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TWO TOPICS IN TRACE ANALYSIS: THE DEVELOPMENT OF A PASSIVE WATER SAMPLING DEVICE AND THE DEVELOPMENT OF A KETONE - SELECTIVE DERIVATIZING REAGENT

presented by

MARY ANN HEINDORF

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Matthew Zabik
Major professor

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TWO TOPICS IN TRACE ANALYSIS: THE DEVELOPMENT OF A PASSIVE WATER SAMPLING DEVICE AND THE DEVELOPMENT OF A KETONE-SELECTIVE DERIVATIZING REAGENT

by

Mary Ann Heindorf

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ABSTRACT

TWO TOPICS IN TRACE ANALYSIS: THE DEVELOPMENT OF A PASSIVE WATER SAMPLING DEVICE AND THE DEVELOPMENT OF A KETONE-SELECTIVE DERIVATIZING REAGENT

By

Mary Ann Heindorf

In the initial portion of this dissertation, the development of a passive water sampling device consisting of a solvent-filled polymeric bag was investigated as an aid in environmental monitoring. The samplers were fabricated from polymeric films such as polyethylene, polypropylene, polyvinylchloride, acetate, and Silastic ^{R} (a silicone elastomer) and filled with solvents that included hexane, 2,2,4-trimethylpentane, toluene, octanol, and methylene chloride. These devices were exposed to aqueous solutions containing a variety of contaminants to determine which solvent/membrane combinations accumulated contaminants most efficiently. Chlorinated compounds such as lindane, aldrin, 2,2-bis-(p-chlorophenyl)-1,1-dichloroethylene (DDE), dieldrin, methoxychlor and 2,4,5,2′,4′,5′-hexachlorobiphenyl were concentrated up to 2,400-fold when the contaminants were present in the water at environmentally relevant levels. Naphthalene was concentrated approximately 4,000-fold. The optimal solvent/membrane combinations were polypropylene or polyethylene filled with 2,2,4-trimethylpentane. No cleanup or preconcentration techniques were required before the contents of the samplers were analyzed by gas chromatography. The effect of environmental parameters on the partitioning process was investigated by systematically altering temperature, turbulence, ionic strength, pH and the content of dissolved organic matter. The successful deployment of

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these devices in diverse field situations indicates their usefulness as environmental monitors.

The feasibility of using 2-diphenylacetyl-1,3-indandione-1-hydrazone (DPIH) as a fluorescent label selective for ketones and aldehydes was addressed in the second part of this dissertation. The DPIH derivatives were separated using microcolumn liquid chromatography and detected by laser fluorimetry. The results of these studies indicate that DPIH is very reactive and forms highly fluorescent derivatives. However, the formation of multiple products during derivatization of certain ketones limits the use of this compound as a precolumn derivatizing agent. For example, the derivatization of testosterone yielded two major and two minor products. The steric hindrance around the azine functional group caused the formation of E,Z configurational isomers. Also, the proximity of the ketone and azine lone pairs of electrons in the Z,Z and Z,E configurations contributed to secondary adsorption on the silanol sites. Hence, these two products exhibited a greater degree of tailing than did the two minor products. Therefore, dansyl hydrazine and 7-diethylaminocoumarin-3-carbohydrazide were studied as two alternative derivatizing agents. Both formed multiple products upon reaction with testosterone and a single product upon reaction with monoketosteroids. The amino functional groups on the coumarin compound created insurmountable chromatographic problems.

This dissertation is dedicated to my husband, John H. Grant III for his help, support and encouragement.

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I would like to express my gratitude and appreciation to my committee, doctors Zabik, Watson, Nocera and Ledford for their help and assistance. Thanks, Matt for providing so much help over the years to bring this to completion. Thanks to Dr. Watson for stepping into a very much needed role, thanks to Dr. Nocera for being so patient and to Dr. Ledford for his willingness to take part in this committee.

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PREFACE

Trace analysis of complex mixtures is a formidable problem in analytical chemistry. Because sample size is often limited and the composition of the matrix is usually unknown, various forms of chromatography are commonly employed to isolate and identify the analyte(s). Several auxiliary techniques have been developed to further alleviate these difficulties.

Analyte identification may be improved by increasing the selectivity of the detector. General detectors that respond to a broad range of compounds may be replaced with those that respond to one class of compounds or one type of heteroatom, such as thermionic and electron capture detectors. Identification of analytes may be further enhanced if a specific detector that senses a single compound is used. The mass spectrometer is the only detector whose response approximates that of a specific detector. Similar properties of certain components in the mixture may limit the advantages of selective detection in trace analysis.

Additional techniques have been developed to aid trace analysis. High efficiency separation techniques such as capillary gas chromatography, microcolumn liquid chromatography, capillary electrophoresis and supercritical fluid chromatography may simplify trace analysis by improving compound resolution. Improvements may be made in detection by increasing detector sensitivity and developing alternate preconcentration techniques.

In this dissertation, two approaches that aid trace analysis will

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Be considered. In the first part of this research, a device was created that simplifies monitoring contaminant levels in large bodies of water. Traditionally, the extent of environmental contamination has been assessed using biomonitors. However, the scope of their application is limited. A more precise indication of water quality is provided by the passive water sampling device developed in this project. It consists of a polymeric bag filled with an organic solvent; organic contaminants passively diffuse from the water column and are preconcentrated by the device. The contents of the device are subject to analysis without further cleanup. Initially, the effects of the chemical and physical properties of the contaminant, membrane, and solvent on the efficiency of the partitioning process were investigated. Once the solvent/membrane combinations were optimized, the effects of various environmental parameters such as temperature, turbulence, ionic strength, pH, and the presence of dissolved organic material on the partitioning process were examined. Lastly, field tests were conducted at Saginaw Bay, MI, Buffalo Harbor, NY, and Seney Wildlife Refuge, MI, to assess the durability of the sampler.

The second part of this dissertation describes the development of a novel derivatizing agent that enhances detection of corticosteroids. The derivatives were separated by microcolumn HPLC and detected using laser-induced fluorescence. This detection system is promising in trace analysis because the laser's high intensity and narrow spectral bandwidth provides both sensitive and selective detection. The use of a suitable derivatizing agent is required because all compounds are not naturally fluorescent at the wavelengths produced by the laser. Also,

derivatization improves selectivity by reacting with specific functional groups and modifying spectroscopic properties. This research utilized 2-diphenylacetyl-1,3-indanedione-1-hydrazone, 1-dimethylaminonaphthalene-5-sulfonyl hydrazine and 7-diethylaminocoumarin carbohydrazide as potential derivatizing agents in the separation and detection of corticosteroids.

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CHAPTER 1

INTRODUCTION

Many lakes and rivers have been contaminated with organic pollutants. A multitude of contaminants including DDT, methoxychlor, dieldrin, and chlordane (1) have been found in the Great Lakes. Mirex has been detected in Lake Ontario, the Niagara River and the St. Lawrence Seaway (2). Polychlorinated biphenyls have been found in the Hudson and Rhine Rivers (3). The causes of contamination may include point sources, leachate from agricultural applications as well as aerial transport.

Traditionally, levels of contaminants have been assessed using fish (4), birds (5), benthic species (4,6,7), or zooplankton (8) as biomonitors. These species accumulate contaminants to a concentration many thousand times greater than that found in the environment, thus enhancing detection (4). The analysis of mobile organisms such as fish and birds may provide an integrated sample of the quality of the entire ecosystem. The use of sessile species such as clams and mussels provides a time-integrated sample at one location (7). Still, several problems are associated with the use of these organisms as environmental monitors.

Considerable time, expense and effort is spent collecting the organisms. The analysis of many individuals is essential due to the large degree of variation within the population. The interferences from endogenous biological material require that extensive cleanup

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To avoid the problems associated with biomonitors, direct water sampling has been employed to assess water quality. Substantial logistical and analytical problems are associated with obtaining representative water samples. The low concentration of the analyte often necessitates using preconcentration procedures or batch sampling to surpass detection limits. Data derived from batch sampling procedures may be of limited value because they represent the instantaneous concentration of the analyte at a single location (10). Thus, conventional water sampling techniques may have limited use in water chemistry research and contaminant surveillance.

Researchers have experimented with on-site continuous extractors that provide time-integrated samples of large volumes of water. Suffet and coworkers have developed and applied both continuous adsorption (11) and continuous liquid-liquid extraction methods (12) to water sampling. In these methods, the analyte is concentrated by an adsorbent, then removed and analyzed. Problems associated with these methods include background contamination of the adsorbent, system clogging, and limited application at remote sites due to the necessity of a power source to drive the sample and reagent pumps (13).

Passive water sampling devices provide a more reliable alternative to the determination of water quality. These devices are easily deployed at remote sites as a power source is not required. Their lack of moving parts improves reliability. Black et al. (14) have employed polypropylene sheets in a river as passive adsorbents to qualitatively monitor polynuclear aromatic hydrocarbons (PAHs) and to identify point

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sources. This technique is attractive because of its extreme simplicity, however, biofouling and a slow equilibrium rate between the polymer film and the water column limit its application as a long-term integrating sampler.

Mieure et al. (15) dialysed organic compounds through polymeric membranes and into various solvents. Their dialysis system could be configured as both a batch and a continuous liquid-liquid extractor, and was applied as a monitor for PCBs and phthalate esters (15).

In 1987, Sodergren (16) reported that dialysis bags filled with hexane could be used to estimate environmental quality. When the uptake of xenobiotics by the dialysis bags and conventional biomonitors were compared, Sodergren found that the organisms had sequestered more of the contaminants from the water column than had the dialysis bags. Analysis of the contents of the bags was simpler and the devices could be used in areas where conventional biomonitors could not. Thus, passive water sampling devices display great potential as environmental monitors.

The sampler developed for this dissertation consists of a polymeric bag filled with an organic solvent. Model contaminants such as naphthalene, aldrin, dieldrin, DDE, lindane, and 2,4,5,2′,4′,5′-hexachlorobiphenyl are passively partitioned through the membrane and concentrated in a hydrophobic medium, sometimes up to 2,400 times their aqueous levels. Optimization of this device as well as the effect of environmental parameters on the partitioning process were studied. The devices were also deployed in field studies at remote locations, highly contaminated sites and underground areas with high water tables.

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CHAPTER 1

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CHAPTER 2

BIOMONITORS

INTRODUCTION

History contains many examples of the use of living organisms as monitors of environmental quality. Medieval kings utilized their guards as food tasters to detect poisons. Coal miners often used canaries to monitor air quality in the excavation. Today, various fish and benthic species are used to assess environmental water quality (1).

After briefly reviewing the field of biomonitoring, the biological attributes of the organism as well as the chemical and physical properties of the xenobiotic that influence contaminant accumulation will be summarized. Lastly, the advantages and disadvantages associated with the use of biomonitors will be outlined to demonstrate the need for the development of new techniques to estimate environmental quality.

The mere presence of a species may provide an indication of environmental quality. The occurrence of lichens, which prefer a sulpher dioxide-laden environment often indicates contamination (2). Alternately, if the environmental requirements of a species are known, the presence of those organisms signifies the suitability of a given habitat (3).

It is more difficult to draw conclusions about the absence of a species in a certain environment. Environmental conditions may not be suitable for that organism, the preferred niche may be currently

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occupied by another species or the opportunity for the selected species to colonize the area may not have occurred (3).

Various species may be avoiding the area due to the presence of contamination. Cherry and Cairns (4) have demonstrated that Salmonids exhibit avoidance behavior when exposed to chlorine residues at concentrations of 0.05-0.10 mg/l. Certain chemicals evoke avoidance behavior. Sunfish exposed to a variety of agents exhibited avoidance behavior to only eight of the forty chemicals investigated (5). Altered environmental selection has been shown to be an indirect by-product to exposure. Atlantic salmon subjected to chlorinated compounds such as DDT and methoxychlor experienced a change in their temperature preference and selected an alternate environment. No correlation was found between the new location and the levels of contamination (2). Although it is difficult to assess environmental quality by the absence of a particular species, the omission of an entire group of species with similar habitat requirements suggests contamination (3).

A more reliable indication of environmental quality is obtained by analyzing the organism itself. Biomonitors tend to accumulate and concentrate contaminants from their environment (1). Accumulation occurs by bioconcentration (uptake directly from water) as opposed to bioaccumulation (uptake by bioconcentration plus by consumption of contaminanted food) or biomagnification (through increases in the trophic level) (2). Often the concentration of contaminants in the organism is many thousand times greater than that in the environment. The actual magnitude of concentration, however, depends on the identity of the species and the type of contaminant (6). Biomonitors also provide

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an indication of which contaminants have a tendency to partition into biological organisms (2). In addition, risk assessment information can be acquired if the species is used for human consumption (7).

Several factors need to be considered when selecting a species as a biomonitor. Of course, the organism must concentrate the contaminant. The organism should not be excessively vulnerable to the xenobiotic. The biomonitor ought to be sessile (non-mobile) to allow geographical resolution. The population must be large enough to allow the acquisition of replicate samples for statistical evaluation of the process. The species should have a life span that allows studies of greater than one year in duration to be performed. Individuals must be large enough to supply adequate sized tissue samples. The organism should be easy to sample, able to live under laboratory conditions and tolerant of brackish water. It ought to provide a time-integrated estimate of the concentrations in the body of water (water column). The degree of individual variation of contaminant uptake and the degree of variation dependent upon location should be minimized. This broad spectrum of requirements coupled with compound-dependent uptake precludes the use of a single species as the ideal biomonitor.

Although far from ideal, various species of fish and benthic (bottom dwelling) organisms are most commonly used as biomonitors. Fish are a familiar and valuable commodity to society, and there has been substantial public pressure to use them as environmental indicators. Advantages associated with fish include their interrelationship with many other species; their presence implies the existence of other species. Also, as fish grow and develop, they pass through most trophic

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levels and therefore can serve as a monitor of the impact of contaminants on the total ecosystem. Identification of the various species is simplified because their taxonomy has been studied thoroughly (3).

In practice, using fish as biomonitors proves to be difficult. Sampling may be laborious, biased, cumbersome and uneconomical (3). Biases may occur because some species of fish school, while others have a more random distribution. Fish populations are subject to broad fluctuations from year to year. Spawning migrations and diurnal changes in location in the water column will affect the exposure of fish to various contaminants (8). Also, exposure may be influenced by random fish movements (9).

The equipment used to sample fish is very selective and subject to drawbacks that are caused by both the fish and equipment. The size, age, sex, and species of fish influence the efficiency of the equipment. Also, fish of varying species and ages respond differently to the equipment (8). Sampling biases also may occur from the equipment itself. Purse seines and sonar are complex, hard to use and expensive. Yet simple, inexpensive equipment such as bottom trawls and shore seines tend to be the most size selective (8). Therefore, the appropriate equipment must be chosen to conform to the fish found in a given area.

Benthic organisms have been used extensively to monitor the organic contaminants in the water column. Benthic organisms play a role in the Great Lakes food chain, providing additional information about the well being of this ecosystem. Also, benthic organisms can provide a more accurate indication of environmental quality because they tend to

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accumulate polynuclear aromatic hydrocarbons (PAHs) to high levels. Fish and some invertebrates are less sensitive to PAH contamination because they readily metabolize these compounds. Benthic organisms are sessile so collection is simplified. However, some sampling bias may occur because benthic organisms form colonies (10).

Previously, bivalves (organisms, such as clams, which possess a shell composed of two separate parts that opens and closes) were utilized to detect metals in the water column (11, 12, 13). However, in 1968, Bedford, Roelofs and Zabik (14) used mussels to monitor the organochlorine content of the Red Cedar River, East Lansing, MI. They (14) found that organochlorine as well as organophosphate compounds were concentrated by the mussels. The mussels filtered large quantities of water, moved little and had a long life span, with some specimens being over twenty years old. The study demonstrated that bivalves can be used successfully as biomonitors, even in the most remote of sites. Subsequently, this study led Fikes and Tubbs (15) to examine the uptake of dieldrin by benthic organisms in a laboratory environment. This began the rapid expansion of the use of benthic species as biomonitors in the 1960s and early 1970s.

Throughout this time period, the use of benthic species as biomonitors became more accepted. In 1976, the EPA established the Mussel Watch Network, to monitor trace contaminants in sea water using the bivalves Mytilus and Osrea or Crossostrea. Heavy metals, transuranic elements, petroleum residues and halogenated compounds were all readily sequestered by these organisms, which provided an accurate estimate of environmental quality.

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Biomonitoring is required by Public Law 922-500, which was enacted in 1972 (17). This law requires some form of biological monitoring to be performed to assess environmental quality. This legislation was enacted to simplify enforcement of environmental quality and to identify deviations from compliance guidelines. It also serves as a tool in environmental management and policy making decisions (18). Although legislation does require the use of biomonitors, we should be aware of the drawbacks associated with their use.

CHARACTERISTICS OF BIOMONITORS

TIME-INTEGRATED ESTIMATION OF CONTAMINATION LEVELS

One serious question posed about the use of biomonitors has been the accuracy of their time-integrated estimation of contaminant levels in the environment. Differences in kinetics for uptake and depuration of various compounds by the same species hinders generalizations about environmental quality. For example, the uptake of dieldrin by mosquito fish is eight times faster than that of aldrin. Additionally, differences in the accumulation of a single compound by species with similar phylogenetic origins make interspecies correlations difficult (2). Therefore, biomonitors do not provide an accurate indication of environmental quality.

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Lipid content has been related to xenobiotic accumulation in several ways. The total percentage of body fat, tissue lipid content and factors that influence these values have been shown to affect contaminant sequestration. The method used to express data also creates erroneous correlations between contaminant uptake and fat content.

The total lipid content of the organism may have a profound effect on the accumulation of contaminants. Some mammals and fish, such as ringed seals, rainbow trout and salmon (2), show a correlation between body fat content and the concentrations of organochlorine compounds. However, no such relationships are found in other species, such as crabs (2).

Negative correlations between residue levels and body fat content have also been noted. Northern redhorse suckers (19) and dwarf perch (2) exhibit an inverse relationship between fat content and the concentrations of organochlorine compounds. Lu et al. (20) found a negative correlation between the fat content of a variety of species including algae, daphnia, mosquito larvae, snails, and gambrusia fish when examining benzene derivative residues. These data may be explained by rapid clearance rates of xenobiotics from the fat pool.

Despite controversy over these conflicting data, the hypothesis that the uptake of contaminants is related to the amount of body fat is still popular. The increased solubility of organochlorine compounds in lipids over water should lead to their accumulation in fat. In fact,

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preferential absorption of organochlorines by tissue containing a higher percentage of fat has been documented.

Organochlorine accumulation is dependent upon tissue type. The concentrations of polychlorinated biphenyls (PCBs) found in the muscle and skin, and the entire body of pinfish were 1.4 and 3.7 times as great as the levels found in only muscle tissue (19). Correlations between PCB concentrations accumulated in the viscera, gills, muscle, stomach, liver, and whole trout, and fat content of those tissues were found (19). Thus, the fat content of the tissues examined may influence contaminant uptake. However, when goldfish were exposed to DDT and dieldrin, no correlations between the tissue fat content and organochlorine concentration were found (21, 22).

Popular theory still attributes differences observed in the uptake of a compound by different species to the difference in the lipid content. When concentrations are expressed on a per gram of fat basis, sometimes interspecies differences in contaminant uptake are resolved. Again, conflicting reports can be found in the literature. It has been postulated that the rate of contaminant uptake is dependent upon the type of lipids. This could explain differences in organochlorine concentrations found in brain vs. other tissues, even when the data are normalized for fat content. In addition, exposure to some compounds such as DDT and dieldrin, causes an increase in the body lipid content making correlations more difficult (23). Other factors such as age, sex, size and weight, and time in the sexual cycle may influence fat content (1). Therefore, the issue of the effect of fat content on contaminant uptake is clouded by a multitude of interrelated factors and opposing

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viewpoints. The presence of lipids themselves complicates the analysis and increases the time and difficulty involved in collecting data.

AGE

The age of an organism is an important factor in the uptake of organochlorine compounds. As the organism ages, exposure time increases. This has been shown by the increased dieldrin concentrations observed in catfish muscle tissues (2). Phillips (2) reports that increased DDD concentrations in white catfish and large mouth bass were related to age. Also, DDT and DDE levels were increased in older salmon. After these data were corrected for the increased fat content, occurring as the individual ages, elevated xenobiotic levels still remained.

Correlations between length and residue levels have been observed (2). Body surface area may be an additional age-related factor that influences contaminant uptake. Rates of absorption, metabolism and excretion are also influenced by age and life stage, as shown by differences in methoxychlor uptake by juvenile and adult crabs (Cancer magister) (1, 6). Kenaga and Goring (19) also noted a thirteen-fold difference in xenobiotic concentrations in organisms at various life stages, but not all researchers have documented these correlations.

Studies have shown that the DDT concentration in high-fat fish decreased with age, whereas the magnitude in low-fat fish remained the same (1). When Hannon et al. (24) corrected for the lipid content of a variety of fish species, no correlations between age and organochlorine

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compound concentrations were discovered. In other words, contrary to reports in the literature, Hannon et al. found that the correlation between age and residue levels was due to increased fat deposits developed as part of the aging process. Again, this illustrates the wide variation in contaminant accumulation, and the many conflicting results documented in the literature.

SEASON AND SEXUAL CYCLE

Seasonal fluctuations in residue levels are caused by differences in application and drainage of agricultural products and discharge of industrial waste products. Variations in concentrations of accumulated organochlorine compounds observed over a period of time is further complicated by the sexual cycle of the biomonitors. As the fishes (*Teleosts*) used as indicator organisms reach spawning season, their body fat content increases and therefore, contaminant levels are elevated. The body fat content decreases during and after spawning. Changes in body fat content also occurs in mussels, but poor correlations between the lipid content and the concentrations of PCBs have been shown (2). Factors such as spawning along with its associated migration and seasonal fluctuations in contaminant levels influence contaminant uptake by biomonitors.

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MISCELLANEOUS

Additional miscellaneous factors also influence contaminant levels in an organism. Some chemicals interact to alter rates of uptake, metabolism and retention (1). Increased salinity decreased the uptake of organic contaminants in mosquitoes by changing osmoregulation (1). Other factors such as temperature also affect osmoregulation and therefore alter uptake rates. Increased water temperature promoted increased DDT uptake by trout. This variable could not be attributed to the concomitant increase in solubility of DDT at the elevated temperature, as the concentrations used were not close to the solubility limit (1).

Another factor that influences contaminant levels is the food web. A species high in the food web can provide an erroneously high reflection of contaminant levels if its prey has been exposed to sublethal doses of contaminants. In addition, behavioral changes caused by exposure to contaminants can hasten their capture, further elevating xenobiotic concentrations in top-level consumers (9).

Kenaga and Goring (19) have found that species with a high surface-to-volume ratio, such as algae, accumulated greater concentrations of contaminants.

Therefore, the concentration of contaminants observed in biomonitors may not adequately reflect environmental quality. The magnitude of the accumulated xenobiotics is highly variable and is influenced by a multitude of factors including fat content, age, sexual cycle, etc. Individual variation further complicates the estimation of environmental

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Even so, the use of biomonitors is a common practice (25-33). In an attempt to standardize the system, Phillips (2) recommends that sampling should be performed multiple times over a period of time, and results expressed per weight of fat. These recommendations should improve consistency in contaminant monitoring.

EXPRESSIONS OF CONTAMINANT HYDROPHOBICITY

BIOCONCENTRATION FACTOR (BCF)

A multitude of research has correlated chemical and physical properties of xenobiotics with the biomonitors tendency toward accumulation. The bioconcentration factor (BCF) is a widely used term associated with biomonitoring. BCF is defined as the ratio between the concentration of analyte in the organism and the concentration in the environment (3):

$$BCF = \frac{\text{ng cpd/g wet weight organism}}{\text{ng/ml water}} \qquad (2.1)$$

BCFs range from 1 to 1,000,000 (3). Table 1 illustrates the species and compound dependence of BCF's.

Species
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Bluegills
Bluegills
Gambusia
Brook Trout
Fathead Min
Fathead Min
Fathead Min
Alewife
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Table 1

Bioconcentration Factors for Selected Compounds
Using a Variety of Species as Biomonitors

Species	Compound	BCF	Reference/ Conditions	
Bluegills	Naphthalene	212	(2)	
Fathead minnow	Naphthalene	430	(3)	(28-day exposure)
Bluegills	Lindane	497	(2)	
Bluegills	Lindane	768-441 range	(2)	
Gambusia	Lindane	560	(2)	
Brook Trout	Lindane	75	(2)	
Fathead Minnow	Lindane	4000	(2)	
Fathead Minnow	Lindane	180	(3)	(32-day exposure)
Fathead Minnow	p,p'-DDE	51,000	(3)	(32-day exposure)
Alewife	DDE	12,000	(3)	
Smelt	DDE	36,000	(3)	
Sculpin	DDE	25,000	(3)	
Alewife	Dieldrin	13,000	(3)	
Lake Trout	Dieldrin	30,000	(4)	
Lake Trout	HCB	7760	(34)	muscle
Lake Trout	HCB	44,000	(35)	per gram fat basis

The large degree of interspecies variation in BCFs is influenced by metabolic differences between species (9). Thus, as with contaminant accumulation, similar factors affect this mathematical description.

The experimental system influences contaminant accumulation. When static, flowing and terrestrial/aquatic systems were compared, the BCFs obtained in the flowing system were seven times greater than the terrestial/aquatic system. The static system did not reach equilibrium to the same extent due to depletion of the contaminant. Bysshe (9) has suggested that flowing systems should be used to determine BCFs. The organisms should remain alive in the system until equilibrium is reached. Thus, the BCFs are dependent on the design of the incubation system, the type of species and the achievement of a state near the equilibrium.

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Factors such as the octanol/water partition coefficient, vapor pressure, and aqueous solubility of the contaminant also affect bioconcentration. Kenaga and Goring (19) reviewed the literature and summarized the correlations between these parameters using 170 chemicals from the following classes: halogenated hydrocarbon insecticides, substituted and halogenated benzenes, halogenated biphenyls and diphenyl oxides, aromatic hydrocarbons, fumigants, phosphorous-containing compounds, carbamates, thiocarbamates, carbamoyl oximes, carboxcyclic acids and esters, dinitroanilines, ureas and uracils, symmetrical triazines, nitrogen heterocycles and miscellaneous compounds. Although correlations were found, no consistent relationship between chemical structure and any of the parameters was detected. This garbled relationship between BCF and chemical and physical properties of the compound is due to the lack of standardized data describing the uptake of compounds within a single species.

However, interrelationships between these four parameters were found. The nonionic, lipophilic, and aromatic hydrocarbons showed the greatest correlation between the factors that included water solubility, octanol/water partition coefficient, bioconcentration factor and the soil sorption partition constant. Some of these relationships have been noted in other works (2, 3, 4, 9, 10, 19, 34-36). Linear regression equations have been developed to allow the estimation of one property from the remaining parameters.

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OCTANOL/WATER PARTITION COEFFICIENT (K_{OW})

Lipophilic organic compounds partition more readily into nonpolar solvents and into fatty tissues in organisms. Early pharmacological researchers used chloroform, ether or hexane to mimic this partitioning process (37). By 1964, Fujita, Iwasa and Hansch (38) proposed the use of octanol as the standard substance to describe lipophilicity. They developed the octanol/water partition coefficient (K_{OW}) as an expression of the distribution of drugs in a variety of biological systems. The K_{OW} is defined as (39):

$$K_{OW} = \frac{\text{concentration in the octanol phase}}{\text{concentration in the aqueous phase}} \qquad (2.3)$$

Following this initial proposal to reflect the partitioning process, K_{OW}s have been determined for a variety of compounds. Because bioconcentration factors also reflect the lipophilicity of the compound, correlations between K_{OW}s and BCFs have been anticipated and confirmed. As shown in Table 2, the literature (34, 40-43) reports logarithmic relationships between the BCF and the octanol/water partition coefficient. The table describes the conditions for determining the BCFs, the correlation coefficients where available, and the appropriate references.

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Table 2

Relationships Between BCF and K_{ow}

Equation	R^2	References/Comments
log BCF=+0.124 + 0.542 log K_{ow}	0.899	8 chemicals were studied, (chlorinated, nonchlorinated, aromatic & nonaromatic) rainbow trout muscle analyzed, (34)
log BCF=-0.632 + (1.022 + 0.059)log K_{ow}	0.986	(35)
log BCF=-0.869 + (0.997 + 0.056)log K_{ow}	0.986	(35) equilibrium was not reached for all compounds.
log BCF=-0.23 + 0.76 log K_{ow}		84 chemicals studied, and a variety species used (9)
log BCF=-0.70 + 0.85 log K_{ow}	0.897	55 chemicals and pesticides analyzed in blue gill, fat head minnow, rainbow trout, mosquito fish (40)
log BCF= -1.146 + 0.819 log K_{ow}	0.995	acridine, benz(a)acradine, isoquinoline, (3 only) Daphnia Pulex (invertebrate) (41)
log BCF= -0.4362 + 0.7520 log K_{ow}	0.85	PAHs, Daphnia pulex (41)
log BCF= -1.495 + 0.935 log K_{ow}	0.757	26 chemicals that included insecticides, biphenyls, halogenated compounds, in various species (19)
log BCF= +0.124 + 0.542 log K_{ow}	0.948	(34)
log BCF= 2.6 +0.28 log K_{ow}		(44)

These equations have been used to estimate BCFs to within one order of magnitude (9). In some cases, the correlation coefficients shown in the table are surprisingly good. However, as the variety of compounds and species studied is increased, the correlation decreases. This is due to both variation in BCFs and inaccuracies in determining K_{ow}s.

Many problems are associated with the measurement of K_{ow}s. The determination of very large or very small K_{ow}s results in greater error caused by analytical difficulties. Further errors occur from the slight miscibility of octanol and water. Traces of octanol can contain large proportions of lipophilic substances, causing a decrease in the

calculated K_{OW}. Variations also result from the experimental technique. Physical separation of octanol and water emulsions presents additional problems. Concentrations should be measured in both the octanol and water layers, but because this has not always been done, much variation is observed in the reported K_{OW} values. If radiolabelled compounds are used to determine the K_{OW}, radioactive impurities may reside in the aqueous phase and affect the determination of the K_{OW}s (37). Additional factors influencing the values of K_{OW} include temperature, purity of the solvent and chemical, time of mixing, phase separation and pH (19).

VAPOR PRESSURE

G. T. Chiou and V. H. Freed (45) studied the relationship between the octanol/water partition coefficient and the vapor pressure of various compounds by using aromatic hydrocarbons, organohalogens, aliphatic alcohols, aliphatic acids and chlorinated phenols. They theorized that if corrections were made to account for hydrophilic interactions from specific functional groups on the molecule, the partitioning of a compound into the vapor phase, the equilibrium vapor pressure might reflect octanol solubility. Within a class of compounds, very good linear correlations between log K_{OW} and log vapor pressure were found. The organochlorine compounds and aromatic hydrocarbons exhibited the best linear relationships and not much difference was noted between these two groups. When the vapor pressure exceeded 10 millimeters of mercury, partitioning of the compounds into a more lipophilic environment became more pronounced.

AQUEOUS SOLUBILITY (S)

A reciprocal linear relationship has been observed between the water solubility (S) and the BCF for compounds including polyaromatic hydrocarbons, polychlorinated biphenyls and organochlorine compounds (5). These relationships found in Table 3, express the tendency of the molecule to partition from an aqueous environment into a more lipophilic one. The physical process of partitioning a chemical from solution involves transporting the contaminant from the bulk phase into the liquid phase, thus releasing energy. Molecules of the same size, but of different solubility, will differentially partition into the lipophilic layer. Compounds with lower solubility in water will be partitioned more favorably. Kenaga and Goring (19) observed that biomonitors exposed to compounds with lower water solubility require increased time to achieve equilibrium.

Table 3

Relationships Between BCF and Water Solubility

Equation	R^2	References/Comments
log BCF = 3.41 - 0.508 log S (46)	0.93	34 compounds including pesticides, organochlorine and organophosate compounds, hydrocarbons, organic halides, aromatic acids, PCBs
log BCF = 2.791 - 0.5641 log S	0.93	36 compounds, chlorinated and aromatic compounds, in rainbow trout muscle (19)
log BCF = 5.36 - 0.895 log S	0.87	(19)
log BCF = 3.9950 - 0.3891 log S	0.9228	(37)
log K_{ow} = 5.00 - 0.6701 log S	0.985	(46)
log K_{ow} = 5.2 - 0.68 log S	0.94	26 compounds (47)

CONCLUSIONS

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CONCLUSIONS

The accumulation of a contaminant from the water column by biomonitors is reflected by parameters that include the bioconcentration factor, the octanol/water partition coefficient, the water solubility, and the vapor pressure of the xenobiotic. These parameters are expressions of the molecule's hydrophobicity, which favors accumulation by the organism.

The accumulation of contaminants by biomonitors is a very complex phenomenon and is influenced by a variety of factors including species, body fat content, age, season and sexual cycle. In addition, exposure to some xenobiotics causes secondary phenomena to occur and influences the concentrations of contaminants that are detected in the biomonitor. The collection of organisms is often very expensive, time consuming and subject to sampling biases. The development of a simpler system that mimics this process is necessary to monitor contaminant levels more effectively. The partitioning of compounds into a passive sampling device provides a more straightforward method than monitoring contaminant uptake by living tissues. A more accurate, reproducible estimate of environmental quality is obtained as contaminant concentrations will not be affected by metabolic and degradative pathways. The devices are inexpensive, easy to deploy and collect, and are useful in areas that are heavily contaminated, remote, and in underground areas with high water tables.

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CHAPTER 2

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CHAPTER 3

PASSIVE WATER-SAMPLING DEVICES

INTRODUCTION

Traditionally, liquid/liquid extraction or purge-and-trap techniques are used to perform trace analysis of water. When concentration procedures are used, interferences and solvent impurities can mask the analyte (1). When liquid/liquid extraction is used, sensitivity is limited unless further concentration is possible, as with relatively nonvolatile compounds (2). The purge-and-trap technique is very time consuming and reproducibility is hindered by thermal or hydrolytic damage of the Tenax resin. Also, contaminants may be irreversibly adsorbed to or degraded by the resin (3,4). In addition to lengthy extraction procedures and possible sample losses, these methods involve the refrigeration, transport or storage of large volumes of water. Water samples used by these techniques are commonly obtained by grab sampling. Therefore, the data derived from the analysis of these samples represent the concentrations of contaminants at a single point in time and do not adequately reflect environmental quality. Thus, a method of continuous monitoring is desirable because contaminant levels fluctuate over time. Pumps and evacuation chambers are commonly used to provide time-integrated samples. Problems associated with these methods include a limited volume of sample, and inaccessibility to power at more remote locations. With these methods, multisite sampling may pose a

problem (5). The disadvantages associated with these methods have prompted the development of improved concentration techniques for the analysis of water samples. Early researchers utilized these advances in concentration technology to develop passive water-sampling devices.

PRECONCENTRATION AND EXTRACTION TECHNIQUES

Adsorbent cartridges have been widely used for passive monitoring of air contaminants. Initial attempts to apply this technology to passive water sampling were marginally successful. In a typical application, one to one hundred liters of water were passed through a resin and eluted with 25 ml of diethyl ether. A Snyder distillation column further concentrated the extract before analysis (6). Although recovery was poor, additional attempts were made using activated carbon and Tenax to concentrate chlorinated hydrocarbons. Polyurethane foam also has been used to concentrate a limited number of pollutants. To improve recovery and reproducibility, Chadek and Marano (7) used C_2, C_4, C_8 and C_{18} packing material to recover 33 priority pollutants from spiked waste water. The best recovery was obtained using the C_8 resins. Average recoveries by this cartridge filtration method (66%) exceeded recoveries from EPA method 625 (46%).

Many advantages are associated with cartridge filtration techniques. Field application decreases the volume of samples that must be transported and stored. Samples are analyzed more easily and the amount of labor and facilities required are minimized. In addition, the

use of cartridge filtration techniques eliminates evaporative concentration of the sample. Possible disadvantages include adsorption to the packing material which results in lower yields. In addition, degradation of compounds may occur from interactions with the packing material. Finally, time-integrated sampling remains difficult with this method (7).

Black, Hart and Black (8) employed polypropylene sheeting to monitor point sources of polynuclear aromatic hydrocarbon (PAH) discharges. The sheets were placed in aquatic environments and allowed to trail in the current. They were collected 24-96 hours later, wrapped in foil and stored at -10 degrees C. The membranes were extracted using Soxhlet extraction with a variety of solvents, and analyzed using HPLC. The researchers correlated the exposure time of the membranes with the concentrations of PAHs detected in the extracts. Thus, the use of polypropylene sheets to monitor water column concentrations of PAHs provides a time-integrated estimate of contaminant levels. This methodology is useful to track point source discharges, including those at remote sites. However, the extraction technique used in this method is very lengthy and time consuming.

In 1984, Blanchard and Hardy (5) combined both of these approaches in the development of a passive water-sampling device. Their permeation cell consisted of a silicone polycarbonate membrane exposed to an aqueous solution. Contaminants passed through the membrane and were accumulated on an activated charcoal filter that was later eluted with carbon disulfide to desorb the contaminants. The rate of permeation through the cell was dependent on the concentration of the analyte in

the feed water and the flow rate of the solution. At flow rates below 0.50 ml/min, a barrier of molecules formed at the surface of the membrane (concentration polarization) and impeded membrane passage. As expected, an increase in water temperature caused an increase in the rate of diffusion across the membrane. Also, an increase in flux across the membrane was observed when the surface area was enlarged. Alterations in environmentally-related parameters that included pH, ionic strength and presence of humic materials in the feed water had no effect on accumulation of contaminants. The degree of variation between replicate samples ranged from 10 to 12 %, compared to purge-and-trap methodology that has an average reproducibility of 20%. Compounds adsorbed on the charcoal were were stable for 36 days in storage. Although this method is sensitive, reproducible and convenient, problems associated with its use include possible degradation or losses of the compounds on the charcoal.

In 1986, Blanchard and Hardy (9) reported a modified version of the membrane permeation device. The charcoal was removed from the device and sample vapor was collected in a gas sampling loop. This was diverted directly into a gas chromatograph. Detection limits were in the low parts-per-billion range. Though an improvement over the previous attempt, this method requires the transport and storage of large volumes of water. In addition, sequential analysis is required limiting the sample through put of this device.

Mieure, Mappes, Tucker and Dietrich (2) also employed a membrane as a selective barrier. The membrane was used to limit passage into a mass spectrometer. Water flowed across the surface of a silicon membrane, and

organic components dissolved into the membrane and diffused through it into a vacuum in the mass spectrometer. Parts-per-billion detection limits were achieved. This was a convenient, rapid and sensitive method for monitoring water column contamination. However, voluminous samples must still be transported to the laboratory, and time-integrated estimates of contaminant concentrations are not supplied (2, 4).

Westover et. al. (10) and Kallos and Mahle (11) have used polymers to form hollow sampling probes as novel methods of sample introduction into a mass spectrometer. Hollow fibers were formed from silicone rubber, polyethylene, polypropylene, polyethylene vinyl acetate copolymer, Lexan MEM-213, and regenerated cellulose and glued to a needle using Silastic ^{R} adhesive (10). The ends were also sealed, closing the tube at one end. Three to fifteen of these fibers were combined to form a probe resembling a paintbrush. Kallos and Mahle (11) used a slightly different probe configuration when they coated copolymers of poly(α-methylstyrene-dimethylsiloxane) on a screen that was attached to a 1/4" stainless steel tube that was sealed at one end. In all cases, organic components readily permeated the membranes and were detected by the mass spectrometer.

Mieure and co-workers (2, 4) have also developed a glass permeation cell that contains two chambers separated by a membrane. Large volumes of water are pumped through one chamber. Various compounds in the water column pass through the membrane and are concentrated in a solvent that is in the second chamber. The contaminants used to test this device included: phthalate esters, polychlorinated biphenyls and carbon tetrachloride. Membranes tested in these experiments include a silicone

membrane, a silicone polycarbonate copolymer, polyethylene and butyl rubber. Solvents that were used include hexane, benzene and mineral oil. All the compounds studied permeated the cell at the same rate, which was controlled by mass transfer at the surface of the membrane and could be altered by modifications in the stirring mechanism. Permeability was inversely related to the thickness of the membrane, and surprisingly, solvent choice did not affect the permeation rate. One possible advantage of this device is the ability to partition contaminants into alternate solvents that are miscible with water. Matrix interferences are minimized by optimizing membrane and solvent selectivity. This technique was also useful for extraction of aqueous solutions with a tendency to form emulsions. The use of hollow fiber membranes could further increase contaminant concentrations.

In 1985, Kurimoto and Kotake (12) reported on an in-situ passive device for monitoring pore water composition in sediment. They employed a perforated acrylic chamber that contained dialysis bags filled with deionized water. The devices were buried in sediment for approximately 20 days. The concentrations of ammonia and phosphate ions were monitored using these devices. The researchers did not report studies to determine whether equilibrium with the pore water concentrations was approached.

Finally, in 1987 Anders Sodergren (13) proposed the use of solvent filled dialysis bags to mimic the uptake of pollutants by aquatic organisms. Dialysis bags with a molecular weight cutoff of 1000 daltons were filled with 3 ml of hexane and exposed to aqueous solutions of organochlorine compounds for 7 days. Both static and flowing incubation systems were tested. The surface area of the dialysis bags was held

constant at 1260 mm^2 and the water temperature was maintained at 22 and 11 • C for the static and flowing systems, respectively. Gammarus Pulex L., an amphipod, was placed with the dialysis bags in the flowing system, and exposed for 8-10 days. The contents were analyzed without the use of clean up procedures. A relative standard deviation of 8% was achieved in triplicate samples.

The dialysis bags placed in the static system reached equilibrium after 48 hours. Alterations in the concentration of contaminants in the water column were not reflected by changes in the concentration in the bags. The dialysis bags exposed to contaminants in the flowing system attained equilibrium much later, and was explained by the temperature differential. When equilibrium concentrations of DDT, DDE and Clophen A50 accumulated by the dialysis bags were divided by the water concentration, the dialysis bag concentration factors, DCFs were calculated. The DCF values were compared to the BCF values for the biomonitors G. Pulex. Contaminant uptake patterns between the bags and G. Pulex exhibited similar profile shapes but the DCF values calculated for DDE, DDT and Clophen A50 were one order of magnitude lower. Also, the DCFs were 2-3 orders of magnitude lower than BCFs reported in the literature for oysters and fish, respectively.

The dialysis bags were tested in field applications and succesfully indicated poor environmental quality. No evidence of microbial degradation of the membrane was observed. Although the use of solvent filled dialysis bags as biomonitors seems promising, their inability to adequately reflect changes in the composition of the water column limits their application as environmental monitors. In addition, conventional

biomonitors provide a more sensitive indication of environmental quality than do the dialysis bags because contaminant accumulation is enhanced.

In 1991, Johnson (14) attempted to predict the aqueous concentration of contaminants from the amounts that were accumulated by hexane filled dialysis bags. He concluded that the bags were very useful to provide a qualitative estimate, but quantitation remained a challange.

CONCLUSIONS

Previously, attempts have been made to develop passive water-sampling devices to aid in environmental monitoring. Devices have ranged from those that employed trapping resins, to the use of synthetic polymers. The dialysis bags developed by Sodergren show great promise, but the accumulation of contaminants needs to be augmented. Also, the devices need to reflect changes in contaminant concentrations within the water column. The passive water-sampling device developed in this project overcomes these difficulties.

CHAPTER 3

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CHAPTER 4

MEMBRANES

INTRODUCTION

Presently, polymeric membranes are used by industry to separate salts, organic compounds and ions from water (1,2). Common applications include reverse osmosis, ultrafiltration and electrodialysis. In these techniques, the polymeric film separates two aqueous compartments and a a driving force is applied to accomplish the separation. Although the water-sampling device developed in this project does not require the application of a driving force, the solution theory developed to explain transport phenomena through membranes may be applicable. Therefore, to provide a suitable background and introduction into solution theory, the physical and chemical properties of membranes along with a summary of polymer film manufacturing will be presented.

DEFINITION OF TERMS

In the packaging industry, a film is defined as a plastic material of up to 0.01 in. in thickness, with substances having greater thicknesses defined as sheets. Units to express thickness are either gauge or mils. One mil is equivalent to 0.001 in., and a gauge corresponds to 0.28125 in. (3).

To strengthen a film, it is oriented or stretched under carefully

controlled temperatures. This causes the molecules to realign parallel to the film surface (3). Film properties such as water vapor transmission, gas permeability, water absorption, dust attraction, haze, gloss, transparency, printability, percent elongation, impact strength, and notched tear strength are equal for oriented and nonoriented films of the same composition (3).

Proper sealing of the film is essential in the manufacture of sampling devices. Although simple and economical, heat sealing of oriented films is difficult because the film reverts to its original unstretched state along the seal line, ultimately destroying the seal. Sometimes the seal may be retained by using rapid cooling and clamping techniques. Multipoint sealing, which involves the application of heat at multiple points, creates extremely localized heating and prevents shrinking of the whole area. · However, a true seal is not created and molecules can eventually diffuse through it (3).

If the film has a dipole moment, high frequency sealing or dielectric heating forms a seal. In this method, a high frequency oscillating field causes polar molecules to attempt to realign themselves with the field. The oscillations of the molecules cause internal friction that creates heating, and welding of the films can result, especially if pressure is applied. Similarly, ultrasonic sealing involves the transmission of vibratory energy through two pieces of film. At the interface between the films, reflection of energy is small because the films are under pressure. Most of the energy passes through the lower film and is reflected by the lower film support. Under these circumstances, high frequencies tend to hammer the films together,

creating heat and eventually, a seal. Reportedly, this method is the most suitable technique for sealing oriented films. Some oriented films such as polypropylene may have to be coated with a material to facilitate annealing (3).

Composite membranes are formed in two steps. First, a microporous support is cast and a barrier layer is deposited on top of it. In this method, conditions for forming each layer can be independently optimized and result in the formation of a thinner membrane (4).

MEMBRANES USED IN SUBSEQUENT STUDIES

POLYETHYLENE

Polyethylene was developed in the 1930s in England and became commercially important during World War II. Chemically, it is the simplest polymer used in this project. Polyethylene has a straight-chain hydrocarbon with some branching and cross linking from CH_2 units (5). The methylene units in the cross linkages prevent close packing of the polymer chains and result in the relatively low densities observed in polyethylene films (5). Two types of polyethylene are common, high density (0.96 g/ml) and low density (0.92 g/ml) forms (3). Chain branching limits the degree of crystallization to 55-70% for low density polyethylene. In addition, the decreased attractive forces between the chains require the addition of less energy to cause the chains to flow. Hence, polyethylene is easily heat sealable (5), but the seals have only 2/3 the strength of the rest of the material (3). The low absorption

properties of polyethylene make the use of sealing adhesives difficult. High-frequency sealing methods are also difficult to perform (5). Polyethylene is an excellent moisture barrier, but does allow the transmission of some gases. It has good resistance to most chemicals; however, the low density form of polyethylene is especially susceptible to attack by some oils and greases (3).

POLYPROPYLENE

The first polymers with different spatial structures were noted following the discovery of polypropylene in 1954 by Natta (6). Like polyethylene, polypropylene consists of a branched and cross linked hydrocarbon chain formed from 10,000-20,000 monomer chains. The attached methyl groups are oriented in various directions. If all the methyl groups are arranged on the outside of the carbon helix backbone, isotactic polypropylene is formed (3, 5). Syndiotactic polypropylene incorporates methyl groups that alternate from side to side. Atactic polypropylene has a random sequence of methyl groups and cannot be crystallized.

Polypropylene may be oriented or nonoriented (3). Most of the commercially produced polypropylene is nonoriented, or biaxially oriented in various directions (5). The density ranges from 0.902 to 0.907 g/ml. Polypropylene has the lowest specific gravity (3). The permeability of polypropylene is greater than that of high-density polyethylene, but much lower than that of low-density polyethylene (5). Halogenated, aliphatic and aromatic hydrocarbons as well as some oils

may dissolve polypropylene (3), however, it is still more resistant to oils and greases than polyethylene (5). Polypropylene has been used to fabricate films as well as woven and nonwoven fabrics (7).

POLYVINYLCHLORIDE

When vinyl chloride is polymerized with benzoyl peroxide as a catalyst at 54°C a branched polymer results. The most common arrangement of the monomer units is random, which results in atactic polyvinylchloride, although both head to head, or head to tail configurations may occur. Polyvinylchloride is a very stiff, brittle polymer, therefore oily materials or phthalates must be added to increase pliability. As a result, many of its properties are dependent on the added plasticizers (5). Although orientation increases the strength of polyvinylchloride, moisture and gas transmission properties remain the same as those of the non-oriented material. Polyvinylchloride is resistant to attack by oils, greases, alcohols and petroleum solvents, but may react with halogenated hydrocarbons (3). Polyvinylchloride can be sealed by high-frequency techniques (5).

CELLULOSE ACETATE

Cellulose acetate was originally used as a photographic film in 1912, and since then has been widely used in desalinization applications. It is synthesized by mixing cotton fibers with glacial acetic acid, acetic anhydride and a sulfuric acid catalyst. This results

in the formation of cellulose triacetate. The addition of water stops the reaction and forms an acetate precipitate. This polymer solution is cast as a thin film onto a surface and any remaining solution is allowed to evaporate. The film is immersed into a gelation medium (water or ethanol) and then thermally shrunk. Each of these steps affect the pore size and transmission properties of the resultant film (8).

SILASTIC

Silastic is a silicone elastomer manufactured by Dow Corning, Midland, MI. The polydimethylsiloxane polymer is filled with SiO_2. These silicone elastomers are nonporous in nature and may be extruded into very thin films (0.0014-0.0020 inches or 0.005 to 0.007 gauge). The Si-O-Si group has a broad angle and long bonds exist between the adjacent silicon and oxygen atoms, relative to those between the carbon atoms. Hence, there is free rotation around all bonds, and the energy of rotation is almost zero, which results in the formation of a very flexible and porous material. Silastic is readily permeable to O_2, CO_2 and N_2, but no exact measurements have been made (9).

The chemical and physical properties of the membranes used in subsequent studies are summarized in table 4.

Table 4

Chemical and Physical Properties of Selected Membranes

Film Type	Density	Heat Sealing Temperature	Dielectric Sealing Temp.
Cellulose Acetate	1.30	350 F	230
Polyethylene, low density	0.92	250 F	2
Polypropylene	0.90	350 F	5
Polyvinylchloride	1.40, 1.28	225 F	140
Silastic	N.D.	N.D.	N.D.

Film Type	Water Vapor Transmission	Water Absorption	Gas Permeability O_2	N_2	CO_2
Cellulose Aacetate	150	high	35	40	1,000
Polyethylene,low den.	1.3	low	550	180	2,900
Polypropylene	0.7	low	240	60	800
Polyvinylchloride	4.0	low	150	65	970
Silastic	N.D.	N.D.	---H I G H---		

NOTES/UNITS:
N.D.= Not Determined. High density polyethylene has a density of 0.96. Density: g/ml Water vapor transmission: g loss/24h/100in^2/mil at 35° C, 90% relative humidity. Gas permeability: cm^3/24 h/100 in^2/mil at 25° C, 50% relative humidity ASTM D1434-63. Dielectric sealing: loss current tangent x 10^4 at 10^6 Hz high frequency alternating current at 25 ° C (high numbers are easier to seal) (3).

CURRENT MEMBRANE SEPARATION PROCESSES

In 1959, Reid and Burton (10) used cellulose acetate to reject salt from water solutions. These early membranes were very thick and symmetrical (1). The use of asymmetric membranes, developed in 1960, increased water throughput by ten fold. Then in 1965, a double layer of cellulose acetate was used to reject salt from sea water with 99.8% efficiency (11). These early cellulose acetate membranes were

susceptible to biological degradation and were hydrolyzed under both acidic and basic conditions (1). To avoid such problems, thin film composite membranes and polyamide (Aramid) membranes were developed.

The current trend (12) toward the exploitation of novel membrane compositions increases the selectivity of reverse osmosis, ultrafiltration and electrodialysis. These processes will be briefly reviewed, then theory describing the separation by various membranes as it may pertain to the passive water-sampling device will be discussed.

REVERSE OSMOSIS

Osmosis involves the flux of fluids across a semipermeable membrane selective toward certain compounds. The chemical potential on each side of the membrane determines the direction of flow. Pressure, temperature and the concentration of dissolved solids will all affect migration across the membrane. If salt is added to one side of the membrane, decreasing the chemical potential, osmotic flow occurs from the pure water compartment to the salt side until equilibrium is reached. When the pressure difference from the volume change equals the osmotic pressure, equilibrium is reached. If pressure is applied to the salt side of the membrane, water will flow toward the pure water side, resulting in reverse osmosis. This mechanism is not completely understood, but the membrane's greater affinity for the water than the salt results in the formation of a concentration gradient. Water exists at the interface or within the membrane and excludes the solute. Transport of water occurs through the pores and may be different from

one bonding site to another (1).

Concentration polarization acts to limit efficiency. Solutes are deposited at the membrane surface and tend to accumulate there, often resulting in concentrations many times greater than that in the bulk phase. This polarization layer acts as a second skin and inhibits movement across the face of the membrane and flux through the membrane (1, 2, 13). As with the passive sampling device, the natural wave motion in large bodies of water helps to clear membrane surface. A rotating stirrer supplied such agitation during experiments conducted in a laboratory setting.

Generally, reverse osmosis has been utilized to separate ions from Water (1, 14), however Bhattacharyya et al. (15) performed reverse osmosis on dilute solutions of hazardous volatile organics using thin-film composite polyamide membranes. Polynuclear aromatic hydrocarbons, phenol, chlorophenol, nitrophenols and chlorobenzenes were separated from an aqueous solution. Therefore, if membranes more permeable to the solutes in question were chosen, perhaps movement of the analytes through the membrane and separation from the water column could be accomplished.

ELECTRODIALYSIS

Although useful in desalination processes, electrodialysis is not applicable to the separation of non-ionic organics. This technique has been included to supply a complete summary of the applications of polymeric membranes in industry. Electrodialysis is a desalting process

in which ions are removed from water by passage through a membrane impermeable to water. A direct current field is applied as the driving force to cause migration of the ions. Both cation- and anion-selective membranes are used in the process, and are placed in an alternating arrangement in the field. The cation membrane allows penetration of cations, and vice versa. Consequently, some of the cells become depleted in ions while others are enriched. Cross-linked polystyrene is usually used as a cation-selective membrane and quaternary ammonia groups on cross-linked polystyrene enhance anion exchange. The efficiency of the separation also is limited by concentration polarization (1).

ULTRAFILTRATION

Compounds are separated by differential exclusion from a membrane surface based on molecular size. Although the size and shape of the analyte molecule are important, membrane passage is determined by the molecular weight limit of the pores. Thus, the physical nature of the membrane controls the separation process, while a pressure differential acts as the driving force. Typical membranes used in this application include polyvinylchloride, cellulose acetate, polyacrylonitrile, polycarbonate and polysulfone. Problems associated with this separation method include concentration polarization, pore plugging, and the formation of a gel layer by solutes. These difficulties are minimized by using high flow rates of feed water (1).

FACTORS INFLUENCING SOLUTE PASSAGE THROUGH A MEMBRANE

PORE AND SOLUTE SIZE

Pore dimensions along with the size and shape of the solute will influence the passage of molecules through the membrane. Pores may stretch to accommodate the entrance of a molecule. Anderson (16) reports that cellulose acetate membranes with a pore size of 8 to 10 x 10^{-9} m permitted passage of molecules much larger than the nominal pore size. Anderson hypothesized that the average pore size is a dynamic property, and that fluctuations in size occur over periods of time. Various factors including the flexibility of the polymer chains, the degree of crystallinity in the membrane, the amount of membrane swelling, the temperature, and the extent of cross-linking have been shown to influence pore stretching (16, 17, 18, 19).

After the molecule passes through a pore in the membrane surface, it must negotiate passage through channels in the membrane. The tortuosity of the corridor is influenced by the density of chain packing, which is decreased by the presence of bulky functional groups. However, chains may be packed tighter if chain flexibility and stereoregularity are increased (1, 2, 17). The degree of crystallinity of the polymer also influences membrane passage. The chemical nature of the polymer and techniques used in its formation determine the degree of crystallinity. For example, crystal nucleation and growth occur during the evaporative stage of solution casting, wet spinning of the membrane, molten stage extrusion, or melt spinning stages of membrane

formation (1, 2, 17).

The molecular size of the solute also affects passage through membranes. For large, non-spherical compounds, the cross-sectional area and volume may be the most important factors that determine the contaminant's ability to pass through an adequately-sized gap (18). Since molecular weight is not an effective description of molecular size, other parameters such the parachlor, permachlor, the space factor or molar volume have been used in an attempt to correlate molecular properties with passage through a membrane (17).

Traditionally, parachlors have been applied in drug design to estimate structure activity relationships and in physical organic chemistry in structure determination. In environmental science, when parachlors and molar volumes have been regressed against BCF's, correlation coefficients of about 0.84 are obtained.

The parachlor (P_C) is an empirical relationship between density and surface tension and is defined as:

$$P_C = \frac{M}{D - d} \tau = \Sigma P_{ca} \qquad (4.1)$$

where τ is the surface tension, D is the density in a liquid phase and d is the density of the molecule when in the gas phase and is often negligible. The parachlor is also estimated by summing atomic parachlors (P_{ca}) (20).

Two generalized attempts to relate permeability data with structure parameters have been made by Salame (21) and Malachowski (22). Salame developed the permachlor concept that relates all of the facets of a polymers structure to its permeability by using a scheme of additive

(+/-) structural components descriptive of the polymer. These values may change, depending upon the permeating molecule. For homologous series of molecules, good correlation occurs between membrane permeability and the permachlor values. Application to diverse groups of compounds is difficult due to inconsistencies in the structural values.

Malachowski derived the space factor concept to allow for the effects of temperature on the polymer (22). The space factor, Q_{sf} may be calculated by:

$$\ln Q_{sf} = a + b \frac{T_c}{T} + c \frac{d}{T^n} \qquad (4.2)$$

where T_c is the critical absolute temperature for the permeant, d is the molecular diameter of the solute and is temperature dependent, n is the space factor, T is the absolute temperature that contributes to the solubility of the permeating species in the polymer and a, b, and c are constants that depend on the polymer. If an homologous series is studied using the space factor, good correlations between membrane permeation and molecular size are not achieved (22).

Molar volume is estimated by summing various structural contributions, both positive and negative. These contributions as well as modifications suggested by LeBas are shown in Table 5 (19). The table also lists the partial molar volumes calculated by simple summation techniques for the xenobiotics used later in this research (19).

Table 5

Molar Volume, V_B (16)

Atom	Increment (cm^3/mol)	Ring Size	Increment
C	16.5	Aromatic and	
H	1.98	Heterocyclic Rings	-20.2
O	5.48		
Cl	19.5		

LeBas Molar Volume, V_B' (16)

Atom	Increment (cm^3/mol)	Ring Size	Increment
C	14.8	3-Membered	-6.0
H	3.7	4-Membered	-8.5
O (methyl ether)	9.1	5-Membered	-11.5
Cl	24.6	6-Membered	-15.0
		Naphthalene	-30.0

Compound	Calculated Molar Volumes LeBas Molar Volume V_B' (cm^3/mole)	Molar Volume V_B (cm^3/mole)
Aldrin	324.8	330.84
p,p'DDE	305.2	324.84
Dieldrin	313.4	328.40
2,2',4,4',5,5'-Hexachlorobiphenyl	310.0	322.92
Lindane	243.6	227.88
Methoxychlor	354.3	363.16

Properties other than size influence passage through membranes. The marked differences in partition coefficients that are observed with similarly sized molecules illustrate this point (16). Forces such as hydrodynamic drag, dispersion and electrostatic forces as well as membrane/molecule interactions also hinder passage (23).

POLARITY AND CHARGE

The membrane can be visualized as an immiscible organic layer in contact with the aqueous phase. Molecules diffuse from the bulk water phase and enter a thin, highly structured layer of water molecules at the membrane surface. Once passage through this aqueous boundary layer has been negotiated, partitioning into and through the membrane can occur. The formation of this boundary layer will be examined. Following this, the effects of membrane and contaminant polarity on aspects of the separation process will be reviewed.

Increases in the availability and accessibility of the dispersive, hydrophobic sites on the membrane surface will enhance water clustering near the film and form a less dense water layer. The capacity of this layer to dissolve the solute may be limited by the energy required to break interactions between water molecules. As a result, membrane passage may be promoted by inducing the loss of the analyte from this boundary layer. Conversely, membrane transport may be hindered by limiting the concentration of analyte in this film. Water molecule clustering is common near the surface of polyolefin, vinyl plastic and elastomer (such as Silastic) membranes (23, 24, 25, 26, 27).

Water clustering decreases the equilibrium concentration of water on the membrane surface, but enhances the mobility of water molecules within the membrane phase. Although water dissolved within the membrane phase would be expected to limit the passage of solute through the membrane, solvent molecules within the membrane may counteract the

phenomenon.

The importance of the hydrophobicity of the analyte has been confirmed experimentally in reverse osmotic applications (16, 28). For some molecules, variations in pH may influence water solubility, alter the diffusion constant from the bulk phase and the membrane solubility. The presence of dissolved humic materials may affect partitioning properties in a similar fashion (29).

The physical and chemical nature of the membrane are important in solute interactions. In general, polymeric membranes are hydrophobic and interact to a greater degree with nonpolar solutes. Analytes may be sorbed to the membrane. The presence of the solvent molecules within the membrane matrix will add an additional equilibrium and aid desorption of the molecule from the membrane and into the bulk phase of the solvent contained within the sampling device.

Other possible interactions between the molecule and the membrane include dipole/dipole, dipole/induced dipole, and London dispersion interactions. Dispersion forces are the most important because some polymers such as polypropylene and polyethylene possess long hydrocarbon segments, and therefore have strong dispersive character (17, 23). These polymers are expected to interact with hydrophobic contaminants in the water column and to facilitate their uptake by the solvent. When the length of the hydrocarbon chain is increased, the dispersive character also increases, as does the molecule's ability to interact with the hydrophobic portions of the membrane. The dispersive character is greater for saturated hydrocarbons than for their unsaturated counterparts, and greater for cyclic than for aliphatic analogs (23).

TEMPERATURE

Temperature influences diffusion of the solute from the bulk phase. Temperature may affect the thickness of the boundary layer at the membrane surface by altering the strength and number of hydrogen bonds in the clusters of water molecules. The concentration of contaminants in this stagnant layer may become elevated through increased random movement of the solute molecules and decreased hydrogen bonding. Enhanced contaminant levels in the boundary layer can establish a greater equilibrium concentration within the membrane (23, 24, 25).

CONCENTRATION

Increases in the concentration of the analyte in the water column will elevate the number of molecules in the stagnant layer at the membrane surface and improve the likelihood of membrane passage. Hwang et al. (17) have demonstrated that the rate of passage through a dialysis membrane is dependent upon analyte migration through the boundary layer. Increases in turbulence maximized the rate of passage. In addition to the solution concentration and temperature, pressure and electric current density also can affect this transfer. However, if the analyte concentration in this stagnant layer is too great, concentration polarization will limit the efficiency of the transfer.

The presence of solvent in the sampling devices, will further influence this partitioning process by interaction with the membrane. When exposed to the solvent, the polymer swells and becomes more

permeable because its glassiness and crystallinity have been reduced. The propensity of the solvent and the membrane to interact with each other may be further described by the solubility parameter.

THEORY

The preceeding factors are considered mathematically by the permeability of the membrane, Q, which is expressed as the product of two factors:

$$Q = \bar{D}\, S_m \qquad (4.3)$$

where $\bar{D}$ is the diffusivity and S_m is the solubility of the permeant in the membrane. S_m is an equilibrium property, whereas the diffusivity is a dynamic property describing how fast the molecules traverse the membrane. The overall rate of uptake is dependent upon both of these parameters. The following definition illustrates the temperature dependence of diffusivity:

$$\bar{D} = D^{\bullet} \exp [- E_D / RT] \qquad (4.4)$$

$D^{\bullet}$ is a preexponential factor, E_D is the activation energy for the diffusion process and is dependent upon both the permeating molecule and the membrane. The gas constant is represented by R and T is the absolute temperature. Temperature affects the activation energy. In the following research, activation energy can be considered independent of temperature because temperature fluctuations in large bodies of water are not great.

Other factors such as molecular size and membrane type will affect the activation energy. Figure 1 illustrates the relationship between the diffusion coefficient and molecular size and contrasts membrane flexibility with permeability (16, 17). As the permeating molecules increase in size, diffusion is hindered because more energy must be expended to generate an opening large enough for the molecule to enter. Rubbery membranes require more activation energy for passage than do membranes with more glassy properties. The greater chain mobility and flexibility in rubbery membranes requires more energy be expended to maintain an adequately sized opening for a sufficient time period. Glassy membranes exibit a larger spread in diffusion coefficients than rubbery membranes because of the increased selectivity on the basis of molecular size and shape (11, 15, 17).

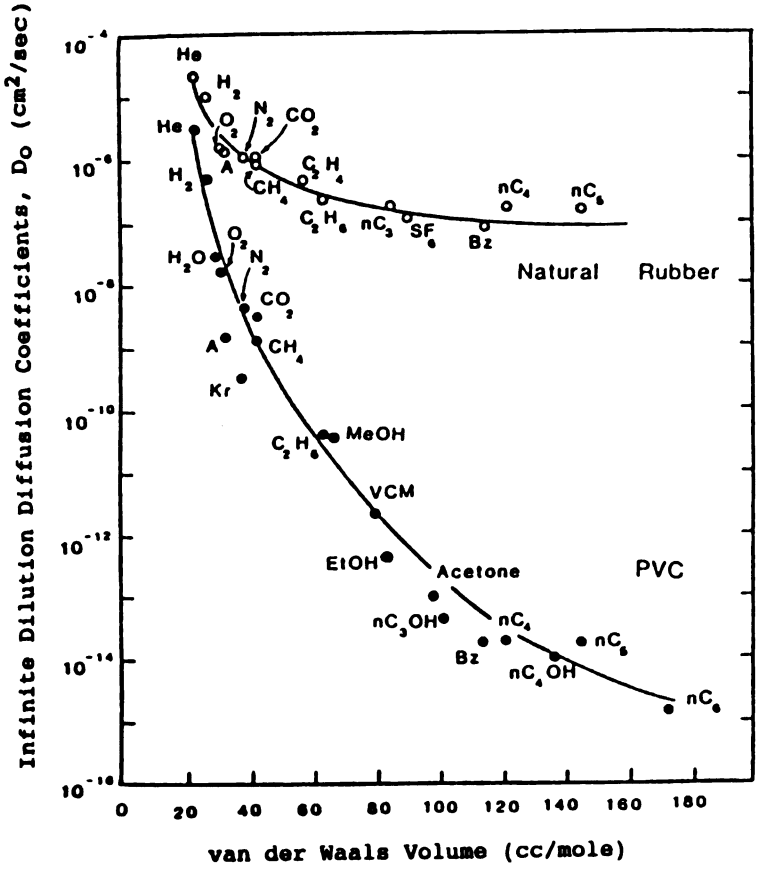
Membrane solubility, S_m, is influenced by polymer and penetrant interactions. S_m can be further defined as:

$$S_m = S\bullet \exp - \Delta H/RT \qquad (4.5)$$

where $S\bullet$ is a preexponential factor and ΔH is the heat of solution. Temperature changes affect the solubility, but this should be minimal in large bodies of water. Interactions between the solute and the membrane also determine solubility. This will be examined further with solubility parameter theory (17).

SOLUBILITY PARAMETER

Hildebrand and Scott (30) developed the solubility parameter to describe the attractive forces between molecules and provide a



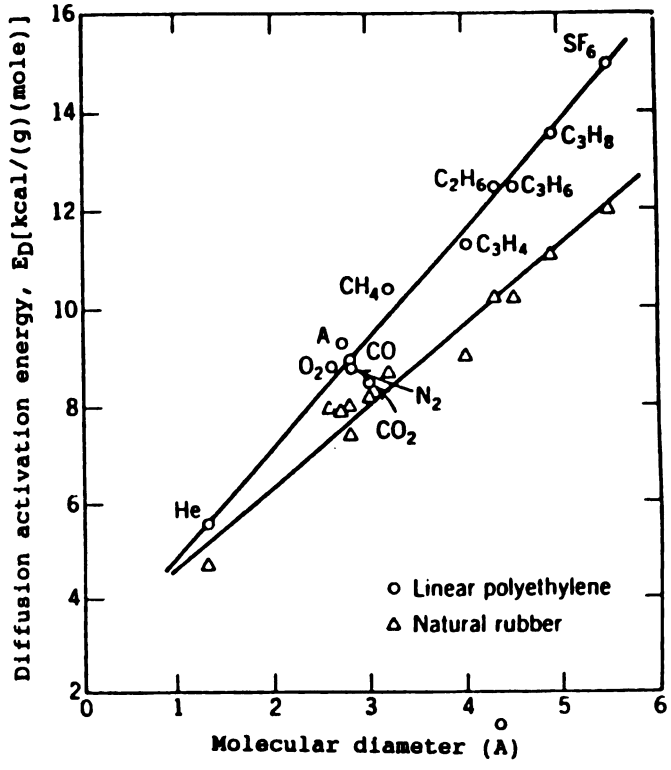


Figure 1: Rate of Contaminant Penetration into a Glassy vs. Rubbery Synthetic Membrane. Chern, Koros, Hopfenberg and Stannett (18), 28 and Hwang and Kamermeyer (17), 285.

quantitative estimate of polarity. After its creation, the solubility parameter has been used to describe a variety of partitioning phenomena. This concept has been applied in pharmacology to study drug solubility, and in polymer fabrication, to choose casting components and composition. Also, the solubility parameter has been used to describe reverse osmosis applications and separations achieved by high-pressure liquid chromatography (23, 31). In this project, the parameter may be useful to predict which solvents will sufficiently swell the membranes, reduce their crystalline and glassy components and promote membrane permeability to the solute. The basic theory of the solubility parameter will be reviewed and then will be applied to the passage of solvents through polymeric membranes.

In condensed phases, there are strong attractive forces between molecules, which results in considerable potential energy for each molecule. This is called the molecular cohesive energy, -E. The solubility parameter, δ, was developed to provide a quantitative meaning to polarity and is defined as (31, 32):

$$\delta = \sqrt{-E/V} \qquad (4.6)$$

where E is the cohesive energy required to transfer 1 mole of a substance from the ideal gas phase to its liquid state, and hence E will always be a negative quantity. The molar volume of the liquid is defined as V (31). Solubility parameters for nonpolar compounds are approximately 7 $cal^{1/2}/cm^{-3/2}$ whereas the parameters for polar compounds are much greater. Water has the maximum solubility parameter of 25 $cal^{1/2}/cm^{-3/2}$. Thus, similar compounds have greater mutual solubility

and smaller difference between their solubility parameters (23).

The cohesive energy (-E) represents the energy of a solute relative to it's behavior as an ideal gas at the same temperature and is composed of two components, the energy $\Delta_i gU$ required to vaporize the liquid to its saturated vapor and the energy to expand it isothermally to infinite volume (33):

$$-E = \Delta_i gU + \int_{v=vap}^{v=\infty} (\delta U/\delta V_t \Delta V) \qquad (4.7)$$

At temperatures below the normal boiling point the equation reduces to:

$$-E \approx \Delta_i gU \qquad (4.8)$$

By assuming ideal gas behavior, and using the following definition for the cohesive energy density, c, where V is the molar volume, equation 4.6 may be derived.

$$c = -E/V \qquad (4.9)$$

The Hildebrand-Scatchard Equation is an empirical relationship to describe internal energy of mixing:

$$\Delta_m U_v = (x_a V_a + x_b V_b)(\delta_a - \delta_b)\phi_a \phi_b \qquad (4.10)$$

where V_a and V_b are molar volumes of the mixture, x_a and x_b are the mole fractions, δ_a and δ_b are the Hildebrand solubility parameters, and

ϕ_a and ϕ_b represent the volume fractions (33). The narrower the differences in the solubility parameters, the smaller the energy of mixing and the greater the mutual solubility.

Several assumptions were made in the derivation of the solubility parameter.

1. Forces of interaction occur between the centers of the molecules, and are additive in nature.
2. Other molecules do not influence interactions between a pair of molecules.
3. Mixing is random.

Various problems associated with the Hildebrand solubility parameter include computational inconsistencies, the invalid assumption that the volume change upon mixing is zero, and the incorrect evaluation of molecular interaction energies (33). In spite of these discrepancies, the Hildebrand solubility parameter provides a good description of mutual solubility when hydrogen bonding is not present.

Bagley and Scigliano (34) developed a two-dimensional form of the equation to describe interaction between dispersive forces and hydrogen bonding. Hanson (32) expanded this equation further when he considered the three modes of interaction which produce the cohesive energy in liquids. These forces are: dispersion or London forces arising from the fluctuating atomic dipole and resulting in the formation of a positive nucleus and an electron cloud; polar interactions that can be subdivided into dipole/dipole and dipole/induced dipole; and specific interactions such as hydrogen bonding. The equation developed by Hanson is (32, 33):

$$\delta^2 = \delta^2_d + \delta^2_p + \delta^2_h \qquad (4.11)$$

Hence, the sum of the squares of the dispersion component, δ_d, the polar component, δ_p, and the hydrogen bonding component, δ_h δ^2, measure the total solubility. To generate a value for the solubility parameter, the contribution of δ_d is determined experimentally using a homomorph to determine the vaporization energies of both molecules. A Homomorph is a non-polar molecule that has the same size, shape and molar volume as the polar molecule. δ_p and δ_h are estimated empirically. Large variation in the estimation of individual components result in inaccurate values for the total solubility parameter (33, 34).

Burrell (35) was one of the first to extend the solubility parameter to reflect interactions in industrial polymer/solvent systems. He classified solvents according to the degree of hydrogen bonding and tabulated δ for 36 polymers. He determined that solvents are compatible with membranes over a range of values of δ. For example, polyvinylacetate is compatible with solvents having a solubility parameter between 8.5 to 14.5 $cal^{1/2}/cm^{-3/2}$.

Flory and Huggins (36-39) further extended solubility parameter theory to describe polymer solutions. The energy of mixing between a polymer and a small solute molecule is greater than expected due to increased entropy contributions as calculated by Flory and Huggins. They proposed a dimensionless solvent interaction parameter, X, which characterizes the difference in the interaction energy of a solvent molecule immersed in pure polymer compared with one in pure solvent. The expression for Gibbs energy of mixing is:

$$\Delta_m G/RT = x_a \ln\theta_a + x_b \ln\theta_b + X\theta_a\theta_b(x_a + V_B x_b/V_a) \qquad (4.12)$$

where x is the mole fraction, θ is the volume fraction, the subscript b denotes the polymer and the subscript a denotes the solvent. Component X is the Flory interaction parameter for that particular solvent/solute pair. The first two terms of this equation result from the entropy of mixing and are always negative. Hence, for $\Delta_m G$ to be negative or for the polymer to be soluble in the solvent, X may be either positive or negative, but must be small. The Flory interaction parameter contains both entropy and enthalpy contributions and may be estimated as follows (33):

$$X = X_s + X_h \tag{4.13}$$

where the enthalpy contribution, X_h, is calculated from Hildebrand Scatchard theory by considering the hydrophobic portion of the molecule:

$$\delta_{hp} = \frac{\Sigma F_i}{\Sigma V_i} \tag{4.14}$$

where F_i are the contributions of the group to the molar attraction constant of the molecule, V_i is the group contribution to the molar volume of a nearby polymer or small molecule (34). The entropy contribution, X_s is usually estimated to be between 0.2-0.6. Hence, for a polymer to dissolve in a solvent, X_h must be small, and δ_a and δ_b must be very similar. The Flory-Huggins parameter is useful when the thermodynamics of dilute solutions are considered, but it is of limited help in polymer formulations. Shortcomings with Flory-Huggins parameter include its concentration dependency, the difficulty of experimental

evaluation, and the influence of hydrogen bonding. In addition, the application to multiple component systems is very difficult because interactions between each component pair must be known (34).

Therefore, solubility parameter theory may be useful in the prediction of which solvents will dissolve the sampler membrane and those that will cause it to swell without complete dissolution. Swelling causes increased permeability of the membrane and enhances solute passage. Solubility parameters for the solvents and membranes used in subsequent studies are listed in table 6 (32, 33). In general, as the cohesive energy density of a membrane increases, its mechanical strength, and the frequency of occurrence of functional groups will also increase.

Table 6

Hildebrand Solubility Parameters

Membrane	δ cal $^{1/2}$/cm $^{-3/2}$	Solvent	δ cal $^{1/2}$/cm $^{-3/2}$
Silastic	7.4- 7.8	2,2,4-Trimethylpentane	6.9
Polyethylene	7.7- 8.2	Hexane	7.4
Polypropylene	9.2- 9.4	Toluene	8.9
Polyvinylchloride	9.3-10.2	Methylene Chloride	9.7
Cellulose Acetate	11.1-12.5	Octanol	10.3
		Methanol	14.5
		Water	18.0

Of course, the solubility of the solute in the solvent and the membrane should also be characterized to predict the uptake from the water. Unfortunately, the solubility parameter concept has not been expanded sufficiently to consider all possible interactions in the passive water-sampler system.

HENRY'S LAW CONSTANT

Although a polymeric membrane is not involved in partitioning of solutes across an aqueous/gas interface, thin films which hinder transport are formed at the interface. Water molecules at the surface are aligned differently than those in the bulk phase. In addition, limited diffusion causes solutes to tend to accumulate on the vapor side of the interface, forming another film. Transfer through these films are discussed here for completeness. The partition coefficient known as Henry's Law Constant (HLC) is defined as:

$$HLC = \frac{C_g\ (eq)}{C_l\ (eq)} \qquad (4.15)$$

where $C_g(eq)$ is the equilibrium concentration in the gas phase and $C_l(eq)$ represents the equilibrium concentration of unionized dissolved gas in the liquid phase. Both concentrations are expressed in units of g per cm^3 of the medium. As long as the solutions approach ideality, proportionality between concentrations in the phases are maintained with alterations in pressure and temperature. In addition, solutes diffuse through the air water interface independently of other compounds (40, 41).

The total resistance to transfer (1/K) is described by the sum of the resistance to transfer in both the aqueous ($1/K_l$) and air phases($1/K_g$) and influenced by Henry's Law Constant.

$$\frac{1}{K} = \frac{1}{K_l} + \frac{R\ T}{H\ K_g} \qquad (4.16)$$

If Henry's Law Constant is greater than 7×10^{-4}, the compound will primarily reside in the liquid phase as do compounds with high molecular weights. Flux across the air/water interface is minimal. Solutes with a value less than 7×10^{-6} will be primarily in the gas phase. Compounds such as polychlorinated biphenyls (PCBs) possess HLCs with median values, partition into both phases and are sensitive to temperature changes (42).

DERIVATION OF AN EQUATION TO DESCRIBE UPTAKE BY THE PASSIVE SAMPLING DEVICE

Studies of the transport of fluids through membranes have assumed no significant resistance to passage, implying the polymers have been significantly swollen. This assumption will be used in the derivation of an equation to describe contaminant uptake by the devices.

Ficks first law (40) described the diffusion of molecules along a concentration gradient and is used to derive an equation to explain the Uptake of contaminants into the sampling device. This derivation uses several assumptions:

1. An infinite bulk concentration of the solute exists in the water column.
2. The membrane is sufficiently swollen, and does not impede solute transfer. Thus, solute concentrations in the membrane and in the bulk phase are equal.
3. The solute is more soluble in the solvent than in the water, hence the driving force toward contaminant uptake is the increase in entropy achieved by dissolution of the analyte in the solvent.

The partition coefficient, ϕ, may be defined as follows:

$$\phi = \frac{C_s}{C_\infty} = \frac{C_s}{C_m} \qquad (4.17)$$

Where C_s is the concentration in the solvent, C_∞ is the bulk concentration and C_m is the concentration of solute in the membrane. Assuming steady state is acheived by the sampler system, the total flux through system will equal fluxes through the boundary layer, the membrane and solvent film. These fluxes are defined in equations 4.18 through 4.20. The flux, N_b, through the boundary layer film is described as:

$$N_b = k_1 (C_\infty - C_1) \qquad (4.18)$$

The existence of two stagnant layers, one on either side of the membrane is proposed. In equation 4.18, where C_1 is the concentration of the analyte in the stagnant boundary layer on the outside of the membrane that is exposed to the aqueous solution. Transport in the bulk film is given by the mass-transfer coefficient, k_1. Likewise, the membrane flux, N_m, is described by:

$$N_m = \frac{D_m}{x_m} (C_1-C_2) \qquad (4.19)$$

where D_m is the diffusion coefficient, x_m is the thickness of the membrane itself as well as the thicknesses of any stagnant layers on either side and C_2 is the concentration of the analyte in the solvent stagnant layer.

Lastly, the flux through the solvent film layer, N_s, is given by:

$$N_s = k_2(C_3-C_s) \qquad (4.20)$$

Where C_s is the concentration in the solvent, C_∞ is the bulk concentration and C_m is the concentration of solute in the membrane. Assuming steady state is acheived by the sampler system, the total flux through system will equal fluxes through the boundary layer, the membrane and solvent film. These fluxes are defined in equations 4.18 through 4.20. The flux, N_b, through the boundary layer film is described as:

$$N_b = k_1 (C_\infty - C_1) \qquad (4.18)$$

The existence of two stagnant layers, one on either side of the membrane is proposed. In equation 4.18, where C_1 is the concentration of the analyte in the stagnant boundary layer on the outside of the membrane that is exposed to the aqueous solution. Transport in the bulk film is given by the mass-transfer coefficient, k_1. Likewise, the membrane flux, N_m, is described by:

$$N_m = \frac{D_m}{x_m} (C_1-C_2) \qquad (4.19)$$

where D_m is the diffusion coefficient, x_m is the thickness of the membrane itself as well as the thicknesses of any stagnant layers on either side and C_2 is the concentration of the analyte in the solvent stagnant layer.

Lastly, the flux through the solvent film layer, N_s, is given by:

$$N_s = k_2(C_3-C_s) \qquad (4.20)$$

where C_s is the concentration in the solvent, C_3 is the concentration in solvent film and k_2 is another mass transfer coefficient. The partition coefficient, ϕ, also equals C_3 divided by C_2 and can be substituted into equation 4.20. Equations 4.18 - 4.20 are summed to yield the total flux:

$$\frac{N}{k_1}_b + \frac{N}{D_m}_m X_m + \frac{N}{k_2 \phi}_s = C_\infty - \frac{C}{\phi}_s \qquad (4.21)$$

Because the system is at steady state, the fluxes must all be equal and terms may be gathered:

$$N\left(\frac{1}{k_1} + \frac{X_m}{D_m} + \frac{1}{k_2} \right) = \frac{C}{\phi}_s - C_s \qquad (4.22)$$

Finally, an overall permeability, K can be defined as:

$$\frac{1}{K} = \frac{1}{k_1} + \frac{X_m}{D_m} + \frac{1}{k_2 \phi} \qquad (4.23)$$

Then,

$$N = K \left(C_\infty - \frac{C}{\phi}_s\right) \qquad (4.24)$$

By assuming equilibrium is reached, the solute concentration in the solvent (C_{eq}) is maximized and can be defined as $\phi\ C_\infty$. The equation is solved for C_∞ and substitution into equation 4.23 yields:

$$N = K \left(\frac{C}{\phi}_{eq} - \frac{C}{\phi}_s \right) = \frac{K}{\phi} \left(C_{eq} - C_s\right) \qquad (4.25)$$

Therefore, the flux through the membrane can be described by a first-order exchange constant that considers the thickness of the membrane and

any stagnant layers on either side. Partition coefficients, the solvent and equilibrium solvent concentrations all affect the flux. These last two parameters are readily determinable in the laboratory. If flux through a membrane is further defined as:

$$\frac{V}{A}\frac{dC_s}{dt} = \frac{K}{\phi}\left(C_{eq} - C_s \right) \qquad (4.26)$$

where V is velocity and A is the area. The concentration in the solvent at equilibrium is assumed to be greater than the concentration in the and integration is performed:

$$\ln\left(C_{eq} - C_s \right) - \ln C_{eq} = -\frac{K A t}{V \phi} \qquad (4.27)$$

Hence, the uptake of the molecules from the water is proportional to the first order exchange constant and therefore should reflect the physical and chemical properties of the molecules.

CONCLUSIONS

The majority of the membranes used in subsequent studies are relatively crystalline in nature. Their high degree of hydrocarbon branching should result in the formation of a layer of water on the outside of the membrane that is highly clustered and less dense in

nature. Solute uptake by the devices will be dependent upon diffusion from the bulk layer, dissolution into the aqueous boundary layer, pore penetration and negotiation through the membrane. Membrane/solvent interactions will cause the membranes to swell, facilitating solute accumulation. Additional factors influencing this process include polarity of the solute, solvent and membranes, water temperature, turbulence, solute concentrations, permeant size and shape.

CHAPTER 4

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CHAPTER 5

STUDIES TO OPTIMIZE SOLVENT/MEMBRANE COMBINATIONS

INTRODUCTION

Miniature sampling devices, consisting of a solvent encased by a polymeric film, were developed to study contaminant accumulation. Initially, the devices were exposed to aqueous solutions of naphthalene at environmentally relevant concentrations. The various solvent/membranes tested were extremely efficient, some accumulating organochlorine compounds to concentrations up to 2,400 times the aqueous concentration. To optimize further solvent/membrane combinations, samplers were exposed to an aqueous solution containing aldrin, dieldrin, p,p'-DDE, lindane and 2,4,5,2'4',5'-hexachlorobiphenyl, also in environmentally relevant concentrations. Optimal solvent/membrane systems were either polypropylene or polyethylene membranes that contained 2,2,4-trimethylpentane.

To gain insight into the separation process, the relative amount of contaminant accumulated from the water column was calculated and compared with respect to various chemical and physical properties of the contaminant. The relative molecular length, the degree of chlorination, and the Henry's law constant are related to the sampler's tendency to accumulate contaminants. To explain these relationships for contaminant uptake by the devices, solution thermodynamics is discussed and a model for contaminant uptake is proposed.

MATERIALS AND METHODS

SOLVENTS

The solvents placed inside the sampling devices were reagent grade solvents obtained from J. T. Baker Chemical Co., Phillipsburg, NJ. They were used without further cleanup and included hexane, 2,2,4-trimethylpentane, methanol, toluene, octanol, and methylene chloride.

MEMBRANES

In most studies, 0.004 gauge polymeric membranes were used. Polyethylene and clear acetate were obtained from Catalina Plastics and Coating Co. (Glendale, CA). Polyvinylchloride was procured from Reynolds Metals Co., (Richmond VA). Bordon Chemical Co. (North Andover, MA) supplied a variety of treated and oriented forms of polypropylene including: polypropylene 100-A, polypropylene 125-A, polypropylene 75 AP-1, polypropylene AP-1, and polypropylene A-60. A slightly thicker, 0.005 gauge silicone elastomer tradenamed Silastic, was received from Dow Corning (Midland, MI).

To determine whether the membranes would withstand exposure to the various solvents, 1.5 cm square pieces of membranes were glued to plastic tubing 0.9 mm in diameter with silicone glue made by Dow Corning. For non-symmetric membranes, tubes were prepared with both possible orientations facing inward. After drying 24 for hours, the tubes were filled with various solvents and allowed to sit for ten minutes. The membranes were then examined for structural integrity.

CONTAMINANTS

Model contaminants used in subsequent studies were aldrin (Nutritional Biochemical Corporation, Cleveland, OH), lindane (City Chemical Corporation, New York, NY), dieldrin (City Chemical Corporation, New York, NY), p,p'-DDE (Aldrich Chemical Company, Milwaukee, WI), naphthalene (Aldrich Chemical Company, Milwaukee, WI), and 2,4,5,2',4',5'-hexachlorobiphenyl (Pathfinder Laboratories, St. Lewis, MO). Contaminant levels ranged from the water solubility limit (in preliminary studies) to environmentally relevant concentrations as noted in Versuchern (1).

EXPOSURE CHAMBERS

Miniature sampling devices, produced by the MSU Scientific Glassblowing Laboratory allowed the exposure of various solvent/membrane combinations to aqueous solutions of pesticides. The glass devices have dimensions of 5.9 cm by 2.0 cm and are illustrated in figure 2. Polymeric membranes were fastened to the bottom of the minature-sampling devices using silicone glue and 10 ml of solvent were added to the interior compartment. A 0.8 mm in diameter septum sealed the top of the tube and allowed easy withdrawal of samples. The area of the membrane affixed to the bottom of the device was 0.603 cm^2. Although, contaminant flux could be increased by maximizing the surface area, the purpose of these initial studies was to compare the various types of membranes and 0.603 cm^2 improved handling in the experimental protocol.

The samplers were suspended from a polycarbonate lid using size six and seven stoppers and allowed to equilibrate for 13 days in the systems

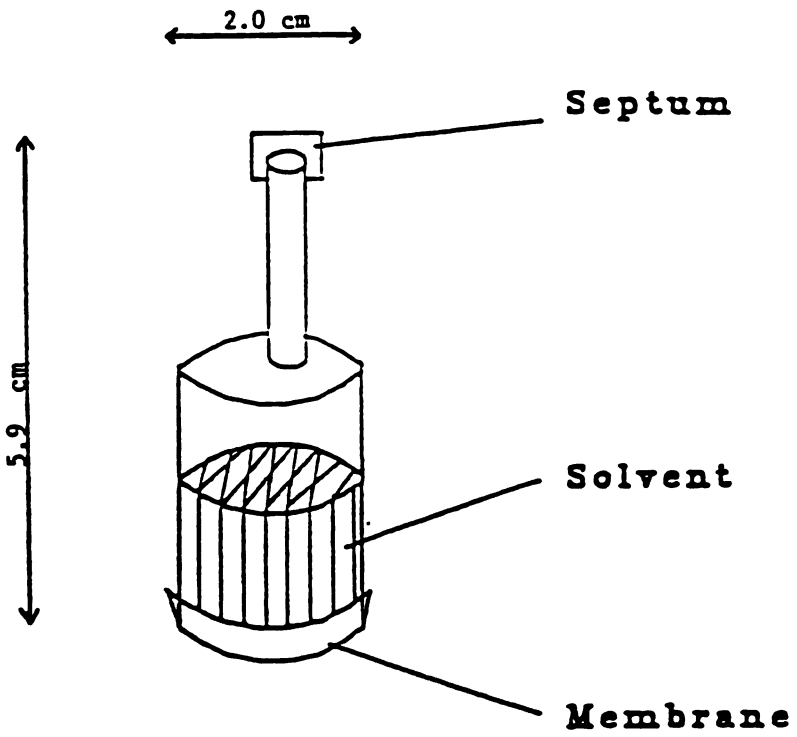


Figure 2: The Miniature-Sampling Devices.

described below. After the contents were mixed using a large 10 ml syringe with a 15-cm long 20-gauge needle, 0.5-ml samples were collected using a 2-ml syringe affixed to the same needle. At 6 and 12 hours, 1, 2, 4, 6, 8, 10, and 13 days, 0.5-ml samples were collected from each miniature sampler. Upon completion of the studies, the miniature sampling devices could be withdrawn through the holes that held the stoppers.

The preliminary exposure system that was developed is shown in figure 3. Exposure chamber (A) was a glass tank 12 L in volume. Air was bubbled through a solution (B) containing an excess of naphthalene. Because the water in B was saturated with the test compound, the gas leaving it, controlled by valve C, has a constant partial pressure or fugacity of test compound. After dilution with the make-up gas, controlled by valve (D), the gas was bubbled through the exposure chamber. Thus, the activity of the naphthalene in the exposure chamber was at equilibrium with the partial pressure of the compound in the test gas and could by varied by adjusting the relative flow rates through valves C and D. The activity in the test tank could be monitored by measuring the partial pressure of the compound in the exhaust line. This system will be referred to as a "static" system, because no water is exchanged in the system.

The flowing exposure system is shown in figure 4. The exposure chamber consisted of a 12-L glass tank. The system was stirred with a Talboy's stirrer. Two Beckman solution metering pumps, pumping at 1.5 ml/min., were used to pump the water solution through 1/8" stainless steeltubing into the incubation tank. A total volume of 200 L was maintained constant in this chamber. A Forma Scientific model 2132 water bath was used to maintain constant temperature.

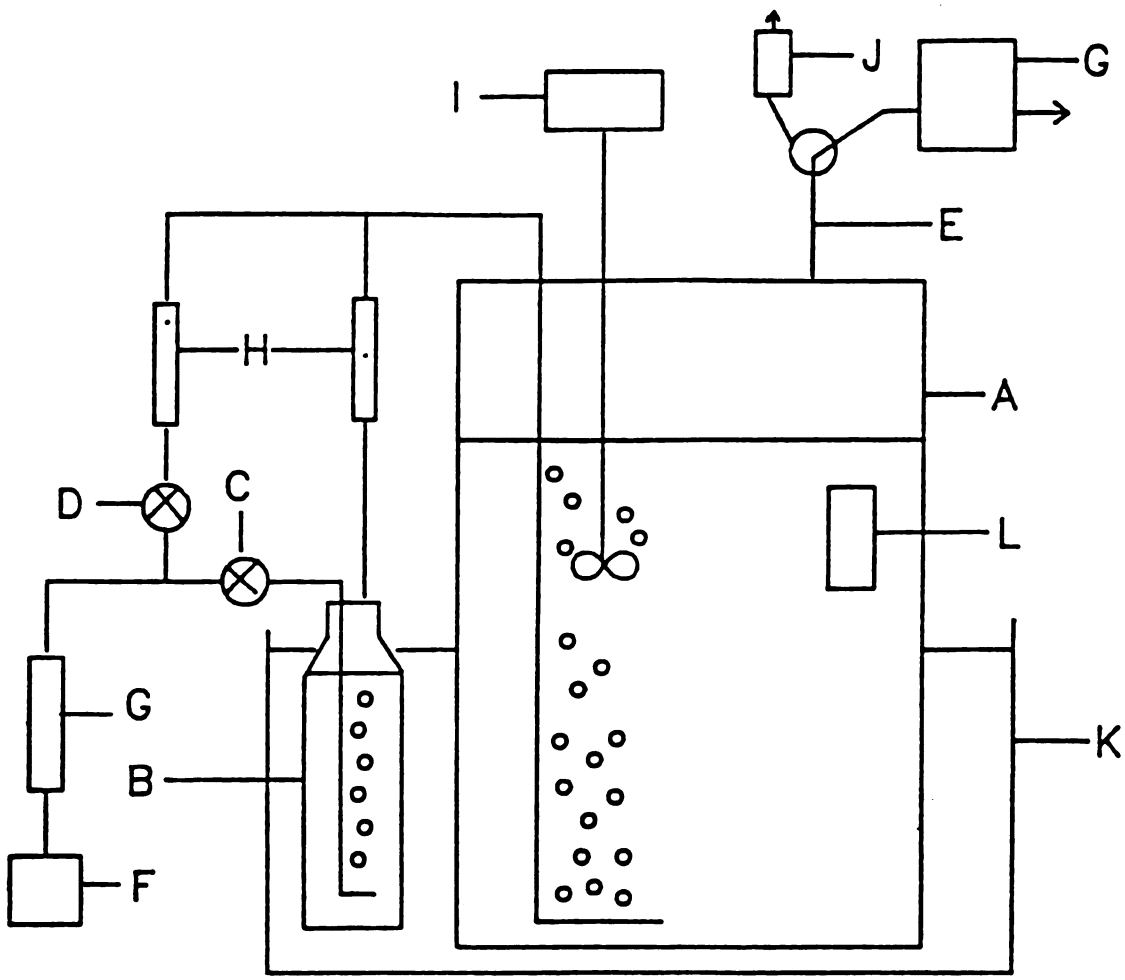


Figure 3: Apparatus for Exposing the Miniature Sampling Devices to Aqueous Solutions with Controlled Activities of Test Compounds. A-exposure tank, B-water solution containing excess solid or liquid test compound, C and D-gas metering valves, E-exhaust gas line connected to two-way switching valve, F-stainless steel bellows air pump, G-activated carbon filters, H-rotometers, I-precision controlled stirrer, J-analytical trap (charcoal) for measurement of the compound in the exhaust gas, K-constant temperature bath, L-sampler being tested.

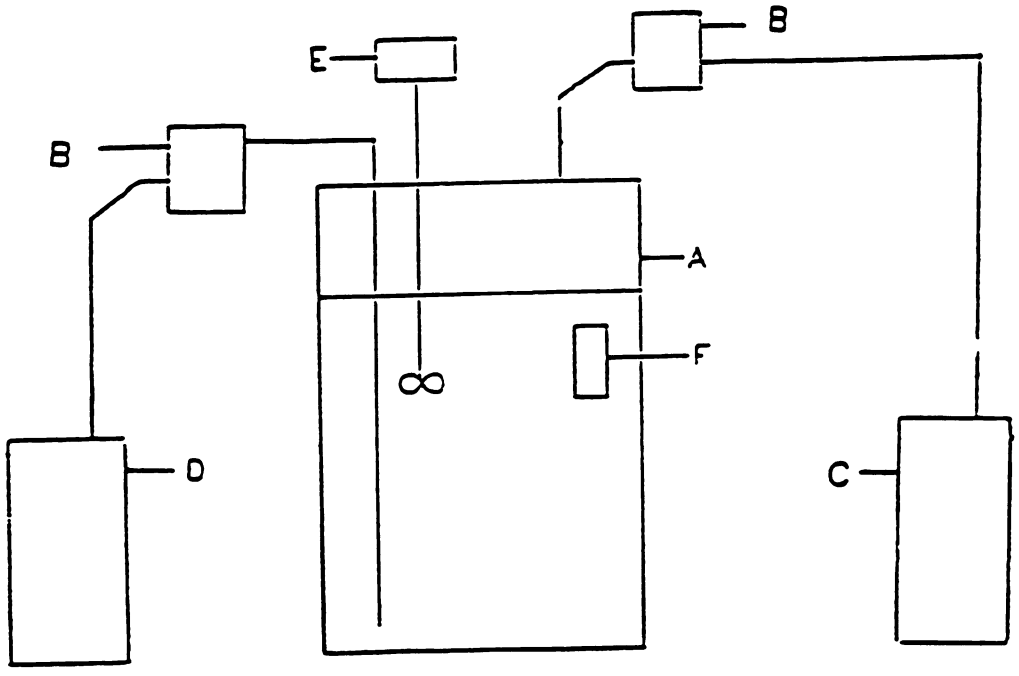


Figure 4: Apparatus for Exposing the Miniature Sampling Devices to Aqueous Solutions of Test Compounds in a Flowing Stream. A-exposure tank, B-water pumps, C-aqueous solution containing test compound, D-waste container, E-precision controlled stirrer, F-sampler being tested.

The concentration of naphthalene accumulated by the devices was determined by direct injection into a Varian model 1700 gas chromatograph containing an open tubular column (0.25 mm i.d. , 30 m length) with DB-5 stationary phase (J & W Scientific, Folsom, CA) and a flame ionization detector. Chlorinated pesticides were analyzed using a Perkin-Elmer 8500 gas chromatograph containing an open tubular column (0.25 mm i.d., 60 m length) with DB-1 stationary phase (J & W Scientific, Folsom, CA) and an electron capture detector. No clean up or preconcentration techniques were performed on any of the samples.

RESULTS USING NAPHTHALENE AS A MODEL CONTAMINANT

In initial compatibility tests, several solvents strongly interacted with and dissolved some of the membranes. These results are summarized in table 7. Strong interactions between some solvent/membrane pairs are predicted by Hildebrand solubility parameters. Theoretically, the more closely the parameters of the membrane and the solvent match, the greater the degree of interaction. This promotes membrane swelling, reduces membrane crystallinity, and increases membrane permeability. Occasionally, the observed interactions created a hole in the membrane. As shown in the table, dissolution of the membranes occurred over a wide range of solubility parameters. This phenomenon has been noted in the literature and may be due to solvent interactions that occur in regions on the membrane with differing polarities (2). These compatibility tests were an essential first step in the design of the sampling devices. All stable solvent/membrane combinations were used in subsequent studies to optimize the system.

Table 7

Incompatible Membranes and Solvents

Membrane	Hildebrand Solubility Parameter	Solvent	Hildebrand Solubility Parameter
	$Cal^{1/2}/cm^{-3/2}$		$Cal^{1/2}/cm^{-3/2}$
PVC	9.3-10.1	Toluene	8.9
PVC	9.3-10.1	Methylene Chloride	9.7
Silastic	7.4-7.8	Hexane	7.3
Silastic	7.4-7.8	Methanol	14.5
Acetate	11.1-12.5	Methylene Chloride	9.7
	Additional Solubility Parameters ($Cal^{1/2}/cm^{-3/2}$)		
Membranes		Solvents	
Polyethylene	7.7-8.2	Isooctane	6.9
Polypropylene	9.2-9.4	Octanol	10.3
		Water	18.0

Initial tests were conducted in a "static" system as shown in figure 3, using naphthalene at a concentration of 30 ppm and hexane as the solvent. The uptake curves (figure 5b) for the hexane/membrane systems indicated rapid acculumation of naphthalene by all the polypropylene systems. Initially, uptake occurred at different rates when different polymers were compared (figure 5a). After three days, a dramatic decrease in analyte concentration was observed in all sampling devices. This was due to a decrease in contaminant concentration in the bulk phase caused by losses due to evaporation, and/or adhesion/adsorption to membrane surfaces. Also, the meager surface area of the bubbling device may have limited the rate of naphthalene introduction into the bulk phase. Thus, these sampling devices reflected changes in the water concentration.

Figure 5b illustrates uptake profiles when oriented and treated polypropylene films are used. The process of orienting the polypropylene does not appear to affect significantly the ability to accumulate naphthalene. Therefore, untreated polypropylene membranes were used in

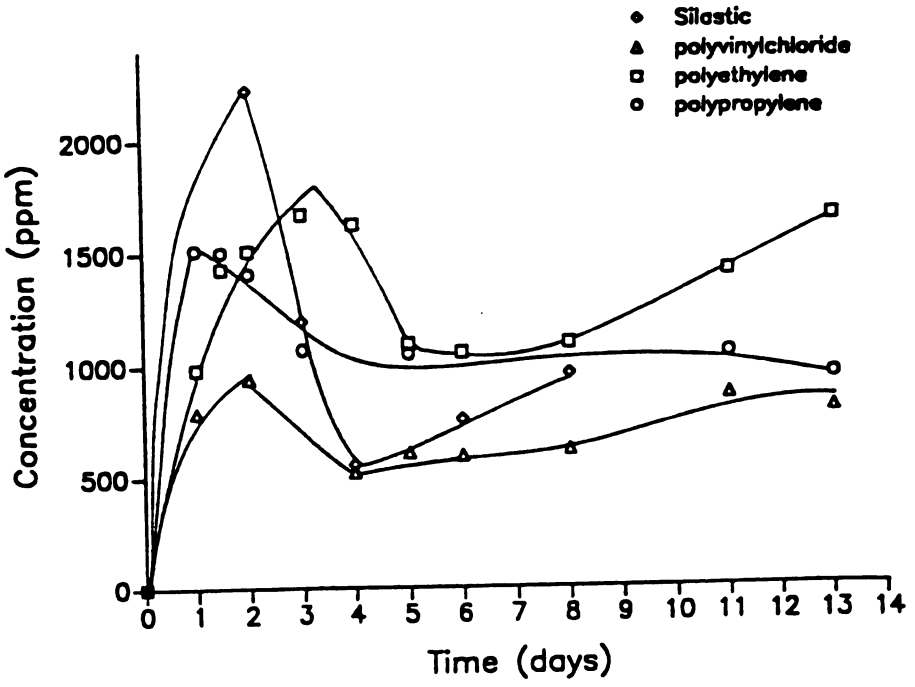


Figure 5a

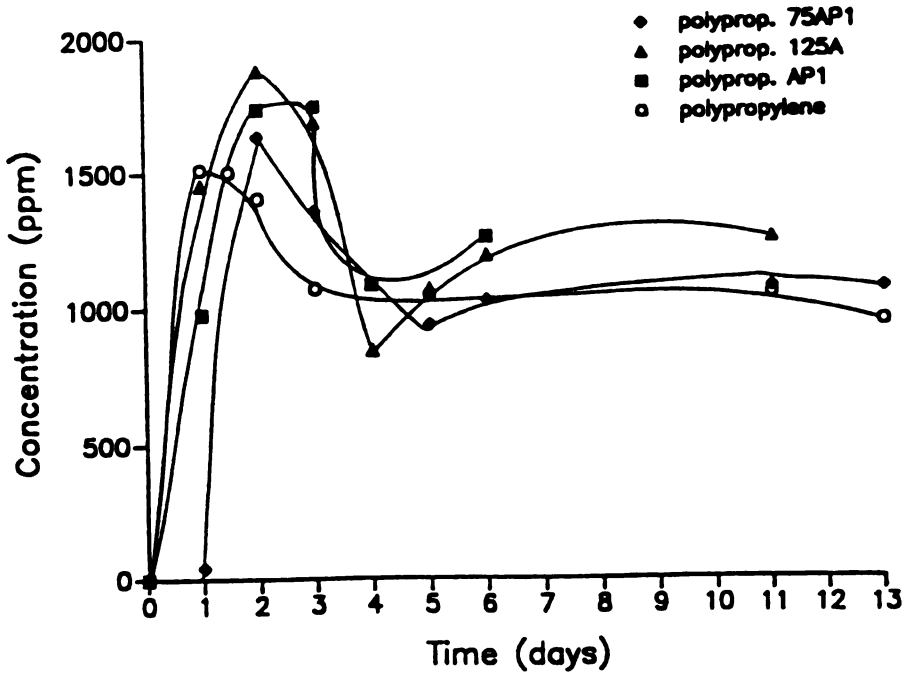


Figure 5b

Figure 5: Naphthalene Uptake by a Variety of Hexane/Membrane Systems in a "Static" Incubation System.

subsequent studies. To maintain a constant concentration of contaminant in the incubation chamber, a continuously flowing system was developed for use in the remainder of the studies.

Naphthalene uptake by sampling devices placed in the system flowing at 1.5 ml/min is shown in figure 6. Equilibrium is approached in the 2,2,4-trimethylpentane/polypropylene, 2,2,4-trimethylpentane/polyvinyl-chloride, hexane/polpropylene, hexane/polyethylene, hexane/acetate, toluene/polyethylene, toluene/polypropylene, octanol/Silastic^{R}, and octanol/ polypropylene systems. Also, the figure illustrates naphthalene loss from four of the devices, namely Silastic^{R}/methylene chloride, polyethylene/methylene chloride, polyvinylchloride/methanol and Silastic^{R}/methanol. These losses were due to a delayed interaction between the solvent and the membrane. The polypropylene/2,2,4-trimethylpentane system was chosen for further study because of its efficient contaminant uptake as well as its rapid equilibrium.

Eighteen polypropylene/2,2,4-trimethylpentane samplers were exposed to a 48 ppb naphthalene solution. Triplicate samples were taken at 0.25-, 0.5-, 1-, 2-, 4-, and 6-day intervals, removing the total contents of each sampler. Thus, the volume of solvent inside a sampler was maintained constant until the sample was acquired. After quantitation of naphthalene in the samples, the relative standard deviation, RSD, was calculated for each time point. The relative standard deviation (RSD) ranged from 5% to 19% with an overall average RSD of 13%. The surface area of film on these test devices is relatively small and variations in the polypropylene are expected to limit precision. To quantitate and compare the partitioning process, membrane concentration factors (MCF), analogous to bioconcentration factors were calculated as follows:

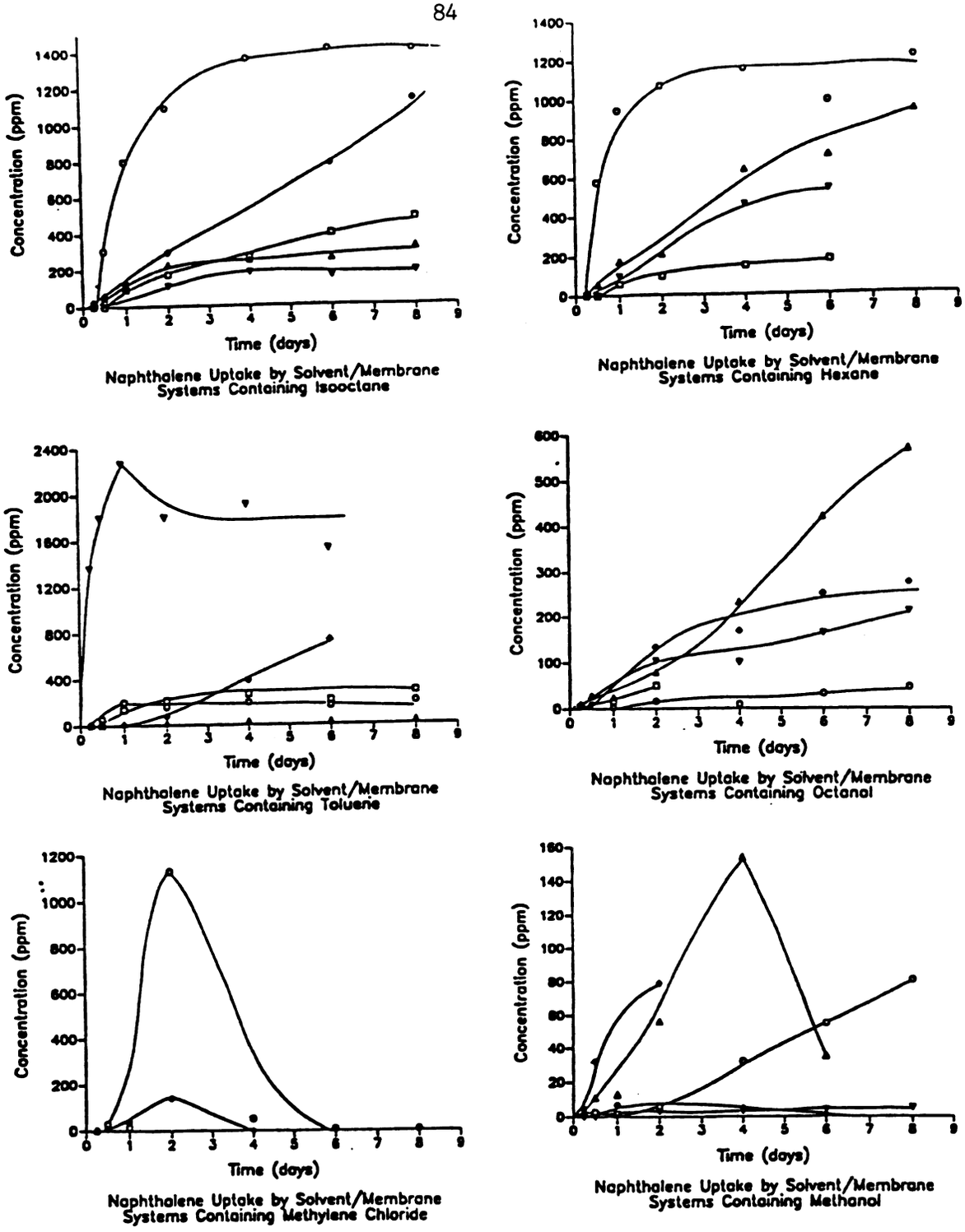


Figure 6: Naphthalene Uptake by a Variety of Solvent/Membrane Systems in a Flowing Incubation System.

key: ○ polypropylene, □ polyethylene, ▼ acetate, △ polyvinylchloride, and ◆ Silastic.

$$MCF = \frac{\text{equilibrium concentration in sampler}}{\text{concentration in water}} \qquad (5.1)$$

In this reproducibility study, the average MCF for naphthalene uptake by the polypropylene/2,2,4-trimethylpentane samplers was 4,300.

To assure that partitioning of the naphthalene was occurring through the membrane material and not the adhesive, a "glue" study was undertaken where polypropylene was bonded to the miniature samplers using silicone glue, Super glue^R and Quickgel^R. Similarly, ethyl acetate membranes were affixed to the samplers using Quickgel^R and silicone glue. Hexane, methanol or toluene were placed in the devices and naphthalene uptake was monitored as previously described. Equilibrium concentrations remained constant at 10 ppm for the hexane/polypropylene systems, indicating no differences in contaminant uptake were caused by using different adhesives. The relative standard deviation was 12%. Ethylacetate did not adhere well.

RESULTS USING ORGANOCHLORINE COMPOUNDS AS MODEL CONTAMINANTS

Organochlorine compounds were added to the bulk water solution to form the following final concentrations: aldrin 0.174 ppb, lindane 0.081 ppb, DDE 6.02 ppb, dieldrin 0.463 ppb, 2,4,5,2′,4′,5′-hexachlorobiphenyl (HCB) 0.405 ppb, and methoxychlor 3.315 ppb. According to Versuchern (1), these concentrations are environmentally relevant. Duplicate samplers consisting of a variety of solvent/membrane pairs were exposed to the solution up to 14 days. Additional samplers, serving as blanks, were exposed to distilled water for 14 days.

In order to magnify the effects of any plasticizer that could leach from the thin film, a figure was constructed that compares the amount of

plasticizers that leach into blanks (sampling devices were exposed to distilled water) to samplers exposed to organichlorines. To further augment the differences, the control illustrates a chromatogram of the componments after an exposure of 14 days, while the exposure of the sample to organochlorine compounds was limited to 24 hours providing a worst case scenario. Figure 7 illustrates results from this analysis by gas chromatography using an attenuation of 4. The top chromatogram, which resulted from the analysis of the contents of a polypropylene/2,2,4-trimethylpentane sampler that was exposed to organochlorine compounds for one day, illustrates how readily the compounds were accumulated. The results from the analysis of the blank, which was exposed to distilled water for 14 days is shown in the bottom. This particular chromatogram was chosen to reveal the effect that extended incubation times have on plasticizer introduction into the sample. As the figure illustrates, after 14 days of contact with the solvent, substantial amounts of polymerizing material were not leached into the sample. The only major contribution from the polymer was represented by the peak eluting at approximately 27 min. Further analysis using gas chromatography/mass spectrometry indicated this peak represented a phthalate ester plasticizer because a peak at m/e 149 was present. The presence of this phthalate ester did not interfere with the chromatographic determination of the xenobiotics because lindane, aldrin, DDE, dieldrin, 2,2′,4,4′,5,5′-hexachlorobiphenyl and methoxychlor eluted at approximately 11.66, 15.60, 19.39, 19.62, 22.56, and 25.37 min., respectively.

Figure 8 illustrates the uptake patterns for various chlorinated contaminants as a function of time for individual solvent/membrane combinations. Again, equilibrium was approached usually after three to

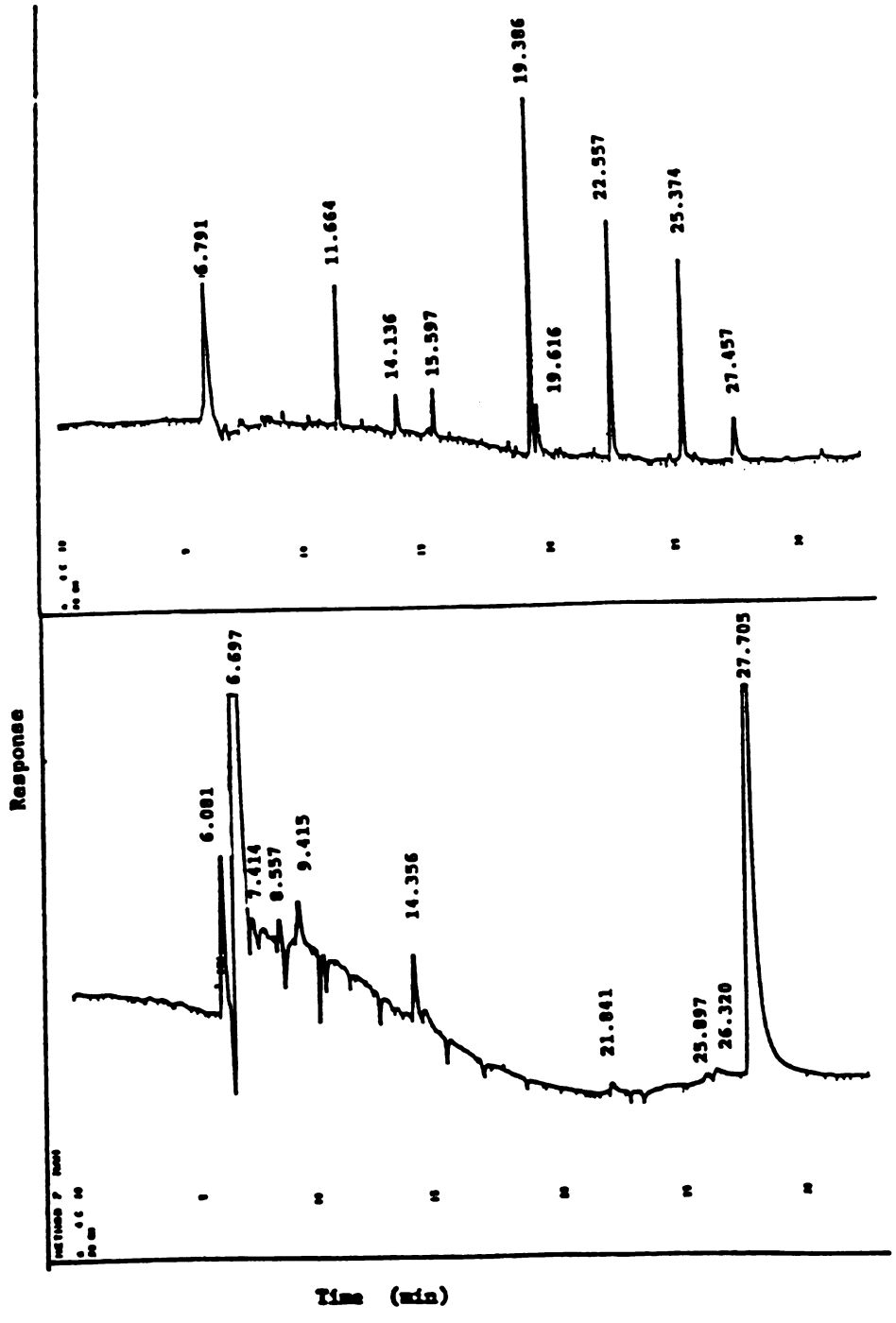
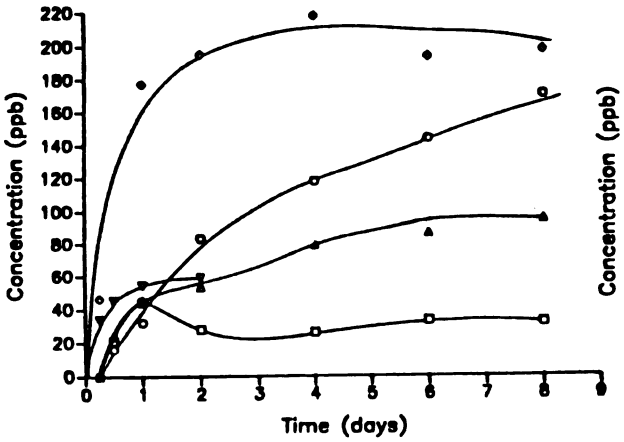
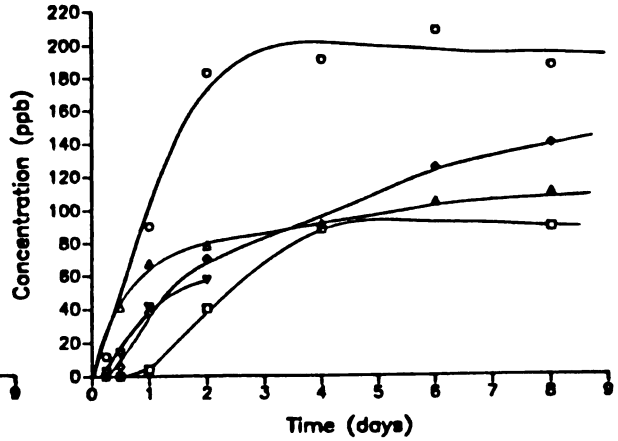


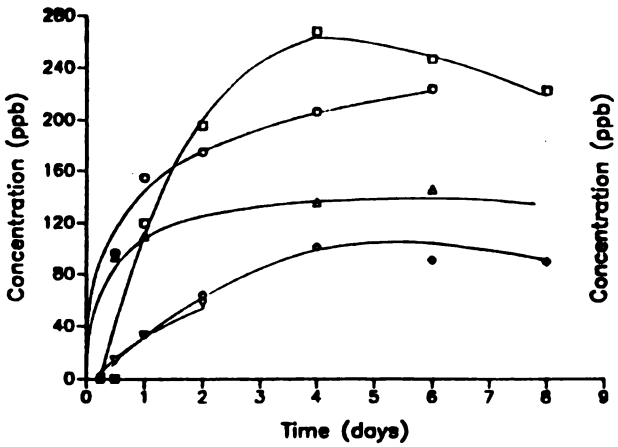
Figure 7: Typical Chromatogram of the Contents of a Polypropylene/ 2,2,4-Trimethylpentane Miniature Sampler (top) Compared to its Blank (bottom). Exposure Conditions: the sample separated in the top chromatogram was exposed to organochlorine compounds for 1 day, the sample blank separated in the bottom chromatogram was exposed to distilled water for 14 days. Chromatographic Conditions: A Perkin-Elmer 8500 gas chromatograph equipped with a 60 m DB-1 column and an electron capture detector was temperature programmed from 170 to 270 ° C at 4°/mim. The attenuation used for both chromatograms was 1.



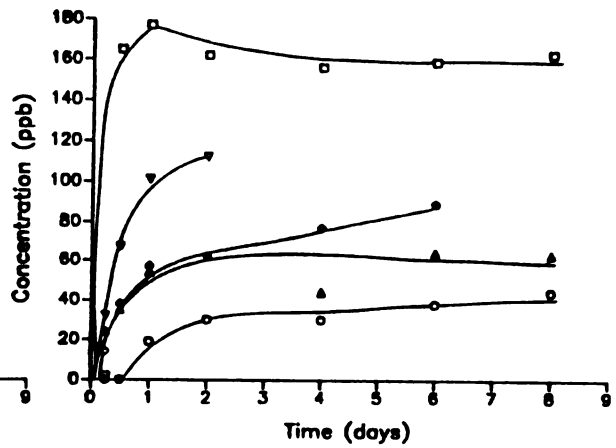
Uptake of Lindane by Solvent/Membrane Systems Containing 2,2,4-Trimethylpentane.



Uptake of DDE by Solvent/Membrane Systems Containing 2,2,4-Trimethylpentane.

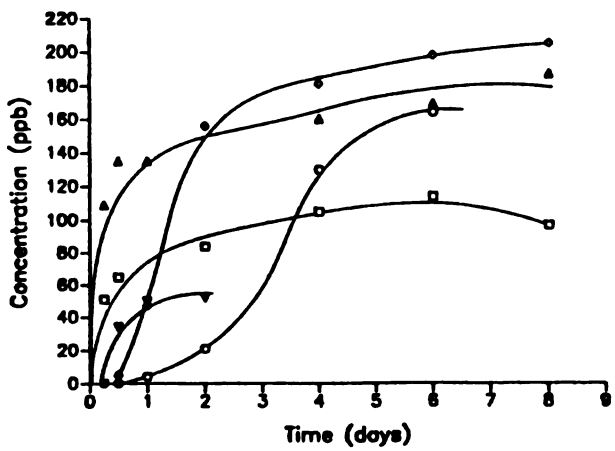


Uptake of Dieldrin by Solvent/Membrane Systems Containing 2,2,4-Trimethylpentane.



Uptake of Aldrin by Solvent/Membrane Systems Containing 2,2,4-Trimethylpentane.

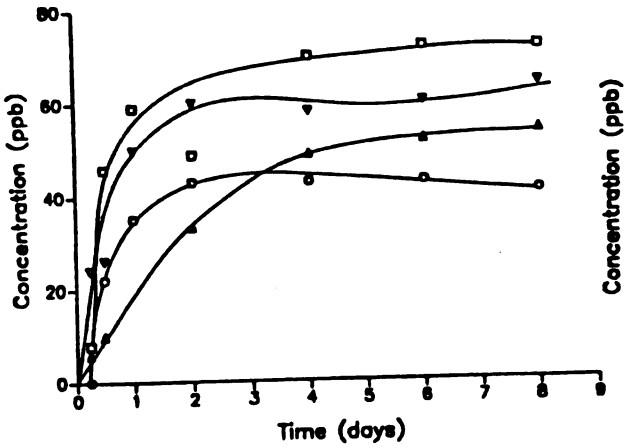
Figure 8: Uptake of Organochlorine Compounds by a Variety of Solvent/Membrane Systems in the Flowing Incubation System.



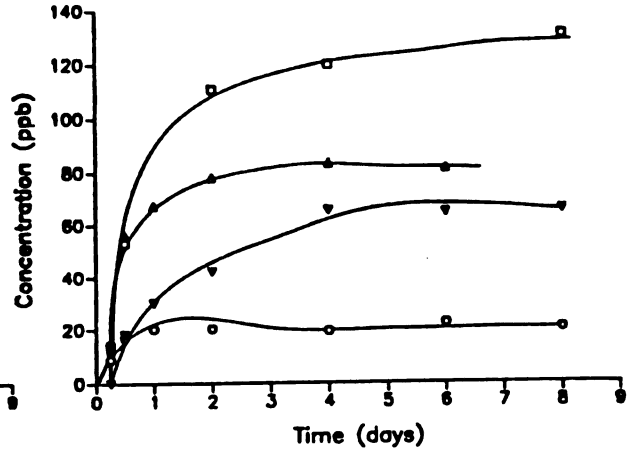
Uptake of 2,2',4,4',5,5'-Hexachlorobiphenyl by Solvent/Membrane Systems Containing 2,2,4-Trimethylpentane.

key:

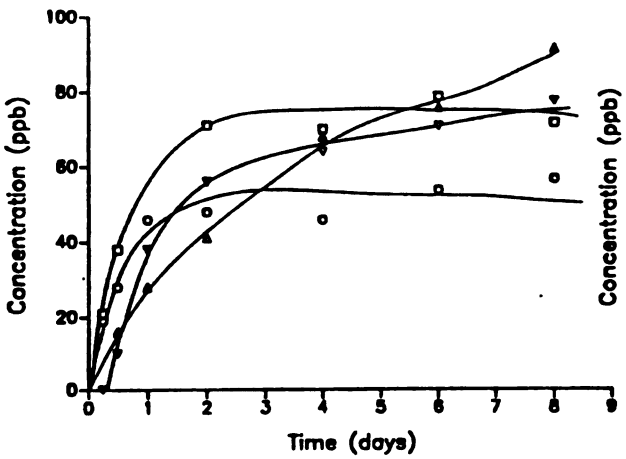
- ▼ acetate
- ● Silastic
- ▲ polyvinylchloride
- □ polyethylene
- ○ polypropylene



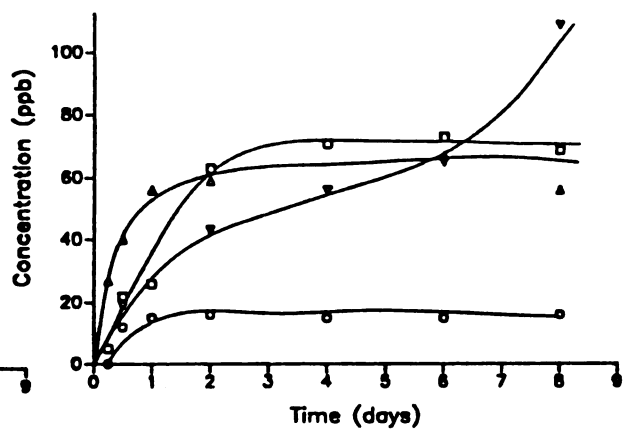
Uptake of Lindane by Solvent/Membrane Systems Containing Methanol



Uptake of DDE by Membrane/Solvent Combinations Using Methanol

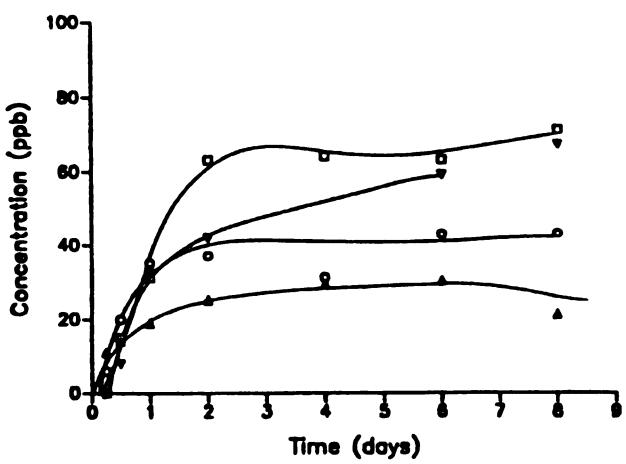


Uptake of Dieldrin by Solvent/Membrane Combinations Containing Methanol



Uptake of Aldrin by Membrane/Solvent Combinations Containing Methanol

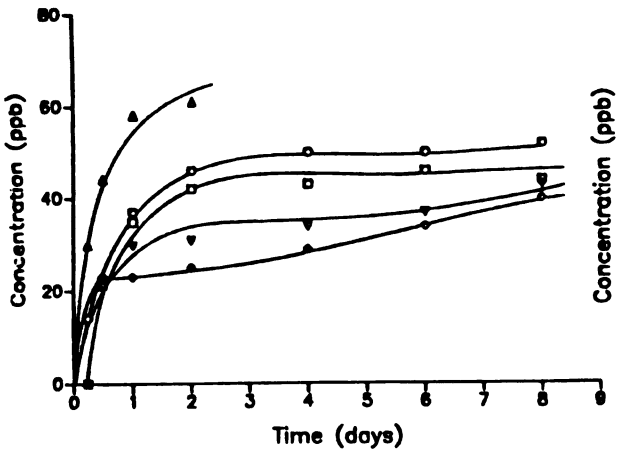
Figure 8: Continued



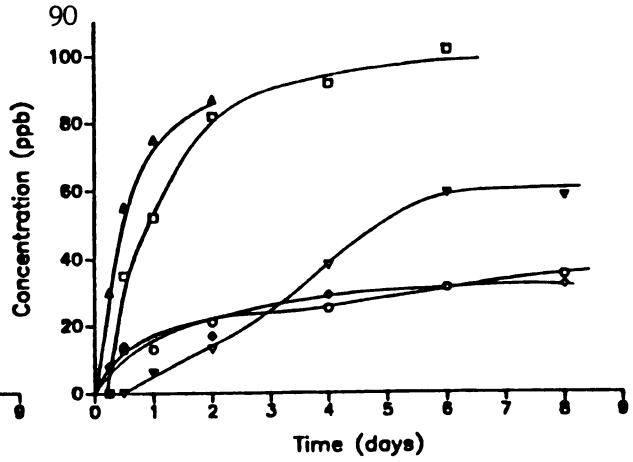
Uptake of 2,4,5,2',4',5'-Hexachlorobiphenyl by Solvent/Membrane Systems Containing Methanol

key:

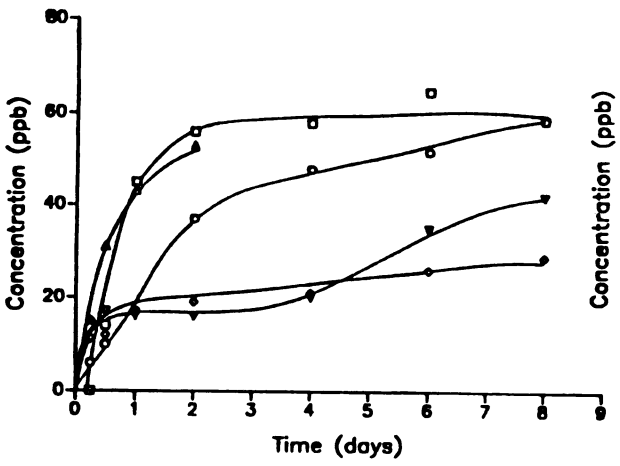
- ▼ acetate
- ◆ Silastic
- ▲ polyvinylchloride
- □ polyethylene
- ○ polypropylene



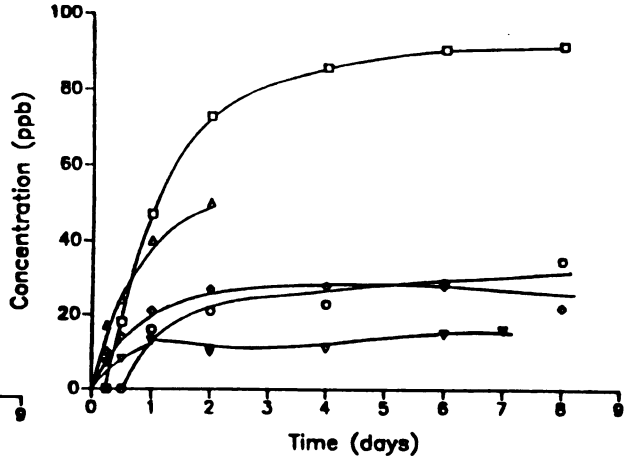
Uptake of Lindane by Solvent/Membrane Systems Containing Octanol



Uptake of DDE by Solvent/Membrane Combinations Containing Octanol

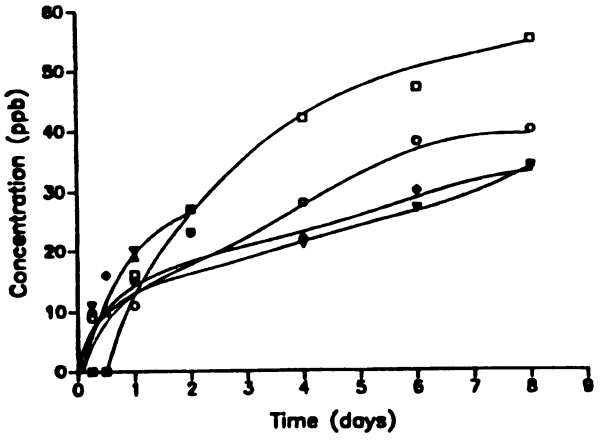


Uptake of Dieldrin by Solvent/Membrane Combinations Containing Octanol



Uptake of Aldrin by Solvent/Membrane Systems Containing Octanol

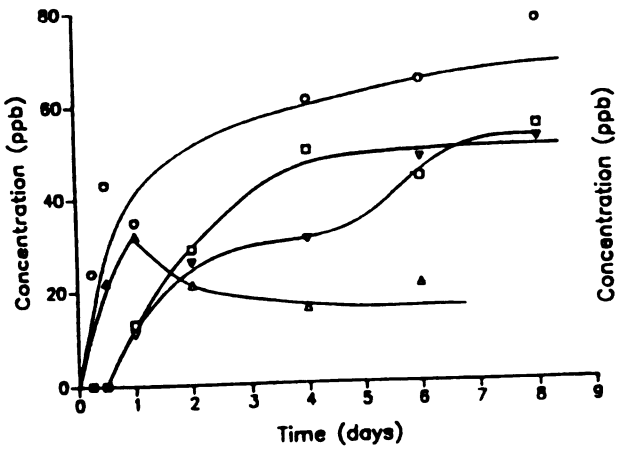
Figure 8: Continued



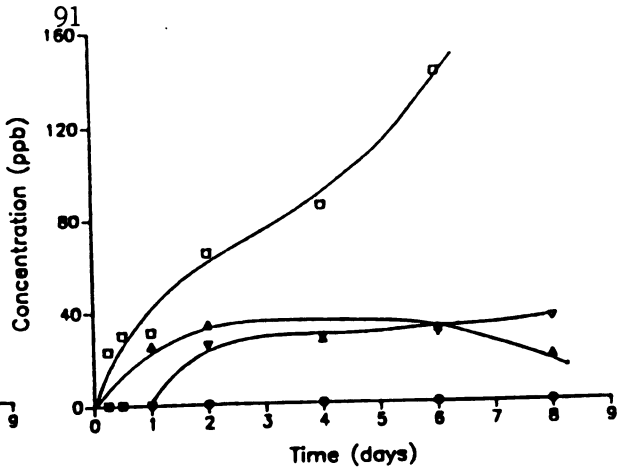
Uptake of 2,4,5,2',4',5'-Hexachlorobiphenyl by Solvent/Membrane Systems Containing Octanol

key:

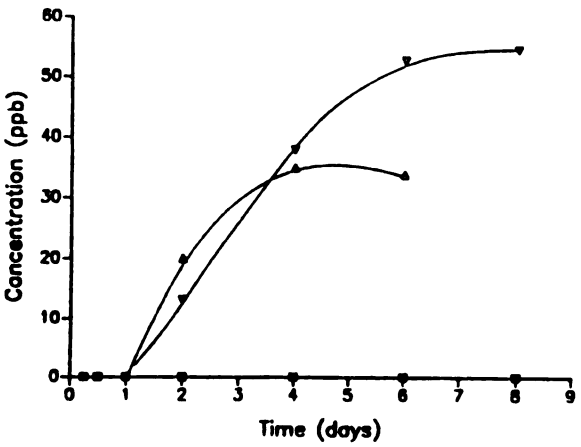
- ▼ acetate
- ◆ Silastic
- ▲ polyvinylchloride
- □ polyethylene
- ○ polypropylene



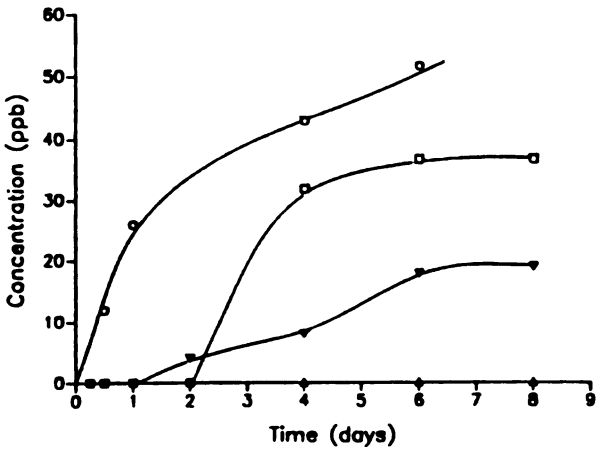
Uptake of Lindane by Solvent/Membrane Systems Containing Hexane.



Uptake of DDE by Solvent/Membrane Systems Containing Hexane

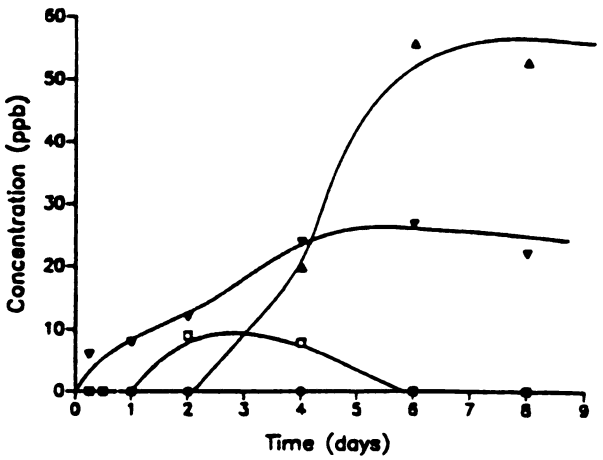


Uptake of Dieldrin by Solvent/Membrane Systems Containing Hexane



Uptake of Aldrin by Solvent/Membrane Systems Containing Hexane

Figure 8: Continued



Uptake of 2,4,5,2',4',5'-Hexachlorobiphenyl by Solvent/Membrane Systems Containing Hexane

key:

- acetate
- Silastic
- polyvinylchloride
- polyethylene
- polypropylene

four days. To compare and quantitate the partitioning process, membrane concentration factors, analogous to bioconcentration factors, were calculated as before. The MCFs calculated range from 2 to 2,400 and are listed in table 8. It is interesting that the system which achieved the greatest MCFs for aldrin, lindane and hexachlorobiphenyl was the Silastic/2,2,4-Trimethylpentane system where the Hildebrand solubility parameters were most closely matched. However, this system did not achieve equilibrium in all instances and therefore was not suitable for all contaminants. Polypropylene/2,2,4-trimethylpentane and polyethylene/2,2,4-trimethylpentane systems also accumulated contaminants to high levels and readily approached equilibrium. The polypropylene/2,2,4-timethylpentane system was more efficient than the polyethylene and approached equilibrium faster. The polypropylene system was more universal in contaminant uptake. This is probably due to greater interaction between the 2,2,4-trimethylpentane and the longer polypropylene subunits, which reduce the crystalline properties of the polypropylene and increase its rubbery characteristics. Glassy membranes have a greater degree of selectivity, at least for molecules in the gas phase. So, the greater universality of uptake for the polypropylene system is not unexpected. Subsequently, both the polypropylene and polyethylene systems were compared in field tests.

Also, first-order exchange constants were calculated using equation 4.25 and are listed in table 8. As shown in the table, the rate constants are within the same order of magnitude because of the similarity in molecular size.

To determine the relationship between the contaminant concentrations in the aqueous and solvent phases, polypropylene/2,2,4-trimethylpentane samplers were exposed to various concentrations of

Table 8

First Order Exchange Constants and Equilibrium Concentrations

System	log Exchange Constant	Equilibrium Concentration	MCF
Aldrin			
pp/meoh	3.22	16 ppb	92
pvc/meoh	3.27	62 ppb	360
pe/meoh	3.52	72 ppb	410
si/oct	3.60	30 ppb	170
pp/hex	3.61	55 ppb	320
pe/hex	3.69	38 ppb	220
ace/meoh	3.71	65 ppb	370
si/iso	3.72	95 ppb	550
pe/oct	3.83	90 ppb	520
ace/hex	3.84	20 ppb	120
pp/oct	3.91	32 ppb	180
ace/oct	3.94	17 ppb	99
Lindane			
pp/meoh	3.10	45 ppb	560
pp/oct	3.54	54 ppb	670
ace/meoh	3.58	65 ppb	800
pe/meoh	3.64	72 ppb	890
pe/oct	3.70	47 ppb	580
ace/hex	3.72	53 ppb	650
pe/hex	3.73	55 ppb	680
pvc/meoh	3.75	53 ppb	650
si/iso	3.77	202 ppb	2500
ace/oct	3.84	36 ppb	440
pp/hex	3.85	70 ppb	860
si/oct	4.13	37 ppb	460
p,p'DDE			
pe/oct	3.75	104 ppb	170
ace/hex	3.76	30 ppb	50
pe/iso	3.80	90 ppb	150
pp/iso	3.83	200 ppb	330
pe/meoh	3.87	130 ppb	220
pp/oct	3.91	38 ppb	63
pp/meoh	3.91	20 ppb	33
si/iso	3.99	145 ppb	240
pvc/iso	4.06	120 ppb	200
si/oct	4.08	36 ppb	60
ace/oct	4.12	62 ppb	100
pvc/meoh	4.13	84 ppb	140
ace/meoh	4.20	65 ppb	110

Sy

Di
pv
pv
pe
ac
ac
ac
p
p
p
p
p
s

Table 8 (Cont.)

System	log Exchange Constant	Equilibrium Concentration	MCF
Dieldrin			
pvc/meoh	3.41	110 ppb	240
pvc/hex	3.64	35 ppb	76
pe/oct	3.66	65 ppb	140
ace/oct	3.72	32 ppb	69
ace/meoh	3.77	100 ppb	220
ace/hex	3.84	58 ppb	130
pp/oct	3.86	52 ppb	110
pe/meoh	3.87	72 ppb	160
pvc/iso	3.88	156 ppb	340
pp/iso	3.95	255 ppb	550
pp/meoh	3.95	54 ppb	120
si/oct	4.01	28 ppb	60
2,4,5,2',4',5'-Hexachlorobiphenyl			
pvc/meoh	3.63	30 ppb	74
pvc/iso	3.71	194 ppb	480
pp/meoh	3.72	44 ppb	110
pp/oct	3.80	40 ppb	99
pe/oct	3.80	58 ppb	140
si/iso	3.82	215 ppb	530
pe/meoh	3.84	68 ppb	170
pe/iso	3.95	100 ppb	250
si/oct	3.98	35 ppb	86
ace/hex	4.01	26 ppb	64
ace/oct	4.11	30 ppb	74
ace/meoh	4.11	80 ppb	200

This chart was obtained using data illustrated in figure 8.

Abbreviations used: pp:polypropylene; pe:polyethylene;
pvc:polyvinylchloride; ace:acetate; si:Silastic.
hex:hexane; iso:2,2,4-trimethylpentane; tol:toluene:
meoh:methanol; oct:octanol.
ppt is parts per thousand, ppb is parts per billion.

contaminants for 4 days in the flowing bath. A linear relationship between the concentration of the contaminants accumulated by the devices and the concentration in the aqueous phase occurred. These data are illustrated in figure 9. Linear regression data for these results are shown in table 9.

Table 9

Linear Regression Data for the Relationship Between Contaminant Concentrations in Solvent and Aqueous Phases

Contaminant	b	m	r
Lindane	2.525	0.464	0.9904
Aldrin	1.862	0.522	0.9985
DDE	1.774	0.594	0.9987
Dieldrin	2.270	0.449	0.9709
HCB	2.194	0.525	0.9958
Methoxychlor	2.484	0.591	0.9998

These relationships were linear to at least 4 orders of magnitude; elevated concentrations were not considered due to solubility limitations. Lower concentrations were not studied due to the minimum detectable amounts observable without using cleanup/preconcentration steps. Therefore, the final equilibrium concentrations obtained by the devices reflect the total mass in the system.

DISCUSSION

The MCFs calculated in this work are less than the BCFs reported in the literature. Sodergren (3) has observed the same phenomenon with contaminant uptake by hexane-filled dialysis bags. Figure 10 illustrates the high degree of variability observed in values for the BCFs (4). The BCF values are influenced by a multitude of factors such as species, sex, age, time in sexual cycle, and time of year (4). Compounds with high BCF values (log BCF > 6) move across membranes very

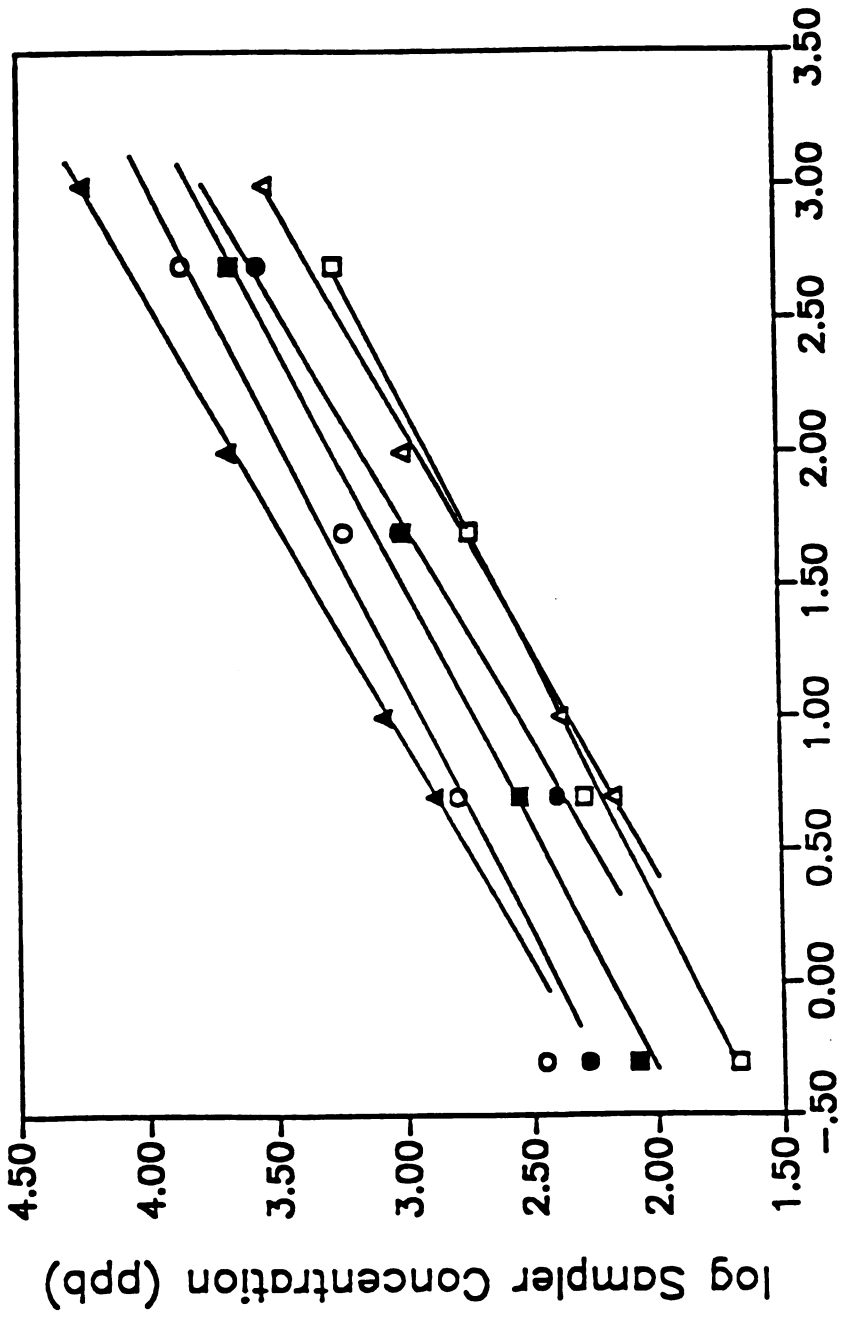


Figure 9: Uptake of Organochlorine Compounds by Polypropylene/ 2,2,4-Trimethylpentane Systems as a Function of Aqueous Concentration.

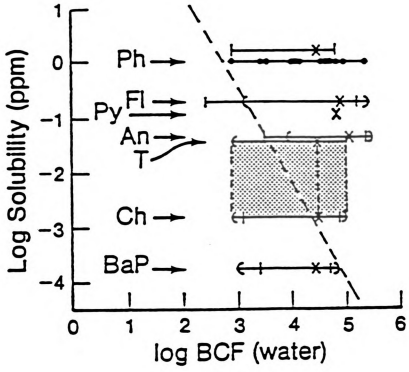


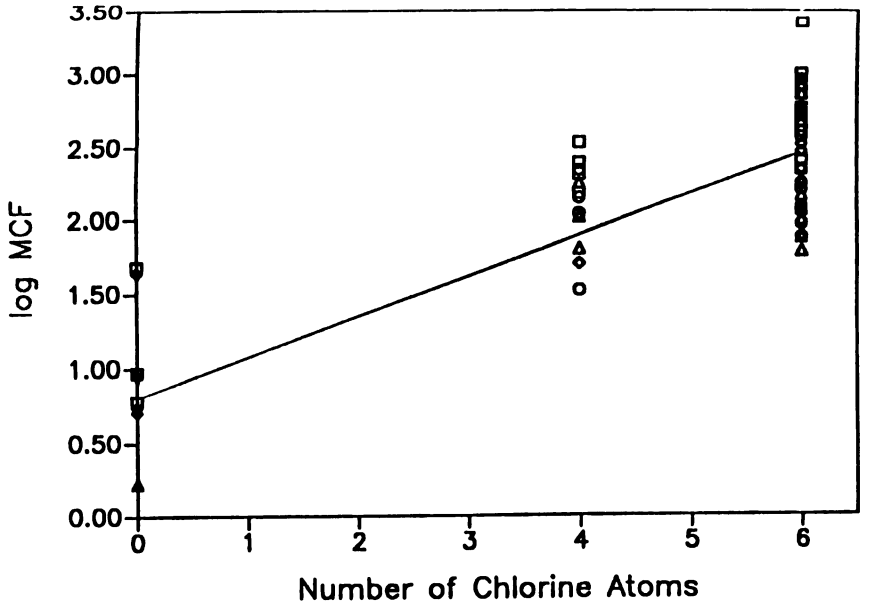
Figure 10: Log BCF (with respect to mean lake water) vs. log Solubility for P. Hoyi. There are 12 values for each compound as illustrated for phenanthrene (Ph). The mean (X) and ranges are plotted for fluoranthene (Fl), pyrene (Py), anthracene (An), Triphenylene (T), Chrysene (Ch) and Benzo(a)pyrene (BaP). The verical bar represents 1 standard deviation. The oblique dashed line is the empiracle relationship of BCF for fish calculated by Kenaga and Goring. From Eadie, Faust, Landrum, Morehead, Gardner, and Nalepa (4), 440.

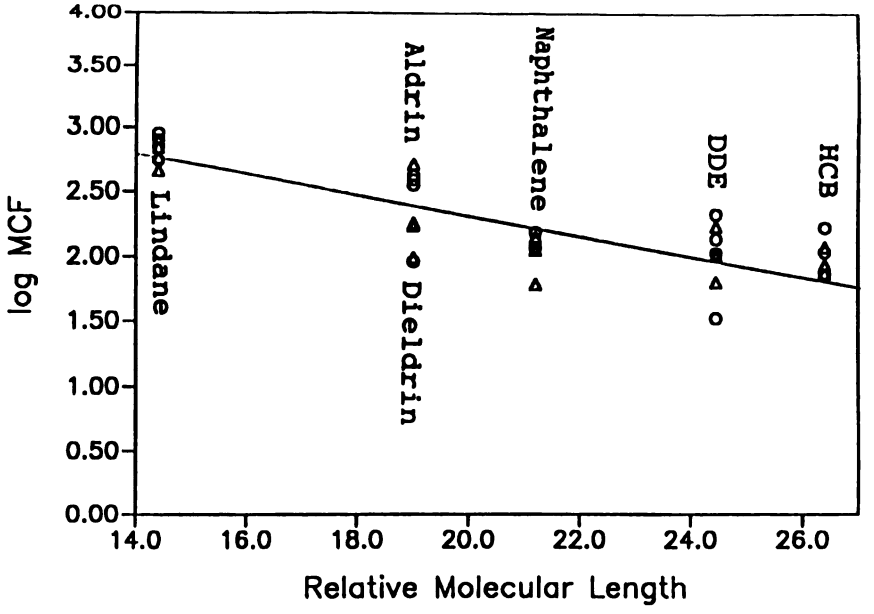
slowly and create errors in the equilibrium concentration of the xenobiotic contribute to further variation in the BCF (5). Therefore, the complex nature of biological systems may preclude comparisons between the uptake of contaminants by biota and by the sampling devices.

The MCFs were also compared to the water solubilities, vapor pressure, and octanol/water partition coefficients of the xenobiotics. Unlike some trends observed with BCFs, no linear correlations were found. This is probably due to the tremendous variation in the literature values of these parameters. For example, one reference lists the aqueous solubility of 2,2′,4,4′,5,5′-hexachlorobiphenyl as 8.8, 1.2, 0.953, and 0.95 ppb at 20 °C (6). When the MCF data were analyzed, physical and chemical data for the seven compounds were tabulated and averaged. Due to wide variations observed in the values, statistical rejection of points was not performed. Therefore, values for the physical and chemical parameters could have been in error, preventing any linear correlations with the MCFs. On the other hand, nonlinear relationships between chemical/physical properties and BCF are prevalent in the literature. For example, various researchers (1, 7, 8, 9) have reported a nonlinear relationship between the log K_{ow} and log BCF.

Neither the pesticide molecular weights, the Hildebrand solubility parameters of solvents and membranes, nor their subtrahend, resulted in a correlation with the MCFs. Yet, when the log MCF was plotted against the number of chlorine atoms, the relative molecular length and Henry's law constant of the contaminant, linear relationships resulted. These data are illustrated in figure 11. The outlying point in the graph of Henry's law constant represents dieldrin, the only oxygenated compound in the study.

DDE
thalene
Lin
3.00
2.50
2.50





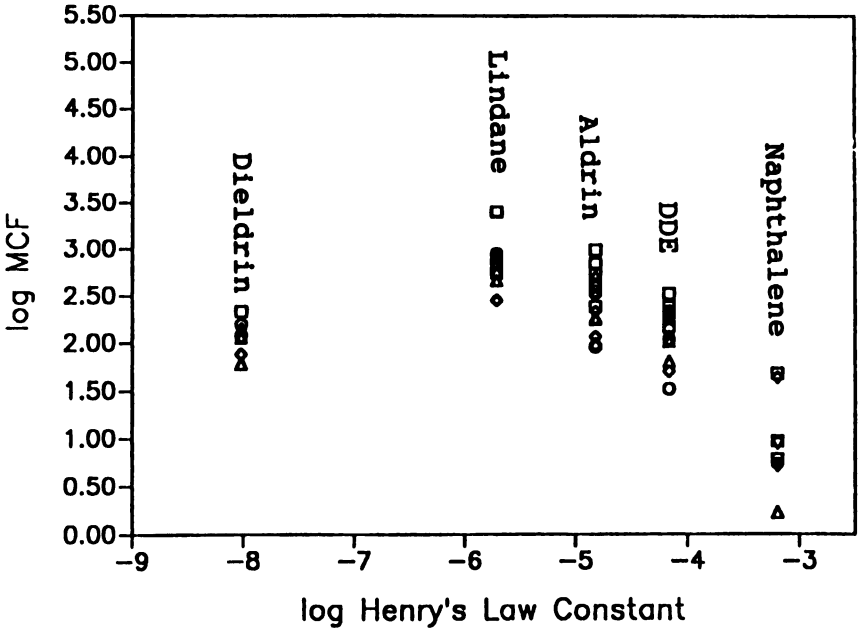


Figure 11:- Relationship Between Membrane Concentration Factor, MCF, and Physical Properties of the Contaminant.
Key: ▽ Toluene, ◇ Hexane, △ Octanol, □ Isooctane, and ○ Methanol.

*Points are for all viable solvent / membrane Combinations.

The number of chlorine atoms, the molecules' relative molecular length, and Henry's law constant affect the equilibrium concentrations of the contaminant in the sampling device. These effects are explained in the following discussion, in which a few assumptions are made (10, 11, 12, 13).

First, we will assume the limiting step in the transfer process is the introduction of the analyte molecule into the film at the aqueous surface. Energy must be expended to move the molecule into the clustered water layer at the surface of the film. Second, the membrane has been sufficiently swollen by contact with the solvent and hence, the pore size and crystallinity of the membrane are not limiting factors. All the pesticides used in these studies are below the exclusion limit of the membranes and should readily penetrate the sampling devices. Therefore, membrane contributions to the transport process are assumed to be negligible.

Solution thermodynamics (11, 12, 13, 14) states that the analyte molecules are uniformly distributed in the bulk phase so the net intermolecular forces on a given molecule approach zero. When the contaminant molecules approach the thin film of water adjacent to the membrane surface, they encounter an imbalance of forces. The water molecules closest to the membrane are farther apart and exhibit smaller forces of attraction for the analyte, creating a tendency for the contaminant to move back into the bulk phase. If kinetic energy is expended, the analyte molecule may enter the film. Once in the film, the analyte molecules move only laterally, instead of three dimensionally. Hermann (12) postulated that this limited motion may be caused by an aqueous film that is a single layer in thickness. This lateral motion has the effect of exerting a two-dimensional pressure on the surface of

the aqueous film, and limits the tendency of other molecules to move into this film. Once molecules are in the film, they may diffuse through the membrane and into the solvent. Eventually, an equilibrium will be established between the analyte molecules in the bulk aqueous phase, the aqueous film, the solvent film, and the solvent phases. Factors influencing the concentration of the analyte molecules in the aqueous film will concomitantly affect the equilibrium concentrations in the solvent inside the sampling devices. Hence, factors that influence the aqueous film concentrations of analyte will affect the MCF.

The concentration of analyte molecules in the aqueous film is always inversely proportional to the concomitant decrease in surface tension of the aqueous film (13). This may be due to disrupting the asymmetry the water molecules and altering the orientation of their dipoles. Alterations in the hydrophobic bonding at the surface of the water also may be responsible for the decrease in surface tension (10, 11, 12, 13).

Anyhow, as the chain length of a series of penetrant molecules is increased, the surface tension of the aqueous film is decreased (13, 14). Therefore, as the length of a molecule increases, a lower concentration is required to cause a given decrease in the surface tension. Therefore, the larger pesticide molecules may have formed a more dilute solution in aqueous boundary layer, decreasing the equilibrium concentration inside the sampling devices. Hence, MCF decreased as molecular length increased. Another possible explanation for this relationship is that as length is increased, there is a greater probability that the analyte molecule will interact with hydrophobic regions on the membrane through dispersion forces (14). Greater molecular length may increase size and limit negotiation through

the pores of the membrane. However, previous studies (15) using reverse osmosis systems containing ethylacetate have shown that molecules exceeding the pore size easily passed through the membrane, possibly due to a fluctuation in pore size as a function of time.

Figure 11 also illustrates a relationship between Henry's law constant and the molecules tendency to be accumulated by the devices. The data respesent the values for all viable solvent/membrane combinations. The outlying point in the graph corresponds to dieldrin, the only oxygenated compound in the study. If additional oxygenated compounds were examined, another series of data points is anticipated that would fall in line with the value for dieldrin, the polarity of the oxygen atom limiting membrane penetration.

If Henry's law constant (H) is defined as a partition coefficient between gas and liquid phases in dilute solutions, a relationship between MCF and Henry's law constant can be developed. McKay (16, 17, 18) has used the concept of fugacity to describe the partitioning of xenobiotics between compartments in the environment. McKay assumed that upon reaching equilibrium, the fugacity in all compartments would be equal. Resistance to transfer between the compartments was assumed to be minimal. Fugacity, f (Pa) is defined as follows:

$$f = C / Z \qquad (5.2)$$

where C is concentration in mole/m^3 and Z is the fugacity capacity in moles/m^3Pa. The fugacity capacity for water, Z_W is equal to:

$$Z_W = C_W (H) \qquad (5.3)$$

where C_W is the aqueous concentration. These equations may be substituted into the membrane concentration factor expression (5.1) to yield:

$$MCF = \frac{C_S}{C_W} = \frac{f_S}{f_W}\frac{Z_S}{Z_W} = \frac{f_S}{f_W}\frac{Z_S}{Z_S}\ (H) \qquad (5.4)$$

Hence, an increase in Henry's law constant should result in an enlarged membrane concentration factor (15, 16, 17, 18, 19) because both quantities are describing the molecules' tendency toward increased entropy. Therefore, for an homologous series of compounds, the tendency for molecules to escape from the film at water-air interface also reflects their ability to permeate the polymeric membrane.

From previous discussions, it is expected that as the degree of chlorination on the molecule increases, the molecular size increases and contaminant uptake should decrease. As shown in figure 11, the molecules with a greater degree of chlorine substitution exhibited an elevated MCF. This relationship may be an anomoly because a nonhomologous series of compounds was examined; molecular shape, size and polarity are all varied. Also, the compounds studied were either substituted with 0, 4, or 6 chlorine atoms and may not supply an adequate set of data. Another explaination is that a non-straightforward relationship exists between size, polarity and uptake by the devices.

The rate of transfer into the sampling devices is proportional to the slope of equation 4.27. When this equation was applied to the preceding data, a linear relationship resulted as shown in figure 12. First-order exchange constants proportional to the slope were calculated, and are listed in table 9. As shown in the table, the rate constants are all approximately the same. This is not surprising because interaction with the solvents has decreased the selectivity of the membranes. Also, the contaminants used in these studies have similar polarities and sizes. It seems as though the same phenomenon is occurring to limit the passage of these molecules through the membranes.


ln(Ceq−Cs)−lnCeq
ln(Ceq−Cs)−lnCeq

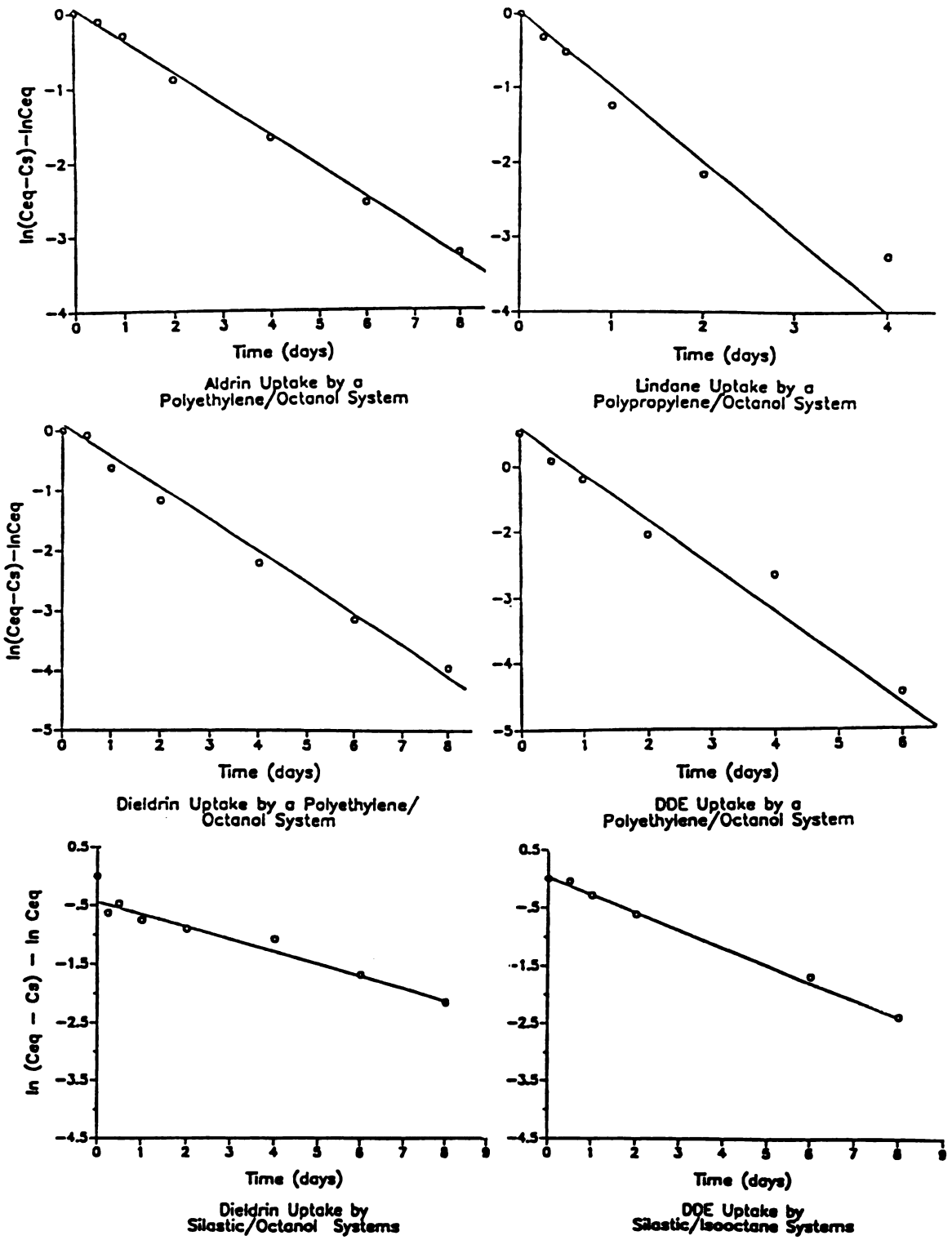


Figure 12: Examples of the Results of the Application of Equation 4.27 to the Contaminant Uptake Data.

No correlation between the first-order exchange constant and any of the preceding physical and chemical parameters was noted. The degree of selectivity based on the rate of uptake is low indicating a decreased degree of crystallization in the membranes.

CONCLUSIONS

Optimal membrane/solvent combinations include polypropylene and polyethylene sampling devices filled with 2,2,4-trimethylpentane. Equilibrium with the bulk aqueous concentration of contaminant is approached after approximately 4 days in the exposure system developed. Alterations in the contaminant levels in the water are reflected by changes in the concentration of the sampler contents; thus, the devices supply time-integrated estimates of contaminant levels. The partitioning phenomenon observed in pilot studies is dependent on the ability of the contaminant molecules to enter the thin aqueous film adjacent to the membrane. Factors influencing the concentration in this layer will affect concentrations in the sampler. Factors which affect membrane passage have not been quantitated. Resistance to transfer, presumably in the membrane, acts to impede solute uptake. Because the membranes used in these studies have a variety of functional groups on the polymer chains, and the same resistance to transfer was observed with all membranes, passage may be hindered by dispersion forces between the xenobiotic and the membrane.

CHAPTER 5

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CHAPTER 6

THE EFFECT OF ENVIRONMENTAL PARAMETERS ON CONTAMINANT UPTAKE

INTRODUCTION

This series of experiments was conducted to ascertain the effects of external parameters on the uptake efficiency of several contaminants into the polypropylene/2,2,4-trimethylpentane sampling devices. The parameters investigated included temperature, turbulence, ionic strength, pH and the presence of humic materials in the incubation system. Background information regarding the effects of humic acids on xenobiotic partitioning into biota will be reviewed.

MATERIALS AND METHODS

The materials and methods used for these studies were identical with those used in previous experiments with the following exceptions:

TEMPERATURE

The temperature of the circulating water bath was varied from 15 to 25°C in increments of 5°. To establish the maximum time required to reach equilibrium at 15°C, contaminant concentrations in the polypropylene/2,2,4-trimethylpentane sampling devices were monitored at various time increments including 0.25, 0.5, 1, 2, 4, 6, 8, 10 days.

For the studies at 20 and 25°C, the samplers were allowed to equilibrate with their surroundings for four days before their contents were analyzed. The quadruplet samples were analyzed as previously described.

Whenever the temperature of the circulating water bath was altered, the system was allowed to equilibrate at the increased temperature for 24 hours before the miniature sampling devices were added. The temperature range from 15° to 25° C represented reasonable environmental temperatures. The temperature in the incubation chamber was monitored daily and ranged +/- 2° C.

TURBULENCE

The turbulence was varied by altering the number of revolutions per minute (rpm) of a Talboy's model 101 stirrer from 0, 30, 45, to 60 rpm. The temperature was maintained at 20°C for all turbulence studies. Sampling devices were allowed to equilibrate for four days before the samples were taken.

IONIC STRENGTH

A 3.765 M solution of sodium chloride was prepared and added to the pesticide incubation mixtures in the following quantities: 10 ml, 100 ml, and 1,000 ml. Distilled water was added to maintain a constant volume of 37 L. The calculated ionic strengths were 1.017×10^{-1}, 1.017×10^{-2} and 1.017×10^{-3}, respectively. The solutions were mixed thoroughly, bottled and stored at 5°C for not longer than two weeks.

They were allowed to attain 20°C before being added to the incubation system. The stirring rate was maintained at 30 rpm. The sampling devices were allowed to equilibrate for four days before the samples were taken.

pH

Solutions of HCl, NaCl and NaOH were made in a concentration of 0.1 M. The pH of the incubation solutions was adjusted to 6.37, 7.26, and 8.48 by the addition of either HCl or NaOH. NaCl was added to maintain constant ionic strength. The solution was filtered through a glass microfiber membrane, bottled and stored at 5°C, as before. The remainder of the procedure was performed as previously described. The pH after the experiment was 5.80, 6.66, 7.58.

HUMIC MATERIALS

Humic laden water was collected from the Betsy River and the Two Hearted River in Michigan's upper peninsula. Additional water containing humic materials was collected from the Black River near Benton Harbor, MI. All the water was stored at 5°C. Three batches of solution were prepared, each with a total volume of 27 l. Each contained 5.71 l of water from the Betsy River, 14.35 l from the Black River, and 7.28 l from the Two Hearted River. These proportions were chosen based on the total available volume of natural waters. Twelve liters of distilled water were added to achieve sufficient volume in the

incubation system. The water was spiked with the organochlorine compounds and the pH was adjusted to 6.25, 7.80 and 8.48. Sodium chloride was added to maintain a constant ionic strength. The water was filtered to remove any precipitating humic materials, bottled and stored at 5°C. The remainder of the procedure was performed as previously outlined.

RESULTS AND DISCUSSION

TEMPERATURE

As expected, increases in temperature enhance contaminant uptake. As illustrated in figure 13, a linear relationship exists between the equilibrium concentration in the sampling devices and temperature. Table 10 summarizes linear regression of these data. The correlation coefficient, r, is greater than 0.99 for all the compounds studied, except DDE.

Table 10

Linear Regression Data for the Relationship Between the Equilibrium Concentrations of Organochlorine Compounds and Temperature

	b	m	r
Lindane	41.7	2.00	0.9983
Aldrin	57.7	2.90	0.9904
DDE	29.0	0.80	0.7559
Dieldrin	-57.0	6.10	0.9901
HCB	-17.3	5.10	0.9969
Methoxychlor	1.33	6.80	0.9930

If the slopes of these lines are compared with the inverse of the LeBas molar volume as shown in figure 14, it is apparent that as temperature

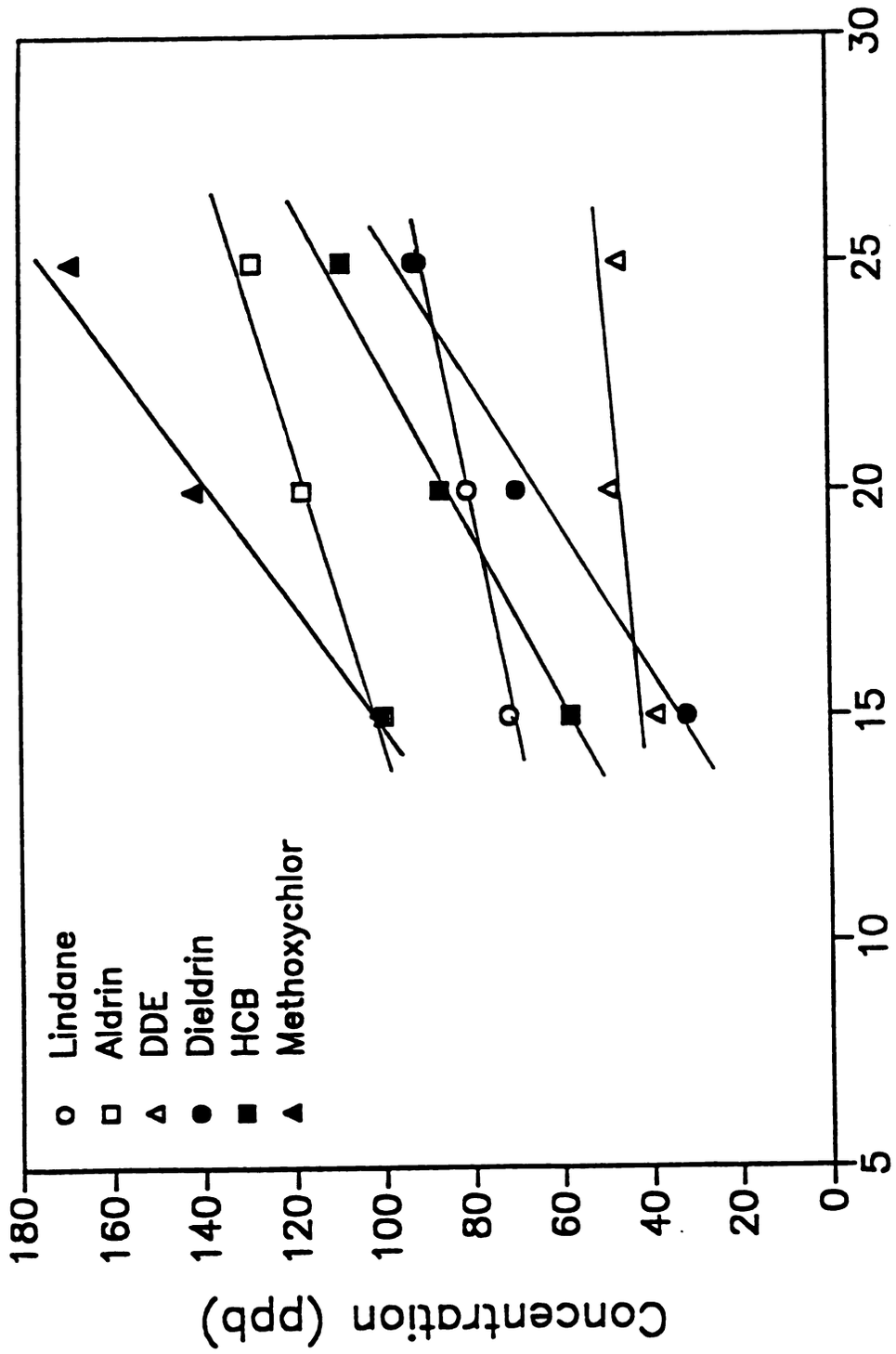

Figure 13: Contaminant Uptake as a Function of Temperature Using Polypropylene/2,2,4-Trimethylpentane Miniature Sampling Devices. The devices were exposed for 4 days and the incubation chamber was stirred at 30 rpm.

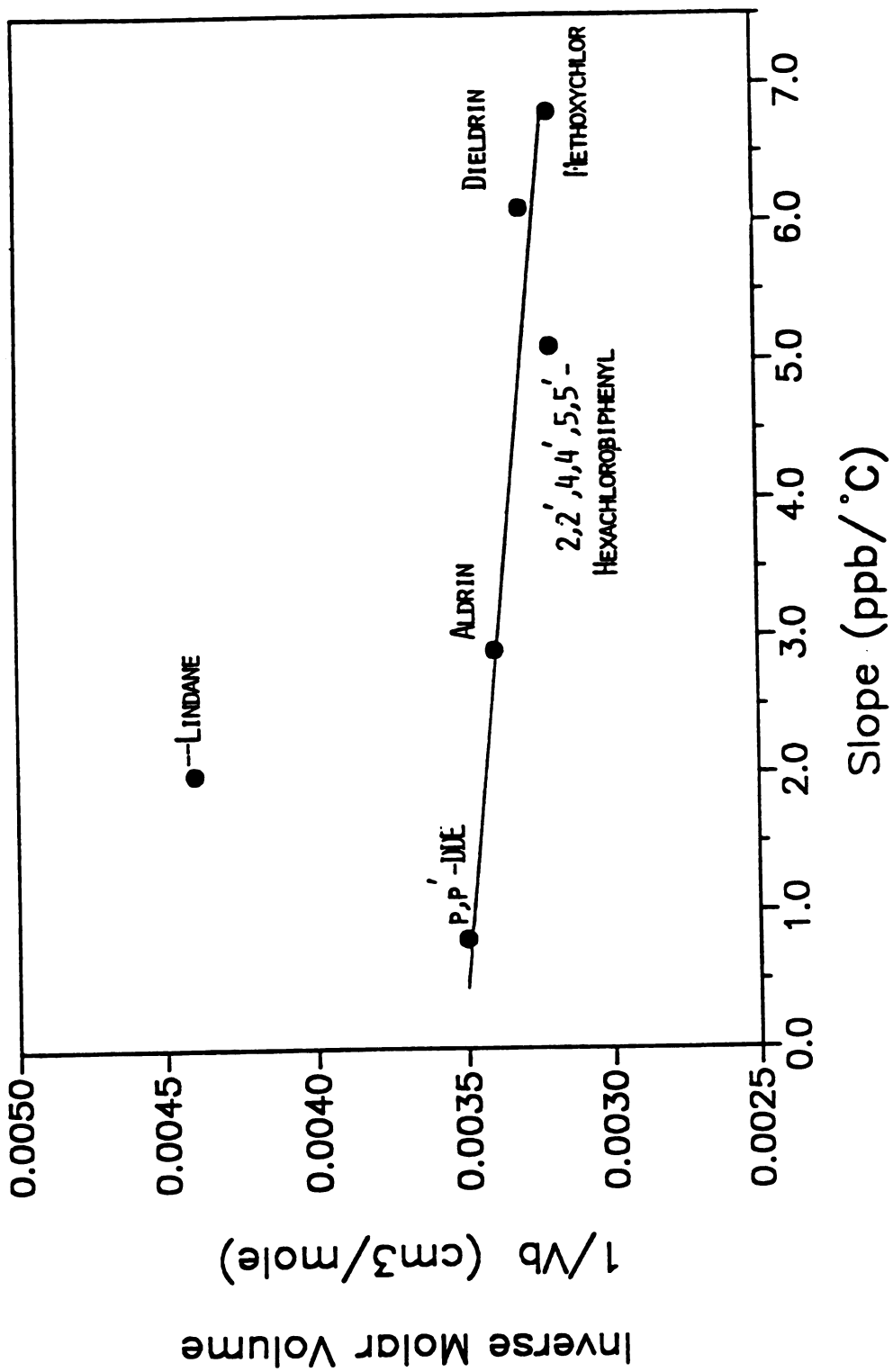

Figure 14: Temperature Dependent Change in Contaminant Uptake as a Function of the Inverse Molar volume.

is increased, larger molecules exhibit a greater change in mobility. Larger molecules may face greater restrictions negotiating passage through the membrane material. The added energy allows them to initiate greater flexibility in the membrane. Lindane is the only nonconforming point and is also the only monocyclic compound studied. The outlying position of this point could be caused by the correction factors used in estimating molar volumes for monocyclic systems. In spite of the data for lindane, increases in temperature supply the molecules with greater energy and enables them to pass through the membrane and into the solvent.

The temperature dependence of the diffusion coefficient, D and the solute solubility in the membrane, S_m is mathematically described below (1).

$$S_m = S\bullet \exp - \frac{\Delta H}{RT} \qquad (6.1)$$

$$\bar{D} = \bar{D}\bullet \exp - \frac{E_d}{RT} \qquad (6.2)$$

the preexponential factors, S• and $\bar{D}$• are of the form shown in equation 6.3 and ΔH is the enthalpy and E_d is the activation energy.

$$D\bullet = e\, \lambda^2 \frac{kT}{h} \exp \frac{\Delta S^{\ddagger}}{R} \qquad (6.3)$$

λ is the distance between activated sites, k is the Boltzmann constant, T is absolute temperature, $\Delta S^{\ddagger}$ is the entropy of activation, h is Planck's constant and R is the gas constant. Although the membrane solubility and the diffusion coefficient influence contaminant uptake, a logarithmic relationship is not observed in the data found in figure 13

as the equilibrium concentration in the devices is not dependent upon the rate of transfer through the membrane.

Instead, the equilibrium concentration is dependent on the concentration of the contaminant in the thin film in the aqueous boundary layer and the ease with which the solute can insert itself into this layer. Opperhuizen, Serne and Van der Steen (2) investigated the distribution of chlorobenzenes from water to octanol or fish and found that the partitioning was described by the Gibbs free energy:

$$\Delta G = \Delta H - T\Delta S \qquad (6.4)$$

where G is the Gibbs free energy, H is the enthalpy and S is the entropy. Entropy is increased through the loss of hydration shells around the solute as the molecule passes from the aqueous layer into the solvent. This term of equation 6.4 becomes more negative as temperature is increased. The Gibbs free energy may become more negative, if the enthalpy for transport is also negative. Opperhuizen et al.(2), report a negative enthalpy for the partitioning of chlorobenzenes into octanol. However, they also reported positive enthalpies for the uptake of chlorobenzenes into fish. Thus, as temperature is increased, the free energy becomes more negative and tendency for transport becomes more probable.

TURBULENCE

Figure 15 illustrates the rise in the contaminant concentration as the stirring rate is increased. The marked upswing in contaminant accumulation after approximately 45 rpm suggests there is sufficient

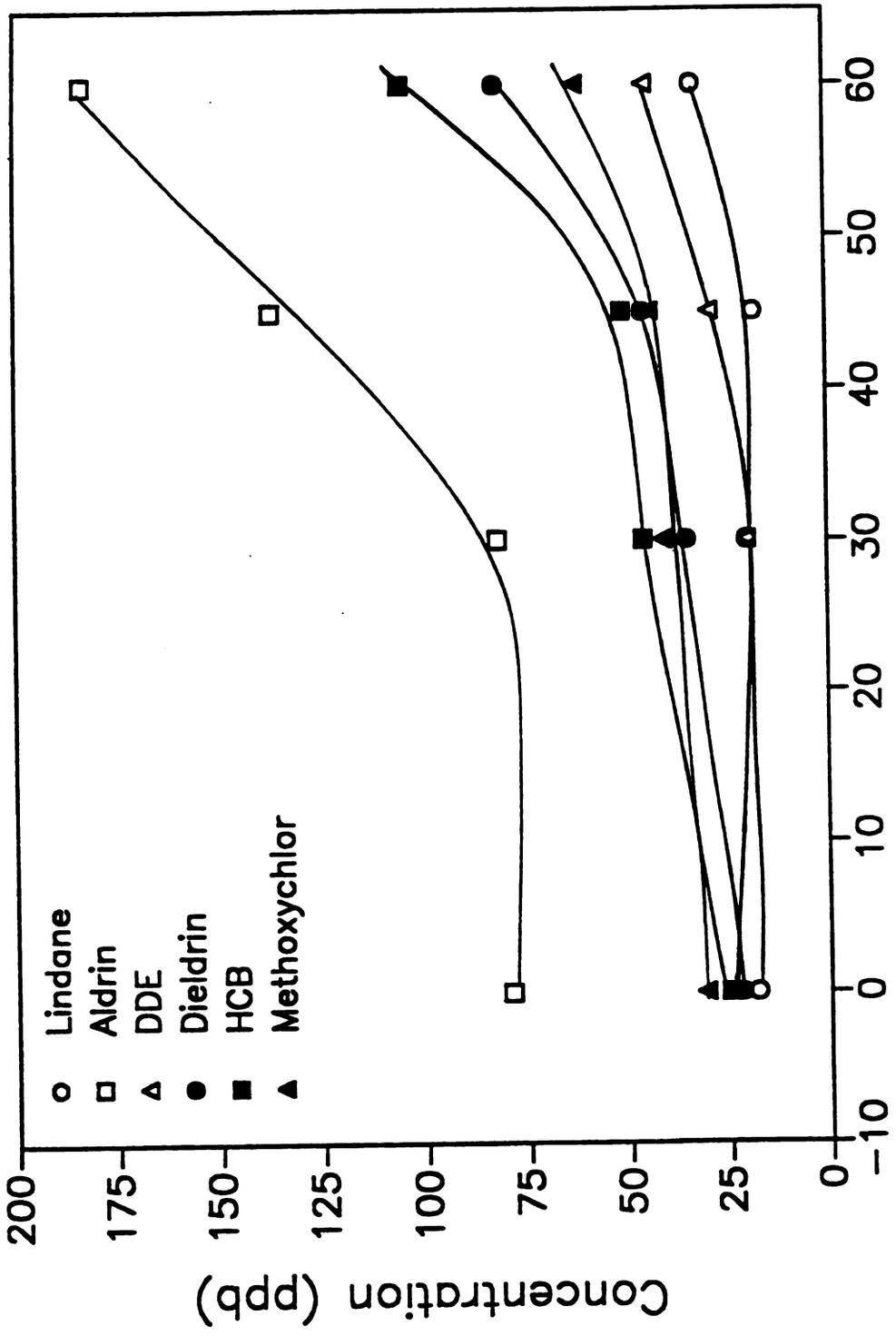

Figure 15: Effect of Turbulence on Contaminant Uptake by Polypropylene/ 2,2,4-Trimethylpentane Miniature Sampling Devices at 20° C.

turbulence to disrupt the boundary layer formed as a result of concentration polarization. This could pose a problem in environmental studies. Wave action may cause irregular fluctuations in the concentration of contaminants in equilibrium with the membrane. However, field studies performed at Saginaw Bay show the linear relationship between aqueous and solvent concentration is maintained, suggesting turbulence may not limit environmental applications of the device.

IONIC STRENGTH

Data in figure 16 show that there was no discernible correlation between the concentration of organochlorine compounds accumulated by the devices and the ionic strength of the incubation solution. Increases in the ionic strength in the aqueous phase should enhance contaminant uptake because of the increased chemical potential in the water. An increased transfer of tetrachlorobiphenyl from the aqueous to vapor phase was observed by Jota (3) as salinity was increased. Similarly, Tulp and Hutzinger (4) found that the addition of electrolytes to an aqueous solution decreased the solubility of nonpolar compounds. However, when a polycarbonate film was used, no differences between solute transfer in the control sample and one with an elevated ionic strength (8×10^{-3}. M NaCl) were noted. Therefore, the sampling devices may prove useful in geochemical applications, especially those that involve monitoring hydrocarbon residues in brine solutions.

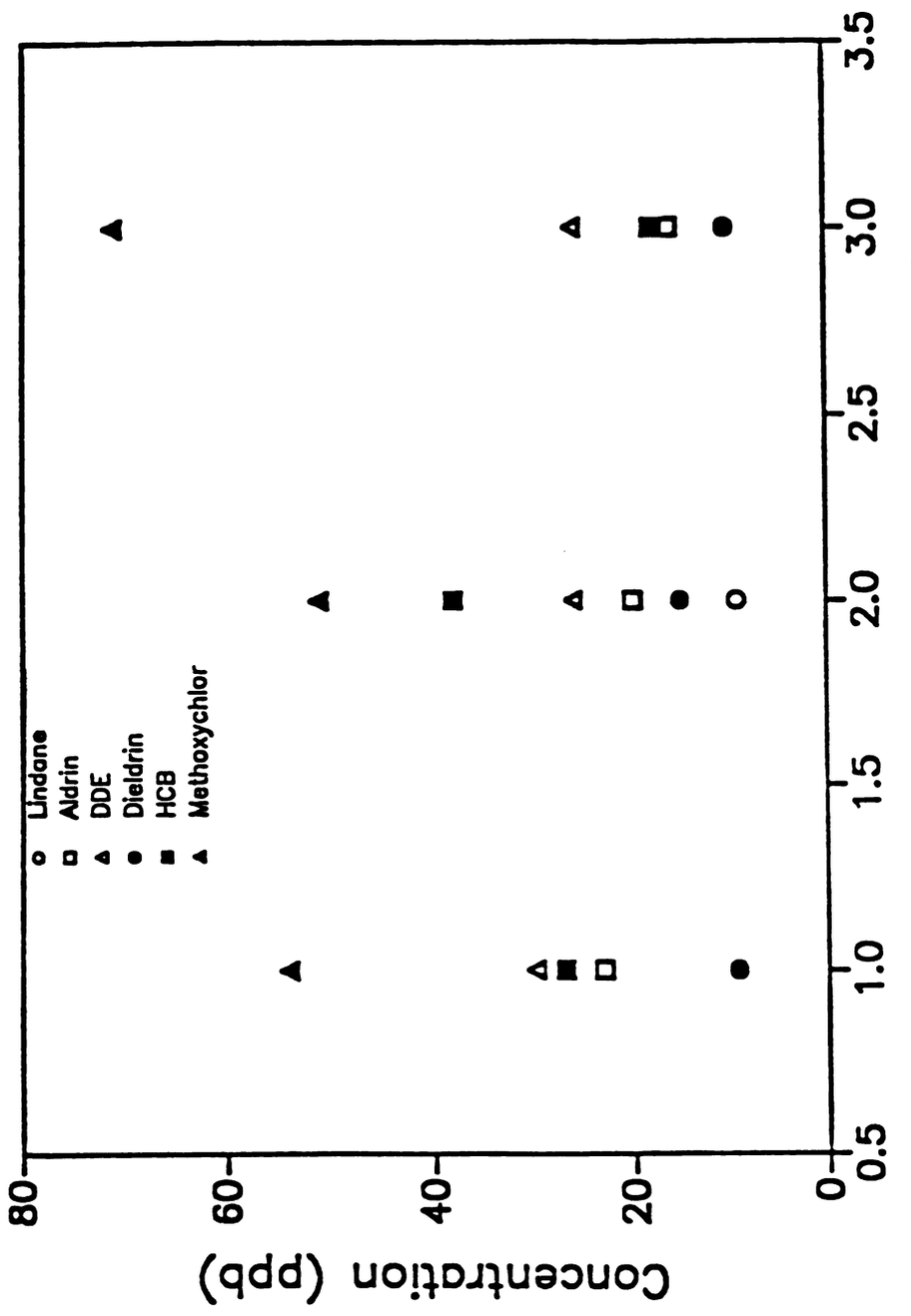

Figure 16: Uptake of Various Contaminants by Polypropylene/2,2,4-Trimethylpentane Miniature Sampling Devices as a Function of Ionic Strength. The temperature was maintained at 20°C and the incubation chamber was stirred at 30 rpm.

pH

Figure 17 shows the uptake of contaminants by polypropylene/2,2,4-trimethylpentane systems as pH is changed and the ionic strength held constant. There is no discernible relationship, but this is to be expected with nonpolar, non-ionizeable solutes. Blanchard and Hardy (5) also performed experiments to examine the effect altering the pH from 3.0 to 8.0 has on the membrane penetration by solutes, and found no relationship. This study was necessary to provide blank samples for the humics experiment.

HUMIC MATERIALS

The pigmentation of natural waters is caused by a class of dissolved substances, called humics, which are by-products of plant decomposition. Their precise chemical structure is unknown, but it has been estimated that 20-30% of the carbon atoms in humic materials are aromatic. The skeleton of humic materials consists largely of hydrocarbon molecules, but carboxyl, phenolic and methoxy functional groups are also present. Nitrogen atoms also may exist in the molecules (1-2%). The molecular weight of humic materials ranges from 800 to 250,000 g/mole as determined by field flow fractionation (6).

Humic substances play a role in the transport and fate of xenobiotics in the environment. Metals are strongly bound to humic materials. These substances also interact with nonpolar organic compounds such as petroleum hydrocarbons, 2,4,5-T, benzo(a)pyrene,

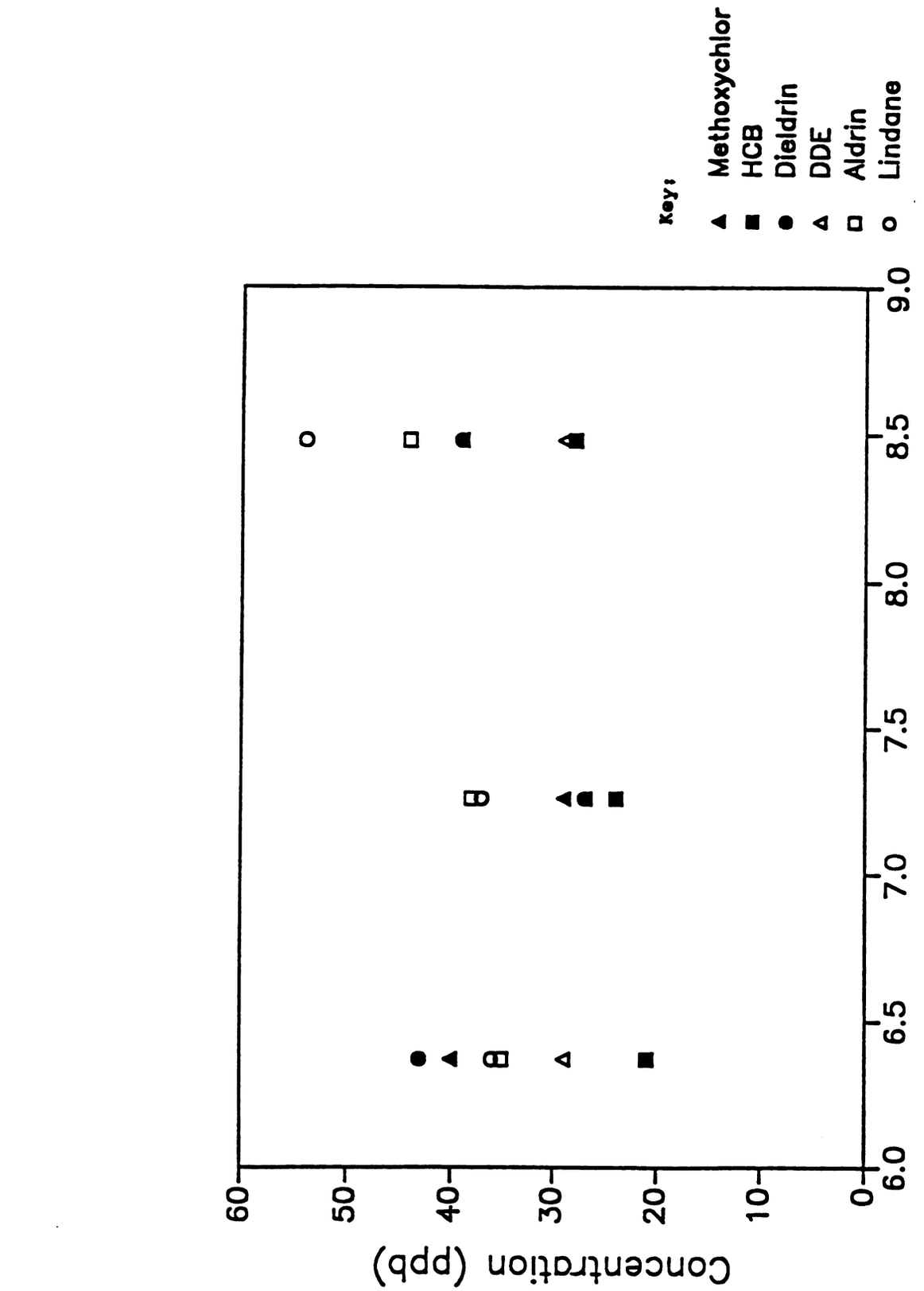

Figure 17: Uptake of Various Contaminants by Polypropylene/2,2,4-Trimethylpentane Miniature Sampling Devices as a Function of pH. The temperature was maintained at 20°C and the incubation chamber was stirred at 30 rpm.

phenanthrene, anthracene, DDT, phthalate esters and alkanes decreasing their bioavailability (6, 7, 8, 9, 10). However, Leversee et al. (10) noted an increase in the accumulation of methylcholanthrene by Daphnia Magna when humic materials were present. This was caused by increased adsorption on the surface of the D. Magna.

Two theories have been proposed to describe the interaction of humic substances with organic compounds. Nonpolar compounds may be adsorbed onto the surface of the humic substances or may be incorporated into a micelle, which is formed from humic materials. Aggregation of humic material may be enhanced by increasing the ionic strength of the solution, the counterions acting to stabilize the micelle (8). The validity of this second theory is doubtful because research has shown that DDT does not interact with micelles (7). In addition, ionic strength may influence the degree of polymerization and coiling of the humic material (11). Therefore, the ionic strength was maintained constant at 1×10^{-6} moles/L throughout these experiments. Thus, the presence of humics should decrease the uptake of contaminants by binding the xenobiotic and altering the equilibrium with the interior of the device. It should be noted that Blanchard and Hardy (5) did not note any effects of humic acids on the efficiency of uptake by their sampling device, which consisted of a polycarbonate membrane attached to two glass flowthrough chambers.

Humic materials have been subdivided further. Humic acid is defined by Wershaw, Burcor and Goldberg (7) as "alkali-soluble, acid-insoluble fraction of humus, while fulvic acid is the alkali-soluble acid-soluble fraction." Humic acid is practically insoluble below a pH of 7 or 8.

Hence, different moieties are soluble at different pHs, and the humic acid concentration can be varied by altering the pH. Thus, at pH 6, the lowest amount of humic binding to the contaminants should occur, maximizing the concentration of contaminants in the sampling devices. However, Carter and Suffett (11) found more DDT bound to humics at lower pH. This may be due to the chemical potential of the solution, although ionic strength and pH information was not given.

Figure 18 illustrates the uptake of contaminants by polypropylene/2,2,4-trimethylpentane miniature sampling devices that were exposed to a humic-laden incubation solution as a function of pH. As shown by the figure, there is no clear relationship between alterations in pH and contaminant uptake by the sampling devices. These data are compared to the concentration of organochlorine compounds accumulated by the devices when humic materials are absent from the incubating chamber in figure 19.

At a neutral pH, the uptake of lindane and dieldrin was markedly decreased by the presence of humic materials, while the accumulation of methoxychlor remained the same. The partitioning of remaining compounds, aldrin, DDE and 2,2′,4,4′,5,5′-hexachlorobiphenyl into the miniature sampling devices was slightly decreased. At this pH, some binding to humic acid should be occurring. The marked decrease in uptake of lindane by the sampling devices may be due to a size or shape specific interaction as lindane is the only mono-cyclic compound studied and has the lowest molar volume. Dieldrin may be interacting with the humic materials via the epoxy oxygen atom.

At a pH of 8, the elevated levels of humic and fulvic materials

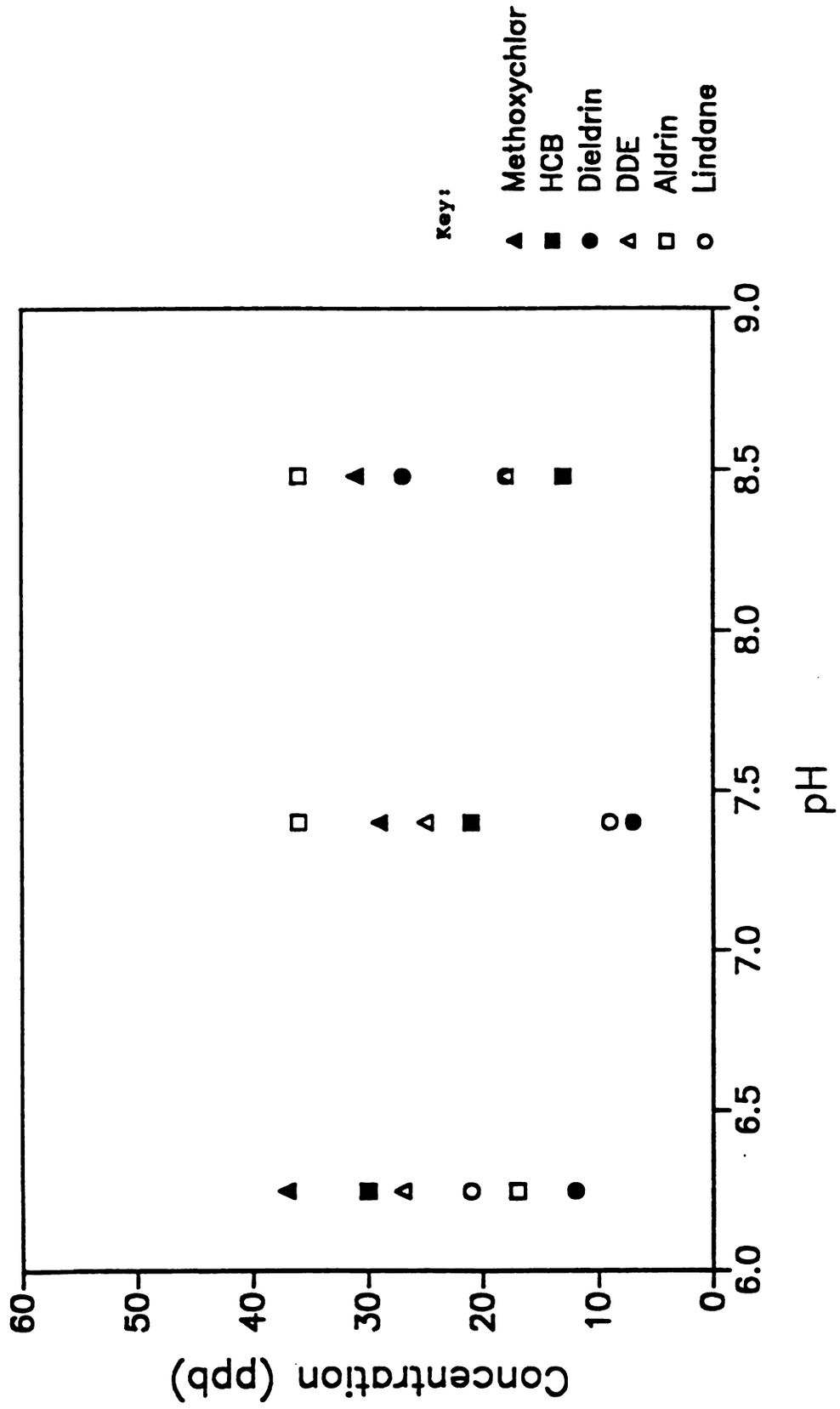

Figure 18: Uptake of Various Contaminants by Polypropylene/2,2,4-Trimethylpentane Devices Exposed to Humic Materials as a Function of pH. The temperature was maintained at 20°C and the incubation chamber was stirred at 30 rpm.

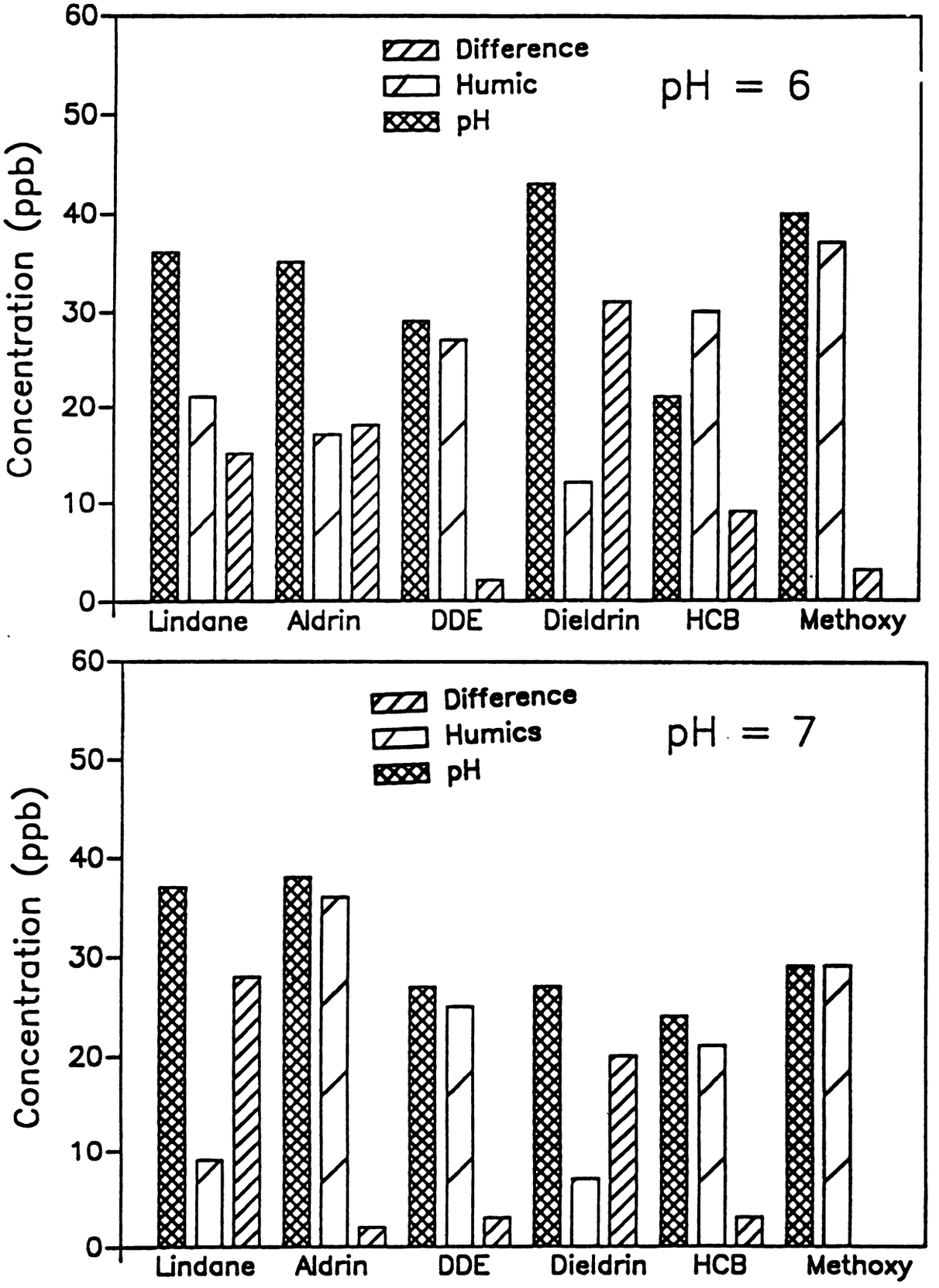

Figure 19: Uptake of Various Contaminants by Polypropylene/2,2,4-Trimethylpentane Devices at Different pHs wth and without Humic Materials. The temperature was maintained at 20°C and the incubation chamber was stirred at 30 rpm.

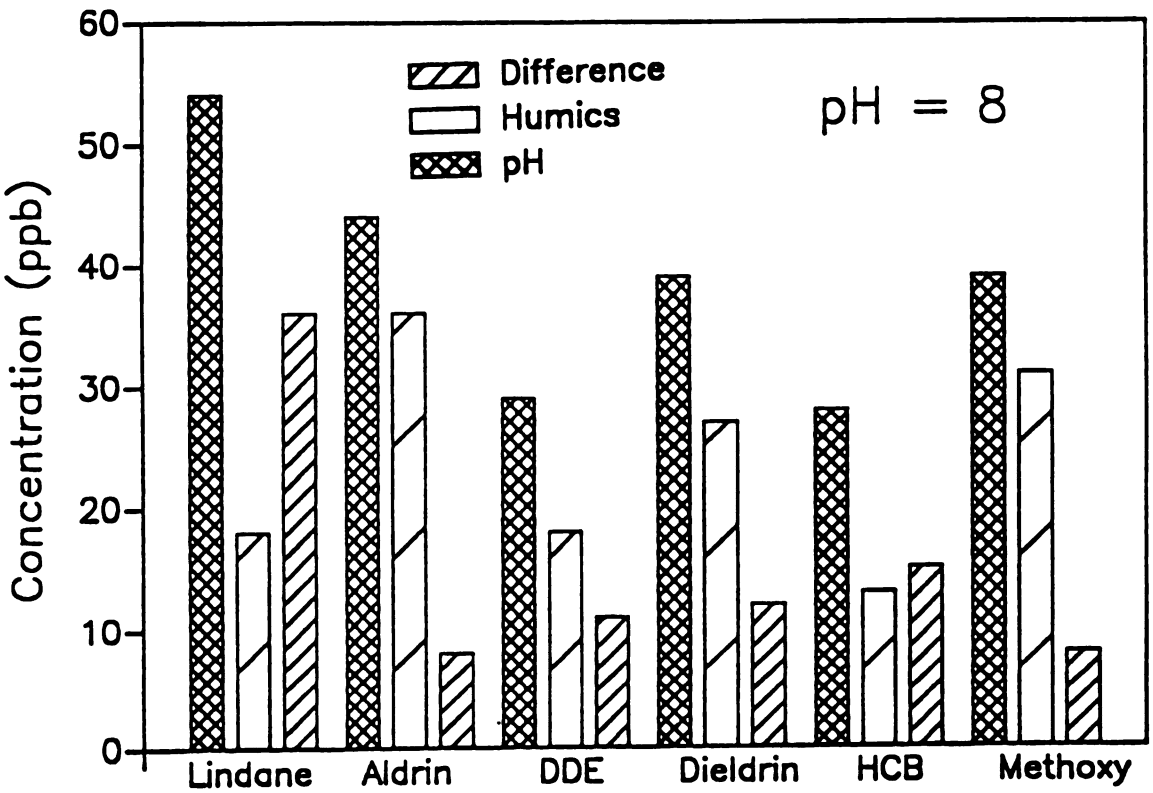

Figure 19: (Cont.) Uptake of Various Contaminants by Polypropylene/ 2,2,4-Trimethylpentane Devices at Different pHs wth and without Humic Materials. The temperature was maintained at 20°C and the incubation chamber was stirred at 30 rpm.

decreased contaminant levels in the sampling devices. The most pronounced reduction in solute accumulation was observed for lindane. The binding of this compound to humic and fulvic substances may be enhanced due to its reduced molar volume. Likewise, lindane and methoxychlor exhibited the least marked change in uptake by the devices and have the largest molar volumes. The remaining compounds that have similar molar volumes showed similar changes in uptake.

At a pH of 6, an increase in the accumulation of 2,2′,4,4′,5,5′-hexachlorobiphenyl was observed. The greatest decreases in uptake occurred for aldrin and dieldrin. This suggests that the shape of these molecules is more compatible with binding to fulvic acid. Lindane uptake into the devices is also markedly limited and may be due to a size-specific interaction with fulvic acids.

Blanchard and Hardy (5) added Aldrich^{R} humic acid to their solution at a concentration of 2500 ppm and found no change in membrane passage. These results may be due to differences between naturally occurring humics and those purchased from Aldrich. Blanchard and Hardy did not specify the pH, which could have been an influencing factor. Jota (3) found that as the pH was decreased, humic acids became more hydrophobic and the cationic sites were no longer charged. Binding to hydrophobic compounds increased, and resulted in limiting transfer from the aqueous phase. This was supported by Carter and Suffet (11). Although the range of molar volumes that were studied is limited, the data suggest a size selectivity. The uptake of compounds with the lower molar volumes is most susceptible to the presence of humic materials, this is due to the humic and fulvic micellular size and the contaminants' ability to enter

the hydrophobic center of the micelle.

CONCLUSIONS

Uptake of organochlorine compounds by the water sampling devices is influenced by temperature, turbulence, and the presence of humic materials. Ionic strength and pH did not affect this partitioning process. Larger molecules were most affected by increases in temperature; the added energy enhancing membrane passage. Although increases in temperature promote contaminant accumulation, the temperature in large bodies of water does not change rapidly over the course of a biomonitoring experiment. Therefore, contaminant uptake in field studies should not be greatly affected by temperature changes. Turbulence-limited concentration polarization and enhanced contaminant accumulation. Although, turbulence experienced in natural waters is expected to have a similar effect on the sampling devices, biomonitors are also susceptible. Exposure to humic materials decreased contaminant uptake in the devices as in biomonitors. The impact of these environmental parameters was studied further in field applications.

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CHAPTER 6

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CHAPTER 7

FIELD STUDIES

INTRODUCTION

To test the sampling devices under various field conditions, a series of feasibility studies was conducted at Buffalo Harbor in Buffalo, NY, Saginaw Bay at Bay City, MI and the Seney Wildlife Refuge in the upper peninsula of Michigan. The Buffalo Harbor study, conducted with Dr.John Adams, was performed to assess possible leakage from contained dredge fills (CDFs) operated by the U.S. Army Corps. of Engineers. The Saginaw Bay study was also performed at a contained dredge fill, housing sediments that contain polychlorinated biphenyls (PCBs). This study, which was conducted with Dr. Russell Kreiss of the Environmental Protection Agency, compared contaminant uptake by the passive-water sampling devices with conventional biomonitors including clams, crayfish, fathead minnows and Cladophora. The Seney Wildlife Refuge study monitored ground water contamination in a location with a history of oil application on the surrounding roadways. Sampling devices were buried at the refuge, which has a very high water table, at 1-2 feet below the soil surface. This study was performed with Mr. Timothy Kubiak of the U. S. Department of Fisheries and Wildlife.

BACKGROUND

The U.S. Army Corps of Engineers has constructed approximately 30 contained dredge fills (CDFs) throughout the Great Lakes Region. The CDFs house potentially contaminated sediment produced by dredging to maintain navigable shipping lanes and limit further environmental contamination. The majority of these CDFs are constructed without using a clay liner so exchange of contaminants with the surrounding environment may occur. One reason the research was performed at Buffalo Harbor was to confirm the containment of contaminants by the CDF wall. These pilot studies also determined whether the sampling devices could withstand environmental conditions and if they were a viable alternative to the use of biomonitors in the field. In addition, these studies allowed the behavior of partially and completely filled CDFs to be compared. The studies performed at Saginaw Bay were designed to evaluate the feasibility of biomonitoring at outdike locations and to develop methodologies to assess CDF performance (1).

The sampling devices were also utilized to assess environmental quality in an area with water saturated soil. The devices were buried at the Seney Wildlife Refuge to determine levels of petroleum hydrocarbons that had migrated from the soil surface. This study showed the utility of the sampling devices in diverse situations.

MATERIALS AND METHODS

SAMPLER PREPARATION

Polypropylene and polyethylene bags were prepared according to the following techniques. Polyethylene bags (Whirl-pak, 6 oz. size) were partially annealed in a diagonal fashion at the top. A Dazey ^{R} Seal-a-Meal II was used to apply heat for 9 seconds to form the seal. The solvent, 2,2,4-trimethylpentane (50 ml) was added to the bag using a funnel to guide the solvent through the small channel remaining in the top of the bag. After filling, the excess solvent residue in the channel was removed using cotton swabs. Air was evacuated from the sampler and another diagonal seal was made with the Seal-a-Meal II. The seals were tested by inverting the bags and inspecting them for leaks. Wide-mouth 16-oz bottles were filled with distilled water and one sampling device was placed in each jar. The bags were forced into the water using foil lined caps. These bottles served three functions: to cushion the bags, to prevent evaporation of the solvent and to pre-equilibrate the bags with water.

Polypropylene bags were formed using 100A polypropylene film (100 gauge) from Borden Chemical Company, North Andover, MA. The film was cut into 2 7/8" x 6" rectangles and a vertical seam was made using Silicone rubber sealant. The most effective method of glue application was to fill a disposable plastic syringe with the sealant and draw two parallel lines across the material, allowing a 3/4" seam. The bags dried for 24 hrs before the bottom seal was made in a similar fashion. The bottom

seal was secured by clips and allowed to dry for an additional 24 hours. A partial top seal was formed, and allowed to dry. The bags were filled with fifty milliliters of 2,2,4-trimethylpentane. A few drops of silicone glue sealed the filling channel, which was secured and allowed to dry. After 24 hrs the seals were tested by inversion and inspected for leaks. Properly sealed bags were placed in 16 oz bottles containing distilled water as were the polyethylene bags.

SAMPLER DEPLOYMENT AT BUFFALO HARBOR, BUFFALO, NY

Contaminant levels were assessed at four contained dredge fills (CDFs) that were in various stages of completion. Duplicate sets of the polypropylene and polyethylene bags were placed inside and outside the contained dredge fills, and in the CDF wall itself. Additional samplers, acting as blanks, were suspended in the water column inside the outer harbor wall. The samplers located outside the CDFs were held in place by mesh bags that were attached to weights and marked with buoys. Devices placed into monitoring wells inside the completed CDF at the small boat harbor, were weighted and lowered into the wells using polyethylene twine. The samplers remained in place for 14 days.

DEPLOYMENT OF SAMPLERS AND BIOMONITORS AT SAGINAW BAY, SAGINAW, MI

Three metal cages, containing four compartments and a lid were covered with chicken wire. Twelve polyethylene devices were constructed as previously described and filled with 50 ml of either 2,2,4-

trimethylpentane or hexane. Four bags were tethered in one of the compartments of each of the cages to prevent abrasion. Crayfish, fathead minnows, and clams were placed into each remaining compartment. Cladophera, an aquatic plant, was added to the compartment housing the crayfish. The cages were submerged at a control site (Buoy 29), as well as inside and outside the contained dredge fill. The cages remained in place for 8 days.

SAMPLER DEPLOYMENT AT SENEY WILDLIFE REFUGE, UPPER PENINSULA, MI

Twenty-two polyethylene/2,2,4-trimethylpentane sampling devices were buried at various sites at the wildlife refuge. Careful placement ensured that half the devices were fully submerged below the water table, while the remainder were just above their counterparts. Although these bags were not placed below the water table, they were surrounded by very moist soil. The samplers were carefully retrieved after 7 days.

SAMPLER COLLECTION

The sampling devices were removed from various sites, rinsed in water to remove soil and wiped dry. The top was cut and the contents were transferred to glass bottles, and stored on ice.

Half the samples from the study at Buffalo Harbor were sent to Aquatech Environmental Consultants, Inc., for analysis. Duplicates were analyzed at Michigan State University.

Water samples, biomonitors and the contents of the bags were placed

on ice and transported to EPA's Large Lakes Research Station (LLRS) where the polychlorinated biphenyl (PCB) content was determined by isomer specific analysis.

The samplers left at the Seney Wildlife Refuge were carefully retrieved and the contents removed as previously described. The samples were placed on ice and returned to Michigan State University, where they were analyzed.

ANALYTICAL METHODS

The samples from the study at Buffalo Harbor were subject to analysis without the use of cleanup or preconcentration procedures. A Varian gas chromatograph containing a 30 m DB-1 column, and an electron capture detector was used to perform out the analysis. A temperature program from 130 to 270 °C at a rate of 0.5 degrees/minute was employed.

At the EPA's Large Lakes Research Station the biomonitors were weighed, mixed with sodium sulfate, and extracted with n-hexane. Gel permeation chromatography was used to clean the extracts from the biomonitors as well as solvent from the sampling devices. The water samples were subjected to a three-step liquid/liquid extraction process and were also cleaned using gel permeation chromatography. All the extracts were concentrated before they were subjecteed to analysis. Isomer specific analysis was conducted using a Varian Model 3700 gas chromatograph that was equipped with a 60-m DB-5 capillary column and an electron capture detector (1, 2, 3, 4).

Samples recovered from the Seney Wildlife refuge were anaylzed by gas chromatography and/or by gas chromatography/mass spectrometry. A Perkin-Elmer 8500 gas chromatograph, equipped with a 60-m DB-1 column and an electron capture detector was programmed from 188 to 280°C at a rate of 2°C/min and used to perform the analysis. A Nermag 1010-C mass spectrometer was also used.

RESULTS AND DISCUSSION

BUFFALO HARBOR, BUFFALO, NY

Although wave action against the rocky surfaces of the CDFs abraded some of the devices that were primarily located on the outside and within the walls, enough samplers survived to test the integrity of the CDFs. As a result, alternate deployment procedures for unprotected areas were developed. All the devices placed in access wells within the filled CDF and those located in the harbor itself, were recovered. None of the devices exhibited signs of algal growth or biofouling of the membrane surface. This was due to the formation of a thin film of solvent on the surface of the bag.

The most striking results were obtained from devices that were recovered from the filled CDF at the Small Boat Harbor. When the samplers were removed from the interior of this site, the contents were a brightly-colored fluorescent yellow. The solvent contained in samplers recovered from within this wall and from outside the CDF appeared clear. The contents of the devices were subjected to analysis. Typical

chromatograms are shown in figure 20, which compares the contents of both polypropylene (top) and polyethylene (bottom) samplers from the interior of the CDF.

Not only were the polypropylene devices more efficient at accumulating contaminants than the polyethylene bags, but separation of the contents showed more components. The contents of these polypropylene bags as well as water samples were subject to analysis by gas chromatography/mass spectrometry. The results of the analysis of two duplicate samples from one test well were averaged, the corresponding MCFs were calculated and are listed in table 11. The relative standard deviations (rsds) ranged from 3.9 to 22.8%. In order to fit within the access well housing, the bags were tied head to tail forming a long chain. This vertical stratification contributed to the observed rsd. Polynuclear aromatic hydrocarbons (PAHs) were readily accumulated by the polypropylene bags, resulting in MCFs from 2,000 to 4,000.

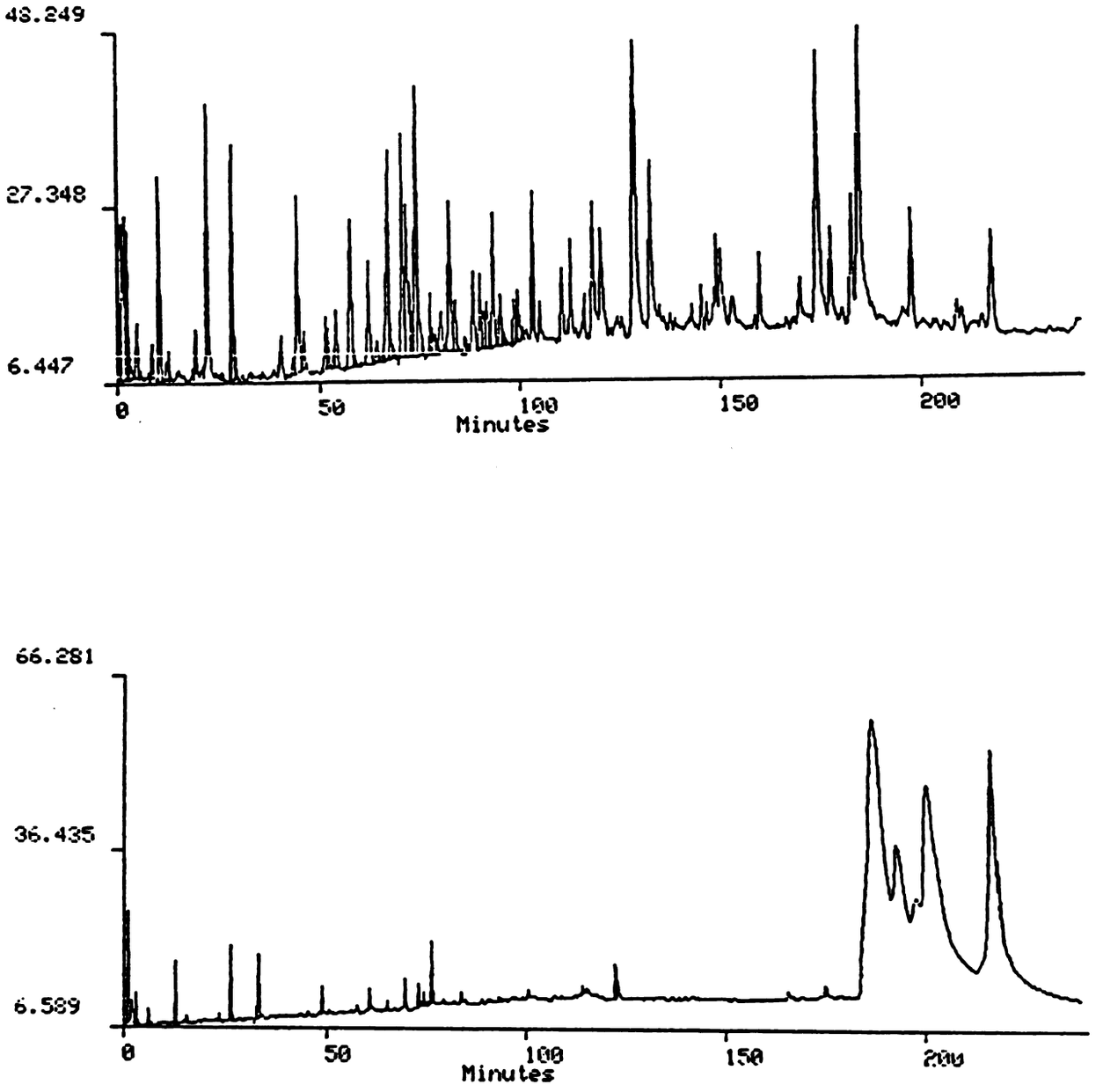

Figure 20: Uptake of Contaminants by Sampling Devices Placed Inside a Completed Contained Dredge Fill at Buffalo Harbor, Buffalo, NY. The top chromatogram was obtained from the analysis of the contents of a polypropylene/2,2,4-trimethylpentane. The bottom chromatogram was obtained from the analysis of the contents of a polyethylene/2,2,4-trimethylpentane sampling device. The temperature was programmed from 170 to 260°C at a rate of 2°/min. A 30-m DB-5 column and an electron capture detector were used.

Table 11

Summary of Results From Polypropylene/ 2,2,4-trimethylpentane Sampling Devices Placed Inside the Filled CDF at Buffalo Harbor, Buffalo, NY

Compound	Concentration (mg/ml) Water	Average Sampler	rsd, %	MCF
Acenaphthene	0.04	126	10.2	3150
Anthracene	0.03	118	14.4	3930
Fluoranthene	0.03	245	13.3	3300
Fluorene	0.034	96.2	8.5	2830
Phenanthrene	0.081	189	16.1	2330
Pyrene	0.025	84	11.6	3360
1,2-Dichlorobenzene	0.021	19.3	19.4	919
1,4-Dichlorobenzene	0.12	88.8	11.1	740
Benzo(a)anthracene	< 0.08	44.4	11.8	> 555
Benzo(a)pyrene	< 0.03	21.6	8.8	> 720
Benzo(b)fluoranthene	< 0.025	49.3	6.2	> 1970
Chrysene	< 0.03	31.9	15.5	> 1060
1,3-Dichlorobenzene	< 0.02	5.9	22.8	> 230
1,2,4-trichlorobenzene	< 0.019	3.6	3.9	> 340
Octyl phthalate	0.051	2*	---	40

*=single sample

Chlorinated benzenes were also sequestered by the bags, resulting in an MCF of approximately 800. More polar compounds such as octyl phthalate were not accumulated to such an extent, illustrating the selectivity associated with the bags.

The bottom chromatogram in figure 20 shows a group of broadly tailing peaks that eluted at 200 min. Even after blank samples were concentrated ten-fold, peaks at this retention time were not detected, nor were they present in any of the other samples. The identity of the compounds represented by these peaks remains unknown.

Similar compounds were present in the samples taken from the

interior of remaining CDFs. The data also indicated that compounds were not leaking from the CDFs.

SAGINAW BAY, SAGINAW BAY, MI

The polyethylene/hexane bags requested by the EPA were placed in the Saginaw Bay according to the map shown in figure 21. The control site, at buoy 29 was located near shipping lanes. The remaining cages were placed on either side of the CDF, near a small cove. Analysis of the isomer specific data did not yield any relationships between the shape of the solute and uptake by the devices. Yet, when the concentrations of PCBs accumulated by the devices were averaged according to the extent of chlorination and compared to the aqueous levels, linear relationships resulted at all the sites. An example using data from the site inside the CDF is illustrated Figure 22. The large degree of scatter in the data was caused by variation in the determination of water concentrations of PCBs at such low levels.

The tendency of biomonitors to accumulate contaminants is compared with uptake by the sampling devices in figure 23. The bioconcentration factors were calculated using the units of μg/kg whereas the MCFs for the bags were expressed in ng/l. As shown in the figure, no particular trend exists for the biological organisms, but the sampling devices exhibited a logarithmic relationship between the degree of chlorination and the MCFs. The linear regressions shown in figure 24 further illustrate this relationship. Thus, for a homologous series, the concentration of contaminants accumulated by the devices is related to

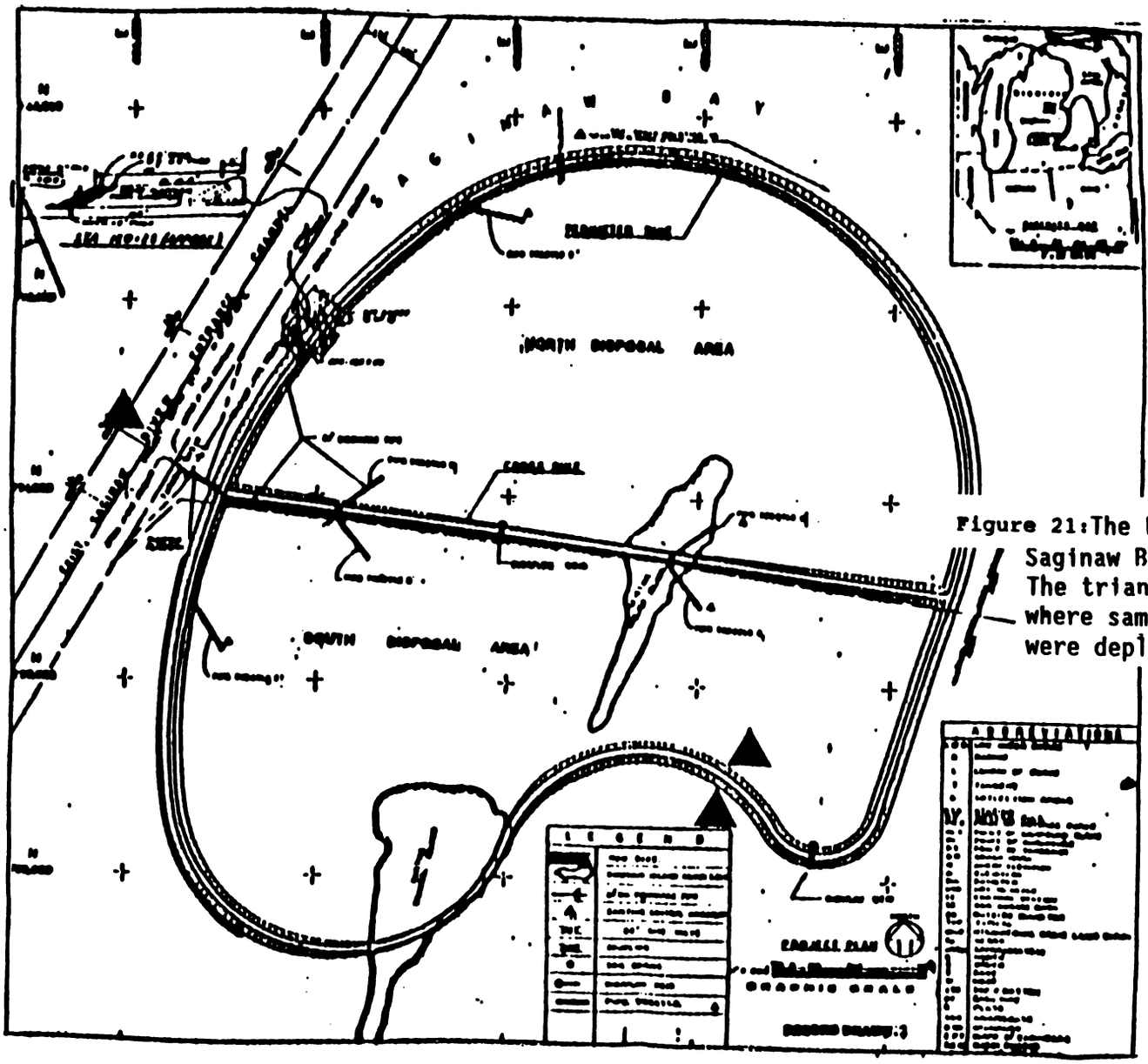

Figure 21:The Location of the Cages at Saginaw Bay Contained Dredge Fill. The triangles represent sites where samplers and biomonitors were deployed.

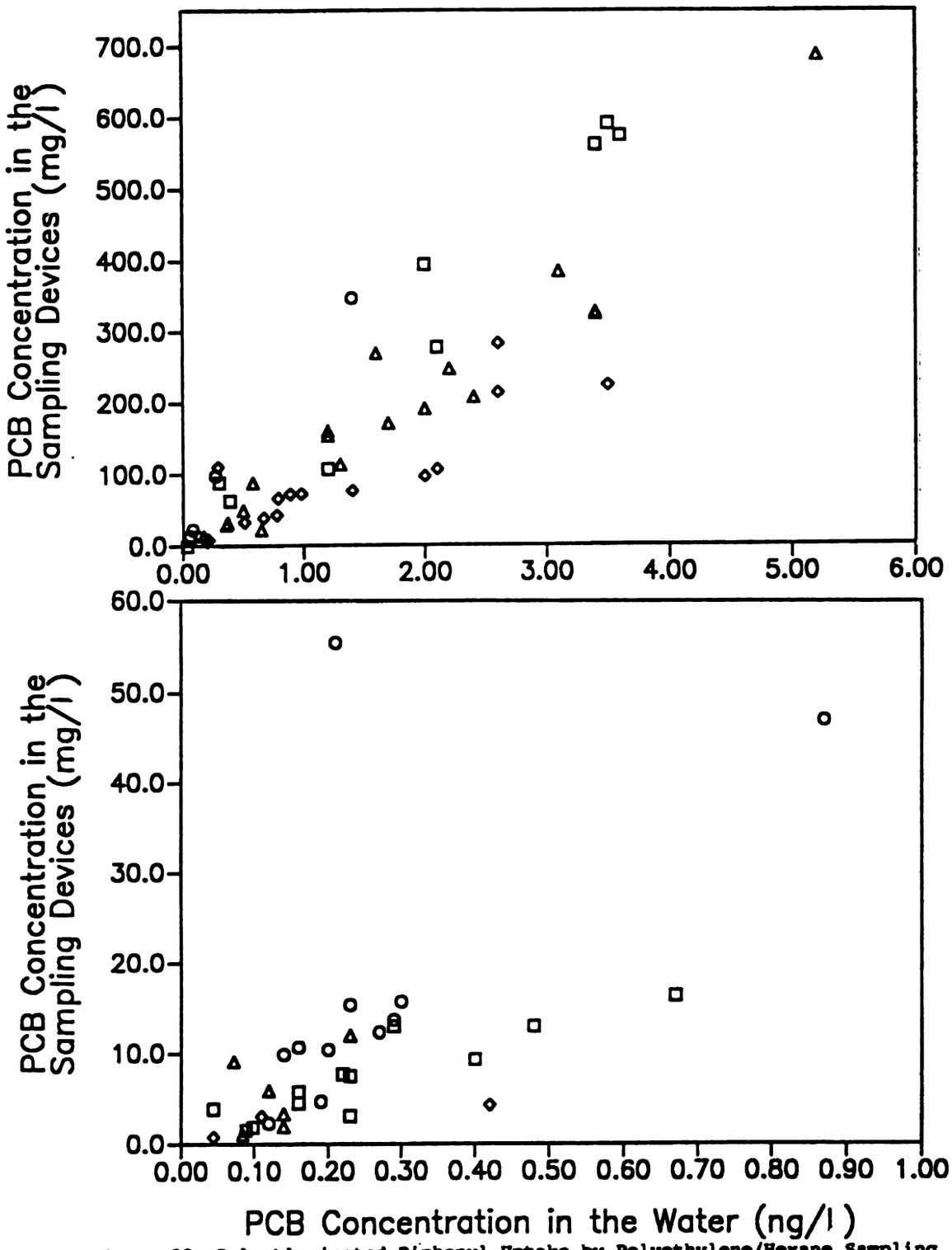

Figure 22: Polychlorinated Biphenyl Uptake by Polyethylene/Hexane Sampling Devices Placed Inside the Saginaw Bay Contained Dredge Fill. The top graph illustrates uptake of dichloro to pentachloro substituted biphenyls.

key: ○ diclorobiphenyl, □ trichlorobiphenyl, △ tetrachlorobiphenyl and ◇ pentachlorobiphenyl isomers.

The bottom graph illustrates uptake of the more highly chlorinated isomers of biphenyl.

key: ○ hexachlorobiphenyl, □ hepachlorobiphenyl, △ octachlorobiphenyl, and ◇ nonachlorobiphenyl.

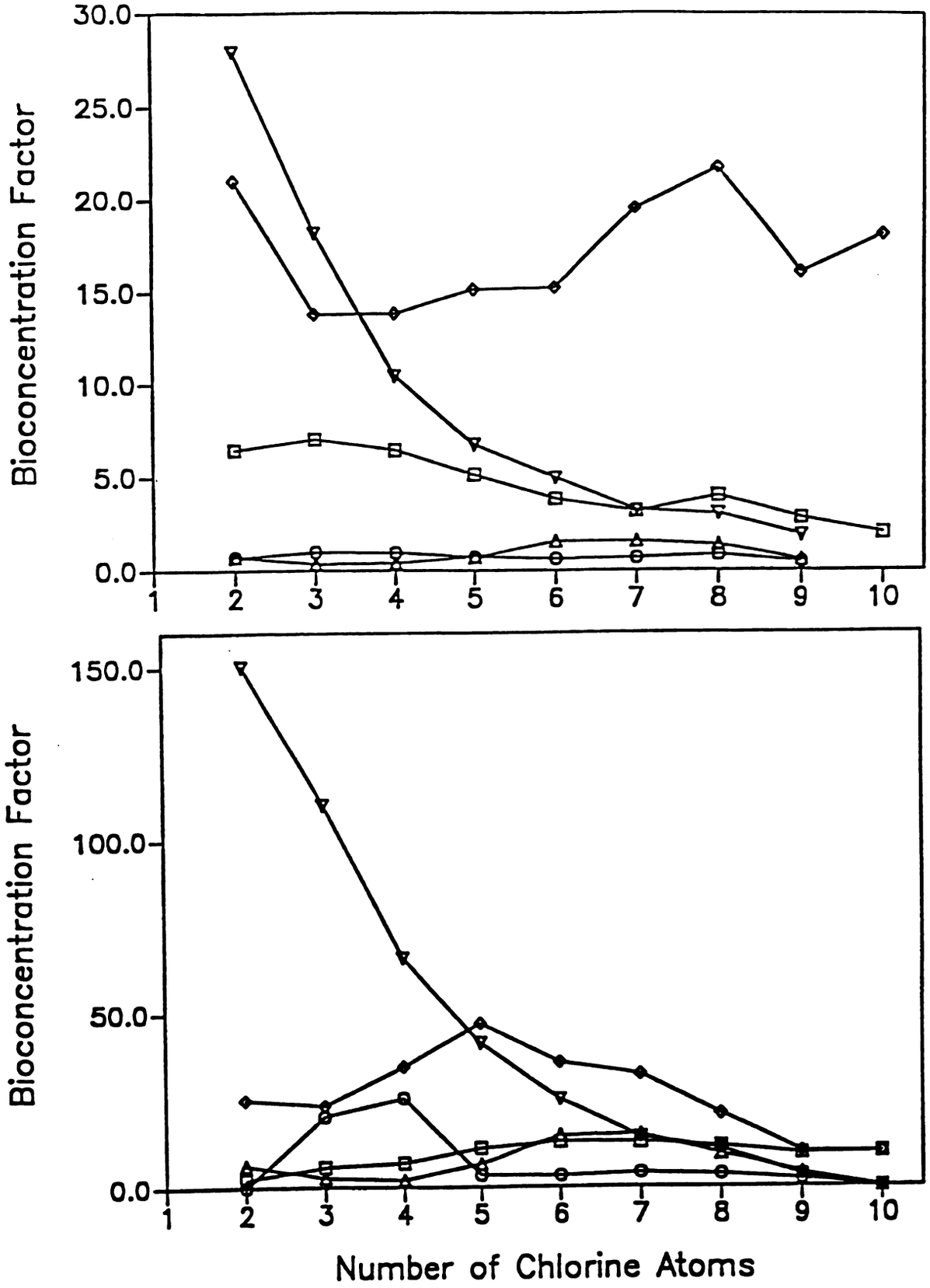

Figure 23--Bioconcentration Factors for a Variety of Species Accumulating PolychlorinatedBiphenyls as a Function of the Degree of Chlorination. The top figure was obtained using samples exposed to the inside of the Saginaw Bay CDF while the samples in the bottom figure were exposed on the outside of the CDF. Key: ▽ Passive water samplers, ◆ Cladophera, △ Crayfish, ○ Clams and □ Fathead Minnows.

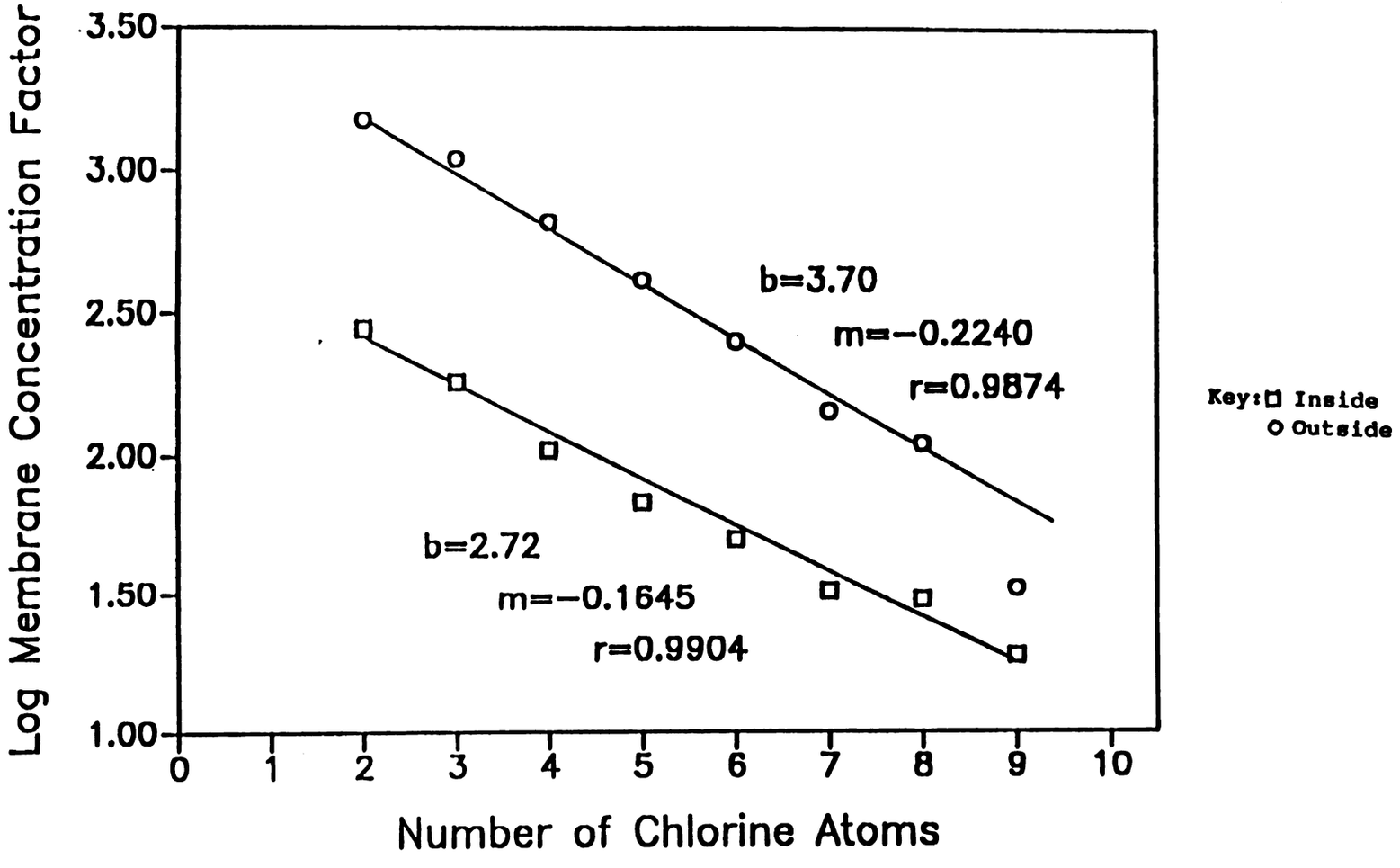

Figure 24: Membrane Concentration Factors as a Function of Degree of Isomer Chlorination Accumulated by Polyethylene/Hexane Sampling Devices Exposed to Polychlorinated Biphenyls at Saginaw Bay Contained Dredge Fill.

the degree of chlorination. Although changes in the degree of chlorination alter more than molecular size, previous studies in this dissertation also suggest that the molecular size is an important factor in contaminant accumulation.

Figure 25 illustrates the average RSD for the various biomonitors and the bags. Although the relative standard deviations of the passive water samplers and the clams were comparable, the devices showed greater precision than did the crayfish, fathead minnows and Cladophera. Again, much of the observed variation is probably due to inaccuracies in the determination of PCB concentrations in the water samples.

It is interesting that the accumulation of PCBs by all the biomonitors at the various sites was greatest inside the CDF and the lowest outside the CDF. The reference site should have shown the lowest levels, as some contaminants may be expected to leach through the CDF wall into proximate locations. The unusually high levels recorded at the reference site could have been caused by increased turbulence near the shipping lanes that access the Saginaw River.

SENEY WILDLIFE REFUGE, MI

Hydrocarbons are especially prone to binding with humic materials and although humic materials were expected to limit the uptake of contaminants and possibly prevent detection, hydrocarbons were found in the devices that were exposed to soil at the wildlife refuge. After retrieving the 36 devices, the contents of 6 were slightly yellow. These samplers were exposed to primarily sandy soil that contains less

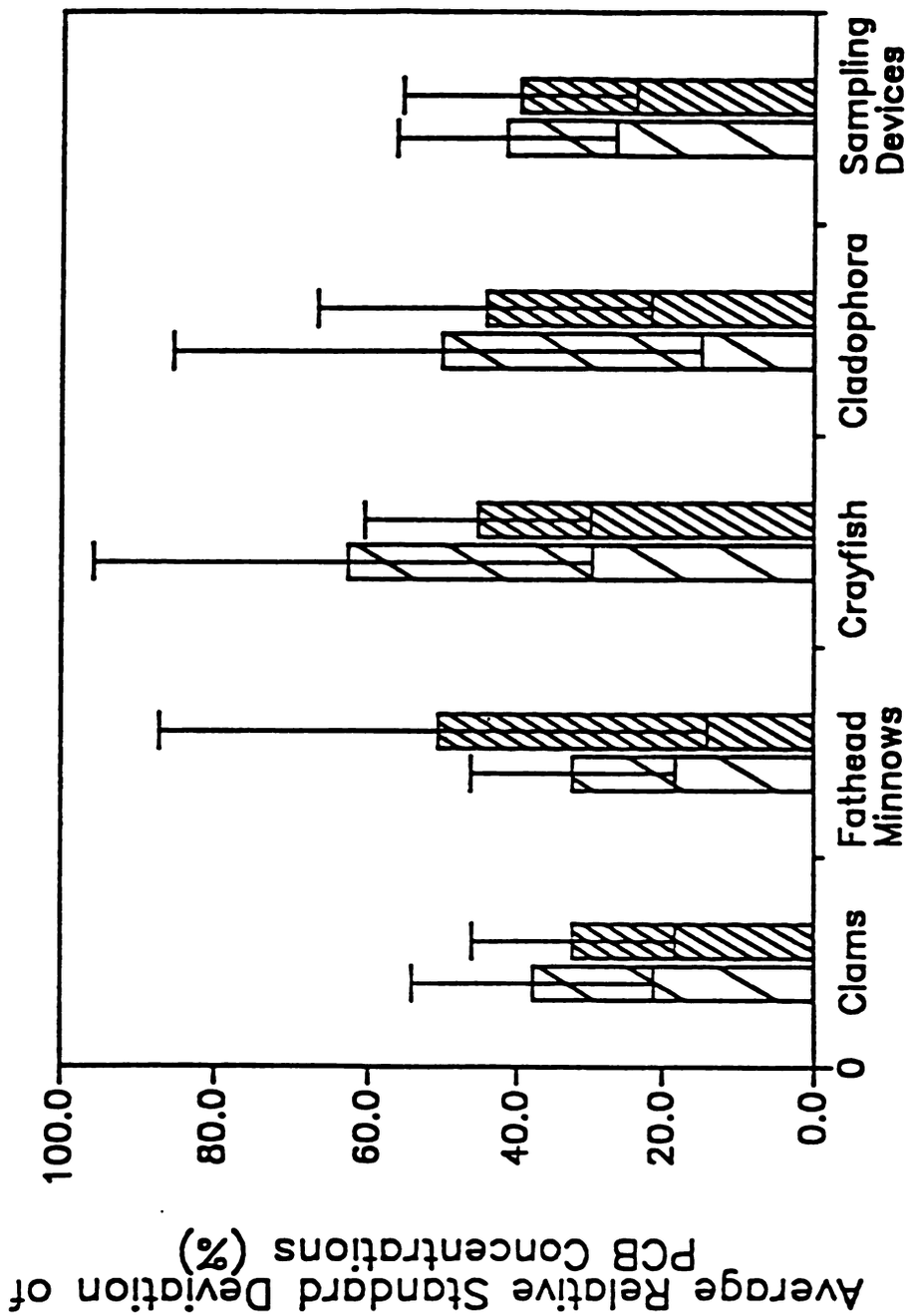

Figure 25: Variation in Polychlorinated Biphenyl Accumulation by Various Biomonitors at Saginaw Bay.

Table 12

Summary of Gas Chromatographic Data from Seney Wildlife Refuge

(Areas Relative to the Area of Peak Eluting at 22.64 min.)

	Retention Time (min)									
Sample	7.45	8.35	10.35	12.0	21.5	23.5	24.9	37.3	43.4	43.8
MSU 1	----	----	----	----	----	----	0.144	-----	0.281	0.233
MSU 2	----	----	----	----	----	----	0.235	0.128	0.075	-----
MSU 3	----	----	0.289	---	----	----	0.417	0.820	0.477	0.193
MSU 4	----	----	0.301	0.506	0.06	----	0.317	0.111	----	----
MSU 5	----	----	0.199	----	----	----	0.132	0.339	0.115	0.087
MSU 6	----	----	----	----	----	----	1.000	-----	0.142	0.059
MSU 7	----	----	0.111	0.034	0.134	0.076	0.153	0.091	0.214	0.147
MSU 8	----	----	----	-----	-----	-----	-----	-----	-----	-----
MSU 9	0.144	----	0.180	----	----	----	0.239	0.419	0.618	0.065
MSU 10	-----	----	----	----	----	----	0.227	0.390	0.141	0.090
MSU 11	----	----	0.075	0.197	0.096	----	0.196	0.297	0.206	0.098
MSU 12	0.425	----	0.176	0.139	0.100	0.042	0.157	0.387	0.075	0.067
MSU 13	0.170	0.492	2.019	0.100	0.097	0.844	0.087	0.598	0.146	0.059
MSU 14	3.816	0.460	0.461	0.089	0.299	0.805	-----	0.421	0.554	0.185
MSU 15	----	----	0.185	0.079	0.060	----	0.272	0.236	0.018	0.059
MSU 16	----	----	0.182	----	----	----	0.258	0.044	-----	-----
MSU 17	----	----	0.172	0.165	0.158	0.082	0.265	0.196	0.047	0.059
MSU 18	----	----	0.096	----	0.055	0.073	0.218	0.179	-----	-----
MSU 19	0.460	0.488	0.397	0.155	0.216	0.354	1.836	1.524	0.686	0.161
MSU 20	4.632	0.235	0.312	0.067	0.189	0.594	-----	0.988	0.361	0.131
MSU 21	5.765	0.703	1.277	1.288	1.162	1.252	0.149	3.303	3.187	0.745
MSU 22	0.435	0.526	0.596	0.658	0.240	1.095	0.042	1.069	0.862	0.181

The even MSU numbers were placed in the moist soil while the odd numbers were placed below under the water table.

humic material than loamy or the clay soil. Figure 26 illustrates mass spectral data from the analysis of one yellow sample. The gas chromatographic data from various sites are condensed in table 12. The peak areas have been normalized to the area of the hydrocarbon eluting

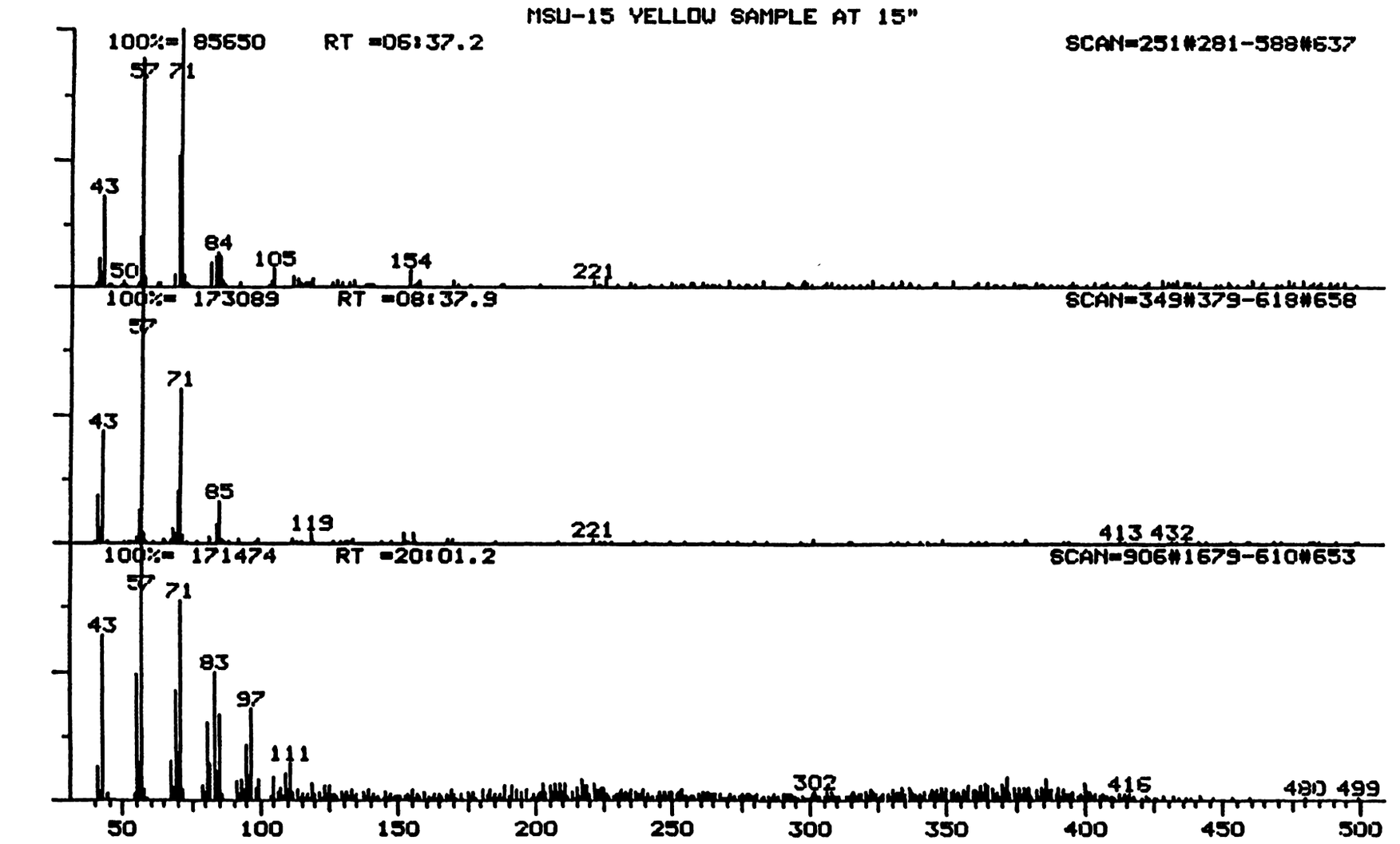

Figure 26.- Mass Spectrum of the Contents of a Polypropylene/Isooctane Sampler from Seney Wildlife Refuge.

at 22.64 min. The data show that the sampling devices placed closer to the surface accumulated relatively more contaminants when compared to their counterparts located under the water table. This is due to limited migration of the hydrocarbons from the surface by interactions with the soil. This study is especially interesting because humic material did not prevent contaminant uptake by the bags and it suggests that the devices may be useful in underground applications with moist soil.

CONCLUSIONS

These studies have shown that the devices are capable of withstanding environmental conditions and provide a useful alternative to biomonitors in the assessment of environmental quality. There was no evidence that biofouling occurred on any of the membrane surfaces. Also, some degree of selectivity toward polynuclear aromatic hydrocarbons was shown by the polyethylene and polypropylene membranes.

Good correlations between the bags and conventional biomonitors were determined in the studies conducted at Saginaw Bay. The sampling devices attained better reproducibility, and unlike the crayfish and mussels, no casualties were observed. Contaminant uptake is logarithmically related to the degree of chlorination on the PCB molecule. Turbulence effected not only the bags, but all the biomonitors involved in this study, therefore the devices still provide a more useful alternative to conventional biomonitors.

The devices may be applied in monitoring situations where the use of biomonitors would be impossible such as ground water and heavily

contaminated areas. The Seney Wildlife study showed that the devices worked well under all types of soil conditions. The bags did not dry out when immersed in damp soil and may be useful to monitor leachate from chemical dumps, degradation and migration of pesticides in holding tanks such as those at the MSU Fennville or Clarksville field stations. Thus, the lengthy soil extraction schemes involved in studying pesticide migration and degradation could be avoided.

CHAPTER 7

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CHAPTER 8

CONCLUSIONS AND FUTURE WORK

The passive water sampling devices developed in this dissertation provide useful alternatives to biomonitors. These devices may be employed in more diverse environments than biomonitors, their only requirement being constant hydration to prevent solvent evaporation. They have been used in test wells to monitor contaminant migration in contained dredge fill sites, even in areas that would be toxic to conventional biomonitors. They have been used to monitor groundwater contamination in a subterranean environment and are a useful alternative to soil extraction. The devices exhibit precision better than or equal to the more conventional biomonitors.

A three-phase process of contaminant uptake has been proposed. In the first step, the solute migrates from the bulk aqueous phase to the thin film adjacent to the membrane. In the second stage, the molecule passes through the membrane and finally dissolves into the bulk solvent. Therefore, the primary factors influencing contaminant uptake will be those affecting the first two stages; resistance to uptake is expected to be minimal at the last stage. Turbulence and temperature act to increase contaminant uptake by facilitating passage through the stagnant layer and membrane, respectively. Molecular size may inhibit migration through the membrane. Additional parameters that relate the solutes' chemical and physical properties with accumulation need to be determined.

Research at the U.S. Department of Fish and Wildlife, Columbia, MO has compared the BCFs of organochlorines into sampling devices that were filled with carp lipid or triolein with the valuse for fish (1). After exposure, the fish extracts were cleaned up, concentrated and subjected to analysis. The department is currently investigating other sampling applications.

Additional studies need to be performed to investigate alternate copolymer membrane materials such as polycarbonate/polydimethylsilicone, and polyvinylchloride/polyvinylidene copolymers. The use of polycarbonate membranes would allow comparisons with other membrane separation processes. Lastly, the use of rubbery membranes such as polyisoprene or silicone DC-23 may provide a sampling device with more universal separation characteristics than the polypropylene system.

Selectivity may be controlled by judicious selection of solvent. If the Hildebrand solubility parameters are not matched too closely, a greater degree of membrane crystallinity may remain. Hence, a greater degree of selectivity may be achieved. Permeation of the membranes by a series of xenobiotics that have different functional groups should be investigated to determine the effect of chemical and physical properties on partitioning. Uptake of a series of compounds with divergent vapor pressures and aqueous solubilities should be studied. Hopefully, the compounds chosen will be more "classical" in nature and a wide range of information about their chemical and physical properties will be available. Also, the effect of solubility of the contaminant in the partitioning solvent should be quantitated. Much care will have to be used in the selection of these compounds and solvents since literature

values are difficult to obtain. Also, a homologous series of compounds that contains dieldrin should be examined to investigate further the influence of Henry's Law constant on the partitioning process.

Studies indicate that these devices will prove to be useful in monitoring pesticide degradation and migration at pesticide holding facilities such as those at Clarksville and Fennville. Extensive extraction procedures could be avoided if the soil at these sites remains damp, allowing the use of the passive water sampling devices.

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CHAPTER 1

INTRODUCTION

Most biological and environmental samples are highly complex mixtures that contain analyte concentrations at trace levels, which complicates the analysis. Furthermore, the choice of the separation method may be determined by thermal instability and/or limited volatility of the compounds. The presence of polar functional groups also limit flexibility in the analytical method. Based on these factors, liquid chromatography is often the best separation method available.

If the sample is extremely complex, resolution of the additional components requires the use of high efficiency columns. Microcolumn chromatography is particularly useful in the analysis of complex non-volatile mixtures. Several types of miniaturized columns have been developed, with different sample capacities and instrumental requirements (1).

To maintain the high efficiencies and limit diffusion and band broadening, dead volume must be minimized in the chromatographic system (2). Injection systems have been developed that can deliver low sample volumes, yet minimize dead volume (1, 3). Sensitive and selective detectors with minimal flowcell volumes also have been constructed (4). Lasers are easily focused into these flowcells and have been used in a variety of applications (5). An especially promising technique is

laser-induced fluorescence detection (6).

Not all compounds are compatible with the laser emission and must undergo derivatization (7). Currently, the existence of a derivatizing agent that is selective for ketone functional groups and amenable to excitation by a HeCd laser is lacking. The intent of this project was to develop a ketone-selective derivatizing agent that could be applied to the analysis of very complex mixtures, such as the analysis of biofluids for corticosteroids.

There are several problems that are associated with the determination of corticosteroids in biological fluids. Corticosteroids are thermally labile and non-volatile. Therefore, they must be derivatized before gas chromatography can be performed. Poor chromatographic properties, instability and the formation of multiple products are common problems associated with the use of corticosteroid derivatives in gas chromatographic separations (8, 9).

Although liquid chromatography is a separation technique better suited to the analysis of corticosteroids, difficulties still arise in the separation of components. The high efficiencies observed using microcolumns could assist the analysis of biological fluids for corticosteroids. In addition, all corticosteroids possess ketone moieties and, therefore could be reacted with a ketone-selective derivatizing agent.

One promising reagent is 2-diphenylacetyl-1,3-indandione-1-hydrazone (DPIH) as shown by Lipari and Swarin (10) when they formed fluorescent derivatives from simple aldehydes and ketones. When this reagent was applied as a derivatizing agent for corticosteroids, the 3-keto-4-ene

moiety caused the formation of multiple products. Analysis of the products formed after the addition of destabilizing agents to the reaction mixture suggests that E,Z isomerization was occurring at this site.

Research using 1-dimethyl-aminonaphthalene-5-sulfonyl hydrazine (dansyl hydrazine), showed that multiple products were formed upon the reaction of corticosteroids. These derivatives were fewer in number and postulated to be syn and anti isomers. A novel compound, 7-diethylaminocoumarin carbohydrazide (DACC) was tested as a potential derivatizing agent for corticosteroids. Multiple products resulted when compounds containing a 3-keto-4-ene moiety were reacted with DACC. The derivatives exhibited severe tailing when separated on a reverse phase column. The use of mobile phase additives could not improve the poor peak shapes. In spite of the difficulties encountered, the novel reagents examined in these studies formed highly fluorescent derivatives that were amenable to excitation by a HeCd laser. These compounds would be better suited as post column reagents or in applications that avoid the formation of multiple isomers.

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CHAPTER 2

MICROCOLUMN LIQUID CHROMATOGRAPHY AND LASER-INDUCED FLUORESCENCE DETECTION

INTRODUCTION

All of the components in a complex mixture may not be resolved by the efficiencies of conventional columns (normally 10^3 to 10^4 for a 5 mm x 22 cm column) used in liquid chromatography. Increasing column efficiencies by 1-2 orders of magnitude may still result in inadequate separations (1, 2). The high-efficiency capillary columns used in gas chromatography have encouraged advances in column miniaturization for liquid chromatographic applications and have resulted in the development of columns with very high efficiencies. These columns are particularly useful in the separation of very complex nonvolatile samples, such as those of biological origin.

Detection of analytes at trace levels is also a formidable task. Specialized detectors with low dead volumes, which are both selective and sensitive have been developed for use in microcolumn liquid chromatography. Laser-induced fluorescence is an especially promising detection technique because of the selectivity in the excitation and emission wavelengths (1, 3, 4). In addition, sensitivity is improved because the laser provides intense radiation that is easily focused into the small flowcells. Not all compounds are naturally fluorescent at a given wavelength, therefore derivatization techniques, which supply an added level of selectivity, are often required. In this chapter, the development of microcolumn technology, laser-induced fluorescence

detection and available fluorescent probes will be reviewed.

TYPES OF SMALL DIAMETER COLUMNS

INTRODUCTION

In the 1960s, the first experimental work began on the application of small diameter columns to liquid chromatography. Horvath and Lipsky (5) used a pellicular material to pack a small diameter column. Due to the meager amount of stationary phase, separation could only be achieved with extremely long columns (6). Interest in small diameter liquid chromatographic columns soon waned because the columns were difficult to pack. In addition, instrumentation that was capable of meeting the stringent low dead volume requirements and able to supply solvent at low flow rates was not yet available (6).

In 1976, Scott and Kucera began the current trend toward miniaturization of liquid chromatographic instrumentation (7). They slurry packed stainless steel columns (1 mm i.d., 1 m length) under high pressure and achieved 30,000 theoretical plates. Unlike conventional columns where a maximum length and efficiency is obtained; coupling these columns together resulted in the achievement of 10^5-10^6 theoretical plates.

The limitation in length and efficiency that is observed when conventional columns are coupled, but not with microcolumns, may be explained as follows. It is postulated that heat generated by forcing the mobile phase through the tightly packed bed of the stationary phase

increases diffusion and, therefore, band broadening. The improved efficiency shown by microcolumns may be due to improved heat dissipation caused by the high surface area (6, 8, 9). Also, the proximity of the column walls may minimize the number of packing irregularities limiting diffusion, band broadening, thus improving efficiency (10).

Scott and Kucera (7, 11) suggested that decreased detector volumes were required to take full advantage of the increased efficiency achieved by the miniaturized columns. Ishii et al. decreased detector volumes through the use of concentric plastic tubes (12, 13). After finding that open tubular and packed capillary columns required flow rates of μl/min or less, Tsuda and Novotny concluded that further instrumental developments were needed to utilize microcolumns adequately (14, 15). After these pioneering efforts, the field of microcolumn liquid chromatography rapidly expanded with investigations into the use of a variety of stationary phases and the partitioning process (10, 16-29). Takeuchi and Ishii (16) confirmed that small columns could be reproducibly packed and investigated the relationship between coiling and efficiency. White and Laufer (30) further expanded the field by using gradient elution to improve the resolution of the later eluting peaks.

Many advantages are associated with the use of small diameter columns over conventional columns. Unlike conventional columns whose length and total number of theoretical plates eventually become limited, small diameter columns may be linked with a minimal loss of efficiency. As a result, very complex mixtures may be resolved (3, 4, 6, 8, 31, 32). Very small amounts of packing material are required, providing economy (33). The low rates of solvent consumption further improve economy and

allow the use of exotic mobile phases (3, 4, 6, 8). The low flow rates also facilitate interfacing the liquid chromatograph to mass spectrometers and Fourier transform infrared spectrometers (4, 8, 33) and may promote the development of new detectors (3, 4). Increased mass sensitivity is more compatible with the micromanipulations required by biomedical applications (4, 8, 33). If thermostated, the high surface area of the column dissipates heat quickly and achieves very precise retention times (6).

The literature abounds with various terminologies that are used to describe small diameter liquid chromatographic columns. The term microbore will be used to describe a column with an interior diameter between 0.5-2.2 mm. Microcolumns are those with an internal diameter of less than 0.5 mm, but generally between 0.2-0.3 mm. Both of these columns are packed with stationary phase. Semipermeable columns with smaller internal diameters can also be fabricated. Open tubular columns are those columns with even smaller internal diameters, which are less than 0.05 mm with a thin film of stationary phase deposited on the wall (15, 32, 33).

Three major types of small diameter columns that include open tubular, semipermeable packed capillary and slurry packed capillary have been developed. They are illustrated in figure 1 and are characterized further below.

OPEN TUBULAR CAPILLARY COLUMNS

Open tubular columns are formed by drawing out a thick walled glass

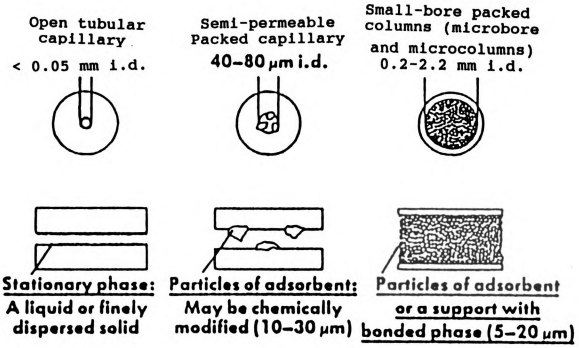

Figure 1- Types of Columns used in Liquid Chromatography
Reference 9, p. 876.

or fused silica tube by techniques conventionally used for forming gas chromatographic capillary columns. The stationary phase, which is either a liquid, a bonded organic layer, or a fine adsorbent, is deposited over the wall of the column. Because glass has wettability problems, the inner surface must be roughened with an alkaline treatment (2, 9). Immobilization techniques can also be used to deposit the stationary phase on the interior surface of the column (9).

Open tubular columns have the most stringent instrumental requirements because of the severe effects caused by dead volume. The detector and injector volumes must be in the low nanoliter to subnanoliter range to minimize peak broadening. As a result, indirect injection techniques such as splitting or heart-cutting techniques are often used (4). Although calculations by Novotny (32) suggest that these columns should have a 100-fold advantage in performance over microbore columns, instrumental difficulties have limited the application of open tubular columns.

SEMIPERMEABLE PACKED CAPILLARY COLUMNS

To form a packed capillary column, a small bore glass tube is filled with packing material and drawn into a capillary tube 70 μm or less in diameter (9). Soft soda-lime glass is commonly used as it has a low melting temperature. Because the heat used to draw out the column may damage the stationary phase, chemical treatments must be performed on the packing material (4, 32). The optimum ratio of the column diameter to particle size is between 2:1 and 3:1. If this ratio is increased, the

packing material will not be as stable while the column is drawn (2). Thus, packing these columns may be tedious and deactivation of the packing material may occur during the heating and drawing process.

Although their efficiency is reduced in comparison to open tubular columns (32), the semipermeable packed capillary columns originally developed by Novotny and Tsuda (14) have more relaxed instrumental requirements and greater sample capacity. One other advantage associated with these columns is that a more stable stationary phase remains uniformly distributed throughout the column because the packing material is embedded in the column wall (4, 32). Additionally, by using packing material with a decreased particle size, column efficiencies are improved (34).

It has been postulated that the packing structure of the stationary phase may cause stagnant pockets of mobile phase within the columns. The hydrodynamics of the separation is unconventional and, therefore, classical equations describing the separation phenomenon cannot be applied to these columns (4, 9).

SLURRY PACKED CAPILLARY COLUMNS

Slurry-packed fused silica capillary columns consist of open tubes filled with conventional packing material. Typically, these columns have an inner diameter that ranges from 100 to 300 μm. A variety of packing materials are suspended in a slurry and packed under high pressure into columns from one to three meters long. Using a 5-μm packing material (Spherisorb ODS) suspended in acetonitrile, Gluckman, Hirose, McGuffin

and Novotny (10) found a slurry ratio of 3.8 parts solvent volume (μl) to 1 part packing material (mg) provided the highest efficiencies when the columns were packed at a pressure of 6000 psi. They also found that as particle size decreased, the columns became more stable, but the level of technology required to pack the column was increased. Fused silica capillary tubing formed more efficient columns than those columns formed from glass tubing. Gluckman et al. postulated that interactions between the glass surface and the packing material hindered the formation of a homologous phase (10).

Typically, 1-meter long columns packed in this fashion can obtain efficiencies of 100,000 theoretical plates (10). Instrumental requirements are less stringent than for the semi-permeable capillary or the open tubular columns (32). Typically, 5-20 μg of sample may be injected onto this type of column (10). Detector volume is commonly in the submicroliter range. These columns have been widely applied to separation of complex mixtures (1) and provide analytical results superior to other small diameter columns (32). Therefore, slurry-packed fused silica capillary columns were chosen for the research conducted in this project.

MICROBORE COLUMNS

Microbore columns, initially developed by Scott and Kucera (7) are made from commercially available stainless steel tubing. They are conceptually similar to slurry-packed capillary columns and warrant discussion. The most common application of microbore columns is in high-

speed separations where a linear velocity of 12 cm/sec may be reached. At this linear velocity, only 1600 theoretical plates/meter are achieved. This application also requires the minimization of dead volume, amplifier time constant, detector response time, and rate of data acquisition (35). The dimensions, typical flow rates, sample capacity and characteristics of the columns are summarized in table 1.

Thus, the slurry-packed capillary columns are the most promising for separations of complex mixtures. Their instrumental requirements are compatible with commercially available instrumentation. Production of this type of column is simple, inexpensive and most importantly, has a high rate of reproducibility.

Table 1

Characteristics of Conventional Columns and Microcolumns used in HPLC

Column Type	Typical Dimensions i.d.	length	N[a]	Volumetric Flow rate	Sample Capacity
Conventional	4.6 mm	10-25 cm	----	1 mL/min	10-100 μg
	4.6 mm	25 cm	25,000	-----	----
Microbore	0.2-1 mm	1-10 m	----	1-20 μl/min	1-10 μg
	1 mm	1 m	100,000	-----	----
Packed Capillary	40-80 μm	1-100 m	----	0.5-2 μl/min	100 ng-1 μg
	0.1 mm	10 m	100,000	-----	----
	0.07 mm	10 m	140,000	-----	----
Open Tubular Capillary	15-50 μm	1-100 m	----	< 1 μl/min	< 100 ng
	0.03 mm	10 m	420,000	-----	----
	0.01 mm	5 m	625,000	-----	----

[a] N=plate number, optimum velocity and plate height are assumed; 5 μm particle size for conventional and microbore columns, 30 μm particle size for packed capillaries. 4, 31.

INSTRUMENTAL REQUIREMENTS

Generally, extra-column dispersion should be limited to a loss of 10% in the number of theoretical plates (15). The volume of the sample, the injector, detector, connections in the system are all factors that contribute to extra-column dispersion and band broadening. These factors will be examined along with commonly employed solutions used in microcolumn chromatography.

CONNECTING TUBING

Tubing used to connect the column to the other parts of the system contributes to dead volume. If solvent flow in this empty tube is assumed to be parabolic due to the increased drag that occurs near the walls of the tubing, an equation describing the variance caused by the connections σ_c^2 can be derived (36, 37).

$$\sigma_c^2 = \frac{\pi^2}{24} \frac{r_t^6 L u}{D_M} \qquad (1.1)$$

where r_t^2 is the radius of the tubing, L is the column length, u is the linear velocity, and D_M is the diffusivity of the solute in the mobile phase. Due to the contribution to variance, the length of all connections should be minimized (36). To form connections of a minimal volume, the ends of any glass or fused silica tubing must be square. This end is inserted into a PTFE tube, in which a hand-drilled hole of the appropriate size has been made. A Swagelok^R fitting joins the two pieces of tubing. If a packed column is involved in the union, teflon

frits or a piece of glass wool inserted into the end will prevent loss of packing material (38). Therefore, these techniques were followed in subsequent research to minimize this contribution to variance.

INJECTOR

The volume of the sample injected as well as the injector itself contribute to band broadening. The variance caused by forcing the sample into the mobile phase (σ_s^2) is dependent upon the sample volume (V_s) as shown by equation 1.2 (37),

$$\sigma_s^2 = \frac{V}{K^2} s^2 \qquad (1.2)$$

and a constant, K that is dependent upon the injection technique. The value of K^2 is usually 4, but may reach 12, if a square shaped plug of sample is introduced into the system. Guichon and Colin (37) have shown that the maximum possible injection volume, V_{max} is dependent upon the column length, L, the particle diameter, d_p and the column diameter, d_c^2.

$$V_{max} = 0.36 \sqrt{L d_p d_c^2} \qquad (1.3)$$

In an attempt to minimize the contribution of the injector itself to variance, alternative injection systems have been developed. Heart-cutting techniques (38, 39), where the central portion of a sample elution volume profile is removed and injected onto the column by timed valves have been developed. Although this technique is very

reproducible, the volumes injected are dependent on the permeability of the column as well as the flow (38). This technique also wastes much of the sample.

Other commonly used methods include valve and split injection systems. Valves with an internal loop from 0.02 to 0.2μl in volume have been successfully employed in small diameter column chromatography (38). Split injection systems where the sample is partitioned between the column and another capillary have also been developed (20, 38). Although this system does not make economical use of the sample and solvent, and maintains irregularities in the shape of the injection profile, it does deliver relatively precise volumes of the sample volumes to the column. Good reproducibility and ease of operation are available with split injection systems and thus, this system was used in subsequent studies.

DETECTOR

The volume of the detector, mixing caused by laminar flow into the flowcell, and diffusion in the cell itself are all factors that contribute to detector variance. If the detector cell is assumed to be cylindrical, an equation may be derived to estimate detector variance σ_d^2 (36).

$$\sigma_d^2 = \frac{V_{del}^2}{12} = \frac{(\pi r_c^2 L_c)^2}{12} \qquad (1.4)$$

V_{del} is the volume of the flowcell that is illuminated, and r_c and L_c are the radius and the length of the cell, respectively. Detector volumes of less than 1μl are required for microbore bore columns (36), whereas packed capillary columns have been coupled with detectors with nanoliter cell volumes. Open tubular columns require detectors with a volume of 0.05 nl or less (40).

Also, the detection system must have a time constant of not more than a few tenths of a second to minimize band broadening that results from electronic contributions (36).

To minimize the contributions of dead volume to band broadening, on-column detection techniques have been developed (41). Yang (42) used fused silica tubing to investigate on column detection and found that no significant contributions to band broadening occurred. In addition to being strong and flexible, the tubing was easy to handle, relatively inert, and the smooth inner surface acted to reduce band broadening by minimizing wall effects. When the polyimide surface was removed, the tubing was optically transparent and could be easily interfaced with the injector (42).

Two approaches to the development of detectors for use in microcolumn chromatography have been made. Specialized detectors that take advantage of the low flow rates used in microcolumn chromatography have been developed (43, 44). The column may be connected directly to detectors that include: plasma (9) and flame-based detectors (38, 45) as well as mass spectrometers (42, 46). Attempts to develop an infra-red detector have also occurred (4, 47).

Miniaturization of components to limit the volume of the flow cell of conventional detectors has also taken place. Ultraviolet/visible (42, 48), refractive index (48, 49), electrochemical (38, 50), and fluorimetric detectors (3, 51-55) have been developed. If miniaturization is coupled with improvements in technology, such as by using lasers (38), increased mass selectivity can result. Laser-induced fluorescence offers a method of detection that is both sensitive and selective. Choice of the excitation and emission wavelengths supplies additional selectivity. The highly collimated source of light is easily focused into the flowcells of nanoliter volume (33, 41, 49).

The high intensity of the laser beam can also improve limits of detection. If the signal is dependent upon the intensity of the incident light beam, as is fluorescence, and the noise is not concomitantly increased (or is filtered out), improvements in the limits of detection result (56). The contributions to noise have been identified and help to minimize the noise component in fluorescent signals. Fluorescence caused by impurities in the solvent and Raman scatter from the solvent itself contribute to noise. Noise is also caused by fluorescence and Raman scatter from the flowcell windows and scattering of the excitation beam by the windows (57). Because the flowcell windows contribute to noise levels and limit sensitivity, a variety of cells have been designed to overcome this problem.

LASER INDUCED-FLUORESCENCE DETECTORS

There have been four major types of on-column laser-induced fluorescence detectors developed to date. The fused silica capillary flow cell, fiber-optic capillary tube flow cell, the sheathed flow cell, and the free-falling jet (droplet). These detectors are shown in figure 2.

FUSED SILICA CAPILLARY FLOW CELL

Yang (42) formed a UV/vis absorbance detector with a volume of a few nanoliters by burning the polyimide coating away from fused silica capillary tubing, as illustrated in figure 2 (D). Research by Guthrie et al. also shows that fused silica is the material of choice for construction of the cell (41). In addition, this design has been used as a cell for a laser-induced fluorescence detector (41, 44, 51 57). This detector employed a HeCd laser that was focused on the column, forming a flowcell with a minimal volume. Noise caused from the scattering of the incident light by the flowcell walls was limited by using optical filters. If the diameter of the laser beam approached the diameter of capillary, shifting and vibrations affected the system (41). In addition, the surface of the silica tubing can become brittle after exposure to the laser beam (40).

Recently, Tsuda and Noda (58) examined the effects that the positioning of the laser beam has on scattered radiation from reflection and refraction at the cell walls. Proper optical alignment was achieved

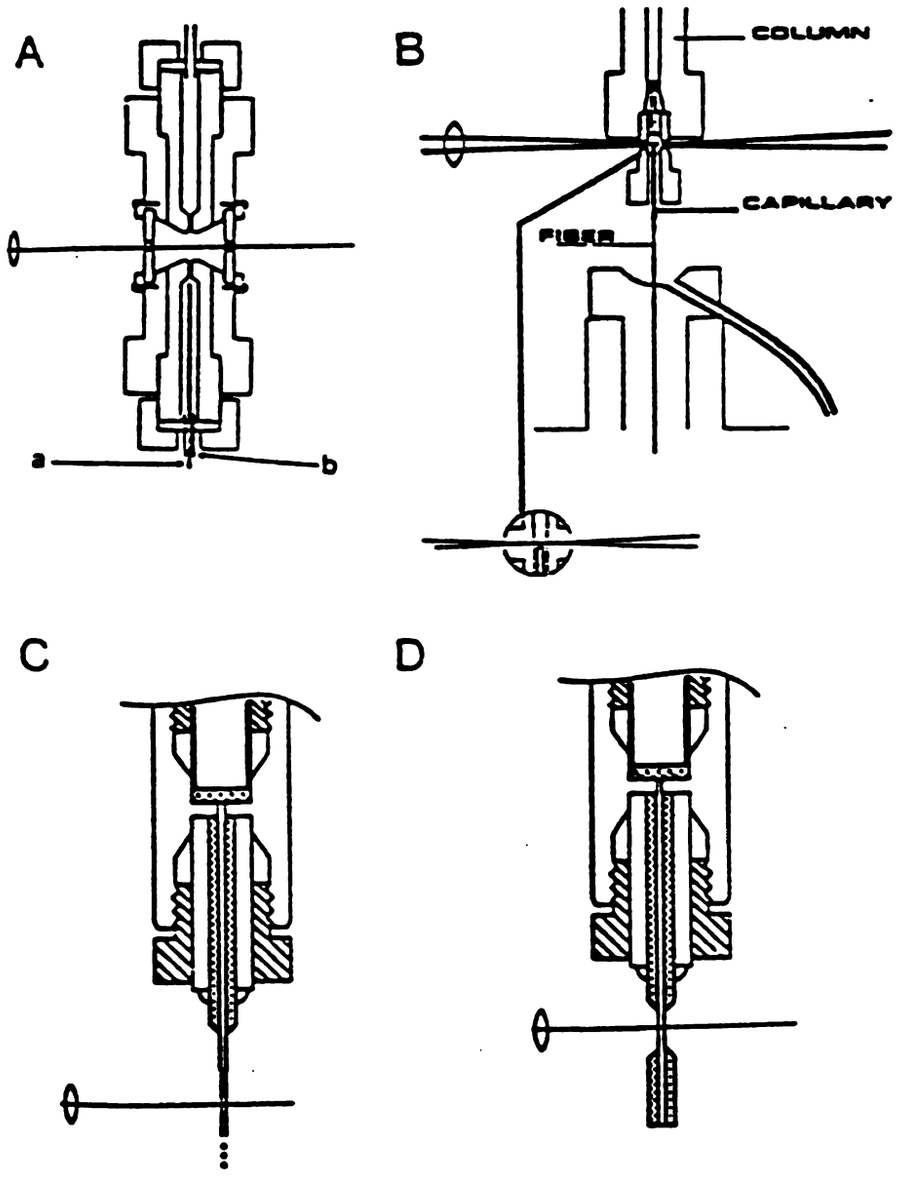

Figure 2: Four Different Laser-Induced Fluorescence Flowcells. A is a sheath flow cell where solvent forms a sheath, a=the eluant entry tube, b= entry tube for the sheath flow; B is a cell based on a fiber optic; C is a free-falling jet (droplet), and D is a detector cell developed from a fused silica capillary. (40), 275.

through the aid of a video camera and allowed the detection of 1.3 fmol of the 4-bromomethyl-7-methoxycoumarin derivative of caproid acid.

McGuffin (44) examined the configuration of this detector for use in microcolumn applications. The limit of detection for Coumarin 440 dye was 2.3×10^{-15} g at a S/N ratio of 7. Also, a linear response of over 8 orders of magnitude was observed. The system was found to be sensitive, stable and easy to use.

FIBER OPTIC-CAPILLARY TUBE FLOW CELL

To reduce scatter and reflected light from the cell walls, Sepaniak and Yeung (59) inserted a fiber optic into the capillary tube to collect emissions. This arrangement is illustrated in figure 2, and resulted in a high collection efficiency, a high sensitivity, and a variable optical path length. The volume of this flow cell was 10 nl.

Gluckman, Shelly and Novotny (43) modified this system using a fused silica capillary and obtained an illuminated volume of 3 pl, but the total cell volume was 98 nl and did not contribute to dispersion (40). The limit of detection was 24 fg of pyrene. A linear dynamic range greater than 5 orders of magnitude resulted. Gradient elution chromatography is compatible with this type of detection.

Edkins and Shelley (53) further modified the detector design by altering the placement of the optical fibers. The linear dynamic ranges and contributions to noise for two novel designs were compared. They also used photon counting to minimize noise.

Finally, in 1990, Tsunoda et al. (60) report the development of a

pulsed fluorescence detector that used a fiber optic flow cell.

SHEATH FLOW CELL

Hershberger, Callis and Christian (61) pioneered the development of the sheath flow cell detector for use with 5-mm diameter chromatographic columns. The detector was based on the principles used in cell sorting. As shown in figure 2, the column effluent is centered on another tube and solvent is introduced to form a flowing sheath around the column effluent. The resulting flow is laminar and hence, the sample effluent and the sheathing solvent do not mix, allowing the chromatographic peak to be isolated from the cuvette walls. The high degree of scattered radiation at the interface between the quartz and the solvent is spatially isolated from the sample fluorescence, so the background from the scattered excitation radiation is reduced. Also, the windows of the flow cell remain cleaner because they are isolated from contaminated samples.

Modification of flow rates in the stream allows the formation of detectors with different cell volumes (40, 61). In the development of this detector, Hershberger, Callis and Christian (61) used a cell volume of 53 nl. In 1984, Dovichi, et al. (57) expanded on this work by modifying a cell sorter to form a sheath flow cell. They achieved an 11-pl cell volume with a flow rate of 0.42μl/s or 25.2μl/min. The detection limit was 8.9×10^{-14} M for rhodamine 6G using a 1-s time constant (40, 57). Clearly, flow rates must be stringently controlled when using this detector (49).

FREE FALLING JET (DROPLET) FLOW CELL

Diebold and Zare (62) eliminated the walls of the flowcell by using a droplet as the cell. As shown in figure 2, the eluent from a conventional HPLC column flowed down a tube to form a droplet that acted as a bridge to a rod, stabilizing the shape of the droplet. The volume of this flow cell was 4μl. The output from a HeCd laser was focused inside the droplet. In this manner, the amount of scattered radiation was minimized, enabling the detection of 7 fg of aflatoxin. The detector exhibited a linear response of over three orders of magnitude. This detection system was subsequently applied to the determination of zearalenone in corn (63) and the determination of insulin in serum (64). The resulting limits of detection were 300 pg and 30 pM, respectively.

Folestad, Johnson, Josefsson and Galle (3) successfully applied the free-falling droplet as a detector for small bore applications. The excitation volume in their cell was about 1 nl. The limit of detection was 20 fg for fluoranthene. The droplet had a stable, smooth surface that minimized background (3, 40).

Maintaining the size and shape of the droplet pose many difficulties in the application of this detector. The droplet may act as a lens (thermal lensing) and may increase reflected radiation if care is not used in the precise positioning of the optics. Studies have increased the signal-to-noise ratio by 6 orders of magnitude through judicious placement of the optics (56). Bubbles may form at the capillary tip from insufficient degassing of the solvent and may be minimized by

notching the tube (56). Also, alterations in solvent composition, temperature, and flow rate as well as vibrations will change shape and size of the droplet and affect the accuracy of the laser focus (49, 56).

Tsunoda et al. (60) have combined two detector designs by employing a free-falling droplet as a detector cell and using a fiber optic to collect the fluorescent signal. They were able to detect sub-femtomole amounts of aminoacids that were derivatized with naphthalene-2,3-dialdehyde.

McGuffin (44) reported recent research conducted with Zare that compared the fused silica capillary, flowing droplet and the sheathed flow detectors. Similar signal-to-noise ratios were obtained for all the detectors. The fused silica capillary detector was used in subsequent work because it is compatible with many solvents, is nondestructive, and is relatively insensitive to changes in temperature. The high degree of sensitivity and reproducibility as well as the innate simplicity make this detector useful in microcolumn chromatography.

DERIVATIZING AGENTS USED IN LASER-INDUCED FLUORESCENCE APPLICATIONS

One disadvantage associated with the use of lasers is the limited number of emission wavelengths. Tunable dye lasers have been used in an attempt to overcome this shortcoming, but the cost was found to be prohibitive (41). Formation of derivatives that have excitation wavelengths compatible with the laser emission wavelengths is an alternate solution that supplies an added level of selectivity. Both pre- and post- column derivatization schemes have been investigated for

use in microcolumn chromatography.

Post-column derivatization reactions should be rapid, reproducible, and form stable derivatives (1, 65). The reaction should not be affected by changes in the mobile phase (1, 65). Another difficult problem to be circumvented is the increased band broadening that may be caused by an in-line reactor (49, 65). Although Kucera and Umagat (65) have developed a postcolumn reaction detection system that meets these requirements, the use of precolumn derivatizing agents may be applicable to a wider range of applications and will be used in this project.

Pre-column derivatization has been used previously in the field of microcolumn chromatography. Derivatives that are compatible with UV-vis detection and separation by microcolumn chromatography will be discussed in chapter 2. Reagents that form derivatives compatible with detection by laser-induced fluorescence are being developed. McGuffin (44) has derivatized amines using dansyl chloride and excited the products using the 325-nm line of a HeCd laser. She also formed derivatives from the reaction of carboxcylic acids, prostaglandins, and phosphoric acids with 4-bromomethyl-7-methoxycoumarin using the same excitation wavelength. Lammers et al. (66) have also used dansyl derivatives in a microcolumn system. Takeuchi and Ishii have derivatized fatty acids with 9-anthryldiazomethane (52) as have Goto et al (67) Novotny et al. (1) synthesized 7-(chlorocarbonylmethoxy)-4-methylcoumarin and reacted it with hydroxy steroids. The derivatives were excited by HeCd laser output at 325 nm. Dansyl hydrazine has been reacted with 17-oxo steroids and separted by microcolumn chromatography (1).

CONCLUSIONS

Slurry-packed microcolumns were found to be the best separation method to obtain the high resolution required in the analysis of complex mixtures at trace levels. Laser-induced fluorescence detection provides a selective and sensitive method to detect the components in the small sample volumes. The fused silica capillary flow cell supplies a detector configuration that is simple, sensitive and reproducible. Lastly, although a number of pre-column derivatizing schemes have been developed for use with laser fluorimetry, there is a need for a derivatizing agent that is selective for ketones and forms a derivative that is compatible with the output from a HeCd laser. The analysis of complex mixtures, especially those of biological origin, could benefit from such methodology.

CHAPTER 2

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CHAPTER 3

CORTICOSTEROIDS

Current derivatization technology lacks a fluorescent probe selective for ketones that is amenable to excitation by a HeCd laser. One method that would benefit from the development of such a derivatizing agent is the analysis of corticosteroids in body fluids.

Sensitive analysis of biofluids for specific corticosteroids presents several problems. High levels of cross reactivity hinder the use of radioimmunoassay and competitive protein binding techniques in the analysis of individual corticosteroids (1, 2). The presence of other, more concentrated steroids in plasma will interfere with the chromatographic detection of corticosteroids unless cortisol, which is more abundant, is determined (3). In addition, corticosteroids have a thermally labile dihydroxyacetone side chain at C-20 as well as a low volatility hindering the use of gas chromatography as a method of analysis (4).

These characteristics necessitate the use of derivatization techniques if gas chromatography (GC) is to be used in the analytical method. Many attempts to form thermally stable corticosteroid derivatives have been made. Poor chromatographic properties, instability and the formation of multiple products are common problems associated with most derivatizing agents used in this GC analysis. As a result, the use of high-pressure liquid chromatography (HPLC) as a

separation technique is superior to gas chromatography for corticosteroid analysis.

Several researchers have used HPLC coupled with UV/visible absorbance detectors to analyze biological fluids for corticosteroids. Greater sensitivity was achieved by using derivatizing agents that enhanced absorption and/or fluorescence. More recently, corticosteroids have been separated by microcolumns coupled with UV absorbance detectors.

In this chapter, corticosteroid nomenclature will be summarized. The advantages and disadvantages of derivatives commonly employed by both gas and liquid chromatographic determination of corticosteroids will be reviewed. Finally, a few examples of corticosteroid separation by using microcolumns will be given.

INTRODUCTION

Corticosteroids are responsible for the physiological reactions associated with the "fight or flight syndrome". They also play a role in the regulation of metabolism, water and electrolyte balance, the proper functioning of the cardiovascular and nervous systems, kidneys and skeletal muscles. Corticosteroids may be divided into two groups, the mineralocorticoids and the glucocorticoids. The mineralocorticoids regulate the sodium/potassium balance, while the glucocorticoids govern all facets of glucose metabolism (5).

Corticosteroids are characterized by a 21-carbon backbone, the numbering of which is illustrated in figure 3 (6). Corticosteroids are

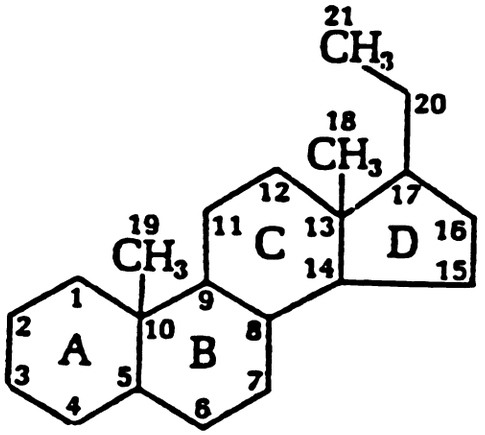


Figure 3-Numbering System for Naming Corticosteroids.
Reference 2, p.533.

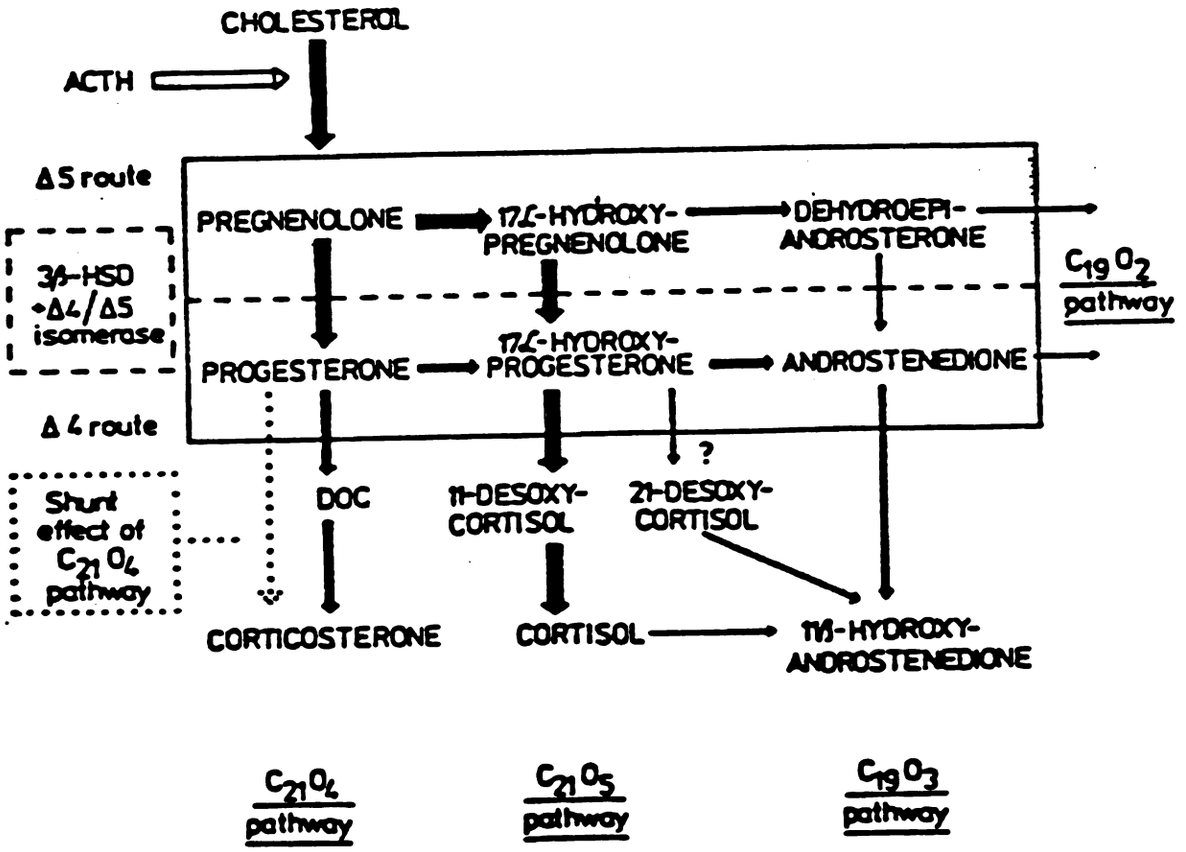


Figure 4-Simplified Synthetic Scheme for Adrenocortical Products.
Reference 3, p.322.

synthesized from a cholesterol precursor as shown by the simplified biochemical pathway in figure 4 (7). The basic corticosteroid structure may contain hydroxyl, ketone and methyl substitutions, forming various corticosteroids (8). Table 2 compares trivial and systematic nomenclature for some common corticosteroids.

Table 2

Trivial and Systematic Nomenclature for Steroids and Corticosteroids

Trivial	Systematic
Aldosterone	11β,21-dihydroxy-3,20-dioxopregn-4-en-18-al
Cholesterol	cholest-5-en-3β-ol
Corticosterone	11β,21-dihydroxypregn-4-ene-3,20-dione
Cortisol	11β,17α,21-trihydroxypregn-4-ene-3,20-dione
Cortisone	17α,21-dihydroxypregn-4-ene-3,11,20-trione
Dehydrocorticosterone	21-hydroxypreg-4-ene-3,11,20-trione
dehydroepiandrosterone	3β-hydroxyandrost-5-en-17-one
11-deoxycorticosterone*	21-hydroxypregn-4-ene-3,20-dione
11-deoxycortisol	17α,21-dihydroxypregn-4-ene-3,20-dione
11β-hydroxyandrostenedione	11β-hydroxyandrost-4-ene-3,17-dione
18-hydroxy -11-deoxy-corticosterone*	18,21-dihydroxypregn-4-ene-3,20-dione
11β-hydroxyprogesterone	11β-hydroxypregn-4-ene-3,20-dione
17α-hydroxyprogesterone	17α-hydroxypregn-4-ene-3,20-dione
pregnenolone	3β-hydroxypregn-5-en-20-one
progesterone	pregn-4-ene-3,20-dione

*deoxycorticosterone = DOC

These characteristics necessitate the use of derivatization techniques if gas chromatography (GC) is to be used in the analytical method. Many attempts to form thermally stable corticosteroid derivatives have been made. Poor chromatographic properties, instability and the formation of multiple products are common problems associated with most derivatizing agents used in this GC analysis. As a result, the use of high-pressure liquid chromatography (HPLC) as the separation technique is superior to gas chromatography for

corticosteroid analysis.

Estrogens and androgens are other steroids structurally similar to corticosteroids; they are synthesized in the adrenal cortex and are present in adrenal cortical and plasma samples. The structural simplicity of testosterone, an androgen, and its structural similarity to corticosteroids suggested its use to optimize reaction conditions in the following studies. Also, pregnenolone, an estrogen, was used to optimize conditions and compare reactivity at the C-20 ketone site with the C-3 site on testosterone. Multiple ketone moieties on progesterone also allowed investigation into the reactivity of various ketone sites.

GAS CHROMATOGRAPHIC ANALYSIS

The use of selected steroids to monitor the reactions developed in this research, suggests a brief review of chromatographic techniques that are commonly used to determine steroids. Analysis of biological fluids for steroids has commonly employed derivatization of hydroxyl functional groups. Acetylation or methylation of the C-3 hydroxyl group has been performed and fluorocarboxylic acids have been used to increase sensitivity by electron capture detection. The most common derivatives formed from hydroxyl groups are trimethylsilyl derivatives (TMS) (4). Silylating agents have been used in the analysis of both steroids and corticosteroids. Commonly used silylating agents will be reviewed. Sample chromatograms that illustrate the results from derivatizing compounds that contain the 3-keto-4-ene functional group will be given. Finally, derivatizing agents that are selective for ketone moieties and

that form products amenable to gas chromatographic analysis will be discussed.

SILYLATING AGENTS USED IN THE FORMATION OF STEROID AND CORTICOSTEROID DERIVATIVES

The use of TMS derivatives offers many advantages including ease of formation, separation and quantitation. Also, the poor chromatographic properties of hydroxysteroids are improved (4). These derivatives have an abundant molecular ion and have mass spectral fragmentation patterns that are easily analyzed. The extent of the derivatization reaction, the degree of silylation and the selectivity of the reaction for various hydroxyl groups may be altered easily by controlling the choice of the derivatizing agent, the use of a catalyst and the reaction conditions (9, 10).

Bis-trimethylsilyl acetamide (BSA), bis-trimethylsilyl trifluoroacetamide (BSTFA) and N-trimethylsilyl imidazole (TMSIM) are the most reactive silyating agents (9, 10). The reaction proceeds more rapidly if these silylation agents are coupled with trimethylchlorosilane (TMCS). Typically, a 3:3:2 ratio of TMSIM, BSA and TMCS is used to react with the steroids at 80°C for approximately 15 hours (11). If only the least reactive hydroxyl groups are to be derivatized, trimethylborosilane or hexamethyldisilane are used instead of TMCS (10). Campbell, et al. (12) have modified this method to be selective for 3 α, 6 α, and 7 β hydroxyl groups by reacting BSTFA without solvent at 37°C for approximately 15 hours. Levels as low as 1 ng of steroid can be detected by mass spectral detection of TMS derivatives and the response is linear over 1-2 orders of magnitude.

However, by using care in the formation heptafluorobutyrate esters, this sensitivity can be surpassed to a limit of approximately 10 pg (13).

Dimethylsilyl derivatives including iodomethyldimethylsilyl, chloromethyldimethylsilyl (CMDMS), t-butyldimethylsilyl and pentafluorophenyldimethylsilyl yield more intense peaks upon mass spectral analysis than do TMS derivatives (4). Chromatographic separation between mono-, di-, and tri-hydroxy steroids is improved when CMDMS is used as a derivatizing agent (13, 14). In addition, the loss of CH_2Cl (M-51) aids in the identification of CMDMS derivatives, while a loss of 57 is characteristic of t-butyldimethylsilyl derivatives. One method of forming dimethyl silyl derivatives is to react 0.5-2.0 mg of the steroid with 50μl tetramethyldisilazane and 50μl dimethyldichlorosilane at room temperature for three hours (13).

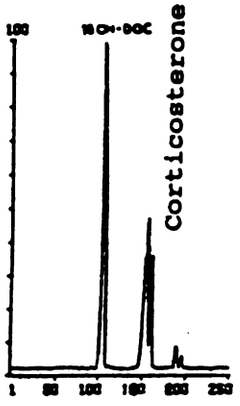
Although the use of silyl derivatives works well in the determination of steroids, keto-enol tautomerization limits the application of this technique toward corticosteroids. To prevent the occurrence of keto-enol isomerization, ketones are usually made into oxime derivatives before the silylation of hydroxyl groups is performed. Methoxime-trimethylsilyl (MO-TMS) derivatives are most commonly formed and have been used in the analysis of biofluids for steroids (15, 16, 17). Although MO-TMS derivatives of corticosteroids are stable, the separation of cortisone and corticosterone is difficult. Also, the 3-keto-4-ene functional group forms syn and anti isomers, which complicates quantitation.

Maume and coworkers (17) investigated the formation of syn and anti isomers of MO-TMS derivatives. Their results for corticosterone and 18-

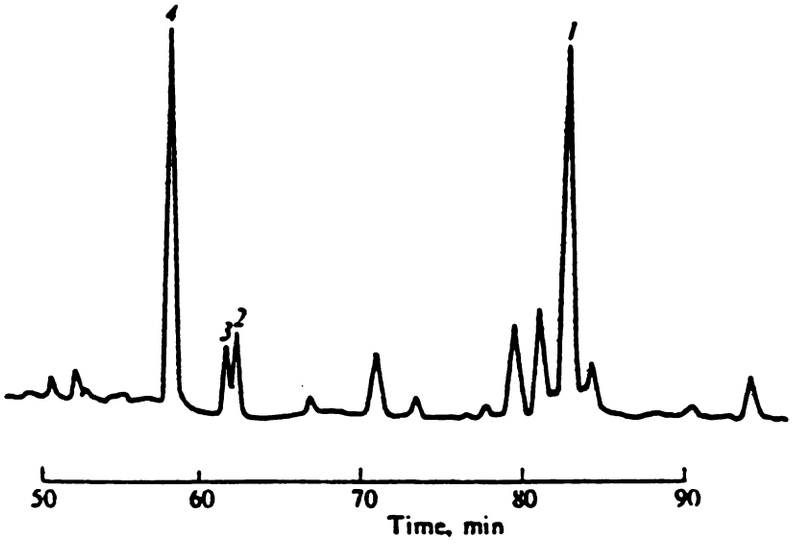
hydroxydeoxycorticosterone (18-OH-DOC) are shown in figure 5 (A). They found the separation of the syn and anti isomers is dependent upon the other functional groups on the corticosteroid skeleton. One product was observed for 18-OH-DOC as well as those corticosteroids that contain 11-oxo or 18-hydroxy groups such as 11-dehydrocorticosterone. When the derivatives of progesterone and related compounds such as 20α-dihydroprogesterone (20α-DHP) as well as 6β-OH steroids including 6β-hydroprogesterone and 6β-hydroxy-DOC were separated, two peaks of equal intensity resulted. Two unequal peaks, the earlier eluting peak giving a greater response than the more retained peak, were observed after the separation of 11-deoxycorticosterone (DOC), and 11βOH steroids such as corticosterone. Separation of 17αOH corticosteroid derivatives such as those formed from 11-deoxycortisol, 17α–hydroxyprogesterone, and 17α-hydroxy-20α-dihydroprogesterone also resulted in two peaks of unequal intensity, with the more retained peak having greater intensity.

The formation of methoximes followed by silylation of 18-hydroxytetrahydrocorticosterone results in the production of four isomers as shown in part B of figure 5 (4, 17). The use of a more potent silylating agent N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) coupled with potassium acetate also resulted in the formation of two products when compounds containing the 3-keto-4-ene structure were studied. Figure 5 C illustrates the chromatogram produced by the separation of cortisone derivatives. The ratio of isomers was dependent upon reaction conditions. Also, the use of a nucleophilic catalyst such as potassium acetate tended to increase enolization (10).

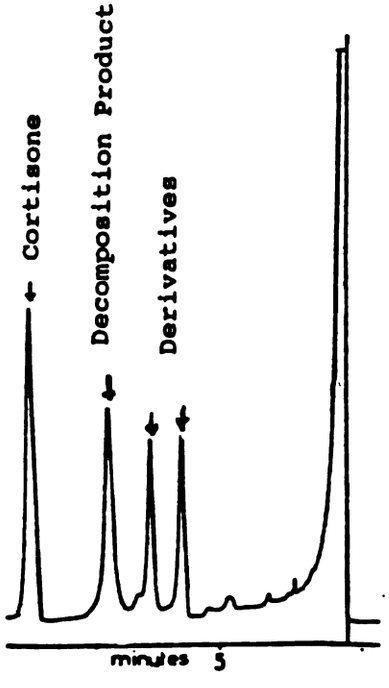
Also shown in the figure are two chromatograms from the separation



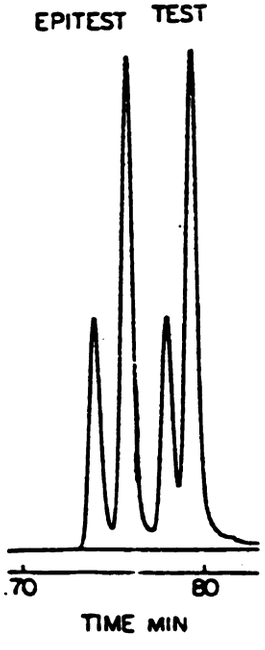
A: MO-TMS derivatives of Corticosterone and 18-OH-DOC (17), 585.



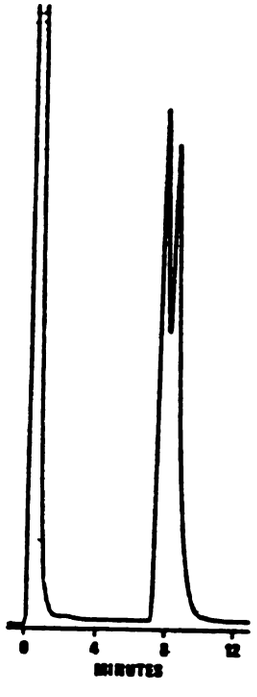
B: MO-TMS derivatives of 18-hydroxy-tetrahydrodeoxycorticosterone (4), 211.



C: TMS derivatives of Cortisone formed by using potassium acetate and BSTFA (10), 1095



D: BO-TMS derivatives of Testosterone (Test) and Epitestosterone (Epitest) (18), 155.



E: O-PFBO derivatives of Testosterone (25), 101.

Figure 5-Gas Chromatograms of a Variety of Derivatives.

of testosterone derivatives. In figure 5 D, benzyloxime (BO) has been substituted for methoxime in the preliminary reaction with ketone functional groups (18). The reaction conditions are very similar to those used in the formation of MO-TMS derivatives. The use of BO-TMS derivatives results in better separation because these derivatives are eluted much later than the corresponding MO-TMS products. The researchers found that steroids with a ketone moiety on the 16-, 17-, or 20-C yielded a single product. Compounds with the 3-keto-4-ene structure, such as testosterone formed two isomers. Figure 5 E is discussed in a later section.

CYCLIC DIMETHYLSILOXANE ALKYLBORONATE DERIVATIVES

Brooks and Harvey (19) formed cyclic esters by reacting corticosteroids with phenylboronic acid, n-butylboronic acid, methylboronic, t-butylboronic or cyclohexylboronic acid. In this reaction, 10μmole of the steroid was reacted with an equimolar amount of the derivatizing agent for five minutes at room temperature. Cyclization was caused by the addition of trimethylchlorosilane and hexamethyldisilazane. They found that all corticosteroids reacted in a similar fashion and formed only one stable product. Heftmann (20) reported derivatives allowed simple separation of cortisol and cortisone and that dimethyldiacetoxysilane can be used in the reaction.

KETONE-SELECTIVE DERIVATIZING AGENTS

Many difficulties in the determination of corticosteroids are caused by the thermally labile side chain (4). Side chain cleavage and thermal decomposition studies have been performed in an attempt to circumvent these problems. In addition to the previously discussed techniques used to form derivatives from hydroxyl groups, several other methodologies have been developed that react with ketone functional groups on corticosteroids. In most of these cases, multiple compounds are formed as a result of syn-anti isomerization at the 3-keto-4-ene structure. Techniques such as side chain cleavage and thermal decomposition studies will be discussed. Also, the use of bismethylenedioxy, methoxime, benzoyl oxime, benzoyl oxime-TMS, and N,N-dimethylhydrazone derivatives will be reviewed.

THERMAL DECOMPOSITION STUDIES

Attempts were made to quantitate corticosteroids on the basis of their thermal decomposition, namely the loss of the 17,21-dihydroxy-20-keto side chain to form the corresponding 17-ketosteroids (4). Grottfield (21) found that a linear relationship between peak area and mass injected did not exist. Thermal degradation of corticosteroids, which varies from 20 to 50% complete and losses of the product due to binding to the column explain this non-linearity (4, 20, 21). Also, the 17α-OH group was required for thermal dissociation and the quantity of the 17-ketosteroid formed may be a function of the number of oxo and

free hydroxyl groups on the molecule. Therefore, thermal decomposition is not a useful method for quantitating corticosteroids.

SIDE CHAIN CLEAVAGE

Because the offending side chain could not be thermally removed, attempts have been made to cleave chemically the C-17 side chain. Periodic acid oxidation has been used to form lactone derivatives from aldosterone and 18-hydroxydeoxycorticosterone (4). Kittinger (14) developed an extensive extraction scheme for use after periodate cleavage of the side chain. Although the analysis is lengthy, tedious, and results in many fractions that must be analyzed, single products were formed from each steroid.

Instead of using oxidative procedures, Szecsenyi and Kecskes (22) reduced ketone groups on corticosteroids with sodium borohydride, then used periodate to cleave the side chain. This method was especially well suited to urine analysis because the glucosiduronic group at C-3 is cleaved to yield a 17-keto steroid or its formate ester.

BISMETHYLENEDIOXY DERIVATIVES

Kirschner and Fales (23) reacted 17-hydroxy-corticosteroids with formaldehyde under acidic conditions to form the bismethylenedioxy derivatives. This reaction added thermal and chemical stability to the side chain. When the products were subjected to analysis, the derivatives of cortisone and hydrocortisone exhibited one major peak and

two other minor peaks as shown in table 3. In general, separation of all the corticosteroids studied showed one major peak as well as either shoulder or smaller peaks. In an unsuccessful attempt to limit the number of products formed, Kirschner and Fales (23) acetylated or recrystallized the steroids before the derivatization reaction was performed.

Table 3

Bismethylenedioxy Derivatives of Various Corticosteroids

Steroid	Number of Major Products	Number of Minor Products
Cortisone	1	none
Cortisol	1	1
Deoxycortisol	1	1 shoulder
Tetrahydrocortisone	1	2
2α-methylcortisone	1	1 shoulder
16α-methylcortisone	1	1 shoulder
16α-methylhydrocortisone	1	1
17,20:20,21-bis-methylenedioxy-3-ethylenedioxy-5α-pregnane-6-,11-dione	1	none
17,20:20,21-bis-methylenedioxy-6-methyl-5-pregnene-11β-ol	1	2
17,20:20,21-bis-methylenedioxy-11α-methyl-5-pregnene-11β-ol	1	1

(23), 1550.

METHOXIME DERIVATIVES

Methoxime derivatives are widely used in the determination of ketosteroids and may be analyzed directly, without further reactions such as silylation. To form these derivatives, 2 mg of the steroid is heated with 8 mg of O-methylhydroxylamine hydrochloride in 0.5 ml pyridine at 60°C for 3 hours. It should be noted that syn and anti isomers may result from this reaction. Another problem is degradation of

the methoxime derivatives when exposed to acidic sites on the chromatographic column (16, 20). In spite of problems associated with their use, methoxime derivatives have been applied to the analysis of urinary steroids (24).

BENZOYL OXIME (BO) AND BO-TMS DERIVATIVES

Another variation in the methoxime reaction can be performed by substituting O-benzylhydroxylamine hydrochloride. These benzoyl oxime derivatives have a high molecular weight and long retention time. Sensitivity of the electron capture detector may be further increased by substitution of fluorine atoms onto the derivatizing agent, as with 2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA-HCl). As shown in figure 5 E, multiple products also result from the reaction of PFBHA-HCl with 3-keto-4-ene functional groups (4, 18, 25). Although, Devaux and co-workers (18) observed syn- and anti-isomers after the formation of benzoyloxime and benzyloxime-trimethylsilyl derivatives from 3-keto-4-ene steroids, a single product resulted when ketone functional groups at C-16, C-17 or C-20 sites were reacted. In addition, unstable products resulted when a hydroxy group was substituted on carbon-17.

N,N-DIMETHYLHYDRAZONE DERIVATIVES

N,N-dimethylhydrazone derivatives are formed by reacting ketosteroids with ethanedithiol and p-toluenesulfonic acid in glacial acetic acid at room temperature for approximately 15 hours. Although these derivatives are easily formed, and have good chromatographic properties, they are not stable when in contact with light and air (20).

COMPARISON OF CORTICOSTEROID DERIVATIVES

Baillie, Brooks and Middleditch (26) compared several types of derivatizing agents to assess their usefulness in the determination of corticosteroids. The derivatizing agents they investigated included cyclic boronate esters, dimethylsiliconides and oxanones as well as MO-TMS derivatives. They had difficulty forming dimethylsiliconide derivatives and were only successful for dihydroxyacetone type and 17α,20-diol type molecules. Also, these derivatives were susceptible to hydrolysis. In addition to the dimethylsiliconide derivatives, the oxetanone derivatives were very difficult to form and required the use of large amounts of steroids (100 mg). Once formed, separation of the the oxetanone derivatives was very challenging. Although the boronate derivatives had stable sidechains, Baillie et al. concluded that the MO-TMS derivatives were the most versatile and stable.

HPLC DETERMINATION OF CORTICOSTEROIDS

The difficulty associated with gas chromatographic analysis of corticosteroids has prompted the development of liquid chromatographic techniques. Both reversed- and normal-phase columns have been employed in liquid chromatography to achieve separation (20, 27). Although many books have been written to describe steroid analysis by HPLC, reviews about corticosteroids have been limited. This section is intended to provide an overview of steroid analysis using HPLC, with emphasis on derivatization and any isomerization that may result. UV/visible absorbance and fluorescence detection are the two most common methods of detection in corticosteroid analysis. If UV/vis absorbance detection schemes are used, derivatization techniques may not be necessary because the 3-keto-4-ene structure enhances the absorption of light (28). If increased sensitivity is required, the derivatization may be warranted. Also, LC-MS has been used in the determination of corticosteroids (29, 30, 31).

UV/VIS ABSORBANCE DETECTION

METHODS NOT EMPLOYING DERIVATIZATION TECHNIQUES

Cavina, Morette, Alimenti, and Gallinella (32) analyzed rat adrenal glands for corticosteroid content using a normal-phase column coupled to a UV absorbance detector at a wavelength of 240 nm. When 1μg of a mixture of seven corticosteroids was subjected to analysis, 36 ng of

aldosterone were easily detected.

Pomoell, Kopu and Adlercreutz (1) employed an octyldecylsilica column, that was eluted with a 52-58% methanol/water gradient and coupled to a UV absorbance detector at 245 nm. The blood samples that they analyzed contained selected corticosteroids in very low concentrations and could not be detected by conventional absorbance detection. These were radiolabeled, and fractions from the column were collected and analyzed by radioimmunoassay techniques.

Siggia and Dishman (33) compared a variety of reversed-phase packing materials to optimize the separation of corticosteroids. They recommended that a column packing of 24% Amberlite (LA-1) on a primarily trifluoroethylene (CTFE) support be used with an aqueous mobile phase.

Both reversed-phase and normal-phase separations were compared by Ballerini et al. (28) using a LiChrosorb SI-100 and a LiChrosorb RP-8 column to analyze corticosteroid extracts. Detection was performed by uv absorbtion at 240 nm. A mixture of cortisol, cortisone, 11-dehydrocorticosterone, corticosterone, 11-deoxycortisol, aldosterone and 11-deoxycorticosterone was successfully resolved by both columns.

O'Hare, Nice, and Capp (34) also compared reversed- and normal-phase columns and separated 18-hydroxy steroids. Severe tailing of the chromatographic peaks was attributed to incomplete capping of the residual silanol groups on the silica packing. Peak symmetry was improved by using packing materials and triethylamine as a mobile phase additive.

DERIVATIZATION TECHNIQUES USED IN UV ABSORBANCE DETECTION

BENZOATE ESTER DERIVATIVES

Hydroxy steroids can be reacted to form benzoate or p-nitrobenzoate esters. Fitzpatrick and Siggia (35) dissolved 0.5 to 50 mg of hydroxysteroids in 4 ml pyridine. A three-fold excess of benzoyl chloride was added. Then the mixture was then shaken and incubated at room temperature for 5 minutes and at 80°C for 15 minutes. An acid wash removed the pyridine and the derivatives were extracted into ether. The derivatives were separated on a reversed-phase column and detected by UV absorbance at 254 nm. The calibration curve for androstanolone was linear from a concentration of 10^{-4} to 10^{-5} M.

2,4-DINITROPHENYLHYDRAZINE DERIVATIVES

Ketone groups can be derivatized with 2,4-dinitrophenylhydrazine by dissolving the steroid in methanol and acidifying the solution with a few drops of hydrochloric acid. A slight excess of the derivatizing agent is added and the mixture allowed to react at 50°C. Using this method Henry, Schmit and Dieckman (36) detected 1 ng of hydroxy steroids by UV absorbance at 254 nm. The derivatives have maximum absorbance at wavelengths of 260 and 350 nm with molar absorbtivity of 8,000 and 11,000 l/cm-mole, respectively. Although these derivatives were readily detectable, the formation of multiple products from the reaction of C-21 steroids still occurred.

DERIVATIZATION TECHNIQUES USED IN FLUORESCENCE DETECTION

DANSYL HYDRAZINE DERIVATIVES

Kawasaki, Maeda, and Tsuji (37) used 5-dimethylamino-1-naphthalene sulfonyl hydrazine (dansyl hydrazine) to form fluorescent derivatives of cortisol in a urine extract. To the extract, they added 0.02% dansyl hydrazine solution (0.01 ml) and 9% hydrochloric acid solution in ethanol (0.1 ml). The mixture was allowed to react for thirty minutes at room temperature and the resulting derivatives were dried under nitrogen before use. The products were separated and excited using a wavelength of 350 nm. The fluorescence emission was monitored at 505 nm. The minimum detectable amount of cortisol was 0.5 ng.

Lawrence (38) reported a variation on this technique that allowed the reaction to proceed at 80°C for 10 minutes. The products were excited at 360 nm, while 510 nm was used as the emission wavelength.

Tomosova reports the determination of 17-oxo steroids using this derivatization technique (39).

ISONICOTINYLHYDRAZONE DERIVATIVES

Isonicotinylhydrazine (INH) has been reacted with compounds containing 3-keto-4-ene functional groups in a post column derivatization scheme to form fluorescent products. Horikawa, Tanimura, and Tamura (40) performed the reaction in a methanolic aluminum (III) solution and monitored fluorescence above 450 nm. An excitation

wavelength of 360 nm was used. Sensitivity was no better than that achieved with UV absorbance at 254 nm.

ACID-INDUCED FLUORESCENCE

Gotelli, Wall, Kabra, and Marton (41) measured serum concentrations of cortisol using acid-induced fluorescence. Serum extracts were reacted with a 70/30 mixture of ethanol and sulfuric acid for 2 minutes at 70°C. The fluorescent products were excited using a wavelength of 366 nm while emission was detected at 488 nm. Derivative formation was strongly time dependent. The reaction was linear over 1.5 orders of magnitude and 10 µg/l of cortisol could be detected. The remaining corticosteroids were present at low levels and were not detected. Gorog (4) reports that the fluorescence intensity for cortisol in a similar study was 100 while aldosterone was 0.21. Similar techniques are currently used to determine corticosterone (42, 43, 44).

MICROCOLUMN CHROMATOGRAPHY OF STEROIDS

Tsuji and Binns (45) used RP-8 microbore column coupled with a commercial Jasco UV/vis absorbance detector at 254 nm to analyze a synthetic mixture of steroids. The results were compared to the separation of a complex mixture achieved on a conventional column. The mixture included cortisone acetate, fluorometholone, hydrocortisone, hydrocortisone hemisuccinate, methylprednisolone, prednisolone and

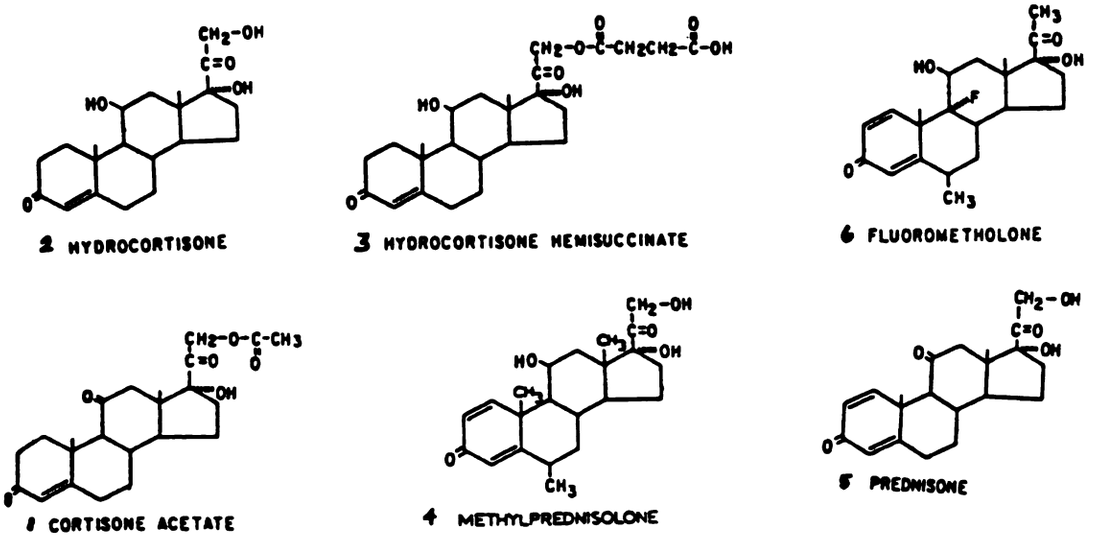
prednisone. As shown by figure 6, these compounds were better resolved by the microcolumn. The microbore system was 16 times more mass sensitive than the conventional system. The conditions used by Patrick and Elliott (46) to analyze bile steroids were very similar. They achieved a limit of detection of 20 picomoles.

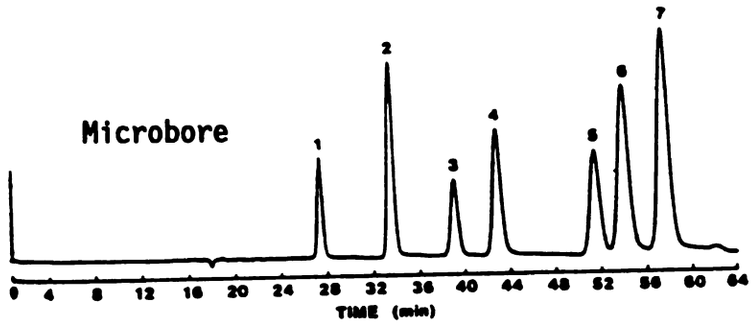
Ishii et al. (47) optimized the separation of corticosteroids by packing a column (145 mm x 0.5mm i.d.) with a variety of 5μm reversed-phase materials. Takeuchi and Ishii (48) employed PTFE tubing with interior diameters ranging from 0.1 to 0.2 mm and studied the separation of antibiotics and steroids that included cortisol, corticosterone, and cortisone.

Novotny et al. (49) used dansyl hydrazine to form fluorescent derivatives from 17-oxosteroids. They also derivatized hydroxy steroids with benzyl chloride and 7-chlorocarbonylmethoxy-4-methyl coumarin. The derivatives were separated on a 1m x 250 μm microcolumn, which was coupled to either a commercial fluorimeter or a UV absorbance detector. They also experienced problems with multiple derivatives.

CONCLUSIONS

The thermal instability of the C-21 side chain and the presence of polar functional groups complicates the determination of corticosteroids by gas chromatography. Although silanizing agents are useful in the derivatization of hydroxyl groups and, if ketones are converted into oximes, can be reacted with corticosteroids, the formation of syn and anti isomers makes the utilization of silyl derivatives difficult.





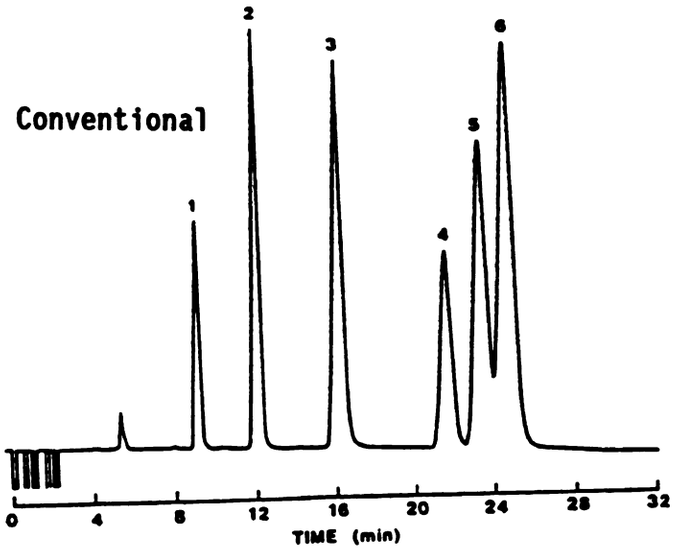


Figure 6-Comparison of Coricosteroid Separations Achieved by a Microbore versus a Conventional Column. Reference 49 , p.233,234.

In an attempt to find a reagent that is selective for ketones, a variety of derivatizing agents has been examined and found to be inadequate. A better approach is the use of high pressure liquid chromatography, which simplifies the determination of corticosteroids. Although UV absorbance detection occurs without derivitazation, greater sensitivity is reached by reacting corticosteroids with a variety of agents. A limited number of fluorescent probes has also been developed.

Improved separation of a corticosteroid mixture is achieved by using a microcolumn over a conventional column. Microcolumns could be coupled with laser-induced fluorescent detection if a reagent selective for ketones and amenable to excitation by a HeCd laser were found.

CHAPTER 3

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CHAPTER 4

FLUORESCENT PROBES DEVELOPED FOR LASER-INDUCED DETECTION SYSTEMS

The reaction schemes studied in this research are based upon the nucleophilic addition of primary amines to carbonyl groups, forming Schiff bases. This reaction is theorized to be a two-step reaction; addition followed by dehydration (1). The overall reaction is enhanced by acid or base catalysis. Dehydration, which is the rate limiting step, is acid catalyzed most effectively between a pH of 2 to 5. The use of oximes, semicarbazones and hydrazones, formed by substituting hydroxyl or nitrogen functional groups on the reactive nitrogen stabilizes the final product (1). Therefore, all the reagents used in subsequent studies were hydrazone-like compounds.

Two novel derivatizing agents selective for ketones were compared to an existing reagent. Reaction conditions were optimized using testosterone as a model C-3 keto steroid. Pregnenolone was used to supply a C-20 ketone site. In this chapter, the use of 2-diphenylacetyl-1,3-indandione-1-hydrazone (DPIH) and 7-diethylaminocoumarin-3-carbohydrazide (DACC) will be examined. These derivatives will be compared to those formed from the reaction of 1-dimethylaminonaphthalene-5-sulfonly hydrazine (dansyl hydrazine).

2-DIPHENYLACETYL-1,3-INDANDIONE-1-HYDRAZONE

INTRODUCTION

Braun and Mosher first synthesized 2-diphenyl-1,3-indanedione-1-hydrazone (DPIH) in 1958 (2) by reacting 2-diphenylacetyl-1,3-indanedione with an excess of hydrazine. In preliminary studies, they showed that DPIH was very reactive with aldehydes and ketones, forming highly fluorescent derivatives. The derivatives were easily crystallized and exhibited melting points over a wide range, thus allowing Braun and Mosher to identify the original carbonyl compounds. Product yields from the derivatization reaction exceeded 95%. As expected, the reaction of DPIH with carbonyl compounds is acid catalysed; a molecule of water acting as a leaving group:

Following their pioneering efforts, DPIH also has been used as a spray reagent in thin layer chromatography to detect alpha-hydroxyacid esters (3) and various insecticides, rodenticides, herbicides and fungicides (4). Additional applications include the use of DPIH as a fluorophore to quantitate ozone (5), as a derivatizing reagent for paper chromatography (6) and for thin layer chromatography (7). Pietrzyk and Chan (7) have

detected fluorescent derivatives from the reaction of DPIH with the following steroids: testosterone, androstan-17-one, estrone, dehydroisoandrosterone, cholestan-3-one, androstan-3,17-dione and androstanolone. Finally, in 1983, Lipari and Swarin (8) used DPIH as a precolumn derivatizing agent for simple aldehydes and ketones found in automobile exhaust. The derivatives were separated using an octadecylsilica column and detected with a fluorescence detector.

These studies have shown that the use of DPIH has several advantages over more traditional carbonyl derivatization reagents such as 2,4-dinitrophenylhydrazone (2,4DNPH) and 3-methyl-2-benzothiazolone (MBTH). DPIH readily reacts with hindered ketone groups (2). The derivatization reaction is rapid (7) and quantitative (9). The limit of sensitivity for DPIH is in the nanomolar range where as 2,4-DNPH MBTH method for aldehydes and ketones is insensitive to compounds with high molecular weights and unsaturated aldehydes (8). Thus, the literature indicates DPIH is a promising candidate as a selective probe for use in corticosteroid analysis.

Braun and Mosher (2) performed derivatization reactions in solvents that included ethanol, ether, dioxane or chloroform using mineral acids, acetic acid, p-toluene sulfuric acid or borontrifluoride etherate as catalysts. Table 4 reviews the optimal reaction conditions found in the literature. From this table, the use of chloroform as a solvent and hydrochloric acid as a catalyst will provide the needed reaction conditions.

Table 4

Summary of Reaction Conditions for DPIH as a Reagent Selective for Carbonyl Compounds

Concentration DPIH	Carbonyl	Solvent	Temp.	Catalyst	Ref.
0.07 M	0.08 M	15 ml CHL	reflux	2 dr HCl	5
0.27 M	0.25 M	20 ml CHL	warm	2 dr HCl	1
0.18-0.27 M	0.16-0.25 M	20-30 ml CHL	reflux	2 dr HCl	1
0.18-0.27 M	0.16-0.25 M	20-30 ml CHL	reflux	2 dr HCl	6
0.18-0.27 M	0.16-0.25 M	20-30 ml ETOH	reflux	none	6
0.06 M	1-3 ppm	20 ml ACN	100°C	5 dr HCl	7

Key: CHL = chloroform; ETOH = ethanol; ACN = acetonitrile; dr = drops

MATERIALS AND METHODS

CONVENTIONAL HIGH PRESSURE LIQUID CHROMATOGRAPHIC SYSTEM

Reaction conditions were optimized by monitoring the intensity of peaks that resulted from the separation of the reaction components by using a Varian model 2010 liquid chromatograph that was equipped with a Brownlee octadecylsilica column (5μm) and a Varian model 2070 spectrofluorimeter. The spectrofluorimeter contained an emission filter that transmitted 310-390 nm, while the excitation filter cutoff wavelength was 460 nm. The signal was displayed on a Linear 261 stripchart recorder.

MICROCOLUMN LIQUID CHROMATOGRAPHY SYSTEM

The derivatives were analyzed using a high-efficiency microcolumn liquid chromatograph coupled with sensitive and selective detection by

laser-induced fluorescence. The analytical system is shown schematically in figure 7, and described further below.

SOLVENT DELIVERY SYSTEM

The solvent delivery system consisted of a reciprocating pump (Beckman Model 114M) that generated flow rates from 1 to 1000 μl/min. Samples of 10-100 nl volumes were introduced by the split injection method using a 1.0-μl valve injector (Valco Model ECIaW1). Microcolumns were prepared by packing fused silica capillary tubing (200 μm I.D., 1-m length) with a slurry of octadecylsilica (ODS) or 3-μm PRP-1 (Hamilton) packing material in an appropriate solvent (10). The microcolumns prepared for this study approached the theoretical limits of efficiency, and achieved theoretical plate numbers in excess of 100,000.

LASER DETECTION SYSTEM

A helium-cadmium laser (Omnichrome model 3112), with approximately 25 mW of continuous-wave output at 325 nm, was used as the excitation source. The laser was focused upon the optically transparent microcolumn that was encased in a square housing and filled with refractive index matched material. This design minimized light scatter and maximized the signal. Sample fluorescence was collected perpendicular and coplanar to the excitation beam. The emission was then isolated with interference filters and focused into a photomultiplyer tube (Centronic Model Q4249B).

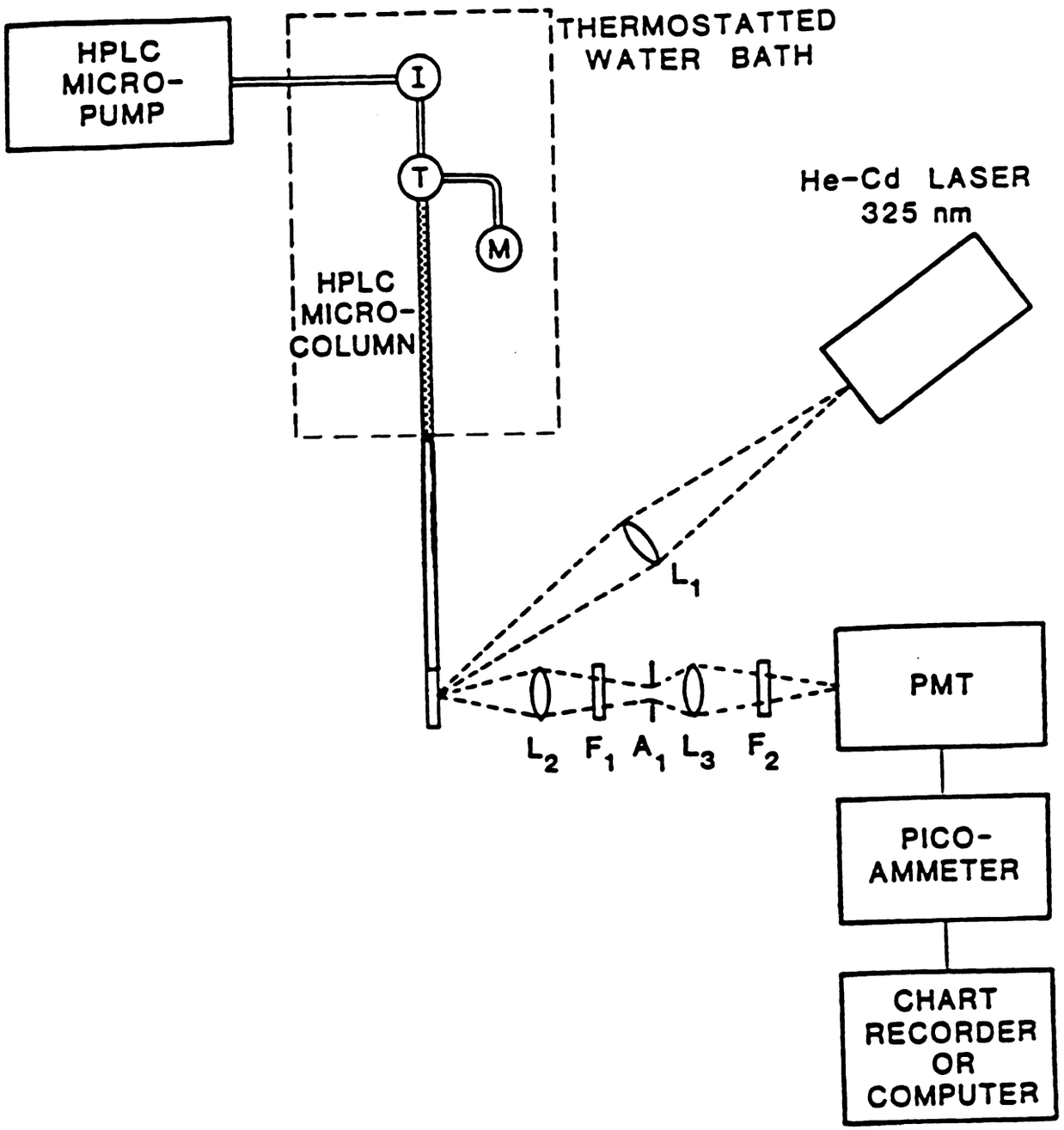


Figure 7-Schematic Diagram of the Chromatographic System Using Microcolumn Liquid Chromatography with Laser-Induced Fluorescence Detection. (I) injection valve; (T) splitting tee; (M) metering valve for splitter; (L) lens; (F) filter; (A) aperture; (PMT) photomultiplier tube.

The photocurrent was amplified by a picoammeter (Keithley model 480) and displayed on a strip chart recorder (Linear model 585).

In preliminary studies the excitation and emission spectra of the steroid derivatives were monitored with a conventional fluorimeter (Perkin Elmer model 512).

GENERAL METHODOLOGY

Standard solutions (10^{-5} M) were prepared by dissolving the ketosteroids (Sigma Chemical Co., St. Louis, MO) in anhydrous methanol. A saturated solution (3 x 10^{-4} M) of the derivatization agent was prepared by dissolving 2-diphenylacetyl-1,3-indandione-1-hydrazone (Aldrich Chemical Co., Milwaukee, WI) in anhydrous methanol. Equal volumes (3.0 ml) of the standard steroid and DIPH solutions were mixed, and 0.14 ml concentrated hydrochloric acid (12 M, J. T. Baker Chemical Co., Phillipsburg, NJ) was added as a catalyst. The reaction was allowed to proceed at 40°C for 30 min to 3 hrs, without stirring. Then, the solution was filtered through Millipore AP glass microfiber filters and the solvent evaporated under a stream of dry nitrogen. Although the derivatized steroids will decompose, they were were stable when stored without solvent at 4°C for several days.

RESULTS AND DISCUSSION

Solubility tests indicated that DPIH was very soluble in chloroform, slightly soluble in methanol, acetonitrile and dioxane, and insoluble in

water. The addition of chloroform to the methanolic solution did not result in appreciable increases in the solubility of the DPIH. Initially, chloroform was used as a solvent, but methanol was later substituted.

In initial experiments, fluorescence spectroscopy was employed to confirm the formation of fluorescent products upon the reaction of DPIH reagent with progesterone, a triketosteroid. Reaction conditions were similar to those used by Braun and Mosher (2), except that methanol, dioxane or chloroform were used as the reaction medium. A bathochromic shift in the wavelength of the emission spectra indicated that DPIH had reacted with progesterone in each solvent. This shift was approximately 70nm, using chloroform as the solvent. Chromatography of the reactant mixture indicated that fewer extraneous products were formed if methanol was used as a solvent. The fluorescence spectra for progesterone in methanol are shown in figure 8 together with the wavelengths selected for analysis.

In preliminary studies, simple aliphatic and aromatic ketones were used to confirm the formation of fluorescent products upon reaction with DPIH. Ketones that were reacted included cyclooctanone, benzophenone, naphthalenone, cyclohexenone, cycloheptanone, methyl-isobutyl ketone, heptacosanone, acetophenone, fluorenone, valerophenone and hydrazone. Benzaldehyde and formaldehyde were also derivatized to confirm reactivity with aldehydes.

Subsequently, a series of monoketosteroids representative of the functional sites in corticosteroids was examined using the same reaction conditions. In general, the derivatization reagent was reactive with all the ketosteroids examined and formed highly fluorescent derivatives.

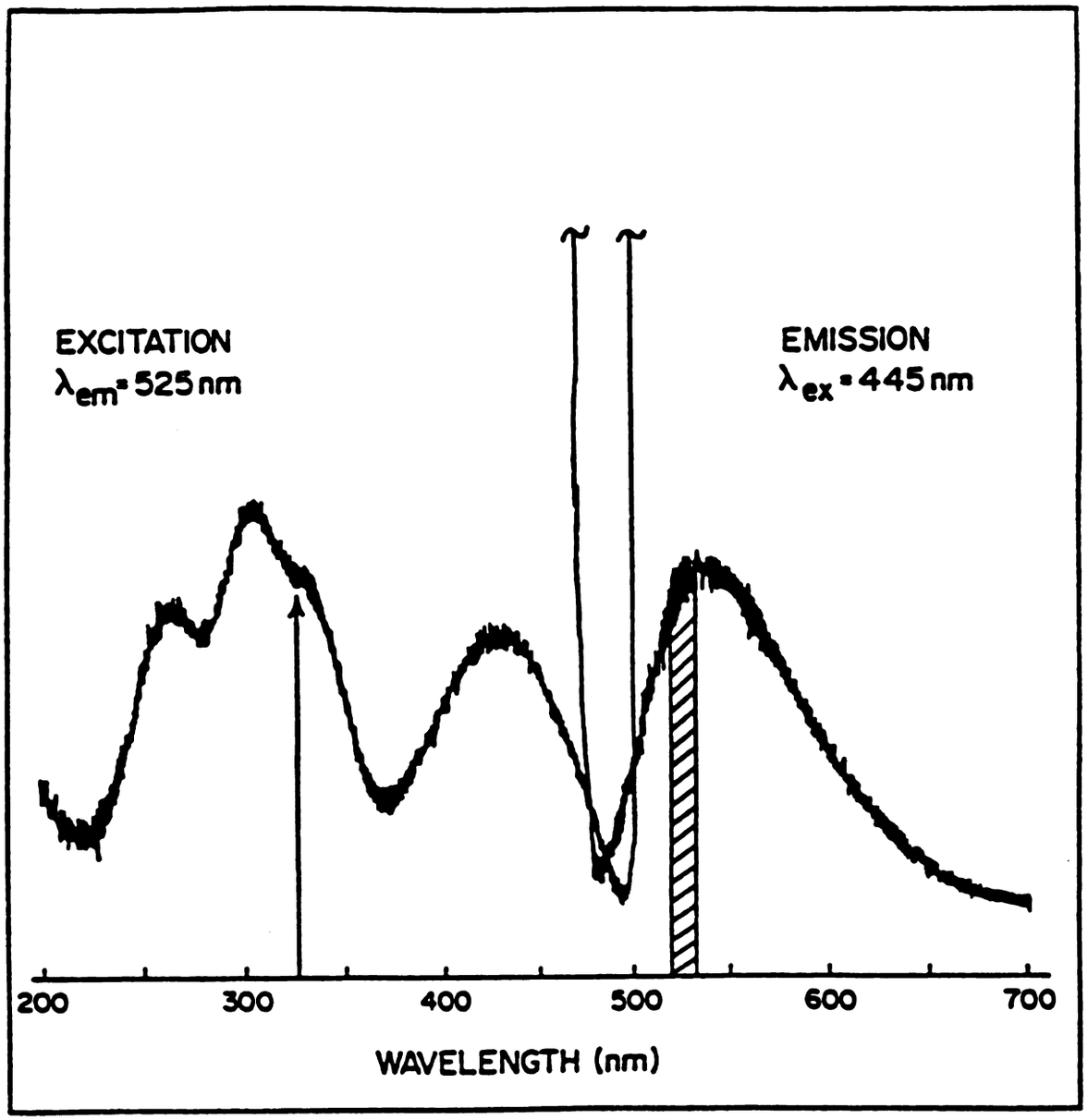


Figure 8-Fluorescence Excitation and Emission Spectra of Progesterone Derivatized with 2-diphenylacetyl-1,3-indandione-1-hydrazone.

Steroids with conjugated ketone sites were more reactive than their unconjugated counterparts. No fluorescent products were formed by steroids that contained hydroxyl groups and were lacking in ketone moieties.

These preliminary results indicated that selective analysis of ketosteroids was feasible using the proposed reagent. However, upon chromatographic separation of the derivatized steroids, multiple fluorescent products were observed for monoketosteroids as well as polyketosteroids, as shown in figure 9. As previously discussed, this phenomenon has been widely reported in the literature for other reagents and is generally attributed to the formation of syn and anti isomers at the 3-keto-4-ene structural site.

Upon the reaction of testosterone with the present reagent, four products were formed with differing chromatographic properties. Two products of approximately equal and low concentration were slightly retained. These products had good peak shape and showed a maximum absorbance at 300 nm. The other two products of greater and unequal concentration were more highly retained and showed excessive tailing under these chromatographic conditions. These later eluting peaks exhibited a maximum absorbance of 320 nm. Because of the differing chromatographic and spectroscopic properties, it was initially believed that the multiple products were the result of impurities in the reagents or by products of the derivatization reaction. Therefore, an extensive purification of the materials was performed and upon completion, no change in the number of products was observed. A thorough optimization of the reaction conditions using testosterone and pregnenolone as model

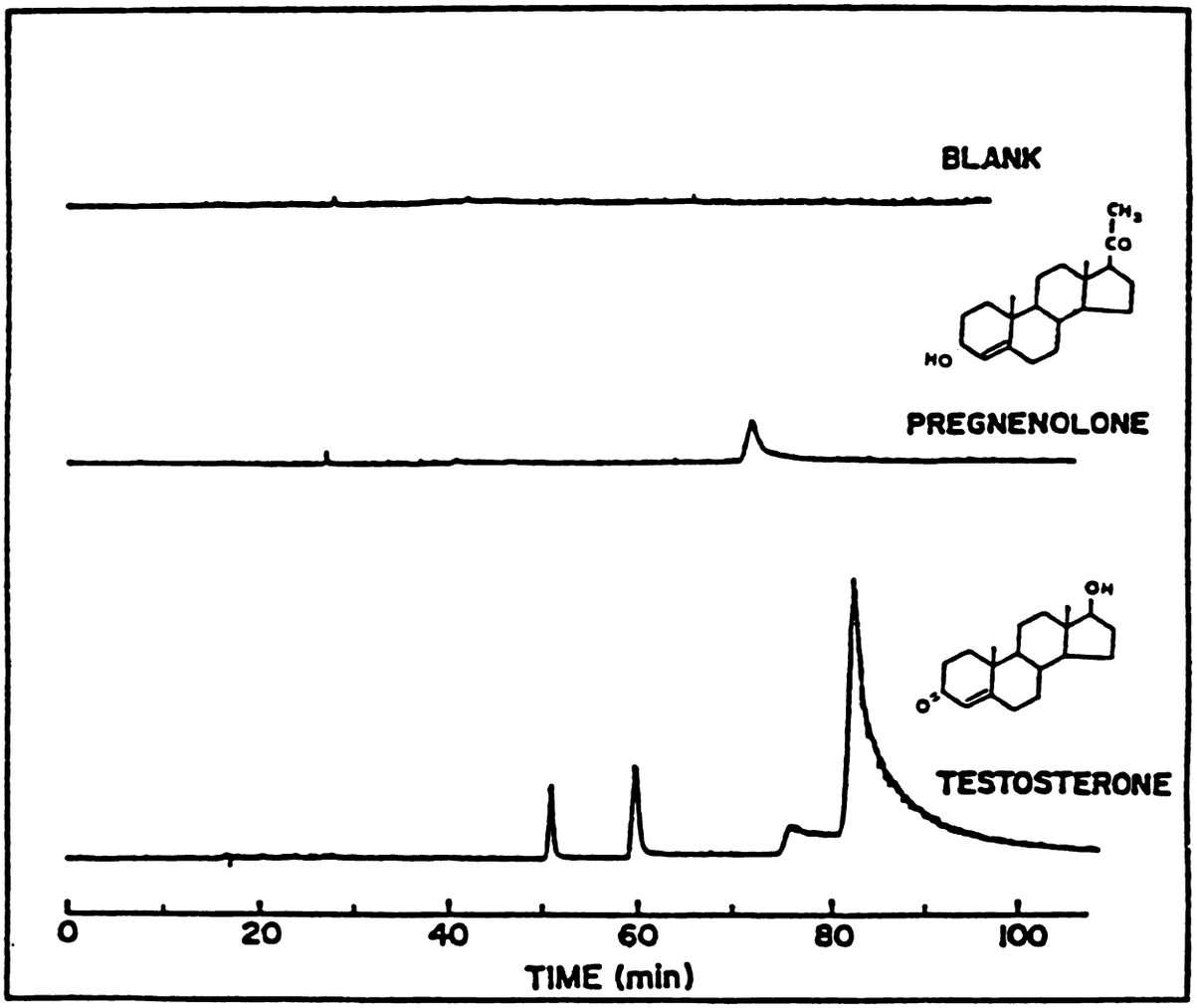


Figure 9- HPLC Chromatograms of pregnenolone and testosterone Derivatized with 2-diphenylacetyl-1,3-indandione-1-hydrazone.

Microcolumn: 200 μI.D. x 89 cm length fused silica capillary, packed with Spheri-5 RP-18. Mobile phase: methanol at 0.9 μL/min. Fluorescence detector: 325 nm excitation, 520 nm emission, sensitivity 1.0 μA/V, 0.1 V full scale.

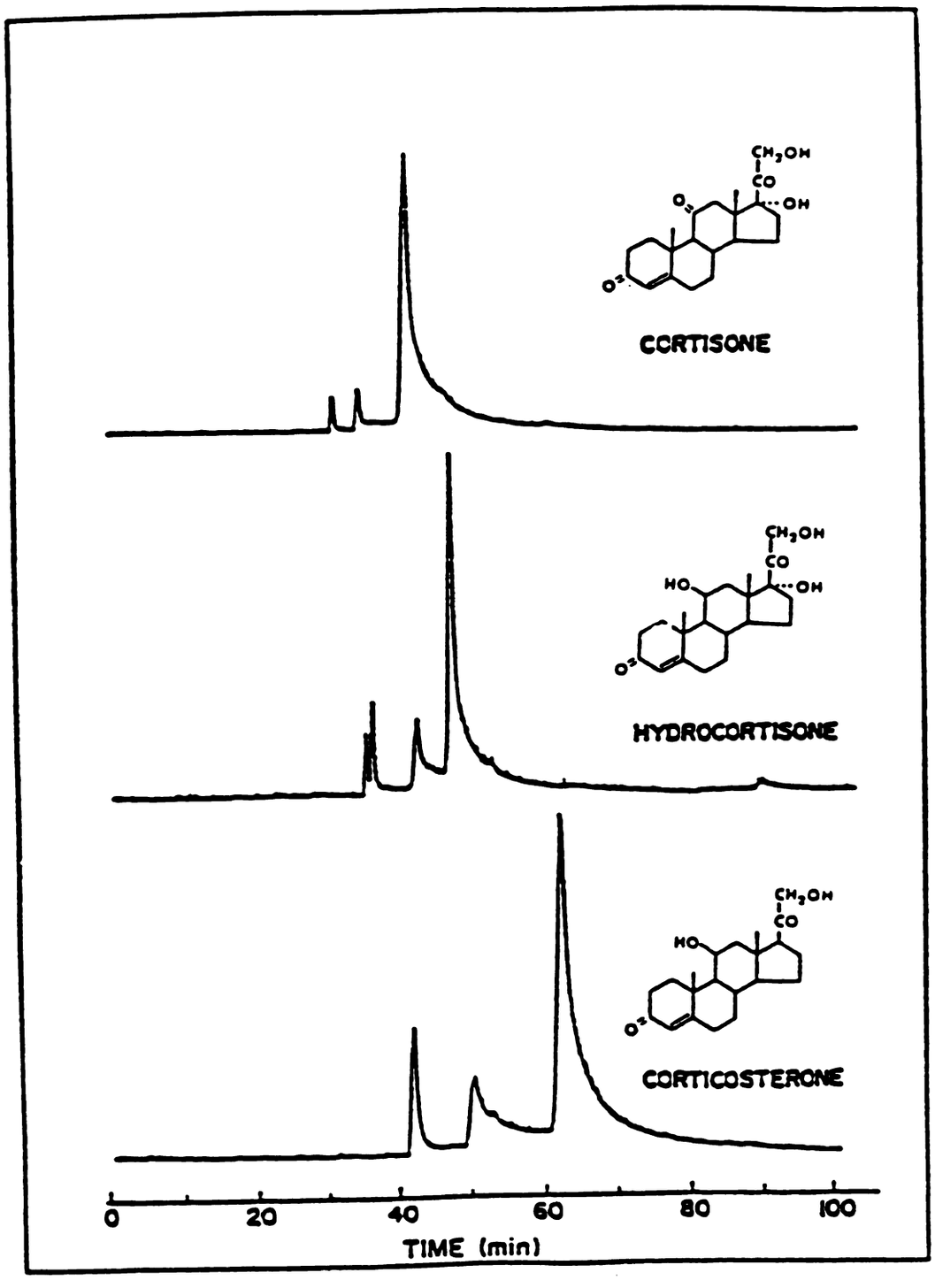


Figure 9- (Cont..) HPLC Chromatograms of pregnenolone and testosterone Derivatized with 2-diphenylacetyl-1,3-indandione-1-hydrazone.

solutes was performed to further investigate this problem and discover more favorable reaction conditions.

CATALYST CHOICE

Various catalysts were used in an attempt to investigate the conditions under which isomerization may be occurring and to limit the number of reaction products formed. Several organic and inorganic acids of varying strength were compared as catalysts for the reaction of testosterone and pregnenolone with DPIH. The catalysts used were hydrochloric acid, sulfuric acid, acetic acid, and borontrifluoride etherate. The results are shown in figure 10 for pregnenolone and the most retained compound formed by the reaction of testosterone as these products all exhibited the same pattern. No fluorescent products were observed without a catalyst nor with acetic acid. The initial rate of the reaction was greater using sulfuric acid than hydrochloric acid, but the decomposition rate of the derivatives was also increased. Thus, hydrochloric acid is the optimum catalyst in both methanol and chloroform. The figure also compares the reaction rates of pregnenolone versus testosterone, the conjugated 3-keto-4-ene reacting at a higher rate than the nonconjucated ketone on the C-20 sidechain.

Boron trifluoride etherate was used as a milder catalyst that could be removed easily by evaporation and was theorized to form more stable products. These results are shown in figure 11. As in previous experiments, the reaction of testosterone yielded four products while pregnenolone formed a single derivative. Although boron trifluoride

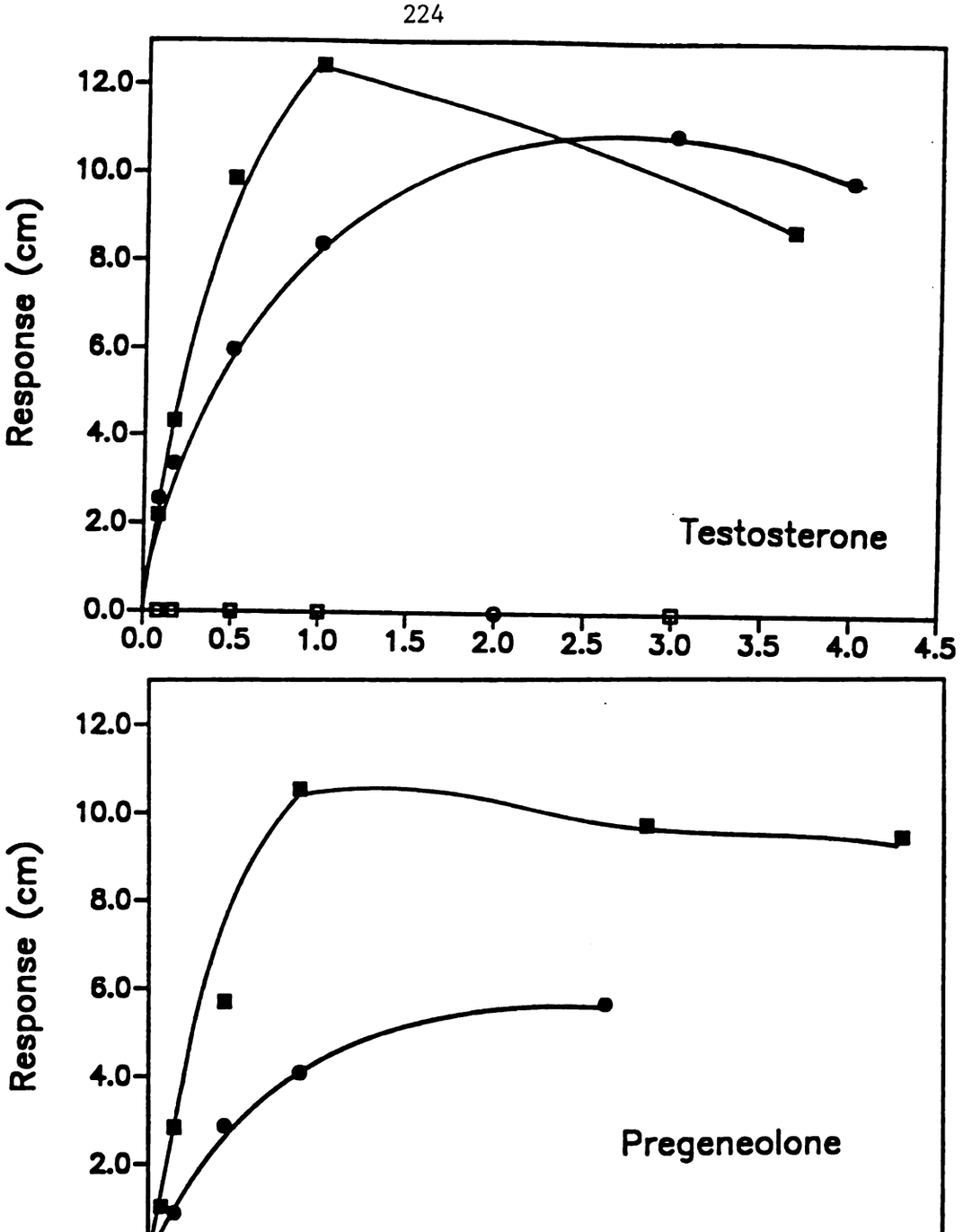


Figure 10-The Effect of Catalyst Choice on the Derivatization of Testosterone and Pregnenolone with 2-diphenylacetyl-1,3-indandione-1-hydrazone. Reaction conditions: DPIH 3.0×10^{-4} M, reacted with 5.2×10^{-5} M testosterone or 5.5×10^{-4} M, DPIH reacted with 3.7×10^{-4} M pregnenolone at 40 °C. o No Catalyst, □ CH_3CH_2COOH, ■ H_2SO_4, ● HCl.

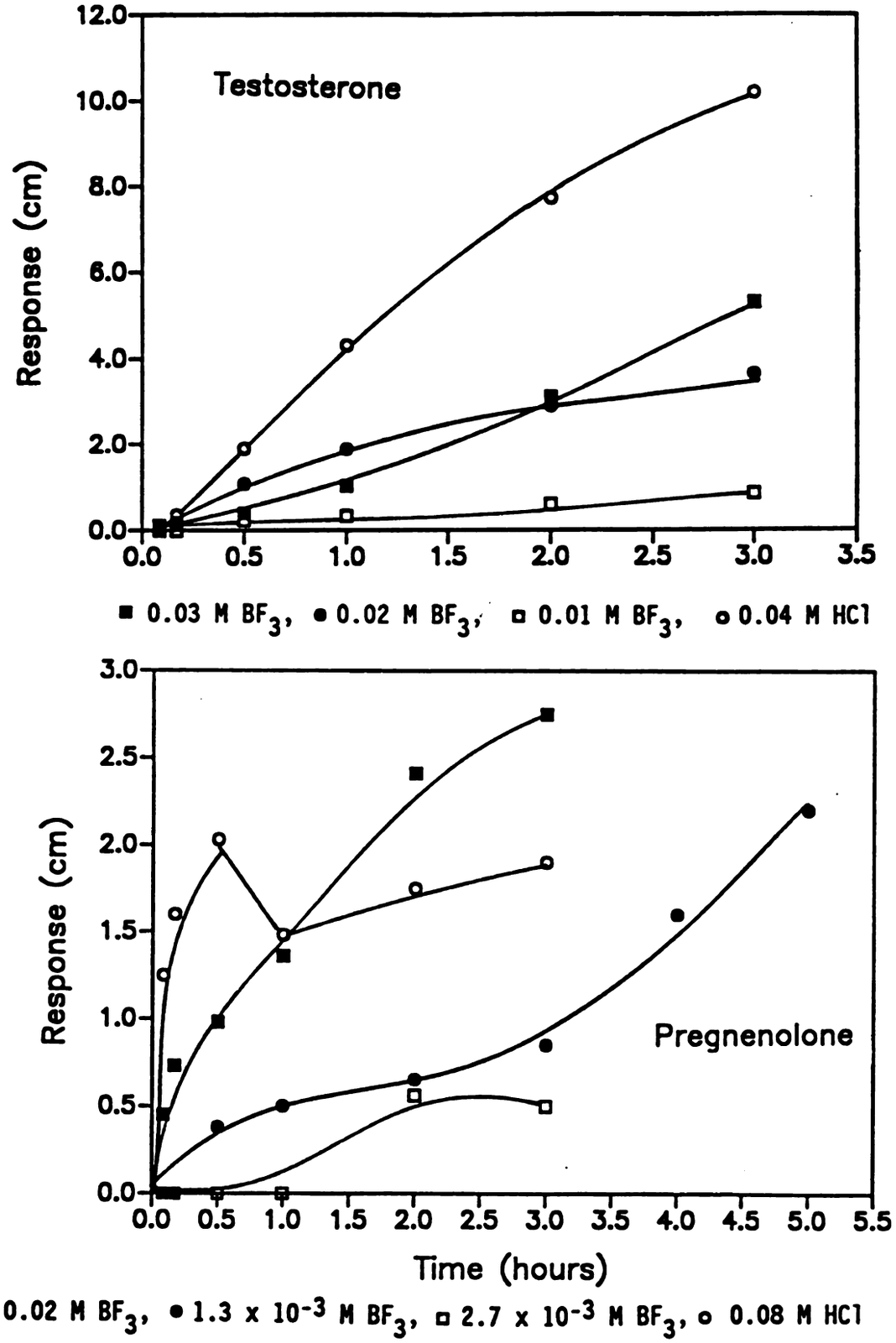


Figure 11-The Use of Boron Trifluoride as a Catalyst for the Derivatization of Testosterone and Pregnenolone with 2-diphenylacetyl-1,3-indandione-1-hydrazone. Reaction conditions: DPIH 3.0 x 10^{-4} M reacted with 5.2 x 10^{-5} M testosterone or 0.5 x 10^{-5} M pregnenolone at 40 °C.

catalyzed the reaction, hydrochloric acid was a more potent catalyst. When borontrifluoride concentrations were increased, no improvement of catalytic action was observed, thus hydrochloric acid remained the catalyst of choice.

The concentration of hydrochloric acid was varied from 20 to 200 μm, as shown in figure 12 with the optimal concentration for the reactions of testosterone and pregnenolone with DPIH being approximately 80 μm. The addition of an excessive amount of hydrochloric acid decreased product formation. This is explained by the pH-dependent step in the reaction, or by the introduction of greater amounts of water causing decomposition or limitation of the reaction. In all instances, multiple products for testosterone were observed.

Various Lewis acids, such as $AlCl_3$, $CrCl_3$, $SnCl_2$, $SnCl_4$, and $MgBr_2$ were added as auxiliary catalysts with hydrochloric acid, as shown in figure 13. The Lewis acids were expected to complex selectively with the conjugated diketone group of the DPIH reagent, possibly reducing the number of by-products formed in the derivatization reaction (11). Although the presence of auxiliary catalysts increased the rate of product formation, multiple fluorescent products were not eliminated.

Aluminum in various forms was also used as a catalyst. Foil, acid-washed foil, 20-mesh granules, acid-washed 20-mesh granules, aluminum wire and aluminum sulfate were tested in a reaction mixture of hydro hloric acid, testosterone and reagent that was heated to 20°C. The vials that contained 20-mesh aluminum washed with HCl and aluminum sulfate exploded. No improvement in the reaction rate nor in the elimination of multiple products was observed in the remaining vials.

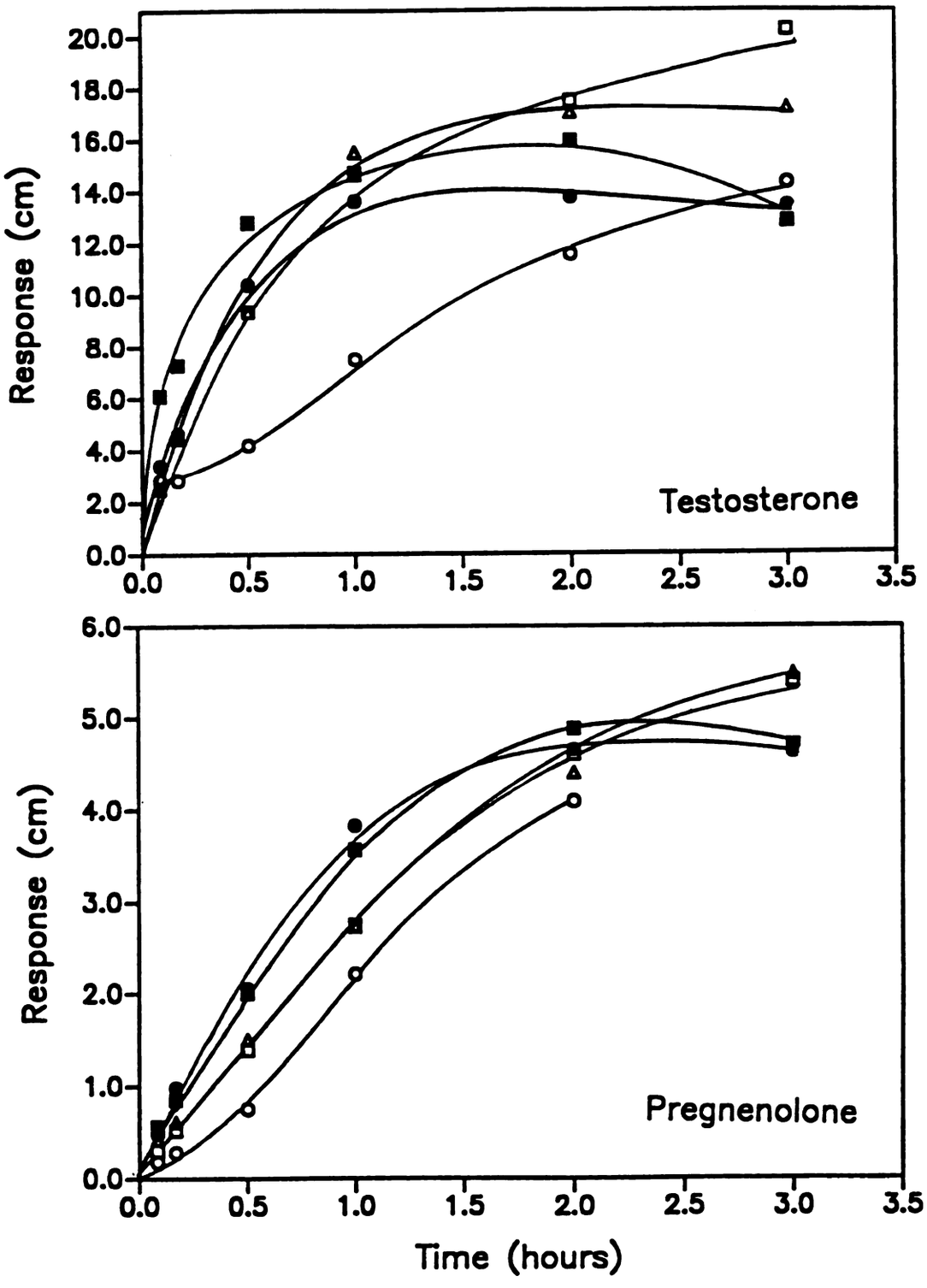


Figure 12-The Effect of Hydrochloric Acid Concentration on the Derivatization of Testosterone and Pregnenolone with 2-diphenylacetyl-1,3-indandione-1-hydrazone. Reaction conditions: DPIH 3.0 x 10^{-4} M reacted with 5.2 x 10^{-5} M testosterone or 5.2 x 10^{-5} M pregnenolone at 40 °C.
○ 0.02 M HCl, □ 0.04 M HCl, △ 0.08 M HCl, ● 0.16 M HCl, ■ 0.20 M HCl.

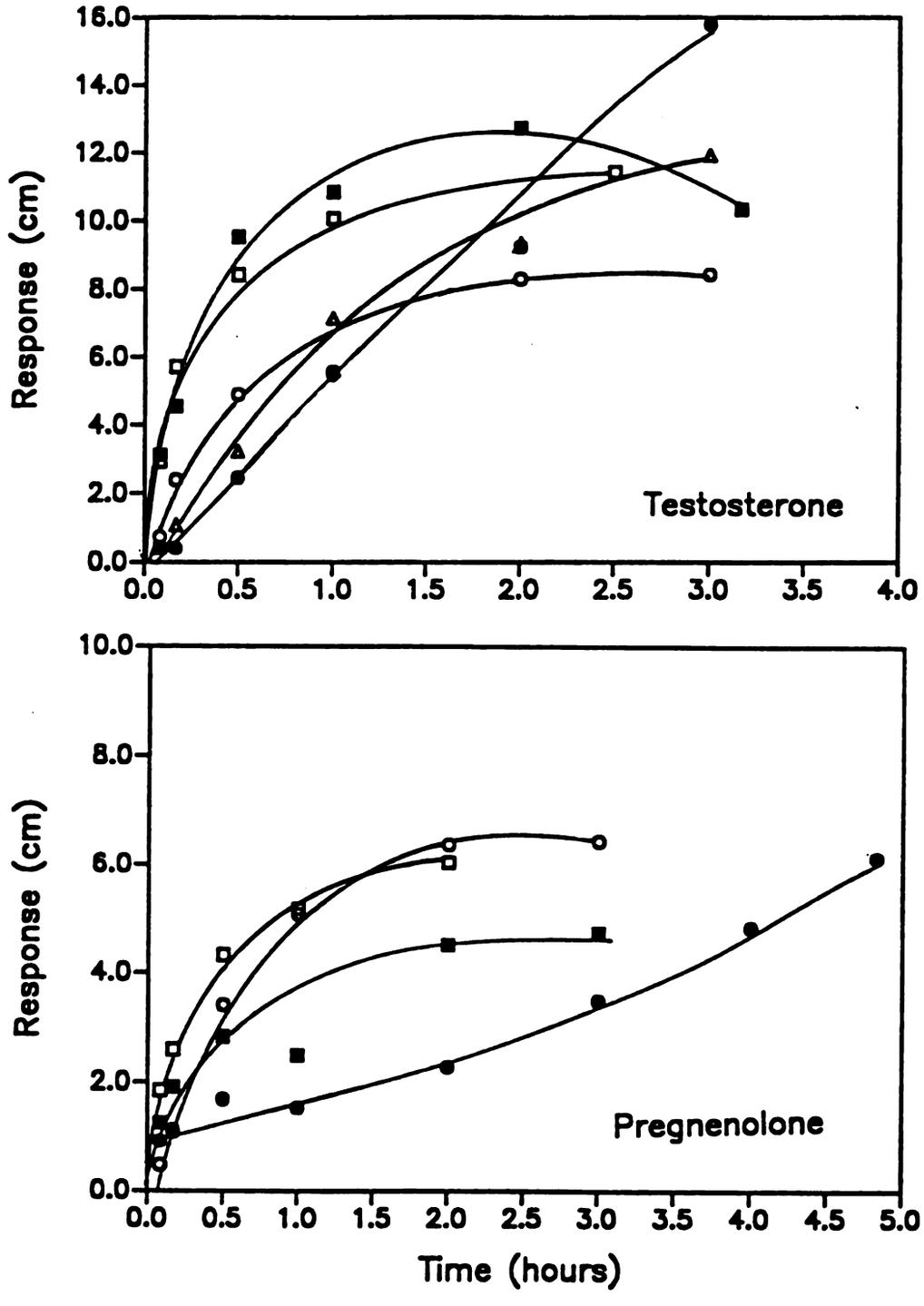


Figure 13-The Effect of the Addition of Lewis Acids (0.05×10^{-3} M) on the Derivatization of Testosterone and Pregnenolone with 2-diphenylacetyl-1,3-indandione-1-hydrazone. Reaction conditions: 6.0×10^{-4} M DPIH reacted with 5.2×10^{-5} M testosterone or 5.2×10^{-5} M pregnenolone and 0.08 M HCl at 40 °C.
○ HCl, □ $AlCr_3$ + HCl, ■ $CrCl_3$ + HCl, ● $SnCl_2 \cdot 2H_2O$, ▲ $SnCl_4 \cdot 6H_2O$.

MOLAR RATIOS

The molar ratio of DPIH to steroid was varied to determine the optimal derivatizing reagent concentration. A 6-fold excess of reagent to steroid proved to be optimal for both testosterone and pregnenolone, as shown in figure 14.

Finally, to evaluate whether interaction of the catalyst with either the derivatizing agent, or the steroid was responsible for the multiplicity of reaction products, the order and timing of the addition of the reagents were systematically varied. There was no change in the amount or number of products formed.

TEMPERATURE STUDIES

The derivatization reaction of testosterone and pregnenolone were examined as a function of temperature from 20 to 50°C. No products were observed when the reaction was carried out at 20°C. As illustrated in figure 15, no significant differences were observed in the rate of product formation or decomposition. This indicates that the rate-limiting step in the derivatization reaction is equilibrium controlled, rather than kinetically controlled, as expected (1). Furthermore, the number of reaction products was invariant with alterations in temperature, indicating that thermal decomposition of the steroid or steroid derivative was not the source of the multiple peaks.

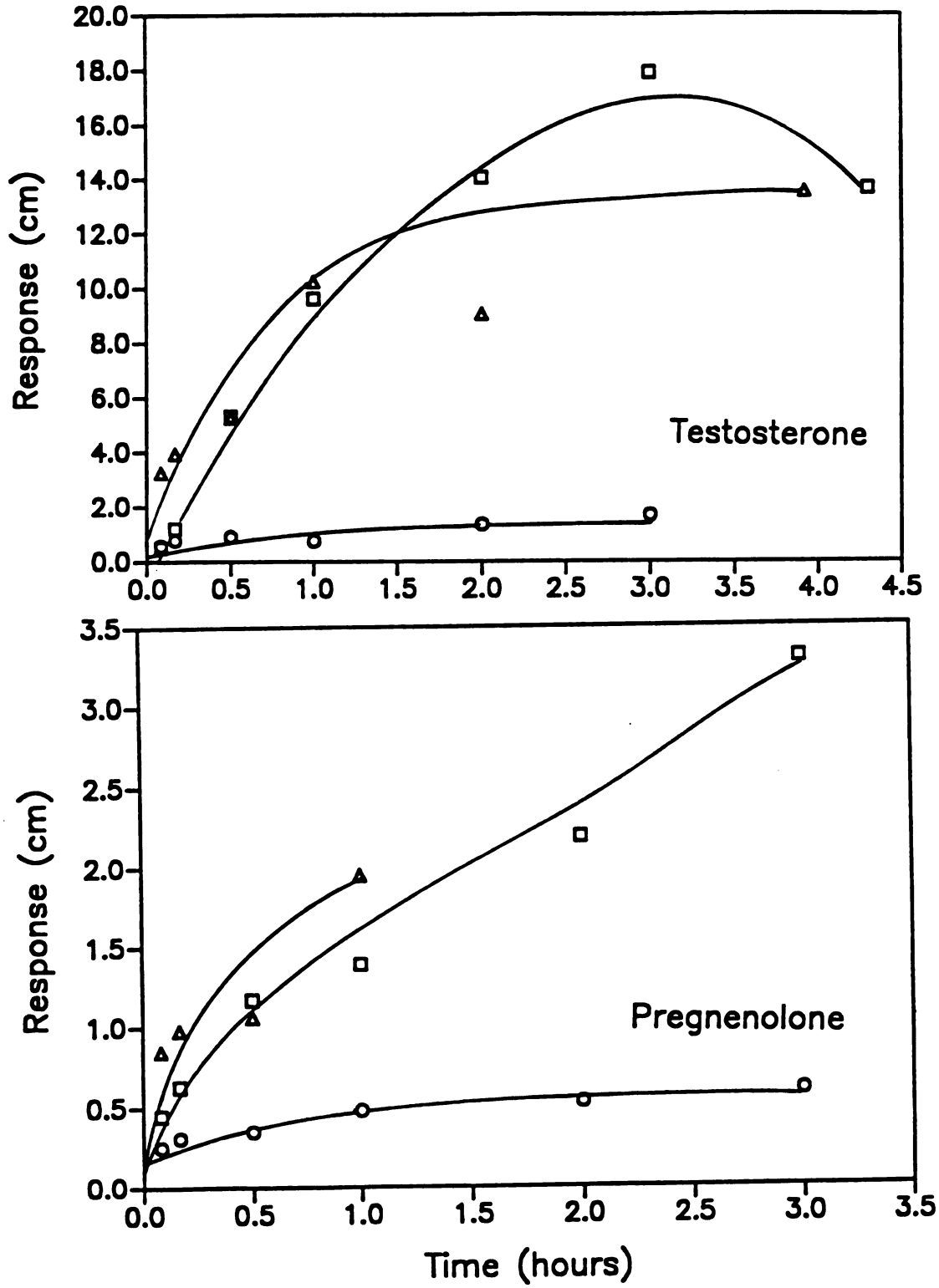


Figure 14-The Effect of Alterations in the Molar Ratio of Reagent to Steroid on the Reaction of Testosterone and Pregnenolone with 2-diphenylacetyl-1,3-indandione-1-hydrazone. Reaction conditions: 5.2×10^{-5} M testosterone or 5.2×10^{-5} M pregnenolone reacted with DPIH using 0.08 M HCl as a catalyst at 40 °C.
▲ 10:1, □ 6.0:1, o 2.0:1.

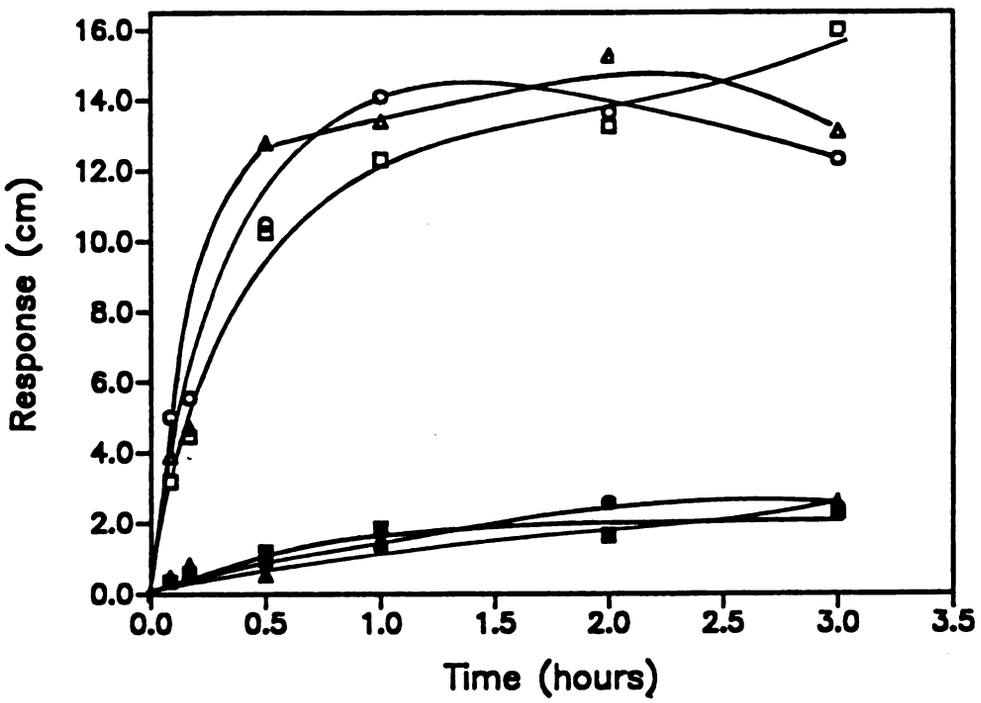


Figure 15-The Effect of Temperature on the Reaction of Testosterone (open symbols) and Pregnenolone (filled symbols) with 2-diphenylacetyl-1,3-indandione-1-hydrazone. Reaction conditions: 5.2 x 10^{-5} M testosterone or 5.2 x 10^{-5} M pregnenolone reacted with 3.0 x 10^{-4} M DPIH using 0.08 M HCl as a catalyst. 50 °C, ◻ 40 ° C, ○ 30 °C.

ADDITIONAL STUDIES

Water may act as a stimulus for derivative decomposition because it is a product of the derivatization reaction. This possibility was eliminated by dehydrating all reagents, solvents and solutions with drying agents such as calcium chloride, sodium sulfate or molecular sieve 4A. As shown in figure 16, no changes were observed in the amount or multiplicity of the products.

To prevent attack of the reagent at the 3-keto-4-ene site, the ketone group was methylated using diazomethane. Despite this attempt to simplify the number of derivatives formed from the reaction with testosterone, multiple products resulted upon derivatization.

Another possible source of interference is the formation and subsequent derivatization of additional ketone sites by tautomerization of hydroxyl groups. This possibility was examined by reacting cholesterol, a steroid with a hydroxyl group in the C-3 position, with the reagent. No fluorescent products were noted. Thus, the possibility of keto-enol tautomerization under the present reaction conditions was eliminated.

In order to limit the number of products formed, all reagents were purified and the order of their addition was systematically varied. The derivatization reaction was also conducted under nitrogen at altered temperatures. The products were either left in solution or evaporated to dryness. All permutations of the above conditions resulted in the formation of multiple products.

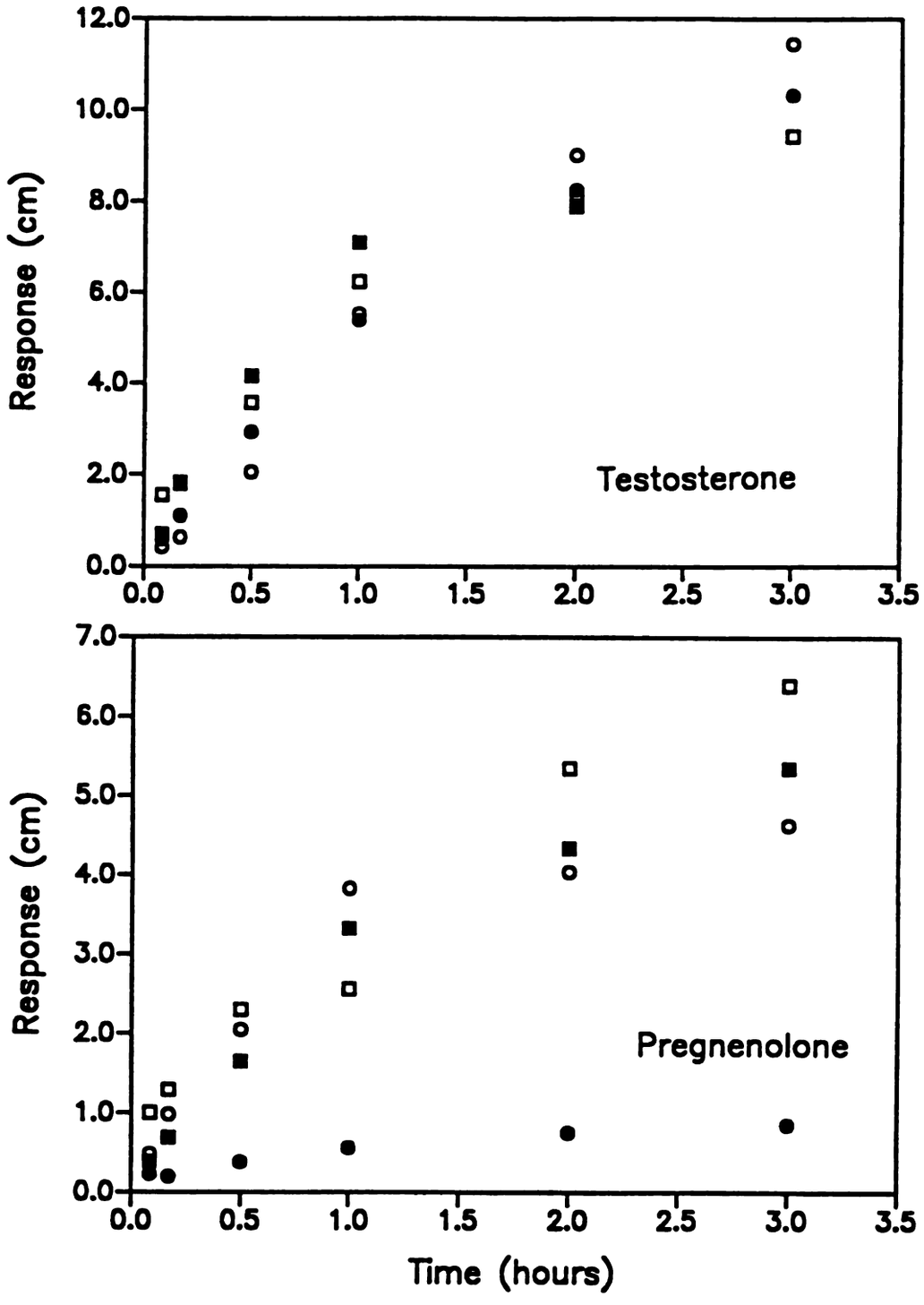


Figure 16-The Effect of Drying Agents Upon the Reaction of Testosterone and Pregnenolone with 2-diphenylacetyl-1,3-indandione-1-hydrazone. Reaction conditions: 5.2 x 10^{-5} M testosterone or 5.2 x 10^{-5} M pregnenolone reacted with 3.0 x 10^{-4} M DPIH using 0.04 M HCl as a catalyst at 36 °C. 0.2 g drying agents added.
o no agent, □ $CaCl_2$, ● Molecular Seive 4A, ■ Na_2SO_4.

Another source of isomeric products is the formation of configurational isomers at the azine bond. As shown in figure 17, four E and Z configurations are possible upon the reaction of DPIH with testosterone. The two early eluting derivatization products of testosterone may be the E,E and E,Z configurations. The examination of molecular models indicates that these products are sterically hindered and are not expected to form in abundance. Furthermore, the lone pairs of electrons on the azine nitrogen atoms are not readily accessible, so tailing of chromatographic peaks due to absorption is not expected nor is it observed. Presumably, the later-eluting products are the Z,Z and Z,E configurations. From examination of molecular models, these products are very stable and are expected to be the predominant products of the reaction.

To substantiate this theory, a weak Lewis acid, $MgBr_2$ was added during the derivatization to act as a stabilizing or protective agent. Configurations with accessible pairs of electrons bind weakly with the Lewis acid and are protected from further interactions with exposed silanol sites. The increased amount of product observed for the later eluting peaks confirmed their identification as the Z,E and E,Z isomers, as greater accessibility of the electron pairs was shown by molecular models. No change in product formation was noted for the earlier eluting peaks, identifying them as the Z,Z and the E,E configurations that lack accessible electron pairs cannot be protected in this manner. Molecular models also suggest that there is less steric hindrance around the C-20 ketone site, and therefore, the derivative of pregnenolone can assume its most stable form and avoid the formation of multiple products.

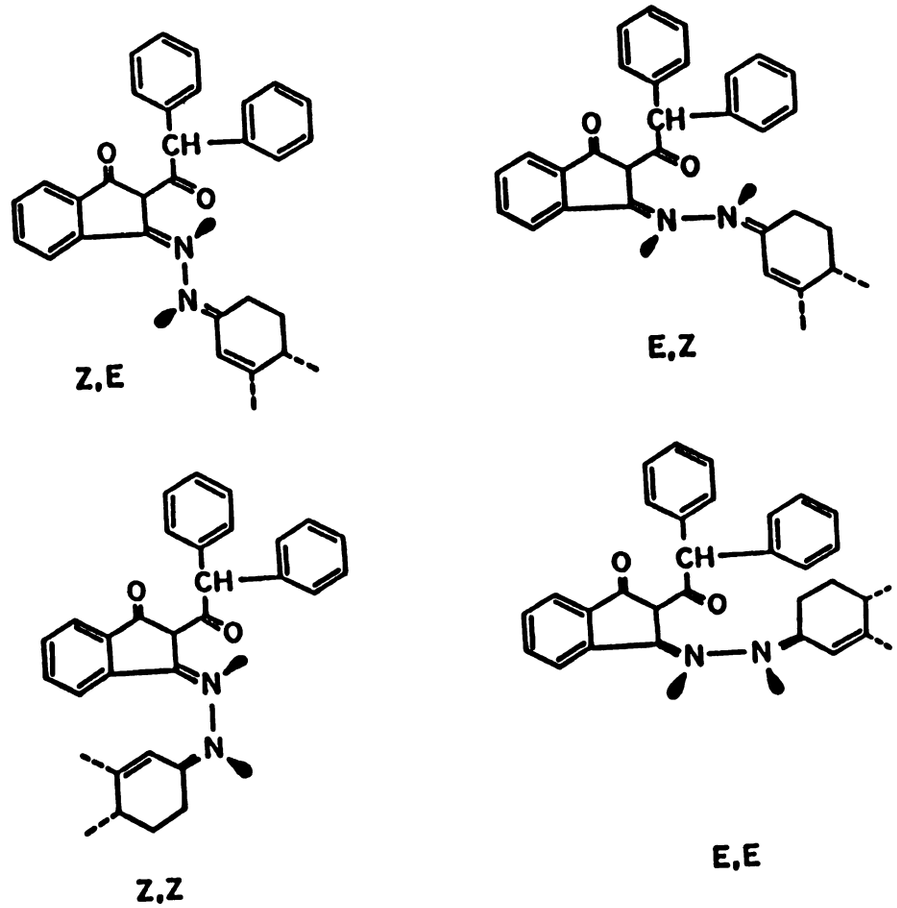


Figure 17-E,Z Isomers of the 2-diphenylacetyl-1,3-indandione-1-hydrazone Derivative of Testosterone.

CONCLUSIONS

The compound, 2-diphenylacetyl-1,3-indandione-1-hydrazone readily reacts with simple aliphatic and aromatic ketones and aldehydes to form highly fluorescent products. Steroids with conjugated and aromatic ketone sites were more reactive than the corresponding unconjugated compounds. Furthermore, sterically hindered ketone sites within the steroid rings were less reactive than the more accessible sites on the side chain. Finally, no fluorescent products were formed for steroids that contained hydroxyl groups but no ketone moieties.

Optimal reaction conditions used HCl (0.08 M) as a catalyst and a molar ratio of DPIH to ketone of 6:1 at 40°C. The reaction proceeded smoothly and yielded fewer by products if methanol was used as a reaction solvent. The reaction was reproducible for both testosterone and pregnenolone as shown in figure 18. Also, the small amounts of water that were introduced by the addition of hydrochloric acid and were byproducts of the reaction did not hinder derivatization.

The use of DPIH as a precolumn derivatizing agent for compounds with a 3-keto-4-ene structure is limited due to the formation of E,Z isomers around the azine bond. However, this compound may be used in other applications such as the formation of fluorescent products for analysis of volatile compounds in food and in carbonyl-containing pesticides. Also, DPIH may be a suitable agent for use in post column derivatization schemes.

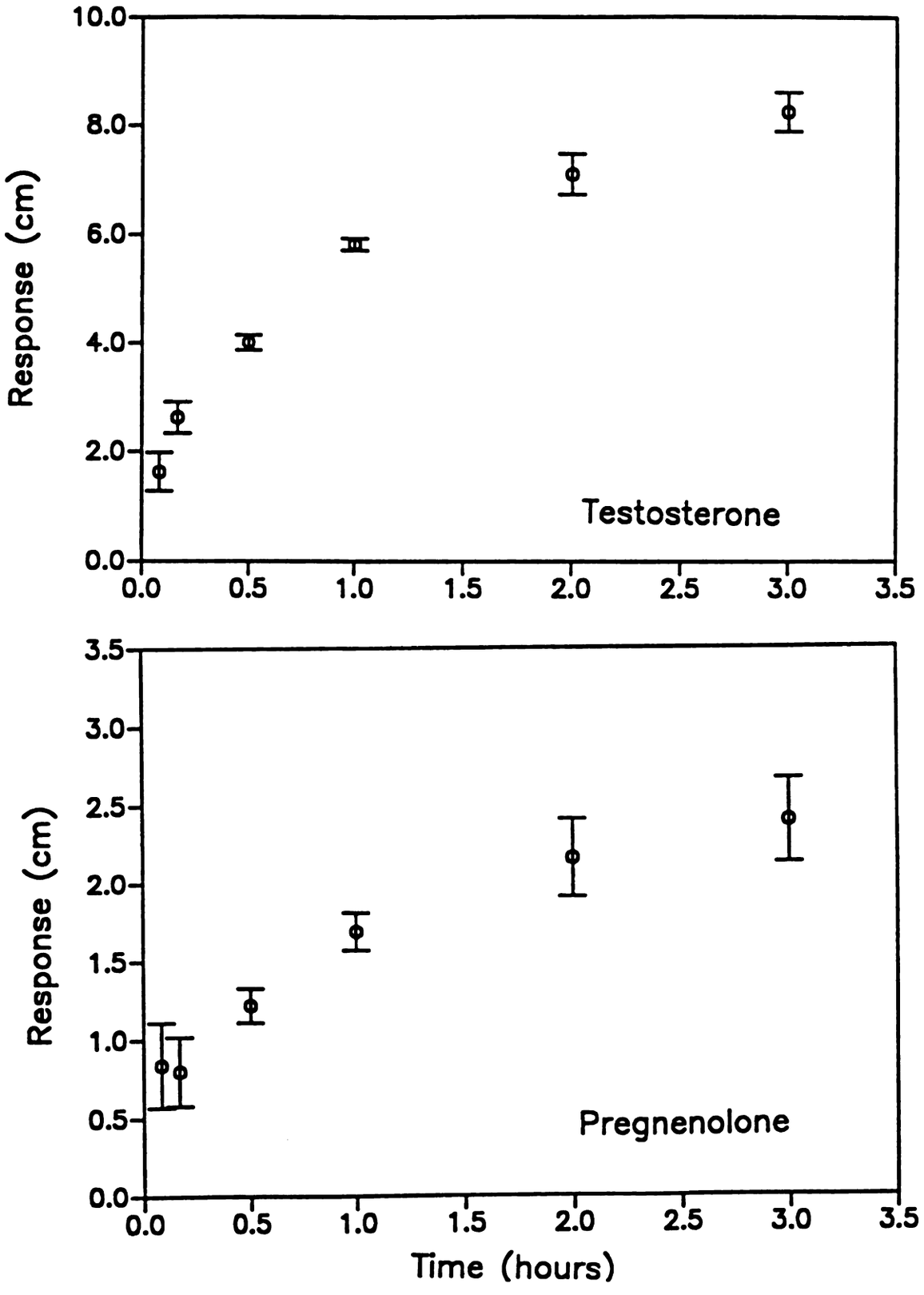
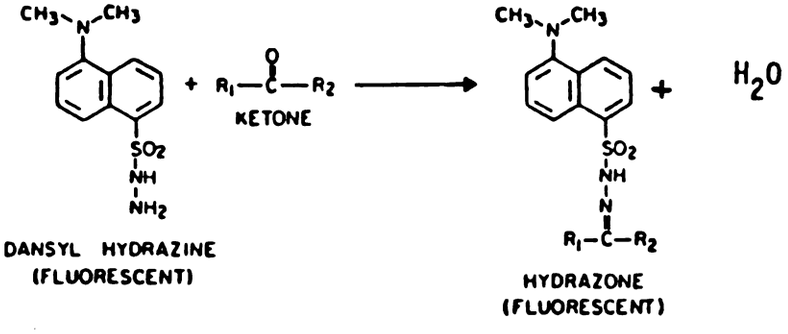


Figure 18-Reproducibility of the Derivatization Reaction of 2-diphenylacetyl-1,3-indandione-1-hydrazone with Testosterone and Pregnenolone. Reaction conditions: 5.2×10^{-5} M testosterone or 5.2×10^{-5} M pregnenolone reacted with 3.0×10^{-4} M DPIH using 0.08 M HCl as a catalyst at 40 °C.

DANSYL HYDRAZONE

INTRODUCTION

The reaction of 1-dimethylaminonaphthalene-5-sulfonyl hydrazine (dansyl hydrazone) with ketone moities is very similar to that of DPIH, also being an acid-catalysed reaction that yields one molecule of water upon derivatization with a ketone group.



The increased length of the side chain at the reaction site should act to minimize steric hindrance and prevent the formation of four E,Z isomers when reacted with a 3-keto-4-ene group.

Conditions for derivatization that are cited in the literature predominantly use a solution of concentrated hydrochloric acid (0.065 ml) that is added to 1 liter of absolute ethanol solution as a catalyst. Dansyl hydrazine (2 mg/ml) is dissolved into ethanol. Steroids (2-20 mg dry wt) are mixed with dansyl reagent (0.1 ml) and hydrochloric acid solution (0.1 ml) and allowed to react at a wide range of temperatures that are dependent upon the reactivity of the ketone group.

Derivatization may occur at lower temperatures as with the very reactive the 3-keto-4-ene moiety of corticosteroids. The 17-keto group is much less reactive and must be heated to 70-100°C for 15-20 min to accomplish derivatization (12, 13).

Other variations in the derivatization procedure include the use of glacial acetic and methanol with a 2:1 ratio of derivatizing agent to ketone (14). Kawasaki, Maeda, and Tsuji (15) employed a trichloroacetic acid (TCA)-benzene (0.5%) solution as a catalyst that was coupled with a dansyl hydrazine-benzene (0.2%) solution. Steroids (0.5 μmol/ml) were dissolved into methanol, evaporated and reconstituted with the TCA (0.2 ml) and dansyl hydrazine (50 μl) solutions. The reaction was allowed to proceed at 60°C for 20 min. Chromatograms of the conjugated 17-keto steroids derivative showed some tailing. Frei and Lawrence (16) report that the reaction also may be performed in DMSO with a trace of hydrochloric acid used as a catalyst. Excitation wavelengths ranging from 340 nm to 365 nm and emission wavelengths from 525 nm to 530 nm have been reported (14, 16) and result in limits of detection from 1 to 10 ng.

MATERIALS AND METHODS

CONVENTIONAL HIGH PRESSURE LIQUID CHROMATOGRAPHIC SYSTEM

A liquid chromatograph (Varian model 2010) that was equipped with an ODS column (5 μm) and a spectrofluorimeter (Varian model 2070) was used as previously described. The mobile phase was altered to various methanol/water solutions. The excitation wavelength was 340 nm and 525 nm

was used to detect emission.

GENERAL METHODOLOGY

Ketone-free methanol (Mallinckrodt, Inc., Paris KY) was prepared by distillation and used throughout the remaining studies. The first and last fractions from the distillation were disgarded.

The dansyl hydrazone solution was prepared by adding 0.4 g dansyl hydrazine (Pierce Chemical Co., Rockford, Il) to 25 ml 95% ethanol (Mallinkrodt, Inc., Paris, KY). A stock acid solution was mixed by adding 0.033 ml concentrated hydrochloric acid (J. T. Baker, Inc. Phillipsburg, NJ) to 50 ml 95% ethanol. Stock carbonyl solutions were prepared by combining the carbonyl (0.5 g) with methanol (20 ml), alternatively, 0.1 ml of the neat solutions were used. In addition to the steroids, simple ketones that included methylethylketone, cycloheptanone, 3-pentanone, acetophenone and acetone (Mallinckrodt, Inc., Paris KY) were derivatized. The various stock solutions were mixed in the following proportions: 0.2 ml hydrochloric acid solution, 0.2 ml carbonyl solution, and 0.4 ml of the dansyl hydrazine solution. The derivatization reaction was carried out in methanol, distilled methanol, ethanol, or chloroform at 20°, 40° or 100°C.

RESULTS AND DISCUSSION

The excitation and emission spectra for the dansyl hydrazine derivative of testosterone in methanol is shown in figure 19; they are

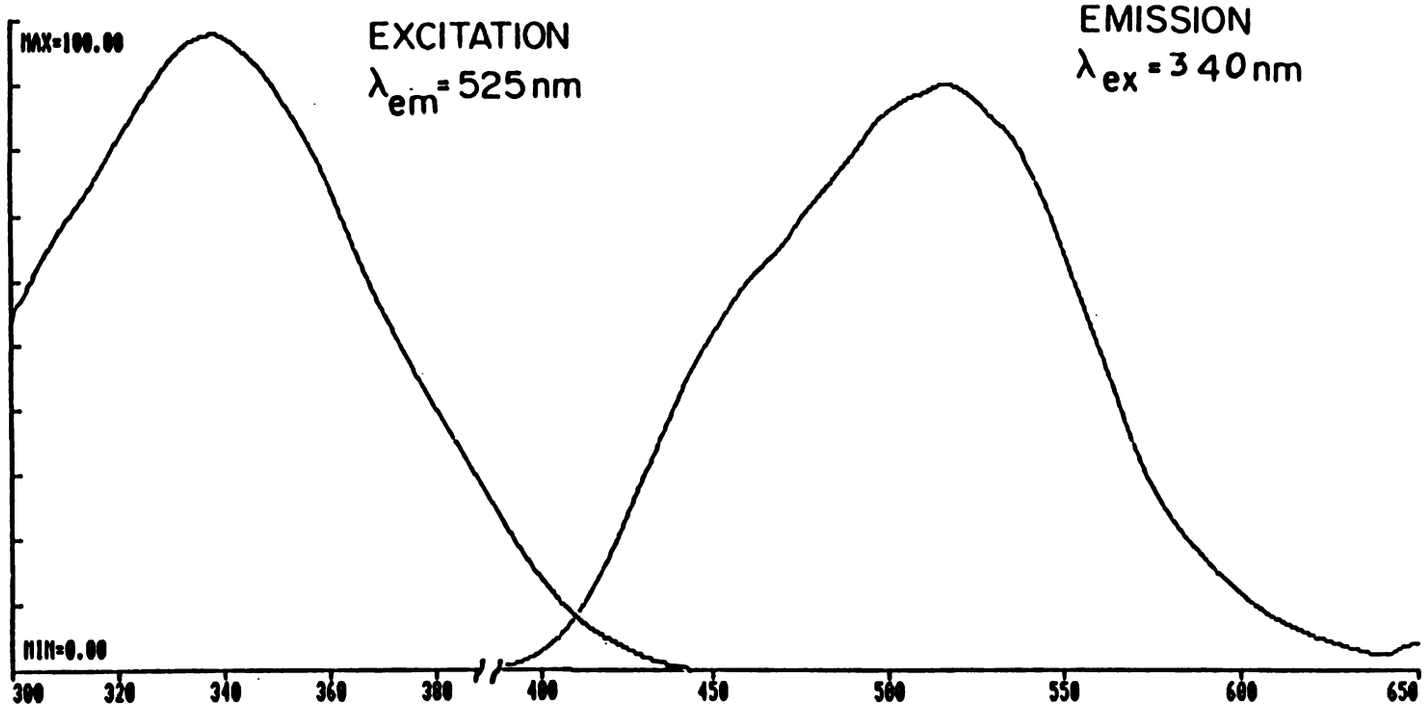


Figure 19-Fluorescence Excitation and Emission Spectra of Testosterone Derivatized with Dansyl Hydrazine.

compared to the spectra for the reagent in figure 20. The intensity and profile of the excitation spectrum of the testosterone derivative (10 x 10^{-6} M) is not markedly different from that of the reagent (50 x 10^{-6} M). Also, shifting the excitation wavelength will not minimize the fluorescence observed from unreacted dansyl hydrazine. In addition, the figures indicate these derivatives are amenable to excitation by a HeCd laser.

The choice of reaction solvent was investigated by comparing methanol, distilled methanol, 95% ethanol and chloroform in the derivatization of testosterone. The solutions were refluxed for 30 min and separated on a reversed-phase column by using a methanol/water (92.5%) mobile phase (1 ml/min). As expected, two products, postulated to be the syn and anti isomers of testosterone were formed. Solvent choice did indeed alter the ratio of these isomers. Methanol was chosen as the reaction solvent because approximately equal proportions of the isomers resulted and would provide continuity with the DPIH studies. The optimal reaction temperature was determined as 40°C.

With the exception of testosterone, which was the only 3-keto-4-ene containing compound that was reacted, single derivatives were formed from methylethyl ketone, cycloheptanone, pregnenolone, 3-pentanone, acetophenone and acetone. The chromatograms that resulted from the separation of the testosterone derivatives is shown in figure 21.

The excessive tailing shown by the peaks in the bottom chromatogram is due to the interaction of the derivatives with exposed silanol sites on the C-18 stationary phase. Reducing the interaction of the lone pairs of electrons with these sites and increasing their association with

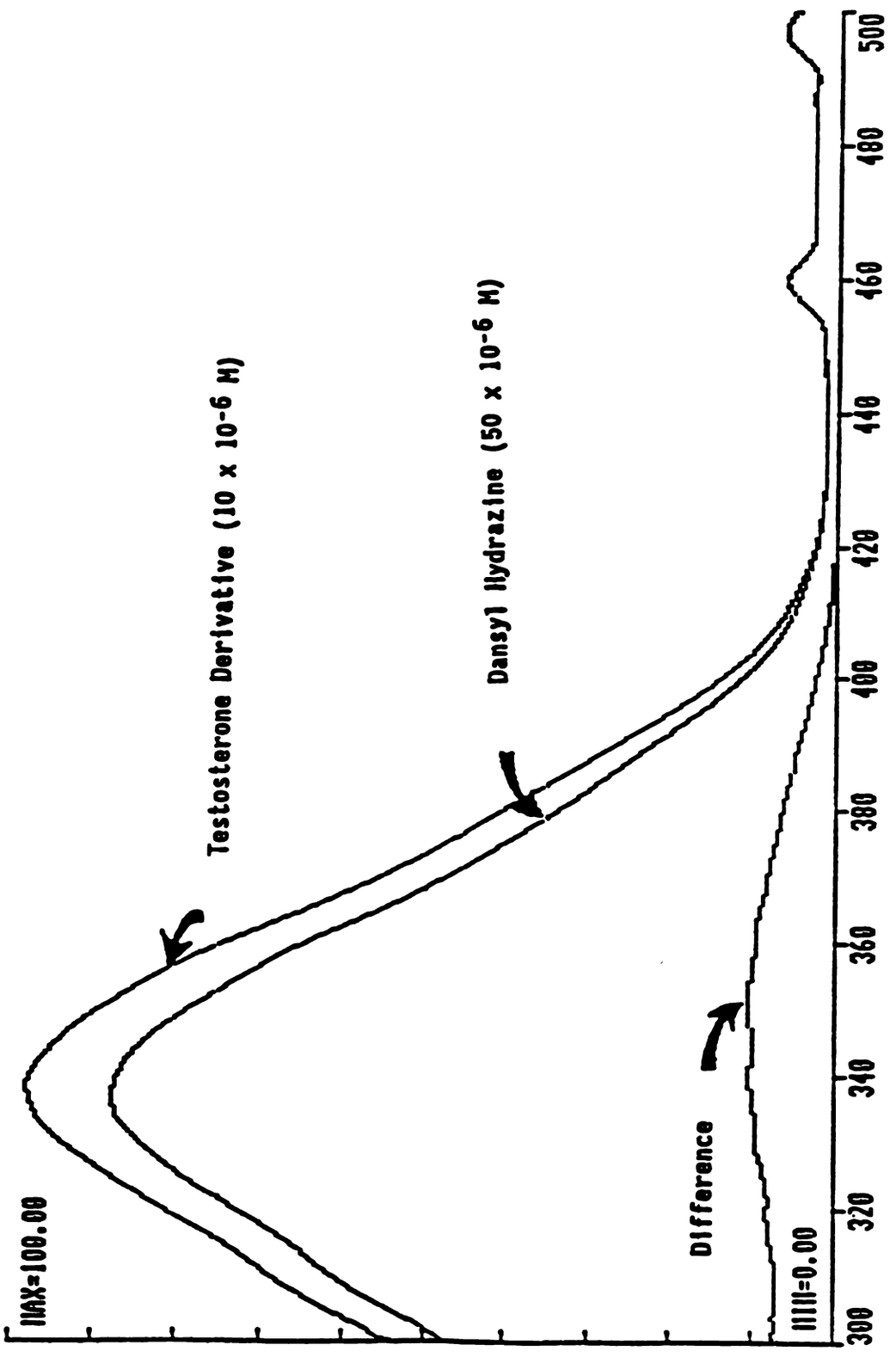


Figure 20-Excitation Spectra for Dansyl Hydrazine and the Testosterone Dansyl Hydrazine Derivative.

Figure 21-Chromatograms of Testosterone Derivatized with Dansyl Hydrazine with and without Sodium Dodecyl Sulfate as a Mobile Phase Additive. Column: 4.6 mm I.D. x 220 mm Spheri-5 RP-18. Mobile Phase: 70% or 75% methanol/water, 1 ml/min. Fluorescence Detector: 340 nm excitation and 525 nm emission. Sodium dodecyl sulfate concentration: 1 mM.

counterions in the mobile phase will improve peak symmetry. Sodium dodecyl sulfate (SDS, Pierce Chemical Co., Rockford, IL) was added to the aqueous component of the mobile phase in concentrations below the critical micellular concentration (3×10^{-3} M) (17) and the pH adjusted to 6. Because extending the length of the alkyl chain of the counter ion increases retention, the mobile phase composition was altered from 70% to 90% methanol. When testosterone derivatives were separated using these conditions, peak symmetry improved as illustrated in the top of the figure.

The use of the asymmetry factor, b/a, allowed the composition of the mobile phase to be optimized further. The asymmetry factor may be defined as the width of the peak after its centroid divided by the width before the center of mass at 10% of the peak maxima as shown below:

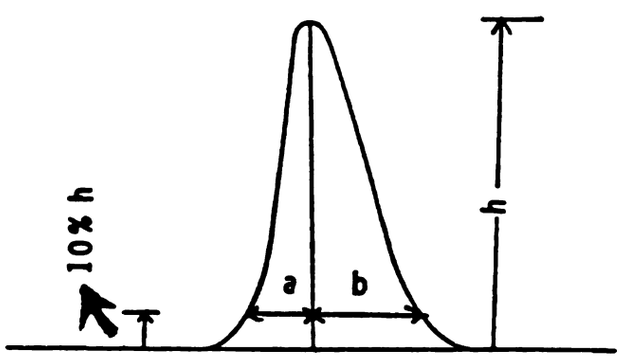


Table 5 lists the data from the optimization of the mobile phase using testosterone derivatives. The early eluting peak was denoted as A and the more retained isomer was assigned the notation of B. Increases in the SDS concentration reduced tailing, the most symmetrical peaks resulting from the addition of 1.5 moles of SDS/L to the mobile phase. Although this

concentration is not near the critical micelle concentration, a 1 M solution of SDS was employed in future experiments to minimize residue in the pumps of the chromatograph and to diminish chromatograph cleanup.

Table 5

The Effect of Mobile Phase Composition on the Asymmetry of Dansyl Hydrazine Derivatives of Testosterone

Peak	Concentration of SDS (M)	% Methanol/ Water	Retention Time (min)	b/a
A	0	75%	13.45	2.00
B	0	75%	17.70	1.29
A	$1x10^{-6}$	80%	8.00	1.71
B	$1x10^{-6}$	80%	9.25	2.79
A	$1.4x10^{-6}$	80%	16.4	1.56
B	$1.4x10^{-6}$	80%	22.3	1.77
A	$1.0x10^{-3}$	75%	8.2	1.45
B	$1.0x10^{-3}$	75%	9.8	1.45
A	$1.5x10^{-3}$	75%	10.9	1.26
B	$1.5x10^{-3}$	75%	13.9	1.31

The reaction of dansyl hydrazine with the 3-keto-4-ene structure contained in corticosteroids was studied by derivatizing testosterone, pregnenolone, androstanolone, and progesterone. Reaction conditions were the same as those described earlier. The products were separated using a conventional reversed-phase column and a methanol/water (75%) mobile phase that contained SDS (1 mM), as illustrated in figure 22. Two products were formed for all the steroids that contained the 3-keto-4-ene moiety where as a single product resulted when the C-20 ketone was reacted. Two of the three products observed for progesterone are from the reaction of the 3-keto-4-ene structure and the later-eluting peak is the

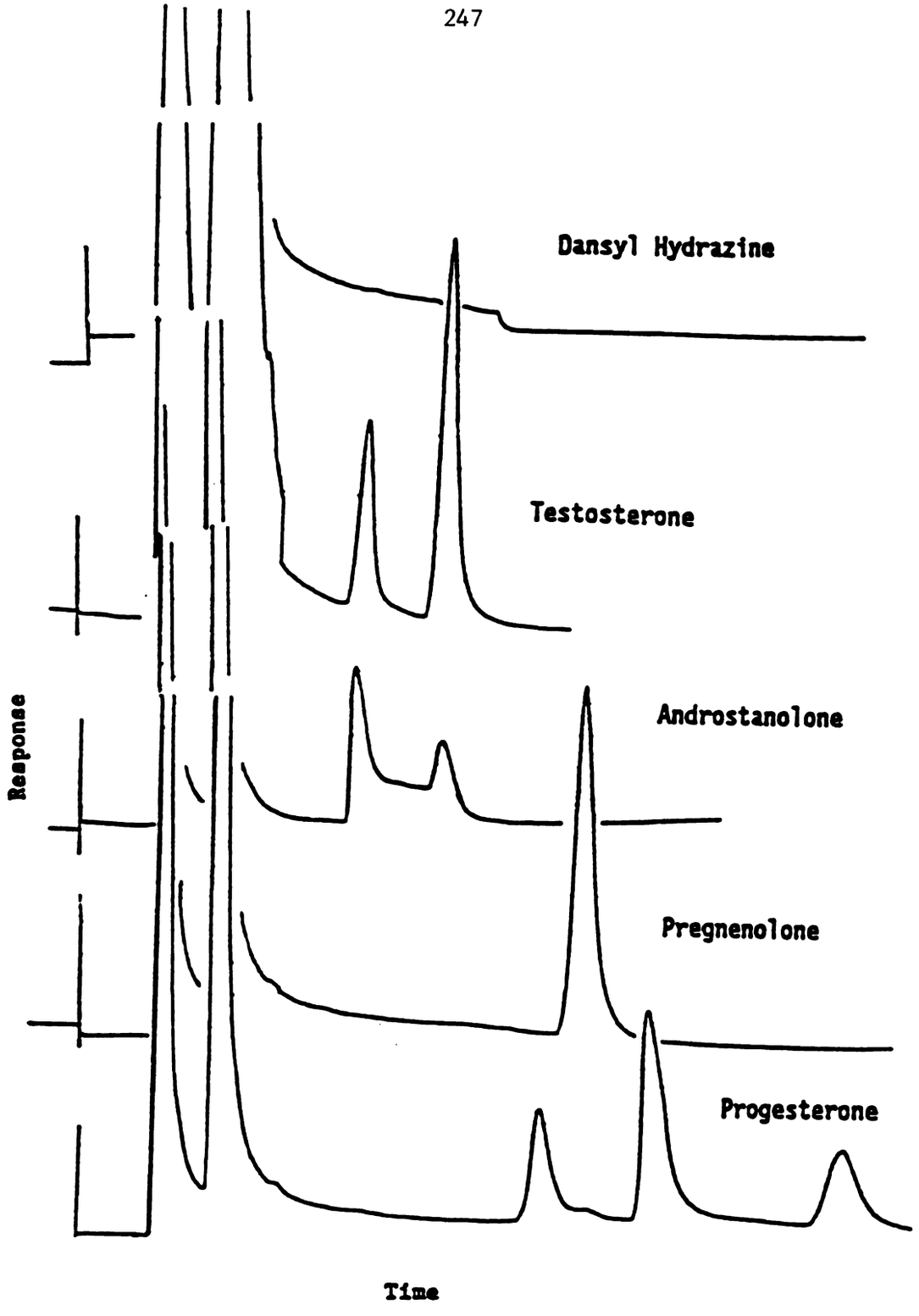


Figure 22-Chromatograms of Testosterone, Pregnenolone, Progesterone and Androstanolone Derivatized with Dansyl Hydrazine. Mobile Phase Additive. Column: 4.6 mm I.D. x 220 mm Spheri-5 RP-18. Mobile Phase: 65-75% methanol/water, 1 ml/min. Fluorescence Detector: 340 nm excitation and 525 nm emission. Sodium dodecyl sulfate concentration: 1 mM.

result of the attachment of dansyl hydrazine to the C-20 ketone.

CONCLUSIONS

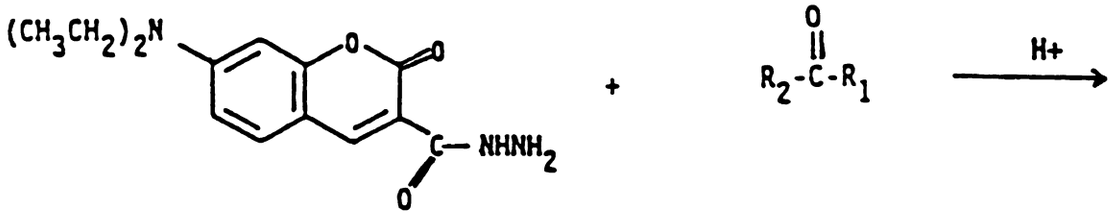
The increased chain length near the reactive site on the dansyl hydrazine molecule limits the formation of extraneous products when derivatizing 3-keto-4-ene moieties. The C-20 ketone reacts to form a single derivative. Despite this reduction in the number of products, dansyl hydrazine is not an ideal derivatizing agent, as the agent itself is quite fluorescent. The similar spectroscopic properties of both the agent and the derivative do not allow selective detection of the corticosteroid.

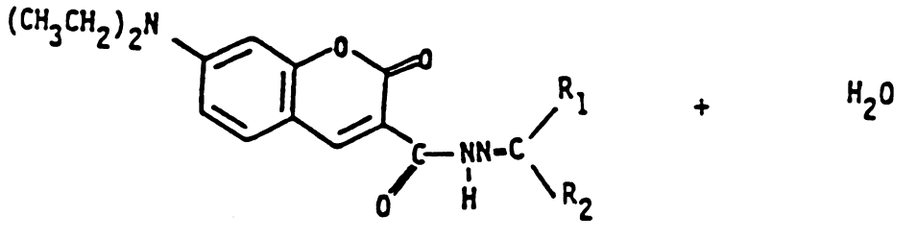
A structurally similar agent to dansyl hydrazine is 7-diethylaminocoumarin carbohydrazide. This agent has a longer side chain at the reaction site than DPIH and so the number of reaction products formed from the derivatization of 3-keto-4-ene moieties should be limited.

7-DIETHYLAMINOCOUMARIN CARBOHYDRAZIDE

INTRODUCTION

A novel derivatizing agent, 7-diethylaminocoumarincarbohydrazide (DECC) was purchased from Molecular Probes. Heretofore, no utilization of this compound for derivatization purposes has occurred. The carbohydrazine moiety should readily react with ketones by a mechanism similar to those of the previous reactions:





The coumarin ring system should also allow the derivatives to be excited by the HeCd laser.

MATERIALS AND METHODS

CONVENTIONAL HIGH PRESSURE LIQUID CHROMATOGRAPHIC SYSTEM

The chromatographic system previously described was modified by inserting an excitation filter passing light from 360 to 460 nm. An emission filter with a wavelength cutoff of 460 nm was also used.

GENERAL METHODOLOGY

Because DECC (Molecular Probes Inc., Eugene, OR) is supplied in 25 mg quantities, the reagent was diluted with methanol (25 ml) and separated into aliquots (1 ml). These were evaporated under nitrogen and stored at -20°C until further use. Methanol readily dissolved the compound and was used in subsequent studies to provide continuity with previous work in this dissertation. Upon use, portions of DECC were diluted to form a stock solution (0.5 g/l). Concentrated hydrochloric acid was diluted and used as a catalyst. Also, stock solutions of steroids (0.9×10^{-3} moles/l) were made and 0.1 ml was mixed with the coumarin (0.20 ml) and the catalyst (0.2 ml).

RESULTS AND DISCUSSION

Using the conditions for dansyl hydrazine as a first approximation, testosterone was reacted with DECC at 40°C for 30 min in methanol. The

fluorescent properties of the product were determined as previously described. The excitation and emission spectra of the derivative (10 x10^{-6} M) are shown in figure 23. Although the excitation wavelength maximum is not particularly well suited to the 325 nm emission of the HeCd laser, the 440 nm line can be used.

The emission spectra for the derivatizing agent (18 x 10^{-6}M) and the testosterone derivative (10 x 10^{-6}M) are compared in figure 24. The quantum efficiency of the derivative is much greater than DECC and therefore, the emission from unreacted derivatizing agent should not be prominent.

As illustrated in figure 25, separation of the derivatives formed from the reaction of DECC with testosterone yielded two products where as pregnenolone forms one. Once again, syn and anti isomerization around the 3-keto-4-ene site in testosterone is occurring. The conjugation at this site also is responsible for the increased reactivity as shown in figure 26. The reaction was systematically optimized to shift the ratio of isomers formed by using only testosterone as the amount of reagent available was severely limited.

CATALYST AMOUNT

A testosterone solution (1.1 x 10^{-4} M) was reacted with DECC (7.3 x 10^{-4} M) in methanol at 40° C using hydrochloric acid concentrations that included 1.9 x 10^{-3} M, 1.2 x 10^{-3} M, 0.81 x 10^{-3} M, 0.64 x 10^{-3} M, 0.48 x 10^{-3} M, and 0.24 x 10^{-3} M. The formation of both isomers was studied as a function of time and is shown in figure 27. Under these conditions, the

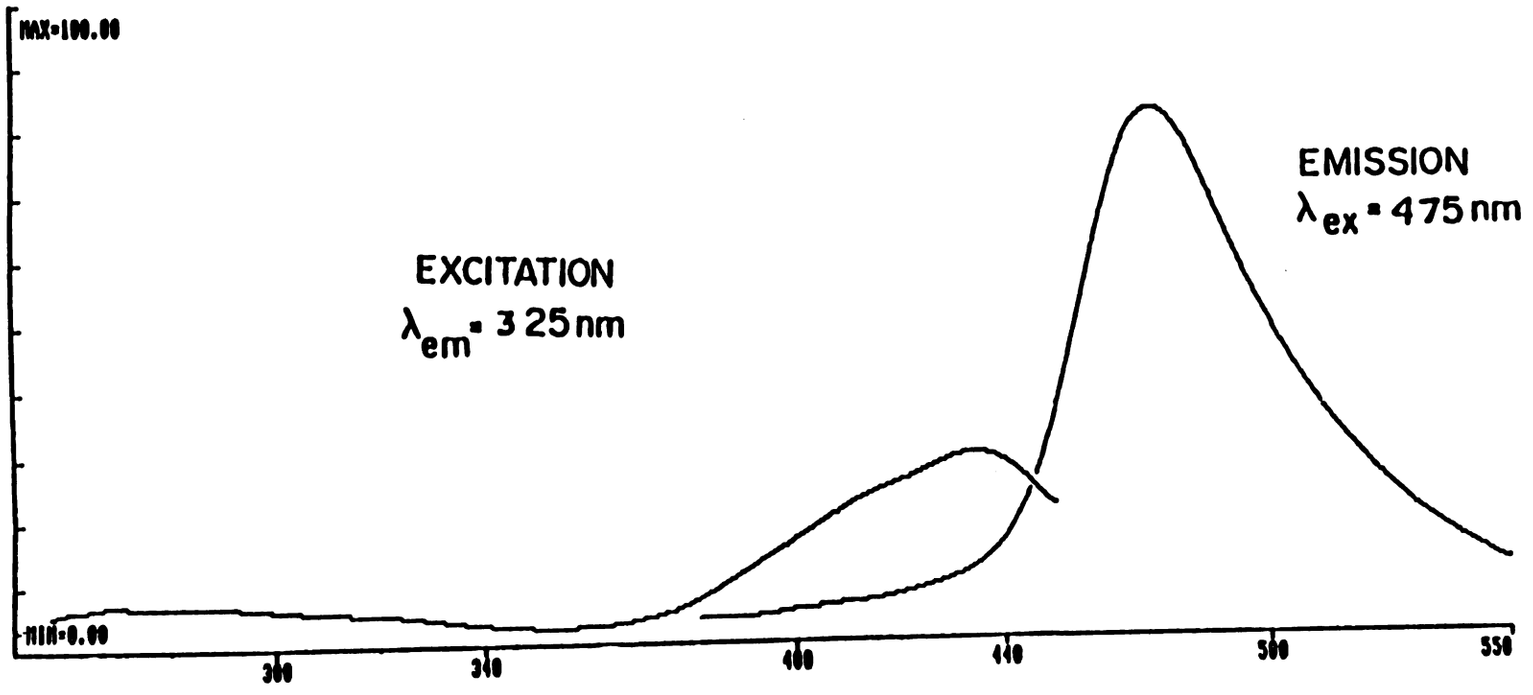


Figure 23-Fluorescence Excitation and Emission Spectra of Testosterone Derivatized with 7-Diethylamino-coumarin Carbohydrazide.

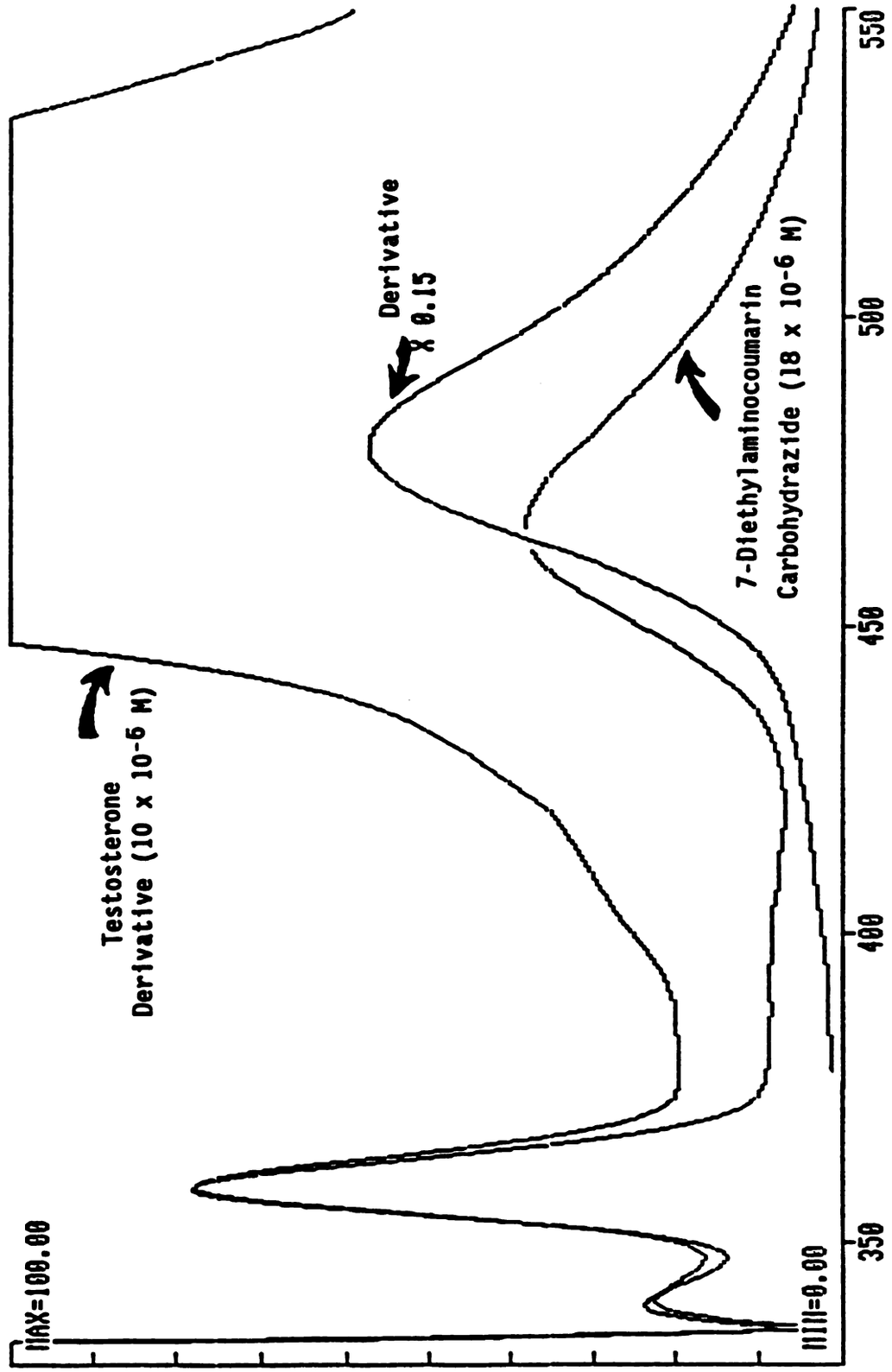


Figure 24-Emission Spectra for Testosterone Derivatized with 7-Diethylaminocoumarin Carbohydrazide and 7-Diethylaminocoumarin Carbohydrazide.

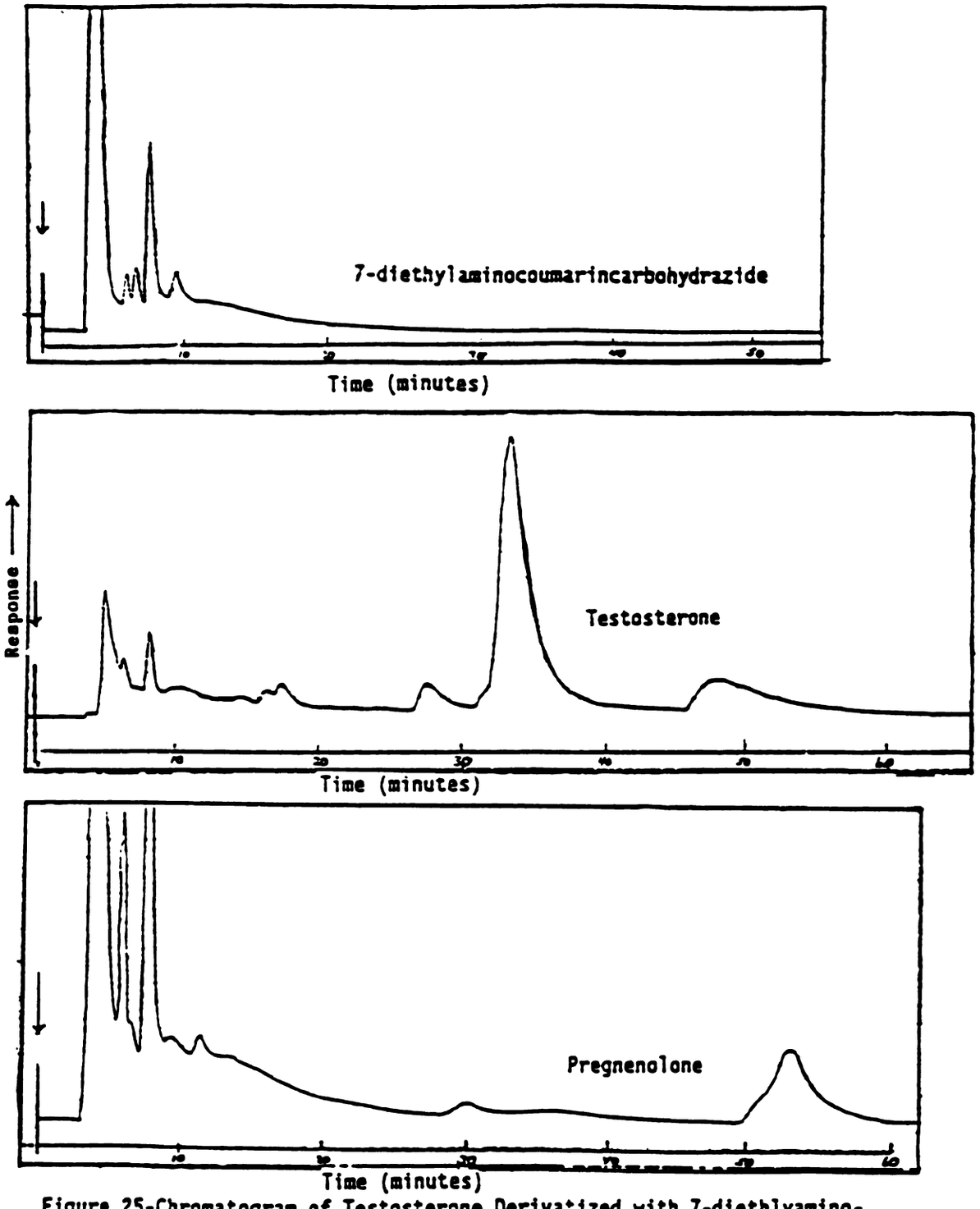


Figure 25-Chromatogram of Testosterone Derivatized with 7-diethlyamino-coumarincarbohydrazide. Column: 4.6 mm I.D. x 220 mm Spheri-5 RP-18. Mobile Phase: 50% acetonitrile/water, 1 ml/min. Fluorescence Detector: 400 nm excitation and 475 nm emission.

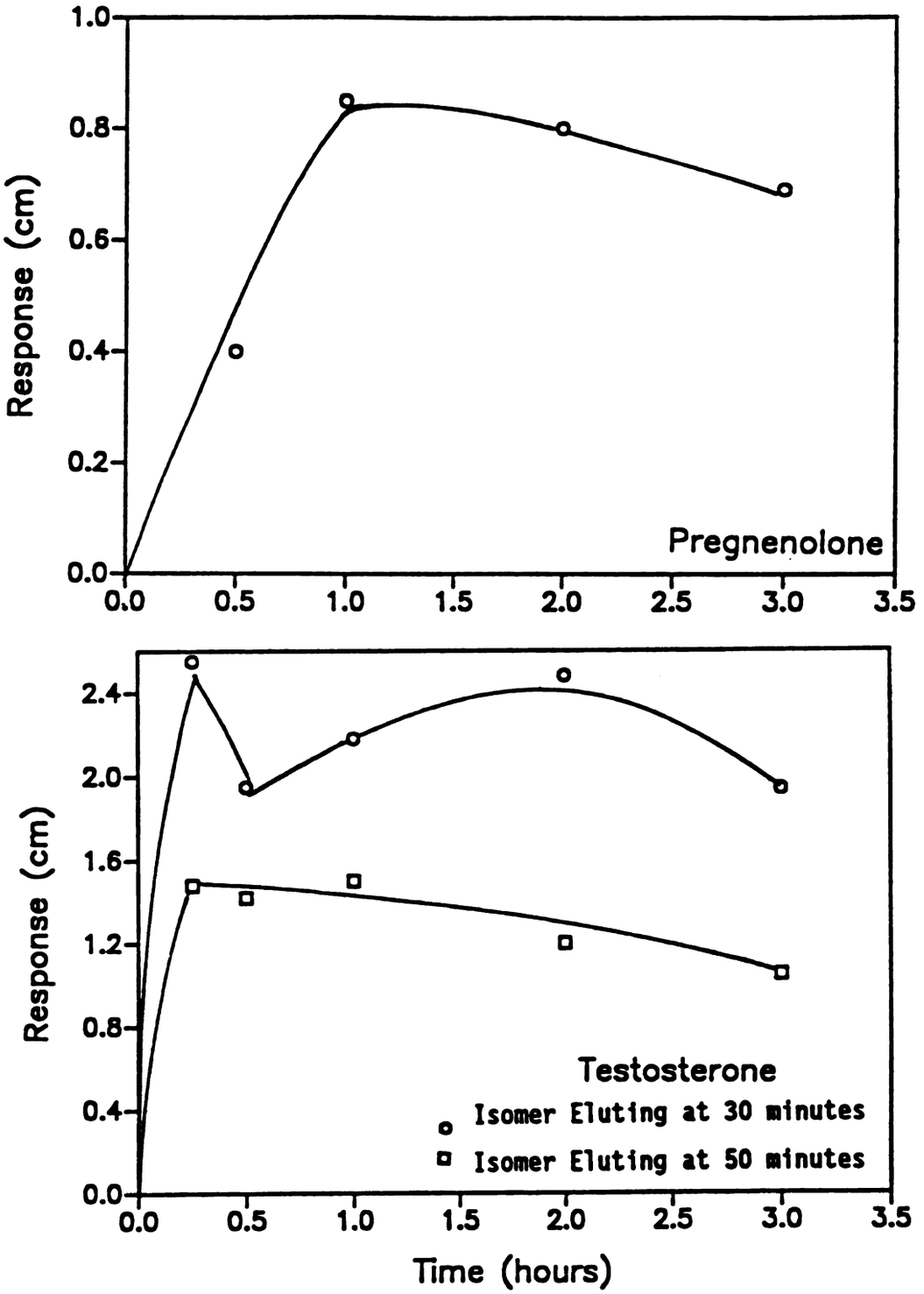


Figure 26-Reaction of Testosterone and Pregnenolone with 7-diethlyamino-coumarincarbohydrazide. Reaction Conditions: DECC 7.3×10^{-4} M, testosterone 9.2×10^{-5} M, pregnenolone 9.1×10^{-5} M, hydrochloric acid 3.1×10^{-3} M, 40 °C.

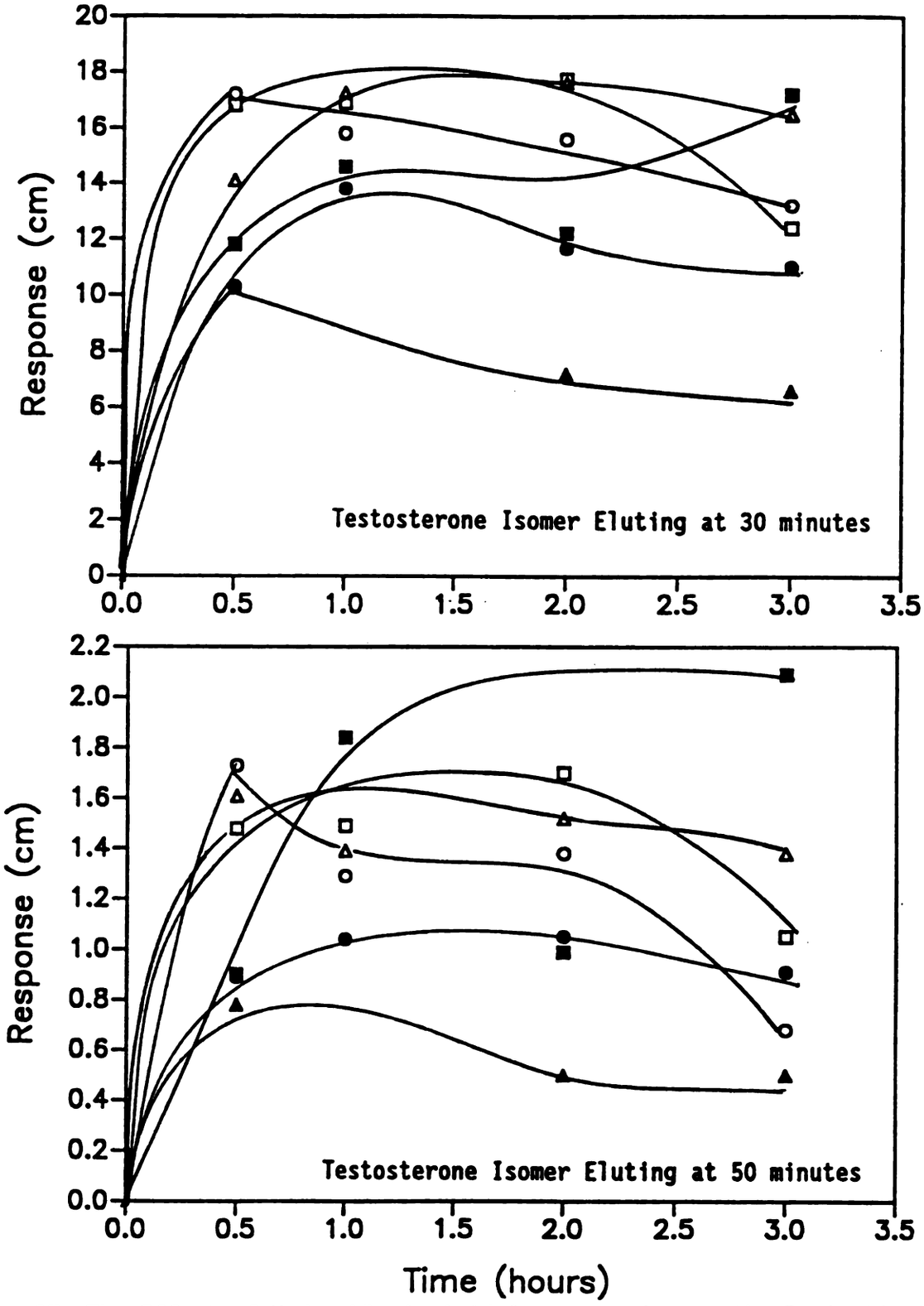


Figure 27- The Effect of Hydrochloric Acid Concentration on the Derivatization of Testosterone with 7-diethlyaminocoumarincarbohydrazide. Reaction Conditions: DECC 7.3 x 10^{-4} M, testosterone 1.1 x 10^{-4} M, 40 °C, hydrochloric acid concentrations: ▲ 1.9 x 10^{-3}M, ■ 1.2 x 10^{-3}M, ○ 0.81 x 10^{-3} M, △ 0.64 x 10^{-3}M, □ 0.48 x 10^{-3} M, ● 0.24 x 10^{-3} M.

formation of the least-retained isomer was favored almost ten fold. Also, the rate of formation of this isomer was enhanced. At higher catalyst concentrations, derivative decomposition began to predominate after approximately 30 min. The optimal acid concentration was 0.48×10^{-3} M and resulted in the most constant levels of product.

MOLAR RATIO

The effect of alterations in the molar ratio of testosterone to DECC was examined by systematically varying the number of moles of DECC added to the reaction mixture. Solutions of testosterone (1.1×10^{-4} M), and hydrochloric acid (4.8×10^{-3} M) at 40°C received aliquots of DECC to vary the ratio of reagent to steroid from 10:1, 7:1, 5:1, and 2:1. As illustrated by figure 28, the reaction proceeded most rapidly using a ratio of 10:1. Also, the greatest rate of decomposition is observed for both isomers when a ratio of 2:1 is used. The most optimal ratio of DECC to testosterone was 7:1 because decomposition was limited and formation of the derivative occurred somewhat rapidly.

TEMPERATURE

The effect of temperature on the derivatization reaction was studied by systematically altering temperature from 25 to 57°C at approximately 10° intervals. In all instances, only two products were formed, which indicated that no additional decomposition reactions were taking place. The earlier eluting peak was formed at the greatest rate when a reaction

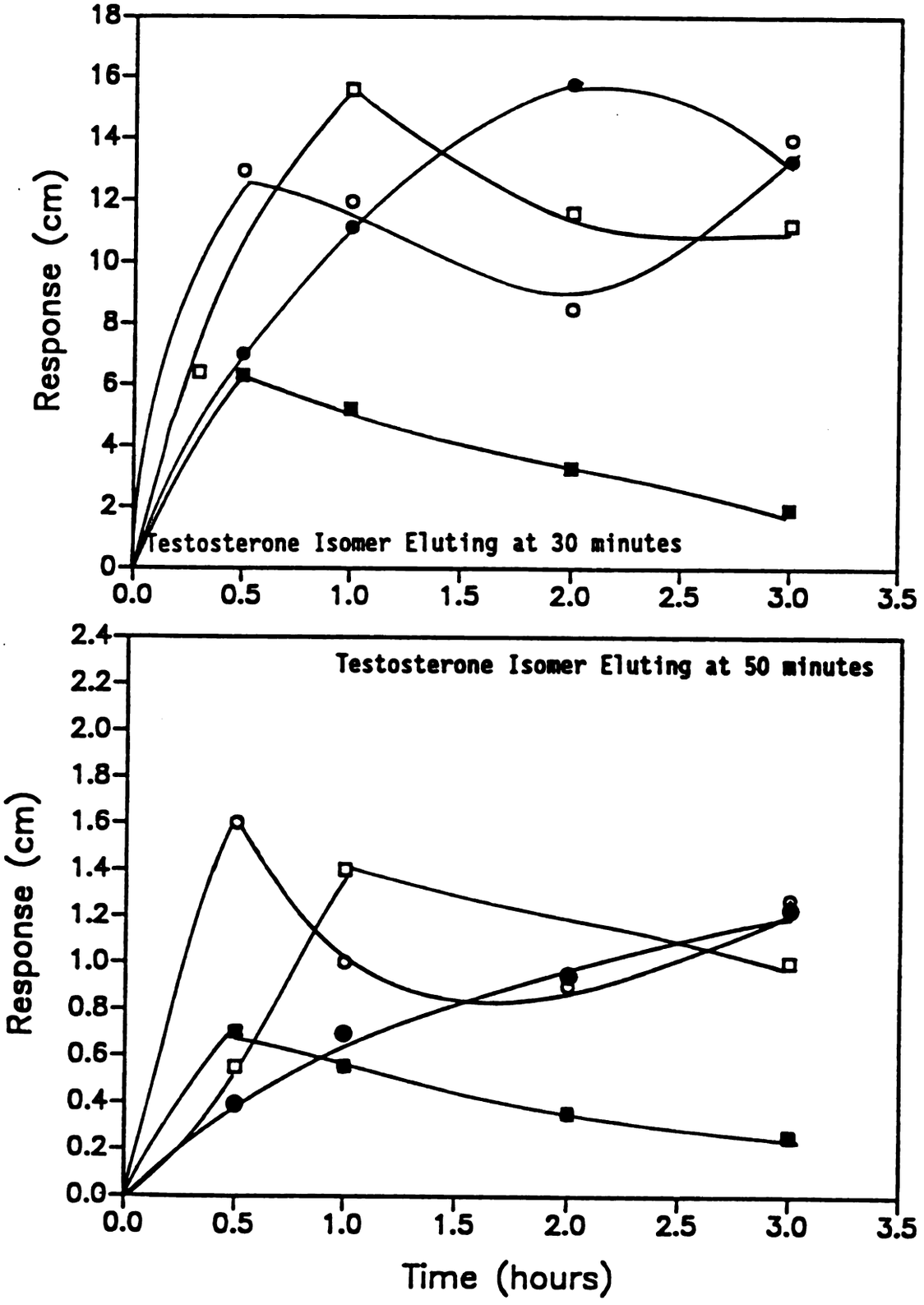


Figure 28- The Effect of Alterations in the Molar Ratio of Reagent to Steroid on the Reaction of Testosterone with 7-diethlyaminocoumarin-carbohydrazide.
Reaction Conditions: testosterone 1.1 x 10^{-4} M, 40 °C, hydrochloric acid 4.8 x 10^{-3} M, DECC: testosterone ratio: ○ 10:1, □ 7:1, ● 5:1, ■ 2:1.

temperature of 45°C was used, as is shown in figure 29. This temperature had the same effect on the formation of the more retained peak. Also, decomposition was minimal for both products at this temperature.

LINEAR RANGE

The linear range of the reaction was studied by reacting testosterone solutions of various concentrations with DECC at 45°C. The molar ratio between DECC and the highest concentration of testosterone was 7:1. Derivative formation was monitored as a function of time as well as concentration of testosterone, as illustrated in figure 30. The response was not linear at extremely low concentrations and could be due to detector response. When the concentration range was extended by one order of magnitude, a linear response was noted as shown in figure 31. A molar ratio of 5:1 DECC to 0.7×10^{-3} M testosterone was used in this study to reduce consumption of the derivatizing agent.

To ascertain whether the nonlinear response observed using lower testosterone concentrations was caused by poor detector response, a 0.09×10^{-3} M solution of the testosterone derivative was diluted and detector response monitored. At these concentrations, the detector did not exhibit a linear response, as shown in figure 32.

The optimal reaction conditions developed in these studies, namely the use of a 7:1 ratio of the derivatizing agent to steroid, a concentration of 4.8×10^{-3}M hydrochloric acid catalyst and a reaction temperature at 45°C for 1.5 hours, were used to form corticosteroid derivatives. The products of this reaction separated using a conventional

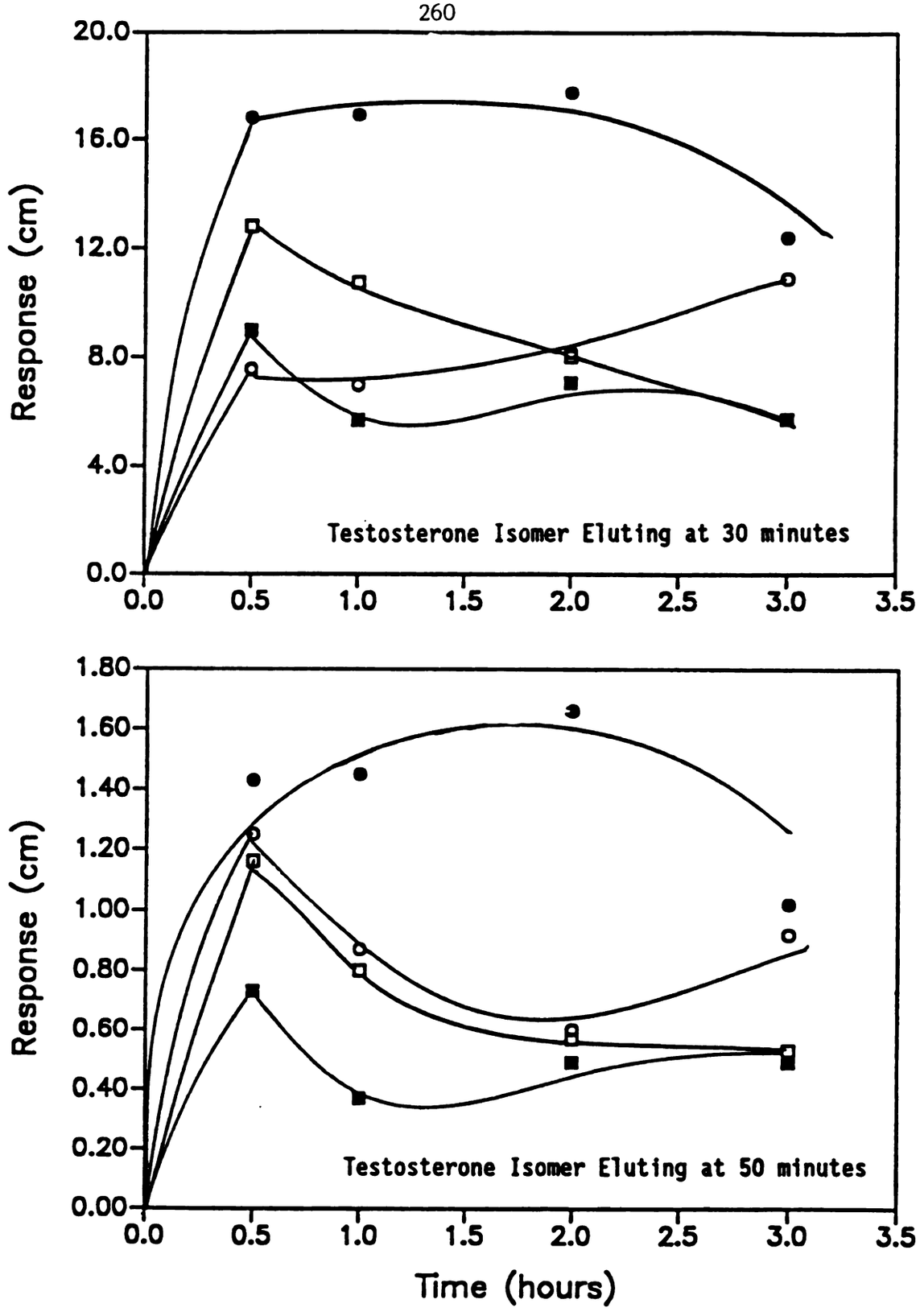


Figure 29-The Effect of Temperature on the Reaction of Testosterone with 7-diethlyaminocoumarin-carbohydrazide. Reaction Conditions: Reaction Conditions: testosterone 1.1 x 10^{-4} M, DECC 5.7 x 10^{-4} M (5:1), hydrochloric acid 4.8 x 10^{-3} M. Temperature: o 25 °C, □ 36°C, ● 45°C, ■ 57°C.

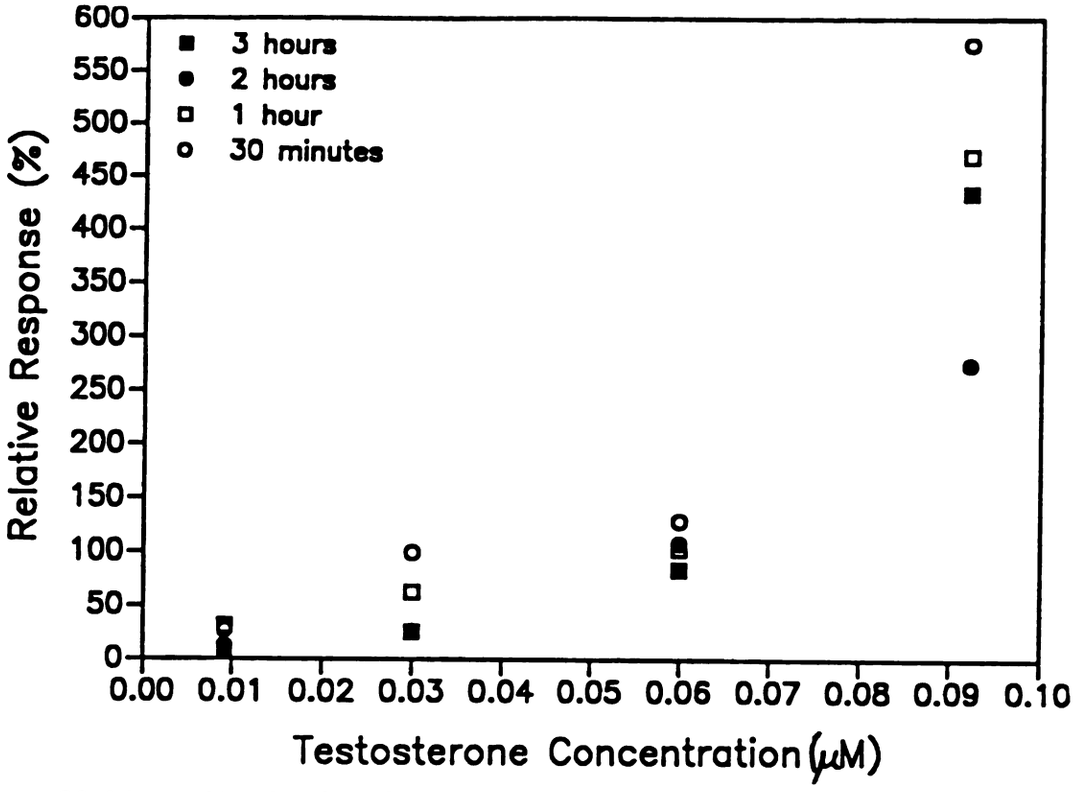


Figure 30-Linearity Study of the Reaction of Testosterone with 7-diethlyaminocoumarincarbohydrazide. Reaction Conditions: DECC 1.3 x 10^{-3} M (7:1), hydrochloric acid 4.8 x 10^{-3} M, 45 °C.

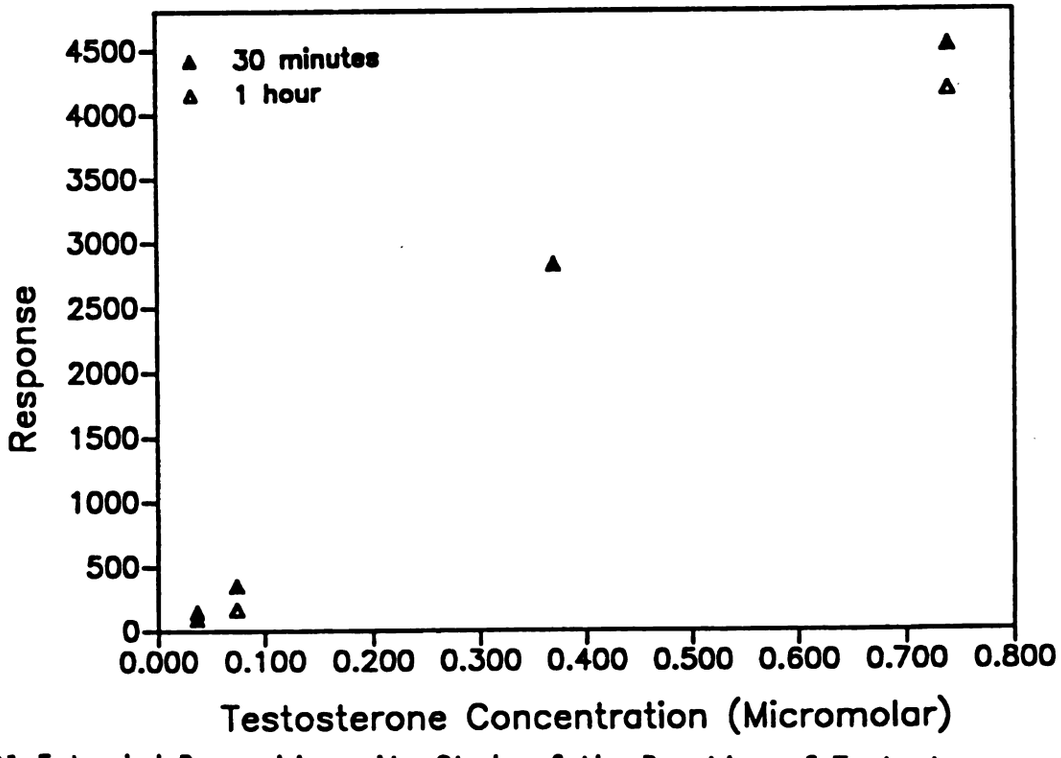


Figure 31-Extended Range Linearity Study of the Reaction of Testosterone with 7-diethlyaminocoumarincarbohydrazide. Reaction Conditions: DECC 10.5 x 10^{-3} M (5:1), hydrochloric acid 4.8 x 10^{-3} M, 45 °C.

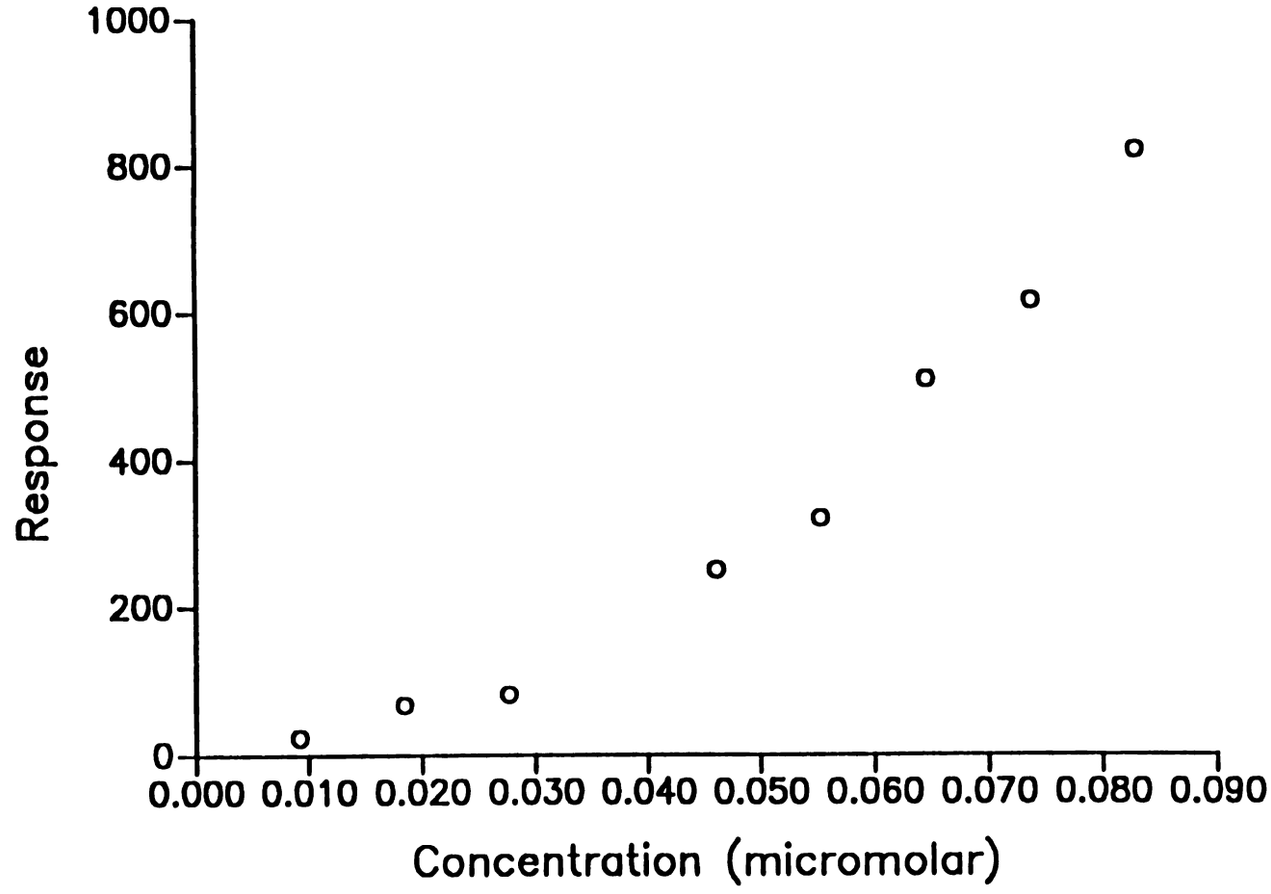


Figure 32 -Detector Response as the Derivative of Testosterone and 7-diethlyaminocoumarincarbohydrazide is Diluted.

column packed with Spheri-5 ODS packing material as shown in figure 33. A 50 % methanol/water solution was used as the mobile phase. The derivatives were excited at 400 nm whereas a wavelength of 475 nm was used for detection. The derivatives were strongly retained by the column and the peaks exhibited severe tailing. In general, steroids possessing the 3-keto-4-ene functionality exhibited the formation of two products, one major and one minor.

Selected derivatives were separated on a microcolumn to compare the peak shapes, as shown in figure 34. Although the peak symmetry for testosterone was not perfect, it was vastly improved over that observed from the conventional column. Of the corticosteroids studied, only corticosterone exhibited severe tailing. The figure also illustrates the separation of the derivatives of testosterone at an increased sensitivity, where the peaks are relatively symmetrical. A systematic study of the addition of various mobile phase additives was undertaken in an attempt to minimize the interaction of the diethyl amine functional group with any exposed silanol sites on the packing material and improve peak symmetry.

MOBILE PHASE ADDITIVES

According to Goldberg and Rowsell (18), two approaches may be used in the selection of an appropriate mobile phase additive. An additive that is structurely similar to the analyte may be used. In this case, triethylamine (TEA, Mallinkrodt Inc, Paris, KY) competes with the diethylamine functional group for the exposed silanol sites, limiting

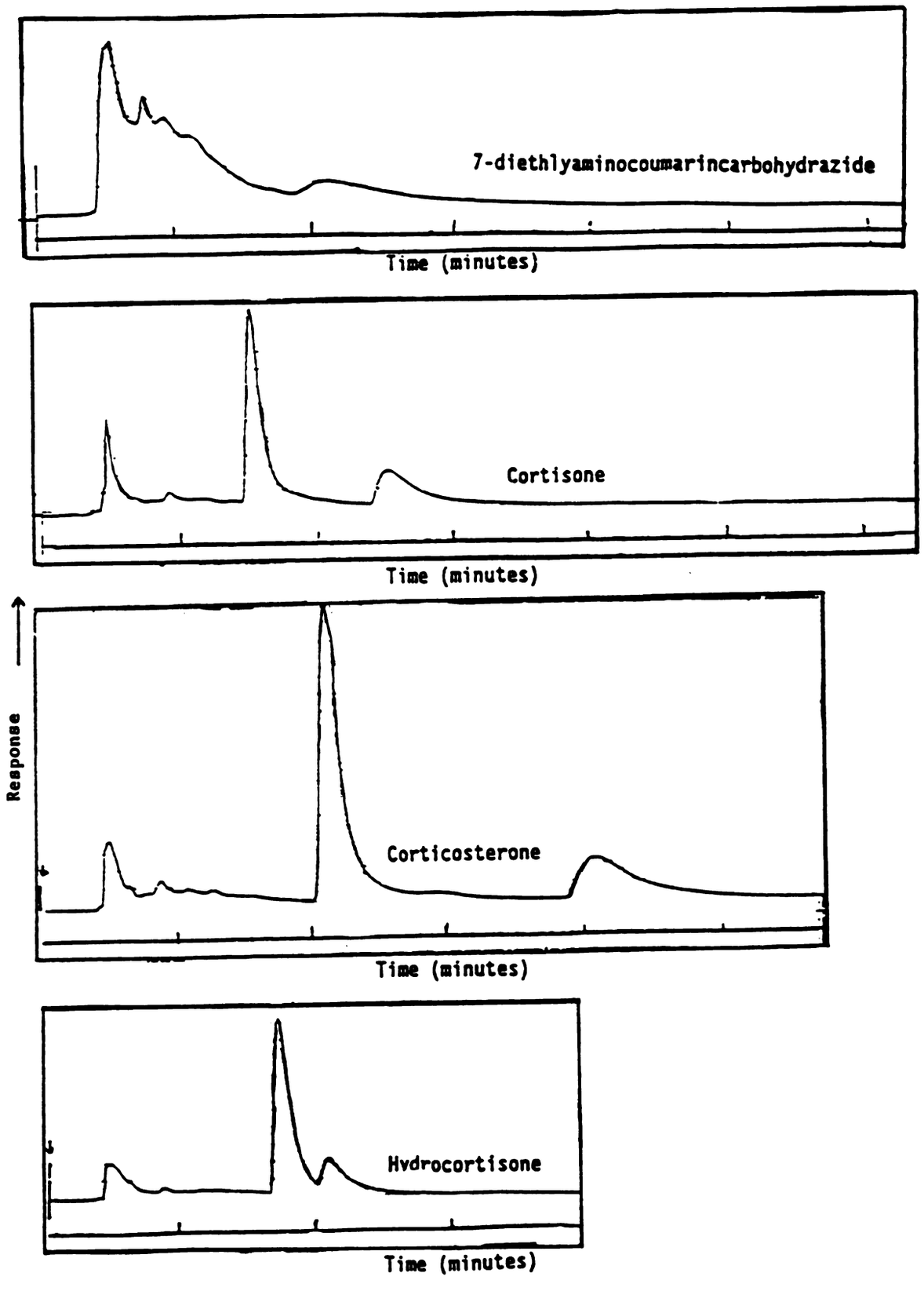


Figure 33-Chromatograms of Steroids Derivatized with 7-diethlyaminocoumarin-carbohydrazide. Column: 4.6 mm I.D. x 220 mm Spheri-5 RP-18. Mobile Phase: 50 % Acetoniltile/Water, 1 ml/min. Fluorescence Detector: 400 nm excitation, 475 nm emission.

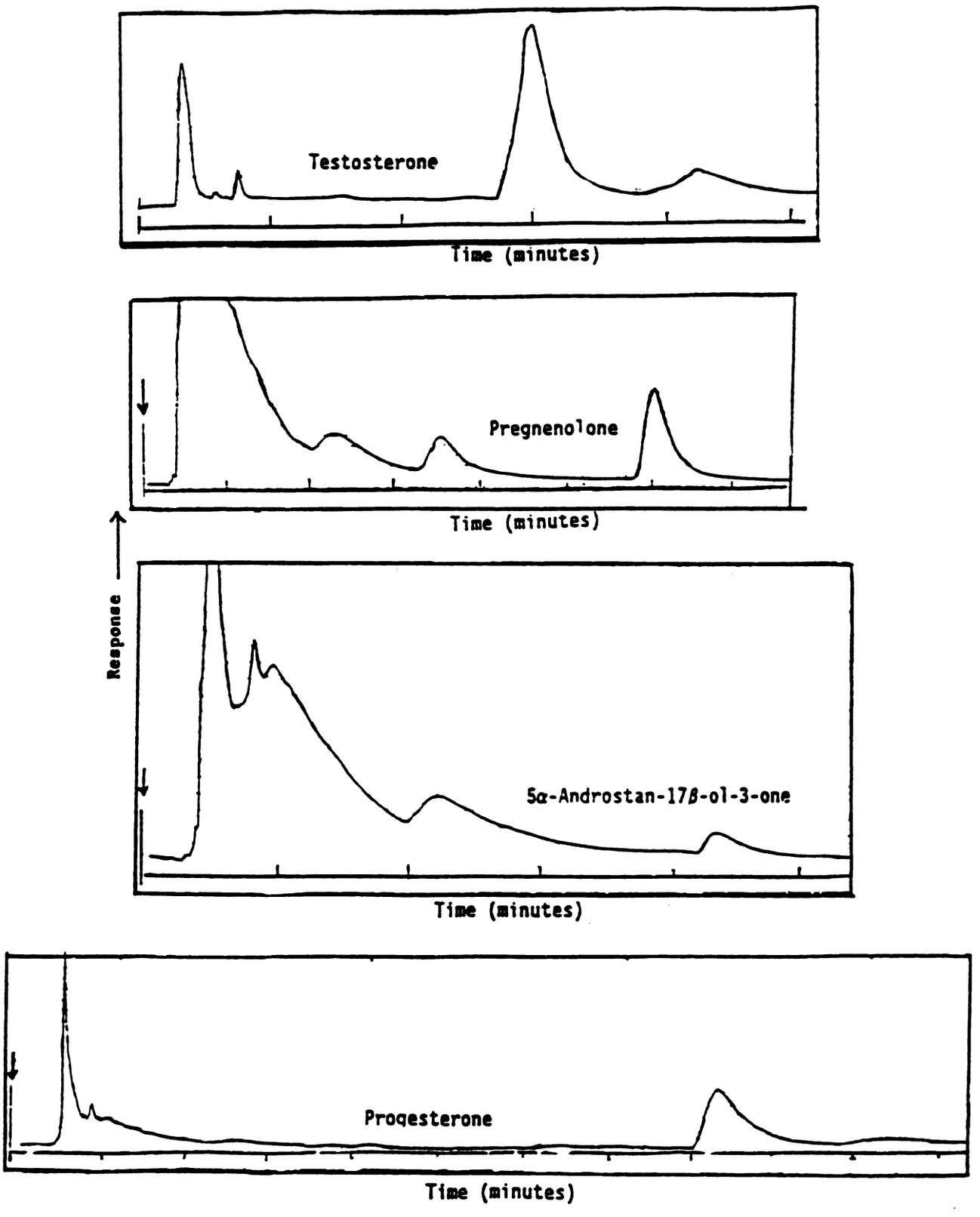


Figure 33- Continued

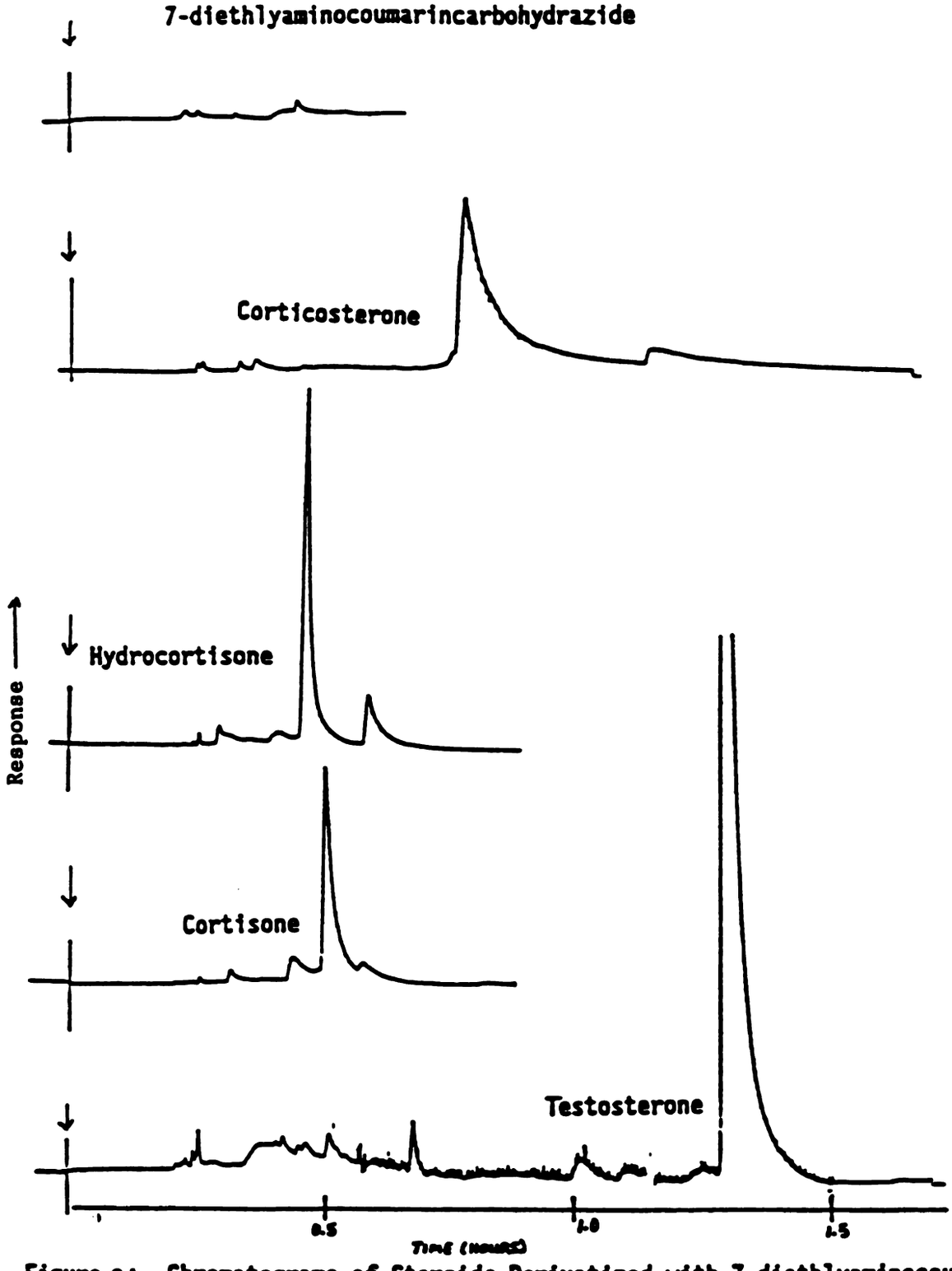


Figure 34- Chromatograms of Steroids Derivatized with 7-diethlyaminocoumarin-carbohydrazide and Separated on the Microcolumn System. Microcolumn: 200 μI.D. x 89 cm length fused silica capillary, packed with Spheri-5 RP-18. Mobile Phase: 70 % acetonitrile/water, 0.85 μL/min. Fluorescence Detector: 325 nm excitation, 480 nm emission.

derivative interaction and peak tailing. Usually millimolar concentrations of the additive are sufficient to improve peak symmetry. Because pH influences amine binding to the silanol sites, the concentration of TEA was varied as a function of pH. To measure the pH of the mobile phase, additives were mixed with an appropriate amount of water, the pH was adjusted and then methanol was added.

The second approach to improve peak symmetry is to use an agent that forms an ion pair with the derivative, preventing attachment to the exposed silanol sites, generally, a 1-3% acid solution. Acetic acid, propionic acid (Mallinkrodt, Inc. Paris KY) and formic acid (Aldrich Chemical Company, Inc., Milwaukee, WI) were used at various concentrations and pHs to study the effect on peak shape. Table 6 illustrates the results of both approaches. As the amount of mobile phase additive is varied, and the length of the hydrophibic side chains is increased, analyte retention is altered. Therefore, in the following studies, mobile phase composition was adjusted to improve separation.

Increases in the TEA concentration resulted in decreased retention of the derivatives and improved symmetry of the peaks by reducing interaction between the diethylamine moiety and silanol sites. When the TEA concentration was maintained constant, and the pH altered, the products eluted more rapidly. In a more basic environment, the diethyl amine moiety has a decreased tendency to be protonated and its interaction with silanol sites is more limited. Interactions between mobile phase additives and the peak symmetry is not straightforward because comparable symmetries were achieved by both lower TEA concentrations at pH 6 and 25 mM TEA at pH 7.0.

Table 6

Asymmetry of DECC-Testosterone Derivatives as a Function of Mobile Phase Additives

Additive	pH	Mobile Phase	Retention Time First Peak	Retention Time Second Peak	b/a First Peak	b/a Second Peak	α
----	4.0	45%	11.1	18.3	1.54*	1.56*	1.23
----	5.0	45%	no peaks- one large undifferentated mass				
----	6.0	45%	no peaks- one large undifferentated mass				
15 mM TEA	6.01	50%	20.5	31.0	2.25	1.48	1.57
20 mM TEA	6.03	50%	20.0	27.9	2.80	1.56*	1.43
25 mM TEA	6.06	50%	19.6	25.8	2.75	1.65*	1.35
25 mM TEA	5.96	45%	11.4	15.5	2.01	1.79*	1.39
25 mM TEA	7.03	45%	11.0	14.5	2.23	1.49	1.34
25 mM TEA	8.68	45%	10.0	13.2	2.40	1.73*	1.35
1% acetate	4.02	40%	5.8	16.7	1.78	0.97 (1.94)	3.64
1% acetate	5.09	40%	10.8	15.1	5.33	1.90*	1.48
1% acetate	6.04	40%	13.4	17.2	3.09	2.33	1.43
1% acetate	7.11	40%	13.0	17.9	3.44	1.89	1.40
1% acetate	8.02	40%	13.5	18.0	0.92 1.57#	1.00 1.33#	1.39
1% acetate	9.01	40%	10.1	12.5	1.29	----	1.27
1% acetate	6.04	45%	14.6	21.6	4.35	1.45*	1.54
2% acetate	6.00	45%	11.7	16.0	1.66	1.68*	1.43
3% acetate	6.05	45%	14.7	20.3	2.75	1.63*	1.42
1% acetate & 15 mM TEA	6.08	45%	15.4	22.5	1.85	1.50	1.53
2% H_3PO_4	6.02	45%	14.0	----	1.30	----	----
1% acetate	8.01	40%	20.8	28.5	2.77	2.10*	1.40
1% acetate	9.07	40%	----	13.4	----	2.00*	1.49
1% propionate	8.07	40%	10.0	12.8	1.00 1.28#	----	1.32
1% formate	8.02	40%	9.80	12.3	1.20 1.54#	----	3.46
2% propionate	8.05	40%	no peaks- one large undifferentated mass				
2% formate	8.02	40%	no peaks- one large undifferentated mass				
10mM MgBr & 2% Acetate & 15 mM TEA		45%	15.6	23.2	3.22	1.89 (3.03)	1.50

mobile phase was various compositions of methanol/water, flow rate 1 ml/min. retention times are listed in min, b/a were calculated at 50% peak maximum due to the extreme tailing. b/a values in parenthesis were calculated at 10% peak height. # assymetry factor at 20% peak height
* indicates that b/a at 50% peak height

At neutral pH, the products were most strongly retained when acetate was added to the mobile phase. The derivatives interact most strongly with octadecyl chains of the stationary phase at these pHs. Although an asymmetry factor close to 1.0 was observed at 50% of the peak height using 1% acetate at a pH of 8, at 20% of the peak height the products exhibited severe tailing. Increasing the acetate concentration to 2% improved peak symmetry somewhat.

The use of alternate carboxcylic acids as mobile phase additives was also studied. Although, a mobile phase using formate (1%) or propionate (1%) at a pH 8 improved the peak symmetry somewhat, a large degree of tailing was present. Increasing the percentages of formate and propionate in the mobile phase did not improve peak shape.

Finally, in an attempt to cover exposed silanol sites, ion pair the derivatives, and minimize the capacity of lone pairs of electrons to interact at silanol sites, TEA, acetate and MgBr were all added to the mobile phase without improving results. Also, the use of sodium dodecyl sulfate (SDS) as a mobile phase additive did not modify peak shape. The SDS concentration was varied from 0.1-2.0 x 10^{-3} M, while the pH was varied from 5-8.

Various end-capped stationary phases were packed into microcolumns in an attempt to reduce tailing. No improvements in chromatographic properties were noted. Therefore, the use of an analogous derivatizing agent lacking the diethyl amine moiety is necessary to reduce tailing of chromatographic peaks.

CONCLUSIONS

7-diethylaminocoumarincarbohydrazide is a very reactive derivatizing agent. The parent compound itself is not very fluorescent, but forms very highly fluorescent derivatives. The reaction conditions are very similar to those used for other reagents examined in this dissertation. Like the other reagents, when 7-diethylaminocoumarin carbohydrazide reacts with compounds that contain 3-keto-4-ene functional groups, multiple products are formed. However, this reagent forms products at a more rapid rate, making 7-diethylaminocoumarin carbohydrazide moreuseful in post column reaction schemes. In addition, the reaction is linear over 1.5 orders of magnitude. The diethyl moiety on the derivatizing agent is responsible for the poor chromatographic characteristics of some of the derivatives. Mobile phase additives and alterations in stationary phase did not reduce tailing. Therefore, the use of a reagent that lacks the diethylamino group could prove to be an avenue of future study to simplify chromatographic separations of corticosteroids.

CHAPTER 4

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CHAPTER 5

CONCLUSIONS AND FUTURE WORK

The use of 2-diphenylacetyl-1,3-indandione-1-hydrazone as a precolum derivatizing agent that is applicable to the separation and detection of corticosteroids is limited by the formation of E,Z isomers around the 3-keto-4-ene functional group. Optimal reaction conditions included the use of hydrochloric acid as a catalyst (0.08 M), and a molar ratio of DPIH to ketone of 6:1 at 40° C. Methanol was the solvent of choice. The reactivity and compatibility with HeCd laser excitation make this compound a potential derivatizing agent useful in the analysis of other complex mixtures of aldehydes and ketones. DPIH could be applied to the microcolumn separation and laser detection of ketones contained in food products and in samples that contain carbamate pesticides. To determine corticosteroids, post-column derivatization techniques should be coupled to the laser-induced fluorescent detection.

Although not a novel derivatizing reagent, dansyl hydrazone is a highly reactive agent also selective for aldehydes and ketones. The number of products formed from the reaction of corticosteroids was reduced because the increased chain length near the reactive site limited E,Z formation. Dansyl hydrazone is not an ideal derivitizatizing reagent because of the high degree of fluorescence exhibited by the agent itself.

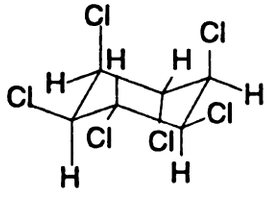
Though the reagent forms highly fluorescent products and is not fluorescent itself, the use of 7-diethylaminocoumarin carbohydrazide in the detection of corticosteroids is limited due to the excessive tailing exhibited when the products are subjected to separation. Mobile phase additives and ion pairing agents were not able to correct the poor peak

symetry. Because the diethylamino group interacts with the silanol sites to cause the tailing, these problems would be prevented by using this reagent in a post-column derivatization scheme.

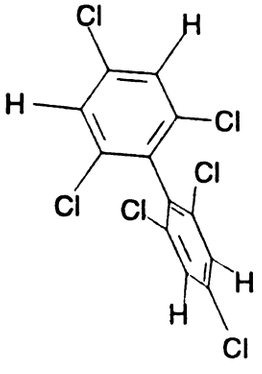
APPENDIX

APPENDIX

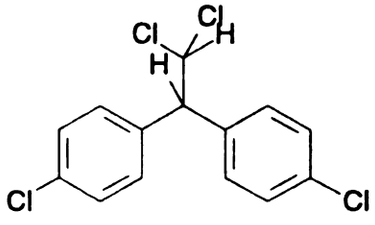
STRUCTURES OF ORGANOCHLORINE COMPOUNDS



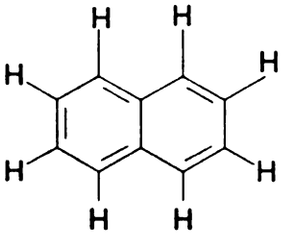
Lindane



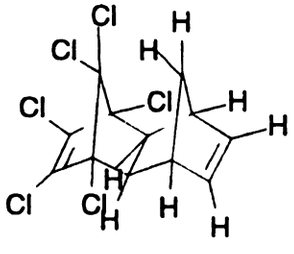
2,4,5,2',4',5'-
Hexachlorobiphenyl



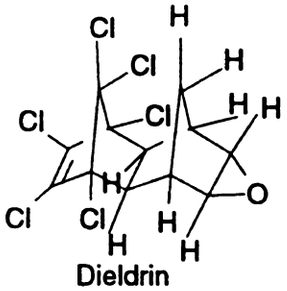
p,p'-DDE



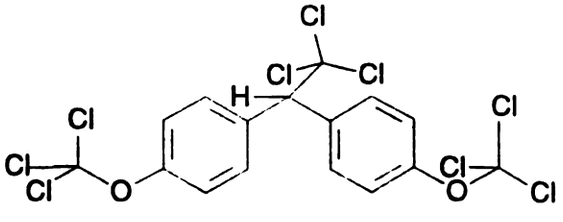
Naphthalene



Aldrin



Dieldrin



Methoxychlor