependent sigmoid-shaped curve. Model parameters were estimated using iteratively reweighted least squares. The variance of each ED_4 also was estimated, using the delta method. The nine ED4 isobol points are given in Table 4.

Table 4. Estimated ED_4 Isobol Points for Percent Liver Weight

Ray	Carbon Tetrachloride (mL/kg)	Chloroform (mL/kg)	Trichloroethylene (mL/kg)
A	0.720	0.00	0.00
B	0.00	1.53	0.00
C	0.00	0.00	4.23
D	0.162	0.162	0.162
E	0.348	0.0833	0.0883
F	0.0666	0.283	0.0666
G	0.116	0.116	0.494
H	0.403	0.0403	0.0403
I	0.0266	0.293	0.0266

From the estimates in rays A through C, the equation for the plane of additivity is given by $1.39x_1 + 0.655x_2 + 0.236x_3 = 1$, which is represented in the parallel axis system in Figure 2. Using the equation for the plane of additivity, $y = 0.438$, $x_1 = 0.494$, $x_2 = 2.32$, $x_3 = 3.18$, and $x_4 = 3.49$. From the nine randomly-ordered points on the ED_4 isobol shown in Table 4, three unique independent (without a common point) three-dimensional planes can be determined. These planes are plotted in Figure 4 using the usual orthogonal axes and are represented in Figure 5 using the parallel axis system. From only three estimates of the plane of additivity, the results of the multivariate sign test for no interaction ($p = 0.39$) are inconclusive. In order to further illustrate the technique, we replicated the nine points on the isobol by generating data associated with each of the points. A normal random number generator (SAS, version 6, SAS Institute Inc., Cary, NC) was used, with mean given by the estimates for the isobol points and variance given by their associated variance estimates.

These ten replicates of the nine isobol points were used in a similar analysis. Figure 3 presents the representation of three-dimensional planes determined randomly from the generated data. Results from the multivariate sign test of the hypothesis of no interaction ($p < 0.01$) indicated that the isobol is different from the plane of additivity. Using these replicate points it seems reasonable to conclude that carbon tetrachloride, chloroform, and trichloroethylene do not have an additive effect on percent liver weight; however, this illustration should not be regarded as a conclusive result about these chemicals.

In an attempt to characterize the departure from planarity, the generated isobol points were compared to a four-dimensional plane where the fourth dimension was the cross-product of the concentration of two of the compounds. These four-dimensional figures are represented in the parallel axis system in Figures 6, 7, and 8 for studying the potential interaction between carbon tetrachloride and chloroform, carbon tetrachloride and trichloroethylene, and chloroform and trichloroethylene, respectively. In all three cases, because the cluster of the numeral

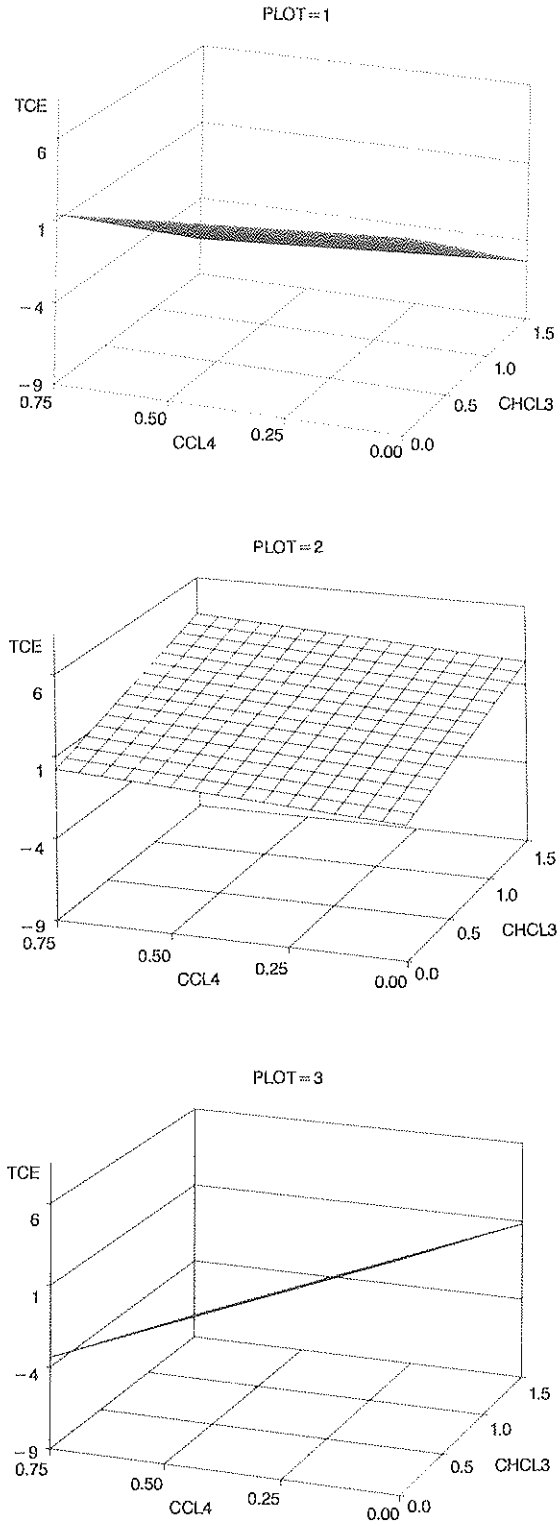

Figure 4. Three randomly selected planes plotted in the usual orthogonal axis system. These planes were determined by random selection without replacing the nine points on the estimated isobol given in Table 4.

5 is to the right of the cluster of the numeral 4, the figures suggest that a positive regression coefficient is associated with the cross-product term. On the basis of a signed rank test on paired data, all three shifts are significant ($p < 0.01$). Therefore, because a positive shift between clusters seems to be associated with a positive interaction parameter, it is reasonable to conclude that there is a synergistic relationship between carbon tetrachloride and chloroform, between carbon tetrachloride and trichloroethylene, and between chloroform and trichloroethylene along the ED_4 isobol of the percent liver weight.

We have demonstrated the use of the parallel axis system in detecting and characterizing interactions. The hypothesis of no interaction is tested using a model-free approach in which points on a specified isobol are compared to the plane of additivity. Isobol curvature indicates the degree of departure from additivity.

Discussion and Conclusions

In the analysis of chemical interactions of a combination of *c* chemicals, two important concentration-response relationships are recognized: the response surface associated with the multidimensional relation, which includes the effects of the chemical interactions; and the additivity surface, which describes the relation under the hypothesis of no interaction. We have made use of the former in the isobolographic analysis and of the latter in the comparison of the observed response at a combination of interest to the response predicted under the hypothesis of additivity. The former is more costly in that combinations on the contour of the response surface associated with a fixed response must be found; the latter is cheaper in that only single-chemical exposnre data are necessary in estimating the additivity model. Further, analysis of the isobologram may result in a claim of global departure from additivity, whereas by design the analysis of the additivity surface results in local (at a specified combination of interest) detection of departure from additivity.

As the number of components, *c*, in a combination or mixture gets large, carrying ont an experiment large enough for estimating the response surface or associated contour of constant response necessary for a *c*-dimensional isobologram may not be practical. The

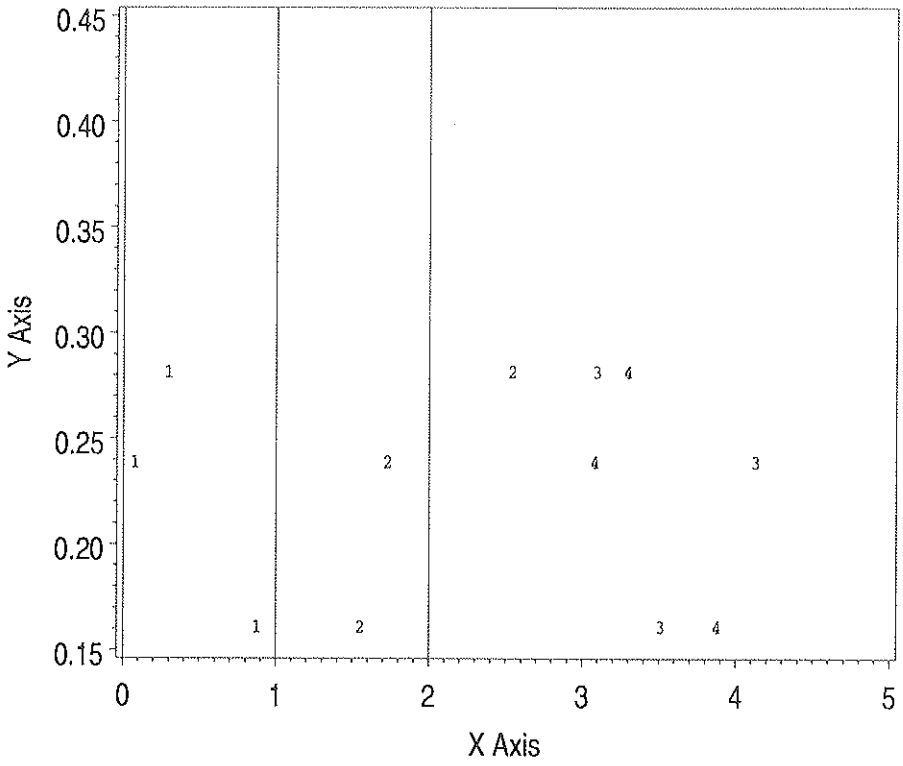

Figure 5. The same three planes plotted in Figure 3 except now represented in the parallel axis system.

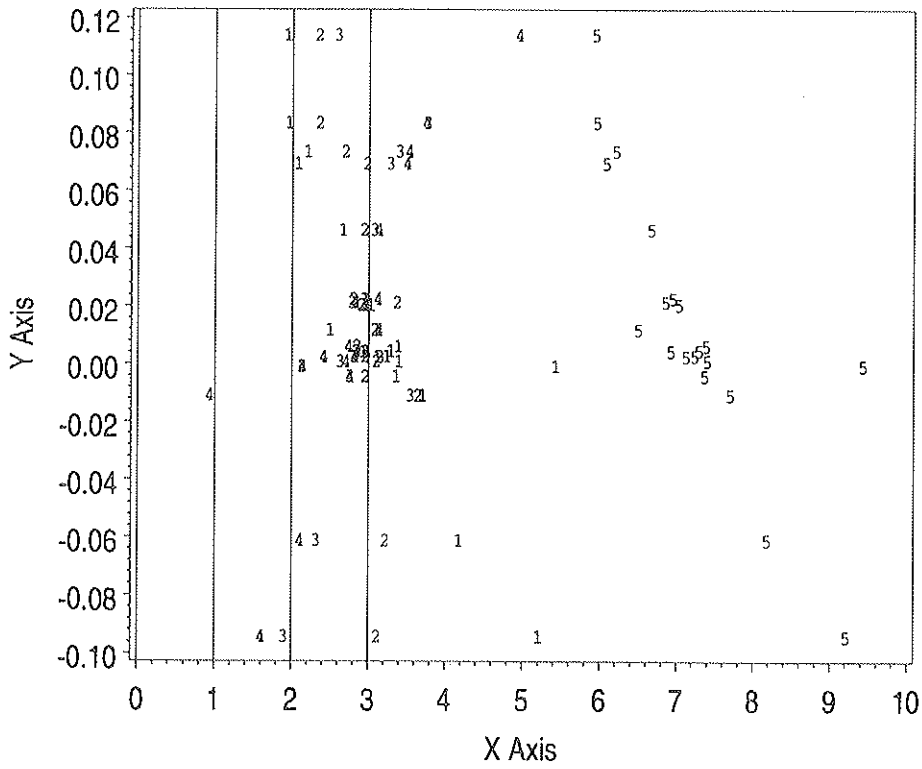

Figure 6. A comparison of the generated data on the ED_4 isobol to a model with a cross-product term associated with carbon tetrachloride and chloroform.

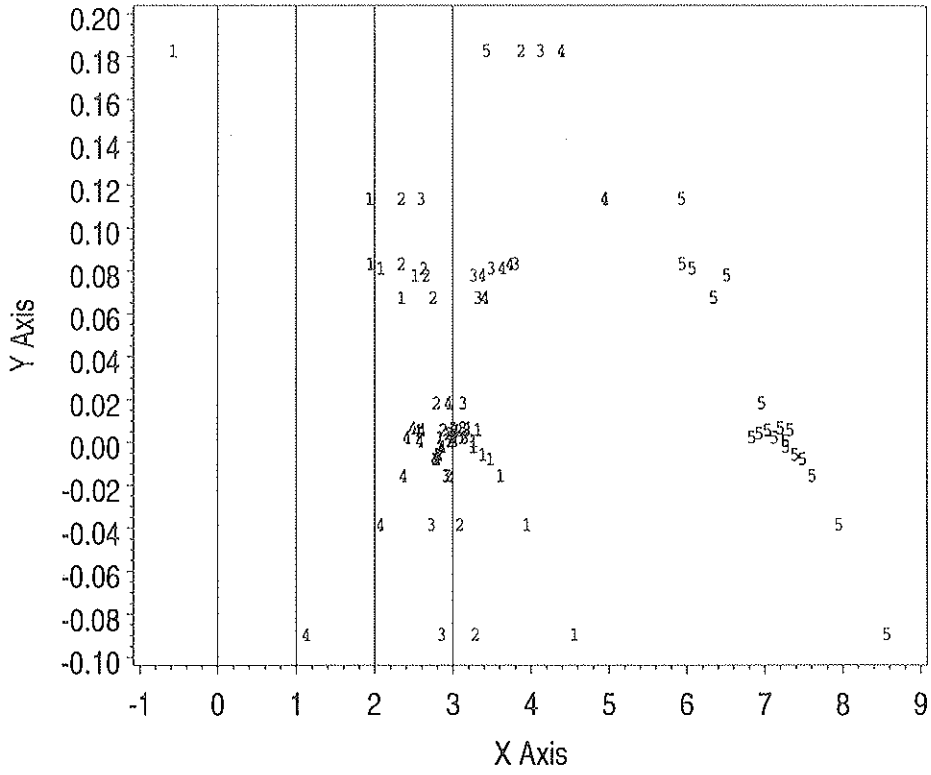

Figure 7. A comparison of the generated data on the ED_4 isobol to a model with a cross-product term associated with carbon tetrachloride and trichloroethylene.

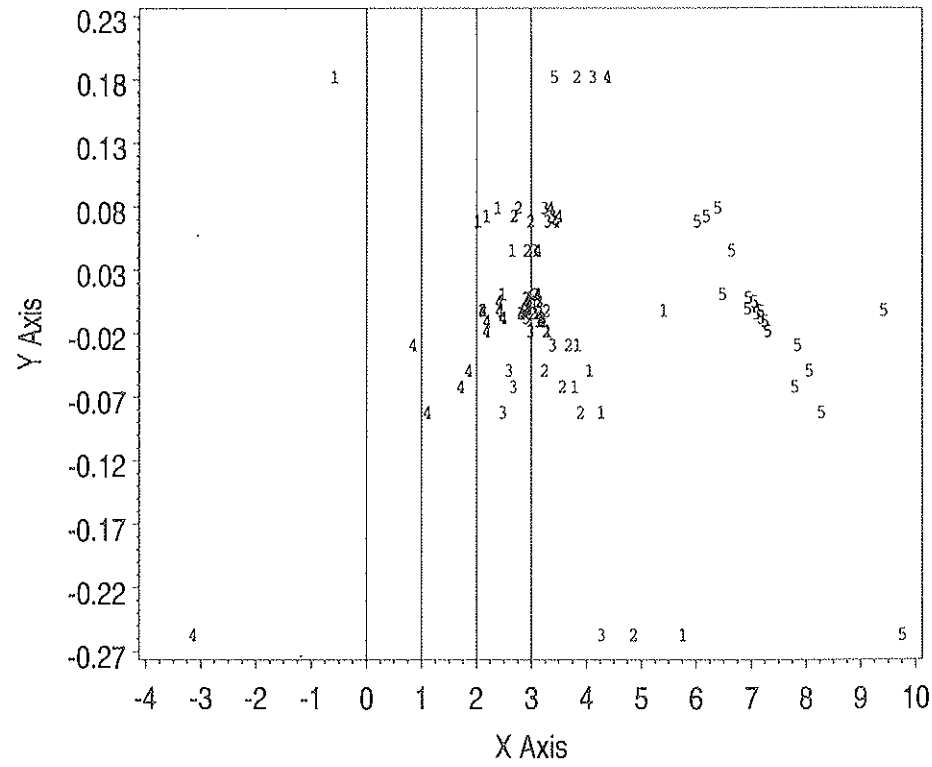

Figure 8. A comparison of the generated data on the ED_4 isobol to a model with a cross-product term associated with chloroform and trichloroethylene.

illustration given for the isobologram method uses a design based on concentration combinations along rays of constant proportion. Although it is possible to estimate points on a contour of constant response associated with each ray, the number of rays must increase rapidly as the number of dimensions in the combination increases. It is not clear how many rays are required to adequately explore c-dimensional space, and the number of rays may be dependent on the complexity of the unknown relation. For this reason we have developed a more economical approach for estimating the additivity surface (described in the first part of the Methods section) using an additivity model, comparing the observed response for a combination or mixture of interest to the predicted response under additivity. This comparison results in a qualitative description of the effect of the overall interaction. Further work is required to identify which components in the combination are important, if there is an interaction. Perhaps important contributors may be identified by taking chemicals out of the combination one at a time.

A common problem encountered in risk assessment is that of extrapolating concentration-response information from a fitted model to a low-concentration region where experimental support is not available. The results of prediction in the low-concentration region often are dependent on the choice of the model. Such is also the case for the methods described herein. In describing the additivity surface we need concentration-response information for each chemical. The parametric form of the concentration-response model can be assumed by the investigator, supported by the data (when available), then combined to estimate the additivity model. This model then is used to estimate the effect of mixtures under the assumption of additivity.

Clearly the analysis of mixtures offers researchers many challenges that as yet are unresolved. An important first step is to recognize that the analysis of single compounds is useful to describe the effect of a mixture only under the assumption of additivity. A comparison of observed and predicted responses using this additivity model results in a qualitative conclusion that the overall effect of the mixture is congruent with a synergism, an antagonism, or is not different from additive. It is our opinion that even such qualitative information about a mixture can be used to increase our understanding of important multidimensional relationships.

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Kathryn S. Dawson is a Senior Programmer Analyst in the Department of Biostatistics at MCV/VCU. She received an M.S. in Mathematics from University of Maine in 1975 and an M.S. in Computer Science from VCU in 1985. She has been a graduate student in the Department of Biostatistics, MCV/VCU, since 1987. Her Ph.D. dissertation topic relates the parallel axis coordinate system to multidimensional statistical issues.

Publications Resulting from This Research

Gennings C, Carter WH Jr. 1995. Utilizing concentration-response data from individual components to detect statistically significant departures from additivity in chemical mixtures. Biometrics (in press).

Abbreviations

PAHs	polynuclear aromatic hydrocarbons
8-AG	8-azaguanine
ED_4	estimates for dose combinations yielding response of 4 units

COMMENTS

Gennings and colleagues took two approaches to evaluating interactive effects: (1) various statistical tests for departures from additivity using generalized linear modeling techniques, random-effects models, and generalized estimating equations, and (2) through the use of a parallel axis coordinate system to depict planes of additivity graphically, where departures from additivity were examined using isobolograms. In the first approach, the authors apply three techniques to the Estimation of Additivity Model (Method I) for a set of experimental data on the mutagenic activity of kerosene soot and its constituents. Because data are available on only 10 of the 21 constituents, accounting for 72% by weight of the mixture, the authors must assume that the remaining uncharacterized compounds (18% by weight) do not contribute to the response. Although the three estimation procedures give somewhat different estimates for the regression coefficients (Table 1), the observed response, 21.7, falls below the 95% prediction intervals obtained from two of the three procedures (i.e., the fixed-effects model [procedure 1] and the approach using generalized estimating equations [procedure 3]). As anticipated, the estimated coefficients obtained by these procedures are quite similar, as are the prediction intervals. Procedure 2 yields a larger point estimate and a wider prediction interval. Although these techniques are largely based on established statistical approaches to nonlinear regression problems, the authors provide little detail about the statistical methodology in the report. A significant limitation of these results comparisons is the lack of information on goodness of fit of any of these models to the data. Lacking information about goodness of fit, we are unable to choose among these models, or even to judge whether the models provide satisfactory fit to the collection of concentration-response studies on the individual chemicals.

Table 1. Prediction Intervals for Regression Response Using Three Statistical Approaches

Procedure	95% Prediction Interval
Fixed effects	(35.1, 49.0)
Random effects	(3.2, 132.5)
Generalized estimating equations	(25.0, 63.3)

Gennings and colleagues attempt to use a c-dimensional Isobologram Model (Method II) to develop tests and graphical techniques to evaluate the additivity of effects. Given nc mixtures of c chemicals which produce the same biological effect (possibly obtained from prior statistical analysis), n sets of c points can be chosen in random order and used to construct n sets of line segments in the parallel axis system. One then can apply the nonparametric test developed by Hettmansperger (1984) to test the hypothesis of additivity of effects. This analysis can be extended to more complex interactions by treating the products of pairs of variables as new variables in the parallel axis system and plotting their values as usual.

To illustrate this approach, consider the three chemicals, carbon tetrachloride, chloroform, and trichloroethylene, studied by J.E. Simmons at the Environmental Protection Agency and discussed in this report. If the concentrations of these three chemicals are given by x_1, x_2, and x_3, respectively, and if the effect of a mixture (x_1, x_2, x_3) of these chemicals depends only on the value of the linear combination of those concentrations given by the expression

$$A_1x_1 + A_2x_2 + A_3x_3,$$

then the effects of these three chemicals are additive in that biological system.

If the sets of mixtures defining an isobologram could be identified exactly, then plotting any subset of three points lying on the isobologram in the parallel axis coordinate system would identify the three points of intersection that characterized the isobologram. When the points on the isobologram are estimated from experimental data, the planes defined by different subsets of points will vary stochastically. The experimental data available to Gennings and colleagues provide estimates of nine points on each isobologram. The authors use these

nine points to construct three planes and plot these planes in three-dimensional space (Figure 3 of their report), and in the parallel axis system (Figure 4 of their report). If the effects of the three chemicals are additive, these three planes are estimates of the same isobologram. The authors then test for additivity using Hettmansperger's test. Although the test fails to reject the hypothesis, the authors note that the test has poor power when based on only nine points. The authors therefore attempt to enrich their data set by stochastically generating ten replicates of the nine points.

When the test of additivity is applied to this simulated data set, the test for interaction is statistically significant. Thus, the authors conclude that the effects of the three chemicals are not additive. Although this may be of some use in illustrating the technique, the simulated data no longer satisfy the independence conditions required for Hettmansperger's test. Thus, the cited p value of 0.01 is invalid.

As the authors acknowledge, this technique is limited by the size of the experiment required to find points on an isobol. Even the rather massive experiment conducted by Simmons and colleagues at the Environmental Protection Agency produces only nine points on a two-dimensional isobol. The power of the proposed statistical test of interaction is not discussed in detail by the authors, but it seems likely that a nonparametric test of this type would require a substantial number of observations, perhaps 100 or more, to be sensitive to moderate departures from additivity of effects.

Finally, other work by Gennings and associates (1990) attempts to demonstrate the use of points of concavity or convexity in the sets of line segments plotted in the parallel axis system to evaluate the additivity of the effects of chemicals. Although the analysis pointing to the existence of points of concavity or convexity is interesting, the reliability and sensitivity of this approach are unknown. HEI

HEI COMMENTS

Concluding Remarks

RFA 90-6 sought to spur the evaluation of complex mixtures by encouraging investigators to develop new approaches that would break mixtures down into subfractions based on functional groups with similar toxic properties. Investigators could then examine interactions among the subfractions without worrying about the identity of specific compounds. Once separation and interactions are understood, investigators could begin to identify toxic components and understand their interactions. Therefore, the RFA requested studies that described potential analytical methods for detecting and separating toxic components, and approaches to interactive analysis.

Three of the four studies funded under this RFA focused on analytical methods. Each describes a potential approach for separating components of complex mixtures. The report by Drs. Bechtold and Hotchkiss discusses an approach that relies on the toxicological literature, which indicates the importance of a specific chemical structure, called the bay region, in the polycyclic aromatic hydrocarbon class of compounds (Sayer et al. 1989). They propose to separate components based on the presence of this feature. This approach, using immunoaffinity chromatography, could potentially subdivide a complex mixture into fractions containing chemicals that are toxicologically similar. Polycyclic aromatic hydrocarbons with bay regions are recognized as substrates by mixed-function oxidases, and thus can be metabolized to chemically reactive species. The interactions between fractions containing a bay region and fractions lacking such a structure could then be evaluated. This study comes closest to meeting the aims of the RFA, and may advance the complex mixture field of science.

The report by Drs. Dorsey and Halsall proposes novel improvements for increasing the resolution power of existing separation techniques. Although these improvements may substantially advance the field of analytical chemistry, particularly when the components of a mixture are known, it is not clear if these methods will yield substantial improvements in the analysis of complex mixtures of unknown substances. The report by Drs. Springer and colleagues also proposes novel techniques for separation, coupled with a bioassay-driven strategy for analyzing toxicity. However, as with Drs. Dorsey and Halsall's strategy, this separation is based solely on chemical properties of the complex mixture components. Thus, although these studies may be useful in the final analysis of the toxicity of complex mixtures, they will not provide information on the interactions of the components of the mixture.

The study by Dr. Gennings and associates explores two statistical avenues for analyzing complex mixtures. The first, using regression methods to examine departures from additivity, is illustrated by using data from a published mutation analysis of kerosene soot exhaust (Kaden et al. 1979). Although the data set is not ideally suited for this analysis (with mutation data available for only ten of the 21 identified components), Dr. Gennings is able to make a few assnmptions, such as that the uncharacterized material does not contribute to mutation, that make this a useful example. The second method for analysis, using the parallel axis coordinate system to examine the planes of additivity graphically, is illustrated using unpublished data from a set of three chlorinated organic compounds and their effect on liver weight. Although this second method of analysis may be useful for mixtures with a small number of components (for example, three), it would be cumbersome for complex mixtures. To fully characterize the response surface or the associated contour of constant response for a c-dimensional isobologram in a complex mixture with c components (where c is greater than 4 or 5), the size of the experiments required would prove impractical.

Given the modest amount of seed money provided, these four theoretical studies resulted in some very promising approaches to the analysis of complex mixtures. The logical next step would be to test some of these approaches experimentally. Ultimately, understanding the interactions in complex mixtures should aid the regulatory process, because people are repeatedly exposed to substances as parts of a complex environmental mixture. In looking ahead,

the most effective approaches will focus on three general issues: (1) What are the tentative identities of compounds or classes of compounds in the mixture? (2) What are the biological activities of the components individually and the mixture as a whole? and (3) What interactions occur within the mixture? Often, these questions operate at cross-purposes. However, it is our hope that the thinking that created these four theoretical approaches may be used effectively to address some of these issues.

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