This is to certify that the

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INVESTIGATION OF THE CHEMISTRY AND TOXICOLOGY OF CHLORINATED BORNANE (TOXAPHENE) RESIDUES ISOLATED FROM GREAT LAKES LAKE TROUT (Salvelinus namaycush)

presented by

Jay William Gooch

has been accepted towards fulfillment
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Major professor

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# INVESTIGATION OF THE CHEMISTRY AND TOXICOLOGY OF CHLORINATED BORNANE (TOXAPHENE) RESIDUES ISOLATED FROM GREAT LAKES LAKE TROUT (*Salvelinus namaycush*)

By

Jay William Gooch

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## ABSTRACT

# INVESTIGATION OF THE CHEMISTRY AND TOXICOLOGY OF CHLORINATED BORNANE (TOXAPHENE) RESIDUES ISOLATED FROM GREAT LAKES LAKE TROUT (Salvelinus namaycush)

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Recent studies have demonstrated the presence of a complex pattern of chlorinated bornane residues , similar to the insecticide toxaphene, in Great Lakes fish. These residues, though structurally similar to toxaphene, are composed of a different mixture of compounds than technical toxaphene. This study provides concrete evidence for the presence of the major toxic components of toxaphene and toxicity associated with chlorinated bornane residues isolated from Great Lakes lake trout (Salvelinus namaycush).

Chlorinated bornanes were isolated from tissues of Lake Michigan and Siskiwit Lake (Isle Royale) lake trout and purified using thin-layer, column and gas-liquid chromatography. The two major toxic constituents of toxaphene, Toxicants A and B, were detected and quantified as a more toxicologically appropriate reference point for residue levels. Capillary gas chromatography/mass spectrometry was used to confirm the identity of the toxic congeners and to demonstrate

the structural similarities of the other components with components in toxaphene. The results show that chlorinated bornane compounds in lake trout from Lake Michigan and Siskiwit Lake on Isle Royale include the two major toxic components of toxaphene at concentrations 10-20 fold less than the estimated total toxaphene.

Twenty-four hour, static, acute, bioassays were conducted using Aedes egypti mosquito larvae and demonstrated that the residues purified from lake trout are as toxic as technical toxaphene isolated in a similar manner. In addition, experiments performed on the GABA (α-aminobutyric acid)-chloride ionophore complex binding site of the central nervous system, show that the toxaphene residues from Great Lakes fish are as potent as technical toxaphene at displacing $^{35}$S-t-butylbicyclophosphorothionate from its macromolecular target site in rat and lake trout brain.

Studies conducted on residues from different tissues and different collection years show no trend toward decreasing toxicity of the toxaphene residue with tissue or time. Pattern recognition techniques did not demonstrate any identifiable change in the composition of the residue over the past three years. Capillary GC-MS of purified residues revealed a relatively pure toxaphene residue composed of a greater degree of structures with nine chlorines than anticipated previously.

## ACKNOWLEDGEMENTS

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# CHAPTER 1

# INTRODUCTION

# INTRODUCTION

Toxaphene is an insecticide which is a complex mixture of compounds made by chlorinating camphene to a 67-69% chlorine content. It has an average molecular formula of $C_{10}H_{10}Cl_8$ with a corresponding molecular weight of approximately 414 (410 nominal). Technical formulations are an amber-colored waxy solid with a characteristic terpene odor (Hercules Chemical Co. Bulletin AP-103B). A similar product, strobane T, is synthesized from α-pinene and was manufactured mainly in Europe (Saleh and Casida, 1977). Originally developed by the Hercules Chemical Company, toxaphene was used from 1947 until 1982 before being banned by the U.S. Environmental Protection Agency for use in the United States. Hercules held the patent until 1971. By 1974, toxaphene was being manufactured by 186 companies producing 817 different registered products (Toxaphene Working Group, 1977). While production and use estimates are difficult to determine, it is clear that toxaphene was the most heavily applied chlorinated hydrocarbon insecticide ever used in the United States. Between 1964 and 1976, usage is estimated to have been 36 million pounds per year. In 1976, the USDA estimated that approximately 31 million pounds were applied to approximately 4.9 million acres of cropland and pasture. Cotton received approximately 85% of the total. In addition, approximately 2.4 million pounds were applied to livestock, particularly cattle. At that time, toxaphene had 277 commodity and other site registrations. State and Federal agencies were recommending use of toxaphene for

control of 167 insect pests on 44 commodities, 40 of which had no alternative control strategy. All of the above statistics have been taken from a report submitted to the Environmental Protection Agency by a Toxaphene Assessment Team in response to the May 25, 1977 Federal Register notice of rebuttable presumption against registration (RPAR) issued by the EPA (USDA, 1978).

While toxaphene was being reviewed, synthetic pyrethroids were registered and their use quickly began to replace toxaphene. In October of 1982, toxaphene was banned for most uses in the U.S. Existing stocks can be used until the end of 1986, though only under limited circumstances. In the cancellation of toxaphene, the EPA relied heavily on hazard to, and persistence in, aquatic organisms (Federal Register, 1982). In hindsight, given the structural and physical properties of toxaphene and the immense quantities that were applied, it is not surprising that toxaphene residues have become problematic. A lack of adequate analytical techniques to deal with toxaphene residues and a gap in our knowledge about toxaphene pharmacodynamics in fish, no doubt contributed to our lack of ability to predict this problem.

**Synthesis and Structural Characterization**

Toxaphene is readily produced by passing chlorine gas through a UV-irradiated carbon tetrachloride solution of camphene until a chlorine content of 67-69% is obtained (Buntin, 1951). This process yields a complex mixture of nearly 200 different constituents which have never been completely characterized (Casida and Saleh, 1978). Saleh and Casida (1977) analyzed toxaphene lots from various sources

around the world and demonstrated that the composition of toxaphene from different manufacturers varied substantially in its capillary column gas chromatography profile. Batches from different years from Hercules, the major manufacturer in the U.S., however, were very consistent. Despite the variable capillary-GC profiles, there was very little difference in toxicity to either mammals or insects.

Four main research groups have taken part in the identification of different toxaphene components. The 17 structures that have been identified are shown in Figure 1. In addition to these structures, Holmstead et al. (1974) and Saleh (1983) used gas chromatography/mass spectrometry to characterize nearly 200 components resolvable by both thin layer chromatographic pre-separation and capillary column GC. Saleh (1983) gives composition estimates of 75% isomeric polychlorobornanes, 18% polychlorobornenes, 2% polychlorobornadienes, 1% other chlorinated hydrocarbons and 3% nonchlorinated compounds. These estimates were derived from both electron impact and negative chemical ionization mass spectrometry. Nearly all studies to date have been conducted with technical or synthesized material; there has been few attempts to characterize the structures found in environmental samples.

**Physical Properties**

Table 1 is a list of the physical properties that have been reported for toxaphene. Because toxaphene is a complex mixture, each component will have its own unique physical properties and therefore, it is very difficult to arrive at good estimates of overall parameters. Different combinations of numbers have been used by several

Figure 1: Structures of toxaphene components and metabolites that have been identified.

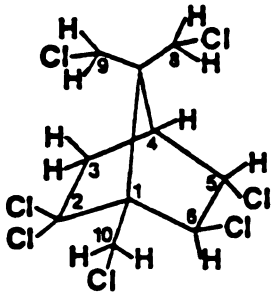
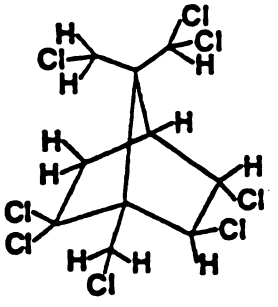
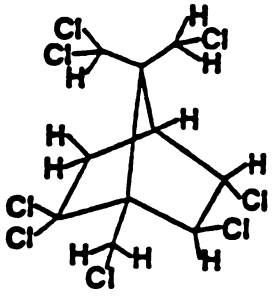
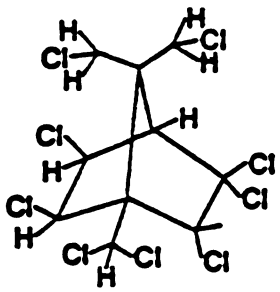
| Structure | Reference |
|---|---|
|  | Casida et al. 1974 |
| 2,2,5-endo,6-exo,8,9,10 heptachlorobornane (toxicant B, I) | |
|  | Turner et al. 1975<br>Matsumura et al. 1975 |
| 2,2,5-endo,6-exo,8,8,9,10 octachlorobornane (toxicant Aα, 8-chloro-I) | |
|  | Matsumura et al. 1975<br>Turner et al. 1975 |
| 2,2,5-endo,6-exo,8,9,9,10 octachlorobornane (toxicant Aß, 9-chloro-I) | |

Figure 1 continued:

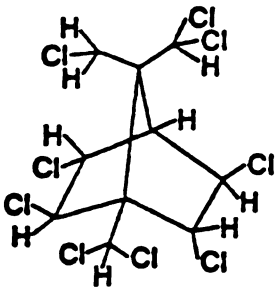
Structure

Reference



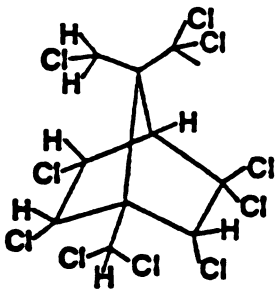
Anagnostopoulos et al. 1974

2-exo,3-exo,5,5,6-endo
8,9,10,10 nonachlorobornane (toxicant C)



Chandurkar et al. 1978

2-exo,3-endo,5-exo,6-endo,
8,8,9,10,10-nonachlorobornane
(toxicant Ac)



Anagnostopoulos et al. 1974

2-endo,3-endo,5,5,6-endo
8,8,9,10,10 decachlorobornane

Figure 1 continued:

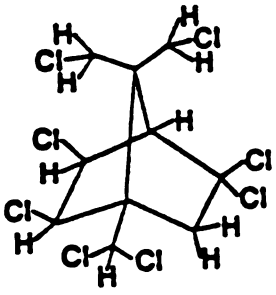
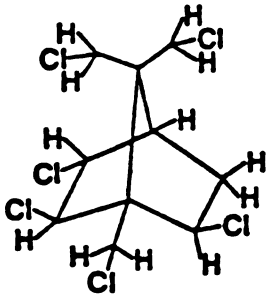
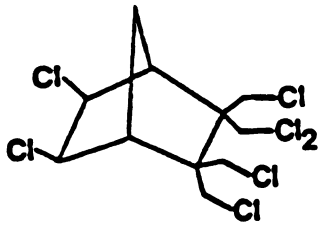
| Structure | Reference |
|---|---|
|  | Black 1974 |
| 2-exo,3-exo,5,5,8,9,10,10 octachlorobornane | |
|  | Black 1974 |
| 2-exo,3-endo,6-exo 8,9,10 hexachlorobornane | |
|  | Landrum et al. 1976 |
| 2,5,6-exo 8,8,9,10 heptachloro dihydrocamphene | |

Figure 1 continued:

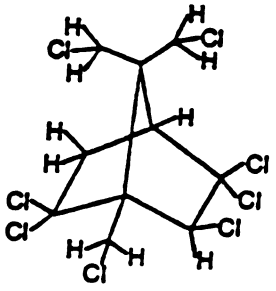
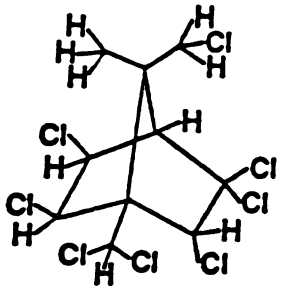
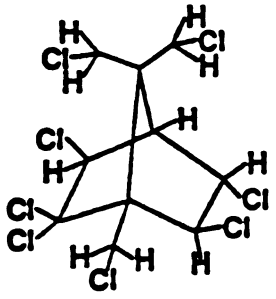
| Figure | Reference |
| --- | --- |
|  2,2,5,5,6-exo8,9,10 octachlorobornane (5-exo-chloro-I) | Turner et al. 1977 [from chlorination of toxicant B (I)] |
|  2-exo,3-exo,5,5,6-endo 9,10,10 octachlorobornane | Anagnostopoulos et al. 1974 |
|  2,2,3-exo,5-endo,6-exo,8,9,10 octachlorobornane (3-exo-chloro-I) | Turner et al. 1977 |

Figure 1 continued:

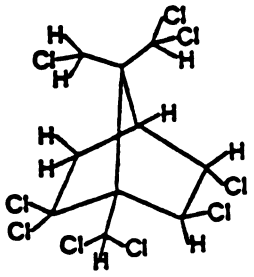
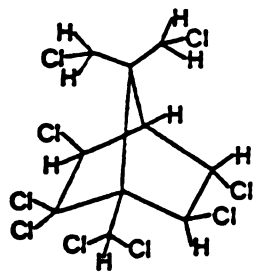
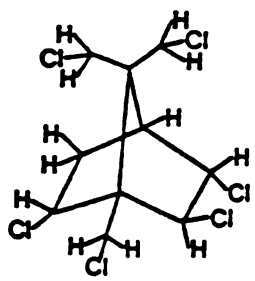
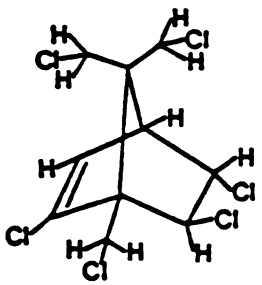
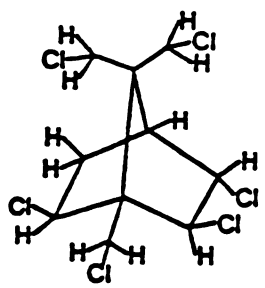
| Structure | Reference |
|---|---|
|  2,2,5-endo,6-exo 8,9,9,10,10-nonachlorobornane (8,10-dichloro-I) | Turner et al. 1977 [from chlorination of toxicant B (I)] |
|  2,2,3-exo,5-endo 6-exo,8,9,10,10 nonachlorobornane | Turner et al. 1977 [from chlorination of toxicant B (I)] |
|  2-endo,5-endo,6-exo 8,9,10 hexachlorobornane | Saleh and Casida 1978 (metabolite) |

Figure 1 continued:

| Structure | Reference |
|---|---|
|  2-endo,5-endo,6-exo 8,9,10 hexachlorobornene | Saleh and Casida 1978 (metabolite) |
|  2-exo,5-endo,6-exo 8,9,10 hexachlorobornane | Saleh and Casida 1978 (metabolite) |

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7 other di, tri and tetachlorobornanes were synthesized and identified by Parlar et al. (1977). These have not been drawn here because they are anticipated to make up very little of the technical products. Most of these structures and more detailed descriptions of identification procedures are contained in Korte et al. (1979), Casida and Saleh (1978) and Parlar and Michna (1983).

Table 1. Physical properties of toxaphene.[1]

| | | Reference |
|---|---|---|
| Molecular Weight | 414 ($C_{10}H_{10}Cl_8$) | (2) |
| Melting Point | range 70-95°C[3] | Brooks 1974 |
| Boiling Point | decomposes > 120°C | Brooks 1974 |
| Vapor Pressure | 0.2-0.4mm Hg(25°C) | Brooks 1974; Hercules AP-103B |
| | 1 X $10^{-6}$mm Hg(25°C) | Korte et al. 1979 |
| | 3 X $10^{-7}$mm Hg(20°C)[4] | Bidleman and Christenson 1979 |
| | 0.3mm Hg(25°C) | Reinert et al. 1982 |
| Aqueous Solubility | 3.0 mg/l | Brooks 1974; Hercules AP-103B |
| | 0.5 mg/l[5] | Paris et al. 1977 |
| | 0.04 mg/l | Reinert et al. 1982 |
| | 0.74 mg/l | EPA 1979 |
| | 0.40 mg/l | Sanborn et al. 1976 |
| Octanol/Water Partition Coefficient | 3.3 $\pm$ 2.5 X $10^2$ | Paris et al. 1977 |
| | 8.3 X $10^2$ | Sanborn et al. 1976[6] |
| | 6.4 X $10^4$ | Reinert et al. 1982 |
| | 2.7 X $10^6$ | Rice and Evans 1984 |
| | 2.0 X $10^3$ | EPA 1982 |

(1) Often values are listed in the references with no obvious source.
(2) Most authors agree on the average molecular weight for the mixture.
(3) Technical toxaphene is an amber waxy semi-solid at room temperature thus melting point is probably very subjective.
(4) Value reported for Strobane T but listed under toxaphene and used in subsequent references by this author.
(5) The only actual value that was measured by the authors.
(6) Measured with C-14 toxaphene. This is probably an overestimate due to a greater abundance of polar constituents compared to unlabelled material (Saleh and Casida, 1977).

investigators in order to try to understand the environmental behavior of toxaphene. In light of the variability in the numbers and the complexity of the mixture, this approach to understanding the environmental fate of toxaphene is inadequate.

**Toxicity-General**

Toxaphene is classified as a general convulsant which acts on the central nervous system. It causes stimulation of the cerebrospinal axis leading to chronic convulsions and tetanic muscular contraction. Death is usually caused by respiratory failure (Hercules Technical Bulletin T-105B). Barbiturates such as phenobarital and pentobarbital are generally effective in controlling convulsions (Stormont and Conley, 1952).

The acute oral toxicity of toxaphene to various mammals ranges from 40 mg/kg (cat) to 270 mg/kg (Guinea pig) and thus would be classified as moderately toxic.

**Toxicity - Structure Activity**

As toxaphene is indeed a very complex mixture, attempts have been made to isolate and identify structures of some of the major toxic components. This process was dictated by following the toxicity of various chromatographic fractions. This method allowed investigators to dwell only on fractions that seemed to be responsible for the toxicity of the insecticide.

The first toxic component identified was 2,2,5-<u>endo,</u> 6-<u>exo</u>, 8,9,10 heptachlorobornane (Casida et al., 1974). This compound would later become known as toxicant B (See Figure 1 for this compound and others to be discussed). Another major constituent was identified in the same

year and identified as 2-exo,3-exo,5,5,6-endo,8,9,10,10 nonachlorobornane (Anagnastopoulos et al., 1974). This component is now known as toxicant C. The third major component identified, toxicant A, was found to consist of two components; 2,2,5-endo,6-exo,8,8,9,10 octachlorobornane and 2,2,5-endo,6-exo,8,9,9,10 octachlorobornane (Nelson and Matsumura, 1975, Turner et al., 1975). There are a number of other minor components that have been identified including a chlorinated dihydrocamphene (Landrum et al., 1976) and chlorinated bornanes with fewer than 7 or more than 9 chlorines (Parlar et al., 1977 and Turner et al., 1977).

In terms of toxicity, toxicant A has been shown by a number of investigators to be the most potent of the three major toxic fractions to mice, goldfish, houseflies, (Turner et al., 1977, Saleh et al., 1977) brine shrimp, and mosquito larvae (Nelson and Matsumura, 1975). Toxicant B is the next most toxic constituent. For structural derivatives of toxicant B, Saleh et al. (1977) lists the toxicity as: 9-choro > 8-chloro > B > 3-exo-chloro > 5-exo-chloro > 10-chloro. Hexachlorobornane and hexachlorobornene metabolites of toxicant B were less toxic than most of the other structures. For many of the compounds tested, pretreatment of mice and houseflies with the mixed function oxidase inhibitor, piperonyl butoxide, increased toxicity 2 to 8-fold, indicating that metabolism plays a significant role in the pharmacologic action of many of the toxaphene components. Isolation and evaluation of all of the components of toxaphene would be virtually impossible. Together, toxicants A and B comprise approximately 8.2% of the technical mixture (Gooch and Matsumura, 1985).

**Toxicity-Mechanistic**

Though the neurotoxic action of toxaphene is well known, the precise molecular mechanism for this effect has not been known. Recently, a number of investigators have found that toxaphene and cyclodiene type insecticides interact very potently at a binding site associated with the GABA-chloride ionophore complex (Ghiasuddin and Matsumura, 1982; Matsumura and Ghiasuddin, 1983; Lawrence and Casida, 1984).

GABA (gamma-aminobutyric acid) is the major inhibitory neurotransmitter of the central nervous system of nearly all vertebrates and functions by stimulating $Cl^-$ flux across nerve membranes. The resultant decrease in membrane resistance inhibits the generation of excitatory post synaptic potentials (EPSPs) (Lund, 1985). Cyclodiene type insecticides have been shown to inhibit $Cl^-$ flux by binding with a receptor (the picrotoxinin receptor) proposed to be located at, or very near, the chloride channel (Matsumura and Ghiasuddin, 1983; Lawrence and Casida, 1984; Abalis et al., 1985). By interfering with the ability of the inhibitory neurotransmitter system to regulate nervous impulses, these insectides produce excessive stimulatory activity.

Two major ligands, $^{3}H$-picrotoxinin and $^{35}S$-t-butylbicyclophosphorothionate (TBPS), have been shown to bind to a site at or near the chloride channel (Ticku and Olsen, 1979; Squires et al., 1983; Ramanjaneyulu and Ticku, 1984). The ability of a compound to inhibit the binding of these ligands to the receptor site is highly correlated with toxicologic potency (Lawrence and Casida, 1984; Matsumura and Tanaka, 1984). This type of activity will be discussed in greater

detail in a later section.

In addition to its acute effects, toxaphene, or some fraction thereof, has been shown to be mutagenic (Hooper et al., 1979) and mildy carcinogenic in rats and mice (Reuber, 1979). It induces rat hepatic microsomal enzymes (Pollock et al., 1983), alters hepatobiliary excretion (Mehendale, 1978), and affects learning behavior in rats (Olson et al., 1980). Histopathologic lesions have been reported for the thyroid, adrenals, pituitary and mammany glands (Reuber, 1979).

**Toxicity - Aquatic**

Toxaphene is highly toxic to nearly all aquatic organisms that have been tested. Fish are very sensitive to toxaphene. Indeed, toxaphene was once tested experimentally as a piscicide in rough fish control programs (Johnson et al., 1966). Hughes (1970) wrote an excellent review of the literature on this topic. Henderson et al. (1959) lists 48-hour LC-50's as: 7.5 µg/l for fathead minnows, 3.3 µg/l for bluegills, 6.8 µg/l for goldfish, and 24 µg/l for guppies. Katz (1961) lists 3.3 µg/l for juvenile chinook salmon, 10.5 µg/l for juvenile coho salmon and 8.4 µg/l for rainbow trout. Johnson and Finley (1980) give 96 hour LC-50's ranging from 2 µg/l to 18 µg/l for 12 major fish species (4 salmonids, 3 cyprinids, 2 ictalurids, 3 centrarchids, and 1 percid). In addition, Johnson and Finley (1980) noted that swim-up fry were the most sensitive and that pH and water hardness did not affect the toxicity. Macek et al. (1969) noted a decrease in tolerance to toxaphene with a decrease in temperature. Invertebrates are less sensitive than fish to the acute effects of toxaphene, though not immensely. Forty-eight hour LC-50 values have

been reported as: 15 µg/l for Daphnia pulex and 19 µg/l for Simocephalus serrulatus (Sanders and Cope, 1966). Johnson and Finley (1980) list 96 hour LC-50 values ranging from 1.3 - 40 µg/l for nine common invertebrate species.

Nearly all of the chronic toxicity data available on toxaphene are from studies by the United States Fish and Wildlife Service, Fish-Pesticide Research Lab in Columbia, MO. On the basis of growth, reproduction, mortality and bone development, maximum acceptable toxicant concentrations (MATC's) were determined to be < 39 ng/l for brook trout, between 25 and 54 ng/l for fathead minnow and between 49 and 72 ng/l for channel catfish (Mehrle and Mayer, 1975a, 1975b). Collagen deposition and bone development were the most sensitive parameters and differences in brook trout fry were evident after only 7 days exposure. Later studies (Mayer et al., 1978) demonstrated a relationship between vitamin C levels (depletion) and the bone anomalies. High levels of vitamin C in the diet reduced whole-body residues of toxaphene and significantly increased the tolerance of fish to the chronic bone effects. The authors proposed a relationship between induction of the hepatic monooxygenase system by toxaphene and a functional vitamin C depletion due to excessive demand from the induction of the biotransformation enzymes.

Bioconcentration factors for various life stages in these studies ranged from 5,000 to 76,000 for brook trout, 10,000 to 69,000 for fathead minnows and 17,000 to 50,000 for channel catfish.

These studies demonstrate that small concentrations (parts per trillion) of toxaphene can cause significant effects on fish. Swain et al. (1982) estimated open water concentrations of 1.5 ng/l for Lake

Huron in 1980 and 1981. Given the uncertainties in measuring low concentrations, these concentrations are not very far from the MATC for brook trout.

**Environmental Dynamics**

**Agricultural** - Nash et al. (1977) studied the behavior of toxaphene and DDT in a model agro-ecosystem chamber. At the end of the experiment 24% of the applied toxaphene had volatilized, 20% was in the soil and the rest was on the plants and chamber. Volatilization half-lives were 15.1 days for toxaphene and 18.8 days for DDT, though losses were non-linear with a large percentage volatilizing in the first 24 hours.

Seiber et al. (1979) studied toxaphene movement in an agricultural field and found that 59% of the toxaphene applied to cotton leaves was lost in 28 days. Analysis of soil and air samples indicated that volatilization was a major loss mechanism with the early eluting, more volatile, GC peaks being lost preferentially. Willis et al. (1982) found that only 5 to 10% of the toxaphene applied to mature cotton plants was washed off by simulated rainfall. Earlier studies by the same group (Willis et al., 1980) had indicated that volatilization was a major loss mechanism.

**Soils** - Hermanson et al. (1971) calculated an approximate half-life of 4 years for toxaphene repeatedly applied to a soil over 5 years. Nash et al. (1973) recovered 45% of the toxaphene that had been applied to soil at a high rate 20 years prior to extraction. Soils, however, had been stored at -5$^{o}$C and would not reflect environmental conditions. Seiber et al. (1979) found a topsoil concentration

decrease from 13.1 ppm to 5.4 ppm in 58 days and suggested that volatilization was the major cause. LaFleur et al. (1973) found a half-life in top soil of about 100 days from a field plot treated with 100 kg/ha, a very high level. They also found a concentration of 1 ng/l in the underlying ground water.

Degradation of toxaphene in soils occurs primarily under anaerobic conditions. Murthy et al. (1977) demonstrated reductive dechlorination of $^{14}C$-toxaphene separated into nine fractions and applied to a metapeak silt loam. $^{14}CO_2$ production and volatility losses were minimal. Parr and Smith (1976) found the order of degradation in a Crowley silt loam after 6 weeks as: flooded anaerobic (stirred) > moist anaerobic > flooded anaerobic (unstirred) > moist. No degradation was found in autoclaved soils.

Toxaphene in anaerobic salt marsh sediments was degraded within a few days (Williams and Bidleman, 1978). Breakdown was also demonstrated in the presence of a $Fe^{+2}/Fe^{+3}$ redox couple operating at a -250 mv potential and pH = 5.6.

Clark and Matsumura (1979) demonstrated significant aerobic degradation of toxaphene in aquatic sediments. They suggested that anaerobic dechlorination coupled with aerobic metabolism would probably be necessary for complete degradation of toxaphene.

Gallagher et al. (1979) found that toxaphene accumulated in anaerobic salt marsh soils and was associated mainly with dead roots. Toxaphene spiked below the soil surface was taken up and translocated by marsh grass (Spartina alterniflora).

**Aquatic Systems** - Much of the work described in this section was done while toxaphene was being tested as a possible poison for use in rough fish control programs. The best review of the early work in this area is contained in Hughes (1970).

Terriere et al. (1966) studied toxaphene dynamics in two Oregon lakes treated with 40 and 90 µg/l. Five years after the application, the deep oligotrophic lake was still too toxic to support fish and had a water concentration of 0.4 µg/l (originally 40). Fish placed in live boxes readily accumulated 3-7 µg/l of residue in 10 days. Both plants and aquatic invertebrates accumulated toxaphene 500 to 1000-fold over water concentrations. Toxaphene residues in the shallower lake, "rich in aquatic life", were detoxified within one year, though fish, plants, and invertebrates still accumulated high residues. Extracts from fish were just as toxic to houseflies as technical material.

Veith and Lee (1971) studied the detoxification of Wisconsin lakes and determined that adsorption to sediments and settling played a major role in the detoxification process. They also reported that greater than 50% of the toxaphene applied to the lakes may have been lost during application through evaporation or co-distillation with water vapor, or was degraded through microbiological activity. Hughes and Lee (1973) found that bluegills in one of the treated lakes accumulated up to 9.4 µg/g and that the residues were highly correlated with fat content.

In a model ecosystem study, Sanborn et al. (1976) found bioconcentration factors of 6902, 9600, 890, and 4247 for algae, snails, mosquitoes and fish respectively. Isensee et al. (1979) tested toxaphene and 3 chromatographic fractions of toxaphene and found

similar results. Neither study found much evidence for metabolism in the fish. Isensee et al. (1979) demonstrated that the intermediate polarity fraction accumulated greatest.

**Global Residues**

It is now apparent that toxaphene, or some assemblage of polychlorinated bornanes, is much more recalcitrant and widely distributed than the early literature would have suggested it was going to be. Now that capillary column gas chromatography and capillary GC mass spectrometry is a routine tool in many analytical laboratories, toxaphene like residues are being detected in places far removed from any known source. Bidleman and Olney (1975) were the first to demonstrate long range atmospheric transport when they detected toxaphene in the air 1200 km out to sea, over the island of Bermuda. Though the chromatographic profiles were relatively enriched in earlier eluting peaks, the profiles observed were remarkably similar to technical toxaphene. This suggests that over longer time frames the atmospheric composition of toxaphene is very similar to technical material, though Seiber et al. (1979) demonstrated more rapid volatilization of early eluting components. In other words, the differences in vapor pressure for the different components of the mixture are not sufficient to create large changes in the relative concentrations of toxaphene components, even far from the source. Later Ziranski et al. (1982) found that experimentally vaporized toxaphene was more similar to a Columbia, South Carolina air sample than to technical toxaphene in patterns determined by n-dimensional euclidean distances. Careful examination of the capillary chromatogram from this

study revealed that only the very latest eluting compounds were not present in the air sample.

Though toxaphene had been in use for many years, reports of widespread contamination of the aquatic environment did not start appearing in the literature until the late 1970's. It is not surprising that this parallels the great increase in analytical capability mentioned previously. The best systematic program for detecting trends in pesticide residues is the National Pesticide Monitoring Program established by the U.S. Fish and Wildlife Service in the mid-1960's. Schmitt et al. (1981) summarized residues found in fish from across the U.S. from 1970 - 1974. This was the first time that toxaphene residues were detected in fish from regions other than the cotton growing areas of the south. Subsequent studies by Schmitt et al. (1983) and Schmitt et al. (1985) have documented an increased frequency of occurrence and levels found in fish throughout the U.S. Ribick et al. (1982) documented a detailed approach to determine part per billion residues in fish.

The more widespread global occurrence of toxaphene became evident when Jansson et al. (1979) discovered residues in herring and seal from the Baltic Sea. Zell and Ballschmiter (1980) reported on toxaphene residues in Antartic cod, North Atlantic salmon, North Pacific salmon, Asiatic clams from the European Alps and sturgeon from the Caspian Sea. Ballschmiter and Zell (1980) found toxaphene residues in a commercial sample of Peruvian fish oil and South Pacific sperm whale oil. Toxaphene or toxaphene like residues have been found in Canadian east coast herring and cod (Musial and Uthe, 1983), seal blubber from the Gulf of Bothnia and the Gulf of Finland (Baltic Sea) (Pyysalo and

Antervo, 1985), surface and deep water fish near the Azores in the North Atlantic (Kramer et al., 1984), and in deep sea rattail collected from the western North Atlantic (Stegeman et al., 1986). Toxaphene has also been detected in recent samples of Swedish breast milk (Vaz and Blomkvist, 1985). The most recent summary of toxaphene residues in the Great Lakes can be found in Rice and Evans (1984) and Sullivan and Armstrong (1985).

# CHAPTER 2

# EVALUATION OF THE TOXIC COMPONENTS OF TOXAPHENE IN LAKE MICHIGAN LAKE TROUT

(Gooch, J. W. and F. Matsumura (1985) J. Agric. Food Chem. 33:844-848)

# INTRODUCTION

Contaminants in Great Lakes fish are well documented and have been widely studied for a number of years (Schmitt et al., 1983). In the majority of cases the contaminants are single components and represent a relatively simple task for quantitation and evaluation. Until recently, analysts have not had adequate techniques for assessment of residues derived from complex mixtures. With the development and routine usage of capillary column gas chrgmatography and capillary GC/mass spectrometry, there has been an increase in the number and complexity of chemicals being detected and analyzed at low concentrations.

One such complex mixture is the chlorinated hydrocarbon insecticide, toxaphene. Toxaphene is a chlorinated organic insecticide composed of over 177 components (Holmstead et al., 1974; Saleh, 1983) which, until recently, was the most widely used chlorinated pesticide in the United States. Recent studies of fish from the Laurentian Great Lakes have shown high concentrations of chlorinated hydrocarbons which appear to be very similar to toxaphene (Schmitt et al., 1981; Rappe et al., 1979; Rice and Evans, 1984). Alterations in composition of the mixture prior to and during deposition as a residue creates difficulty for accurate quantitation and evaluation due to the lack of a suitable analytical standard (Musial and Uthe, 1983; Jansson et al., 1979; Wideqvist et al., 1984).

Since the relative composition of the components in the residue is different from technical toxaphene, concentrations of overall "toxaphene" may not accurately reflect the true toxicologic nature of the material being measured. Studies have shown that toxicity to various organisms is not distributed evenly among various fractions of technical toxaphene. Studies by Nelson and Matsumura (1975), Saleh et al. (1977), and Isensee et al. (1979) have shown that toxicity to invertebrates and fish can be attributed to several components, while mammalian toxicity is apparently more restricted. In addition, Harder et al. (1983) have shown that anaerobic sediment decomposition products are at least as toxic as technical material to two species of estuarine fish.

Because of the complexity of the technical mixture, very few of the components have been isolated and identified. Studies by Turner et al. (1977) and Saleh et al. (1977) contain excellent summaries of several chemical structures that have been isolated and their toxicities. It should be noted that only small quantities of a few of these compounds exist or are available for routine analytical use.

In all of the studies to date, including those of Seiber et al. (1975) and Pollock and Kilgore (1980), two constituents of toxaphene have proven to be the most toxic to all organisms tested. Toxicant B (2,2,5-endo,6-exo,8,9,10-heptachlorobornane, heptachlorobornane I) was originally isolated and identified by Casida et al. (1974). Toxicant A (a mixture of 2,2,5-endo,6-exo,8,9,9,10 and 2,2,5-endo,8,8,9,10 octachlorobornanes) was isolated and identified by Turner et al. (1975) and Nelson and Matsumura (1975). These two components comprise approximately 8% of the toxaphene mixture as manufactured by the

Hercules Chemical Co., Wilmington, Delaware (Casida et al., 1974; Saleh, 1983; Turner et al., 1975). This study was conducted to investigate the presence and potential use of quantitation of toxicants A and B as a more toxicologically meaningful measure of residues derived from toxaphene. Here we report the presence and concentrations of these two constituents in lake trout from Lake Michigan.

## EXPERIMENTAL SECTION

### MATERIALS

The lake trout examined in this study were obtained as incidental catch from commercial fishing nets approximately 5 miles south of Muskegon, Michigan in Lake Michigan on August 6, 1982. Fish (50-60 cm total length) were kept on ice approximately 10 hours before being frozen in individual plastic bags.

### METHODS

For analysis, fish were partially thawed and a sample of the "belly flap" region was removed. This region is rich in adipose tissue and was chosen since it was expected to contain higher levels of contaminants than other tissues. Fish were extracted and apparent toxaphene residues isolated essentially as described by Ribick et al. (1982), with omission of the final nitrification procedure. Briefly, this method involved grinding tissue with anhydrous sodium sulfate and column extracting with methylene chloride at a flow rate of 3-5 ml/min. Lipids were removed via gel permeation chromatography on BioBeads (SX-3) using 1:1 methylene chloride:cyclohexane. The residue

was fractionated on florisil to remove some of the cyclodiene insecticides, followed by silica-gel to remove interfering polychlorinated biphenyls (PCBs). The final extract contained toxaphene, chlordane, DDT (etc.), hexachlorocyclohexane (BHC) and several other chlorinated pesticides. Replicate samples were periodically spiked with $^{14}$C-toxaphene in order to determine recovery. Lipid analysis was not conducted as it has been shown (Schmitt et al., 1983) that differing amounts of lipid do not generally explain variations in residue concentrations. All reagents used were pesticide residue grade. Recovery from spiked samples was generally > 90%. Residue concentrations were not corrected for recovery.

**TLC Purification of Toxicant A**

Toxicant A is a variable mixture of two octachlorobornane components which chromatograph with identical retention times on capillary GC (Saleh and Casida, 1978; Gooch and Matsumura, unpublished data). The A (α) (Nelson and Matsumura, 1975) or A-1 (Turner et al., 1975) component is 2,2,5-endo,6-exo,8,9,9,10-octachlorobornane while the A (β) (B-1) component is 2,2,5-endo,6-exo,8,8,9,10-octachlorobornane. Saleh (1983) provides a good description of the basic structural framework of the chlorinated bornane, bornene and bornadiene components of toxaphene.

Silica-gel 60 F-254 (250 μ, E. Merck) plates were heated at 130°C prior to use. The final extracts from the procedure described above were spotted and plates developed 4 times using n-heptane (saturated chamber). Toxaphene chromatographs as a streak with several distinct spots under these conditions (Nelson and Matsumura, 1975). Authentic

toxicant A was chromatographed under identical conditions to determine the appropriate Rf region to isolate. A region corresponding to an Rf of 0.30 to 0.43 was scraped, placed into a chromatographic tube and eluted with 25% diethyl ether in hexane. This procedure separates some of the toxaphene components from p,p' DDT which migrates with an Rf > 0.60.

**Gas Chromatography and Quantitation of Residues**

Extracts were analyzed with a 30M x 0.25 mm i.d. DB-1 (J and W Scientific) fused silica capillary column using helium as the carrier gas (fixed pressure, 140 kilopascals). Samples were injected (injection temperature 200°C) in the split mode (split ratio 3:1) with the column at 190 °C. The oven was immediately programmed to heat to 260°C at 2°/min. The $Sc^3H$ foil electron capture detector was operated with a nitrogen make up gas flow of 30 ml/min at 280°C.

Data was collected using a Spectra Physics 4270 integrator. Technical toxaphene standards, originally obtained from the Hercules Chemical Co., (Lot # X16189-49) were used for comparison with residues. Areas from 30-32 peaks (after p,O' DDE with the appropriate retention times, window 0.1 min) were used for quantitation of the residue. Retention times used were either absolute or relative to p,p' DDE. Quantitation of "estimated total toxaphene" was done using methods employed by several other authors, whereby peak areas are summed for selected peaks with identical retention times to those of the analytical standard (Ribick et al., 1982; Musial and Uthe, 1983). The same peaks are summed for several concentrations of a known quantity of standard for generation of a standard curve. This method assumes equal

peak response factors for analogous peaks in the residue and standard, a situation which may or may not be true. For this study, 30 peaks eluting after p,p' DDE were used for quantitation. Peak matching was done manually using a light table and overlaying residues on technical standards spiked with p,p' DDE. By using this method we were able to more accurately determine peak matches than by relying on consistent performance of our integrator. After viewing a large number of chromatograms, it is readily apparent that the residue profile, in terms of presence or absence of peaks, is very similar between fish, sites, and years, (this subject will be discussed in more detail in a future report).

Quantification of toxicants A and B was done using peaks matched with NMR certified standards isolated in this laboratory (Nelson and Matusumurs, 1975; Matsumura et al., 1974). Toxicant B was quantitated in the original extract prior to TLC purification of the residue for toxicant A. Because of the limited amount of material available (micrograms), we have used our standards for peak identification purposes only. In order to generate standard curves for toxicants A and B we have used average fractional composition estimates based on available information in the literature. Using the mass spectrometric data of Casida et al. (1974), Turner et al. (1975), Khalifa et al. (1974), Holmstead et al. (1974) and Saleh (1983), we have arrived at estimates of 4.8% for toxicant A and 3.4% for toxicant B.

Masses for the respective toxicants in the mixture can be derived by multiplying the mass of the standard times the proportional composition (0.48 or 0.34) estimate and using the corresponding mass/peak area relationship to generate a standard curve. Although this is an estimate, we feel that the proportional estimates for

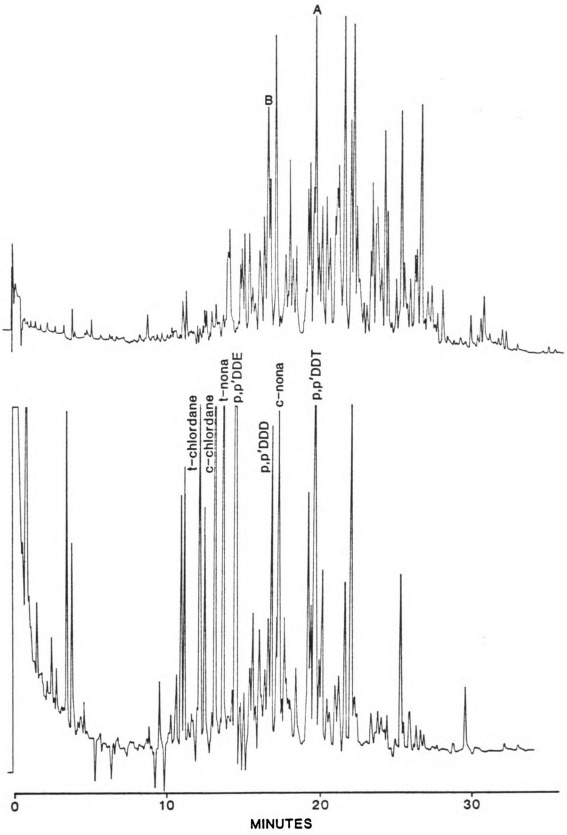
composition are sound and thus can be used by other investigators when no technical standard is available, as long as identification of the appropriate peak can be made.

Capillary gas chromatography/mass spectrometry was done using a Hewlett Packard 5985A quadrupole mass spectrometer operated in the electron impact mode (70 eV). A 30 M x 0.25 mm i.d. DB-1 fused silica capillary column was used and operated essentially as described for routine ECD analysis although injections were done in the splitless mode. Approximately 700 spectra were acquired during each run (approximately 17 scans/minute). Spectra of the individual peaks were analyzed manually for the appropriate characteristics.

## RESULTS AND DISCUSSION

The pattern of toxaphene compounds in Lake Michigan lake trout samples is very different from technical toxaphene (Figure 2). This has also been noted in canadian east coast marine fish (Musial and Uthe, 1983) as well as seals from swedish waters (Jansson et al., 1979). Zell and Ballschmiter (1980) called attention to the widespread occurrence of toxaphene [polychlorinated camphenes (PCC)] in spawn from fish from several different areas, though they only used 4 key reference peaks for quantitation from the 177 different components that toxaphene contains. Part of the difficulty stems from the fact that many toxaphene components are readily degraded by microbes (Clark and Matsumura, 1979) and there are several unrelated compounds in the residue that co-elute and interfere with accurate quantitation of toxaphene components. Most of the compounds found in samples after

Figure 2: Capillary column GC-ECD traces of toxaphene (above) and the toxaphene fraction of an extract from a lake trout from Lake Michigan (below). Conditions are reported under Materials and Methods. Locations of toxicants A and B are indicated.


A
B
t-chlordane
c-chlordane
t-nona
p,p'DDE
p,p'DDD
c-nona
p,p'DDT
0
10
20
30
MINUTES

silica gel purification are related to technical chlordane and the DDT complex. Nitration procedures are available for selective removal of the aromatic compounds, a procedure we feel is useful but not absolutely necessary for reliable "overall" toxaphene analysis. However, as we will show, some type of purification procedure must be used for accurate quantitation of the toxic congeners. In our samples the ubiquity of the DDT compounds was useful for determining relative retention times since they chromatograph in strategic regions. None of the compounds eluting prior to p,p' DDE were considered for quantitation due to interferences from chlordane components. In our system this excludes very few components since the vast majority of the standard toxaphene elutes after these compounds (Figure 2). In general, we were able to match approximately 30 peaks of the residue to the toxaphene standard. Since this is only a fraction of the available peaks, other peaks must either be transformation products or compounds unrelated to toxaphene. The mass spectral data indicates that most of the peaks following p,p' DDE have a polychlorinated bornane type of structure. Since accurate quantitation relies on matched peaks having the same composition and response factor, quantifying this residue can only be considered a rough estimate, a concept agreed upon by many authors (Jansson and Wideqvist, 1983; Musial and Uthe, 1983).

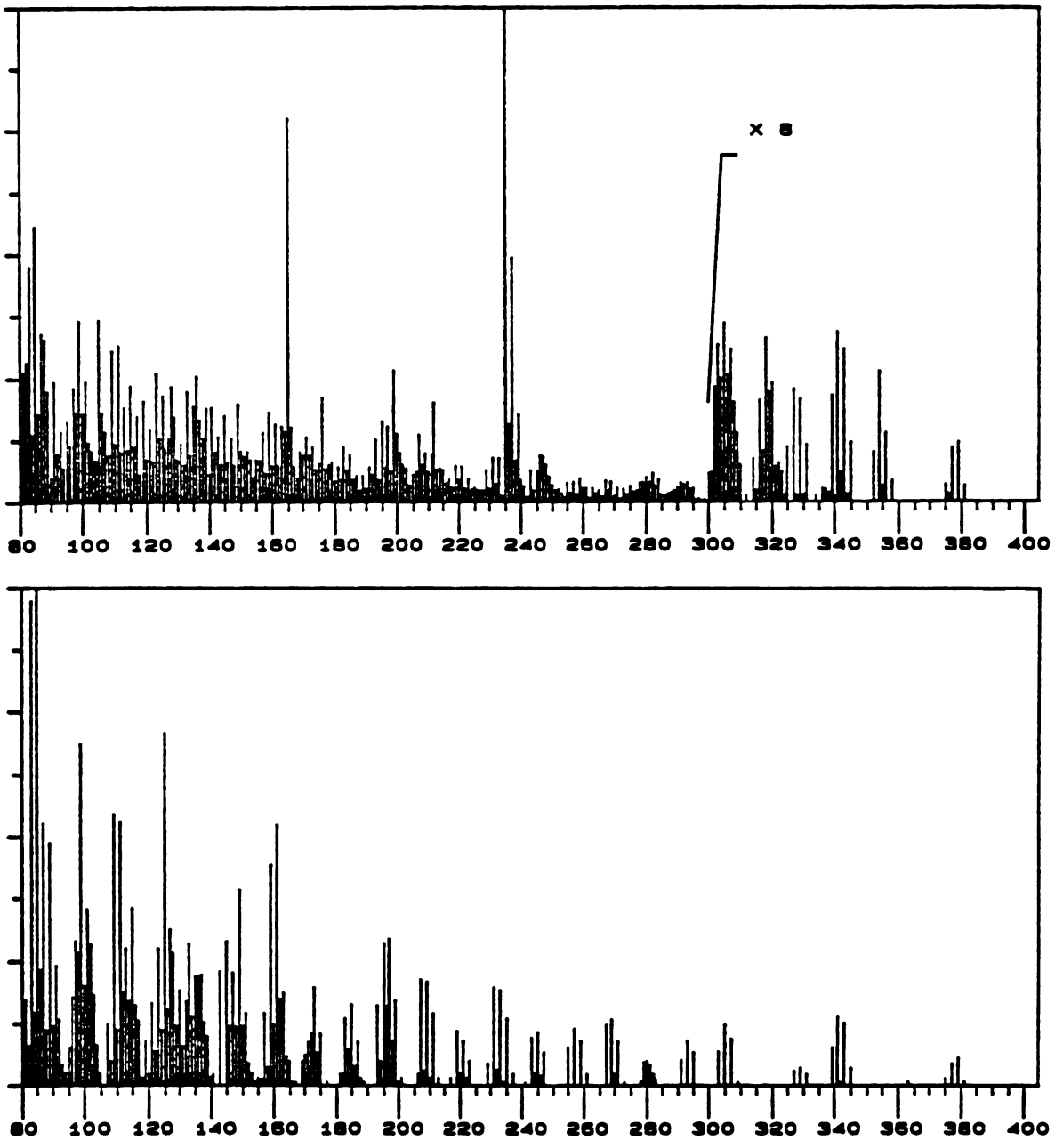
Since toxicants A and B exert substantial toxicity in all organisms investigated (Casida et al., 1974; Nelson and Matsumura, 1975), it is important to quantify these components in relation to the overall total. This should provide a more reliable indication of the toxicologic potential of the residue. Intially, we used thin-layer chromatrography of a lake trout residue and authentic toxicant A to look for this component. A capillary gas chromatogram of a lake trout

residue before and after being fractionated in this manner is shown in Fugure 3. When this procedure is carried out, a peak free of p,p' DDT remains with the same Rf as authentic toxicant A. It also possesses an identical retention time on capilary GLC and has the same retention characteristics on florisil and silica gel. Investigation of gas chromatograms of initial extracts for toxicant B suggested that no post silica-gel purification was necessary (Figure 2).

To confirm the presence of toxicants A and B in lake trout samples, we examined the pesticide fraction from the silica-gel column with capillary GC-MS in the electron (EI) impact mode. The mass spectrum of a toxaphene component co-eluting with p,p' DDT (top) is shown in Figure 4. The clusters at masses 352, 316, 235, and 116 are from p,p' DDT with masses 352 and 316 corresponding to the parent ion ($M^+$) and a fragment from the loss of HCl, respectively. Toxicant A elutes incompletely resolved from p,p' DDT in this region. For identification of toxicant A we have relied primarily on the fragment clusters at masses 375, 339, and 303. The cluster at 375 corresponds to the $[M-Cl]^+$ ion from a compound having a $C_{10}H_{10}Cl_8$ (MW=410) formula. For comparison, a mass spectrum of toxicant A as it elutes from technical toxaphene under the same GC conditions employed for the residue is shown (Figure 4, bottom). The lack of a parent ion near mass 410 is typical of toxaphene components (Saleh, 1983; Holmstead et al., 1974). The results supporting the identification of toxicant A are, the mass spectrum indicating a bornane structure with 8 chlorines, matching Rf's on thin layer chromatography, similar retention on florisil and silica-gel columns, and capillary gas chromatography with authentic toxicant A.

Figure 3: Capillary column GC-ECD traces of the toxaphene fraction of an extract of lake trout from Lake Michigan prior to (above) and after (below) TLC purification for toxicant A. Locations of toxicants A and B are indicated.

Figure 4: Electron impact mass spectra of toxicant A (below) and of toxicant A (above) co-eluting with p,p' DDT from a lake trout residue during capillary GC-MS. The spectrum of toxicant A (below) was acquired during a capillary GC-MS run of technical toxaphene.


x 8
80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400
80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400

As stated earlier, identification of toxicant B in the residue was achieved by matching retention times of an authentic standard to that of a corresponding peak in the extract prior to TLC purification for toxicant A. The mass spectra acquired in this region which support our identification as shown in Figure 5. Toxicant B, labeled in Figure 2 and 3, can be seen to elute just prior to p,p' DDD. With the small loss in resolution we encountered in the GC-MS system and the relatively small amount of toxicant B present, the spectra acquired show a co-elution with p,p' DDD. The masses at 318, 325, and 165 identify p,p' DDD with the mass at 318 corresponding to the parent ion ($M^+$). The lower spectrum of toxicant B as it elutes during a capillary GC-MS run shows a weak $M^+$ ion at mass 376 ($C_{10}H_{11}Cl_7$) and complex characteristic fragments at masses 339, 325, and 303. Identical fragments can be seen in the peak that co-elutes with p,p' DDD. If it is indeed true that these residues are derived from the use of toxaphene, we would expect both of these compounds to be present.

The results of the analysis of 4 Lake Michigan lake trout for polychlorinated bornanes (estimated total) and more specifically for the toxic congeners, are presented in Table 2. The concentration of toxicant A is more than an order of magnitude less than the total residue while toxicant B is 2-3 times less than toxicant A. Since these are two of the most universally toxic congeners, and we have no other good reference point, this concentration may be more toxicologically relevant. We are currently conducting more detailed studies to further explore this suggestion.

Figure 5: Mass spectra of toxicant B eluting from a technical standard (below) and of toxicant B co-eluting with p,p' DDD in a lake trout residue (above).

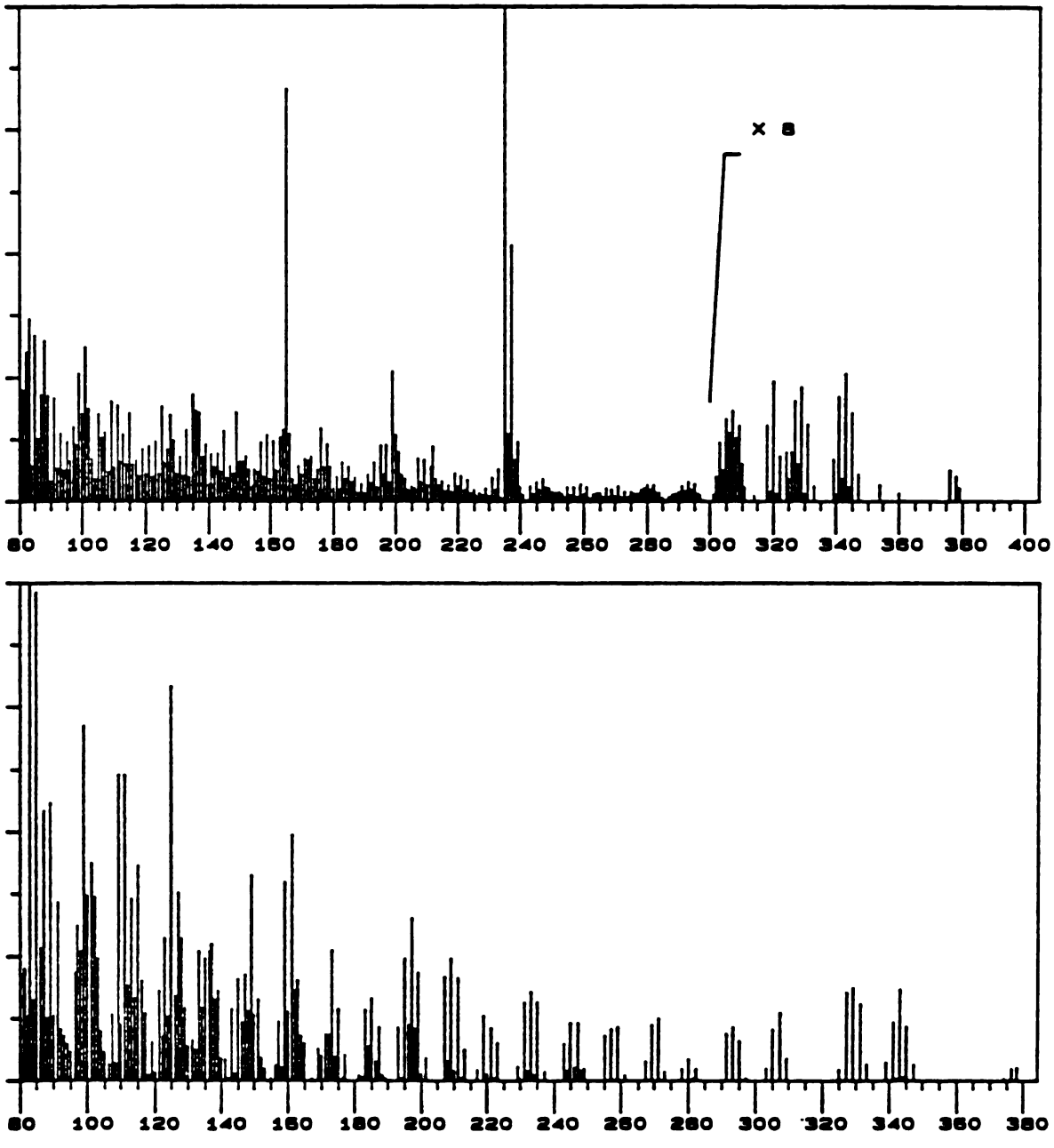

x 8
60 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400
60 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380

Table 2: Concentrations of the two primary toxic constituents and estimated total toxaphene found in lake trout from Lake Michigan.

| Fish | Estimated[a] Total Toxaphene | Toxicant A | Toxicant B |
|---|---|---|---|
| 1 | 10.7 | 0.70 (6.6)[b] | 0.21 (2.0) |
| 2 | 1.9 | 0.08 (4.4) | 0.08 (4.0) |
| 3 | 1.6 | 0.12 (7.1) | 0.06 (3.7) |
| 4 | 2.9 | 0.12 (4.2) | 0.04 (1.4) |
| X ± S.E. | 4.3 ± 3.7 | 0.26 ± 0.26 | 0.10 ± 0.07 |

[a] All residue concentrations are in µg/g (ppm) wet weight. Estimated total toxaphene levels have been derived on the basis of equal electron capture response factors for all components (see text).

[b] Numbers in parentheses indicate the percent composition of the toxic components in the estimated toxaphene total.

Estimates of the amount of toxicant A and toxicant B in technical toxaphene varies from 1.5 to 8.5% for toxicant A and 2.5 to 4.1% for toxicant B. On average, toxicant A is approximately 4.8% and toxicant B is approximately 3.4% (Casida et al., 1974; Turner et al., 1975; Saleh, 1983; Khalifa et al., 1974; Holmstead et al., 1974) of the total mixture. Using these estimates and the mean values from Table 2, it appears that the environmental residues of toxaphene have nearly the same relative amounts of the toxic components as the technical mixture. This is somewhat surprising, and perhaps coincidental, since the estimates for the total apparent toxaphene is subject to such large assumptions during quantitation. Indeed, since the toxaphene we measure as a residue has been altered by various processes (Musial and Uthe, 1983; Jansson et al., 1979; Zell and Ballschmiter, 1980), this similarity of ratios is unexpected. Perhaps the rate of environmental transport and metabolic degradation of toxicant A and B may be similar to the rest of toxaphene residues as a whole, an observation suggested by Clark and Matsumura (1979) when they demonstrated that the rate of degradation of toxicant A by microorganisms and in sediment was roughly identical to the overall mixture. The presence of toxicants A and B suggests that the residue should be examined for its toxicologic properties.

# CHAPTER 3

# TOXICITY OF CHLORINATED BORNANE (TOXAPHENE) RESIDUES ISOLATED FROM GREAT LAKES LAKE TROUT (*SALVELINUS NAMAYCUSH*)

# INTRODUCTION

## I. Acute Toxicity and Mammalian Neuroreceptor Binding

Chlorinated bornane residues, most likely derived from the pesticide toxaphene, have now been found in aquatic biota from around the world (Jansson et al., 1979; Zell and Ballschmiter, 1980; Musial and Uthe, 1983; Wideqvist et al., 1984). Recently, residues were also found in the Great Lakes (Ribick et al., 1982; Rice and Evans, 1984; Gooch and Matsumura, 1985). Because these residues are found in places far from any known use, chlorinated bornanes have joined DDT and the polychlorinated biphenyls (PCBs) as a class of global pollutants (Zell and Ballschmiter, 1980).

Studying toxaphene residues is difficult because of the complex multicomponent composition of the material. Toxaphene is an incompletely defined mixture of at least 177 different components formed during the process of chlorinating camphene (Turner et al., 1977; Saleh et al., 1977). Until recently, analytical techniques were unable to detect toxaphene residues in an unambiquous fashion. The routine availability of capillary column gas chromatography (capillary GC) and capillary GC-mass spectrometry has provided great strides in our ability to identify residues derived from such complex mixtures. This increased capability now allows us to ask more detailed questions about the nature of the changes that are occurring with these materials during movement to and in the aquatic ecosystem. In particular, since

most investigators have noted the dissimilarity between toxaphene residues and analytical standards (Musial and Uthe, 1983; Wideqvist et al., 1984), it is unclear whether the toxaphene residues found in the environment still possess a broad spectrum of toxicological activity. Since the toxicity of toxaphene is not distributed evenly throughout the mixture (Nelson and Matsumura, 1975; Saleh et al., 1977; Isensee et al., 1979), more detailed analyses and studies need to be conducted in order to clarify the potential significance of toxaphene derived residues.

Our recent work (Gooch and Matsumura, 1985) demonstrated the presence of toxicants A and B, two of the most toxic constituents of toxaphene, in residues from Lake Michigan lake trout (Salvelinus namaycush). The presence of these two components suggests that the residue may possess significant toxicological properties. This study was conducted to examine the toxicity associated with toxaphene residues derived from Great Lakes lake trout using acute toxicity and neuromolecular receptor site binding affinity as endpoints.

## MATERIALS AND METHODS

### Extraction and Purification of Residues

The lake trout examined in this study were taken from the southern half of Lake Michigan. In 1982, fish (50-60 cm total length) were obtained as incidental catch from commercial fishing nets near Muskegon, Michigan, on August 6th. In subsequent years, fish were obtained from late summer (September) sampling surveys conducted by the U.S. Fish and Wildlife Service near Saugatuck, Michigan (approximately

45 miles south of Muskegon). Lake trout were also obtained from Siskiwit Lake on Isle Royale in Lake Superior in July of 1984. Fish were kept on ice for approximately 10 hours before being frozen in individual plastic bags at -20° C.

For analysis, fish were partially thawed and a sample of the belly-flap region was removed from an area immediately anterior to the pelvic fin between the lateral line and the ventrum. This region is rich in adipose tissue and was chosen since it was expected to contain higher levels of lipophillic contaminants than other portions of tissue. Concurrently, a sample of muscle tissue (fillet) was removed from an area immediately dorsal to the belly-flap and frozen for later analysis. All tissues were examined with the skin removed.

Individual 10-20 gm samples were weighed, minced, and ground with 4-6 volumes of anhydrous sodium sulfate using a mortar and pestle until a uniform preparation was obtained. Extraction, gel permeation lipid clean-up and preliminary fractionation for toxaphene was done using the multi-residue procedure described by Ribick et al. (1982). The final nitration procedure was not done at this point. This protocol uses florisil and silica-gel to separate toxaphene from the PCBs and the ubiquitous cyclodiene insecticides (dieldrin, endrin, etc.). The appropriate silica-gel column fraction was analyzed for toxaphene components using capillary GC with electron capture detection. GC conditions were as follows: 30 m DB-1 column (J and W Scientific) .25 mm id,0.25 μ film, head pressure 130 kpascals, precolumn split 5:1, injector temp 190°C, detector 270°C, and the oven was temperature programmed immediately following injection from 190-260°C 2°/min. Data collection and integration was done using a Spectra-Physics 4270

integrator. Chromatograms were checked and all quality control comparisons made manually. Comparisons and quantitation were done using technical grade toxaphene (originally obtained from the Hercules Chemical Co., Lot # X16189-49) with methods described previously (Gooch and Matsumura, 1985). Samples at this point contain several major components of chlordane and some of the components of the DDT complex. These compounds preclude simple interpretation of toxaphene derived residues and are not desirable for toxicity testing.

For separation of chlordane and toxaphene, samples were applied to a charcoal column similar to that originally described by Underwood (1978) and further modified by Farrell (1985). For this procedure, 2 g of a 1:1 mixture of Super A activated carbon (AX-21, Anderson Development Co., Adrian, MI 49221) and Celite 545 (Supelco Inc.) was placed into a 22 mm id X 500 mm id chromatographic column plugged with glass wool and 1 cm of anhydrous sodium sulfate. Another 1 cm of sodium sulfate was carefully added to the top of the charcoal mixture and the column was rinsed with 1 liter of pesticide grade benzene followed by 200 ml of diethyl ether (2% ethanol). Samples (1-5 ml) were placed on the column and eluted with 80 ml of diethyl ether (chlordane fraction) followed by 200 ml of benzene (toxaphene fraction). The chlordane fraction contained approximately 5-10% of a $^{14}C$ toxaphene spike and the toxaphene fraction contained cis-nonachlor and the DDT complex as major contaminants. The charcoal column can be washed with 200-300 ml of ether and used again until an excess of charcoal penetrates the sodium sulfate layer.

Charcoal column purified toxaphene samples were reanalyzed using the same peaks that were used in the first analysis. Samples were usually pooled before the final nitration purification for removing

compounds related to DDT. Nitration was done using a modification of the methods developed by Klein and Link (1970) and Holdrinet (1979). Solvent evaporated samples were treated with a 1:1 mixture of concentrated sulfuric:fuming nitric acids at 70°C for 1/2 hr. The mixture was quantitatively transferred to a 500 ml separatory funnel with 3X 10 ml portions of methylene chloride. The acidic water layer was extracted twice with 50 ml of methylene chloride and the solvent was pooled into another separatory funnel. One-hundred ml of a 5% sodium bicarbonate solution was added and the mixture was shaken. The organic layer was filtered over anhydrous sodium sulfate and evaporated to near dryness using a rotary evaporator. The sample was placed on a 5 g florisil column similar to that used in the first part of the procedure and eluted with 40 ml of 6% diethyl ether in petroleum ether.

The final extract was reanalyzed using the same peaks as previously with the addition of toxicant A (now removed from p,p' DDT interference). This sample contains almost exclusively chlorinated bornanes as judged by capillary GC negative chemical ionization mass spectrometry. The major notable exception is the presence of cis-nonachlor, a minor component of technical chlordane, but a major component of chlordane residues in fish (Ribick and Zajicek, 1983). This final fraction contains both toxicants A and B, the two major toxic toxaphene components found previously in lake trout from Lake Michigan (Gooch and Matsumura, 1985).

Recovery studies were done using $^{14}C$ toxaphene spikes (= internal standard) and were generally greater than 75% after the charcoal column. Reagent blanks were periodically checked for possible system contamination. Finally, a spike of technical toxaphene was added to

hatchery reared lake trout tissue and carried through the entire procedure in order to determine the changes that may be occurring due to the procedure alone (= procedural standard).

**Acute Toxicity Tests**

Acute toxicity tests were conducted using 3rd and 4th instar Aedes egypti mosquito larvae (Rockefeller strain) reared in this laboratory. Tests were conducted in 16 x 100 mm disposable culture tubes containing 5 ml of distilled water and 10 larvae per treatment. Technical toxaphene positive controls were run for all tests and analyzed separately for statistical heterogeneity. Because no statistical difference was detected among tests for the positive controls, the data were pooled and used as a separate treatment for analysis of variance procedures. $LC_{50}$ calculations were done using probit analysis on data conforming to the distribution with at least a 50% probability.

**[$^{35}$S]-t-Butylbicyclophosphorothionate (TBPS) binding**

The ability of toxaphene residues extracted from lake trout to inhibit the binding of $^{35}$S-TBPS (New England Nuclear, Lot #2101-118, original specific activity 69.3 Ci/mmole) to the picrotoxinin receptor site in the GABA (gamma-aminobutyric acid)-chloride ionophore complex of the central nervous system, was examined using rat brain microsomes (synaptic membranes + endoplasmic reticulum) prepared by the method of Ramanjaneyulu and Ticku (1984). The cerebella and cerbral cortices from 3 male Sprague-Dawley rats were removed, weighed, and homogenized in 25 volumes of cold 5 mM Tris, 1 mM EDTA (pH = 7.1) using 14-16 strokes of a teflon-glass homogenizer. The homogenate was centrifuged at 1000 g

for 10 min. The supernatant was then centrifuged at 140,000 g for 30 min yielding a mitochondrial plus microsomal pellet. The pellet was homogenized in 25 volumes of 5 mM Tris, 1 mM EDTA (pH = 7.1) and dialyzed overnight against 50 volumes of double distilled water at 4°C. The dialyzed preparation was centrifuged at 140,000 g for 30 min afer which the pellet was either resuspended in .32 M sucrose or 5 mM Tris/1 mM EDTA and frozen in 3 ml aliquots at -80 C.

For binding assays, tubes were thawed, membranes precipitated at 140,000 g twice and homogenized in 5 mM Tris/1 mM EDTA/0.2 M KBr (ph = 7.5). Binding assays were conducted essentially as described by Abalis et al. (1985). One-hundred µl of the membrane preparation was incubated in Tris/EDTA/KBr (pH = 7.5) containing 2 nM $^{35}$S-TBPS in a final volume of 1 ml. Non-specific binding was determined in the presence of 50 µM cold t-butylbicyclophosphate (TBP) or picrotoxinin (PTX). Specific binding was determined as the total (2 nM $^{35}$S-TBPS alone) minus non-specific (2 nM $^{35}$S-TBPS + 50 µM TBP or PTX) and was generally 40-70%. Residues and toxaphene were tested for their ability to inhibit $^{35}$S-TBPS binding by incubation for 10 min before the addition of radioactive ligand. A concentration/inhibition curve was plotted to determine the binding characteristics and the optimum concentration for comparisons among residues. Tubes were incubated for 90 min at room temperature and the reaction was terminated via dilution with ice cold reaction buffer and rapid filtration through Whatman GF/B glass fiber filters. Filters were rinsed twice and counted using liquid scintillation. Quench correction was done using curves generated for $^{14}$C standards since the beta energies are nearly identical for the two isotopes. All statistical analyses were done using Statistical Analysis System (SAS) software (version 4).

## RESULTS AND DISCUSSION

Residues were purified from Lake Michigan lake trout and compared to technical toxaphene (Figure 6). The lake trout residue is essentially free of interferences from chlordane and DDT related compounds; cis-nonachlor, a minor component of technical chlordane (retention time near 18 min) is the major notable exception. The large peak with a retention time near 23 minutes has not been identified. Mass spectral data is consistent with a chlorinated bornane with 8 or 9 chlorines. Because this peak is disproportionately large compared to the rest of the chromatogram, it has not been used for quantitation. Careful inspection of other available literature on purported atmospherically deposited toxaphene (Zell and Ballschmiter, 1980; Kramer et al., 1984; Vaz and Blomkvist) reveals a large peak in the residues with a retention time similar to the one mentioned here. In all cases, this peak is characteristic of a toxaphene component, through no structural identification has been made. This conformation is apparently quite stable compared to the rest of the components. The peaks that were used for quantitation have been noted in Figure 6. A further description of the characteristics and levels of these residues will be the subject of a future publication.

The peaks used for quantitation of the purified residues were plotted as normalized histograms (Figure 7) in order to facilitate visual comparisons. Normalization was to peak with the greatest area in each case. While it is difficult to generalize, interpretation of

Figure 6: Capillary gas chromatogram of technical toxaphene (above) and purified lake trout residue toxaphene (below). Peaks designated (●) have been used for quantitative and comparative purposes. For conditions, see Materials and Methods.

Figure 7: Normalized histogram of the peaks used for quantitation of the residues and standards compared in this study. For a description of abbreviations, see Table 3. Peak numbers can be established from designated (●) peaks in Figure 6 by moving from left to right in sequence.

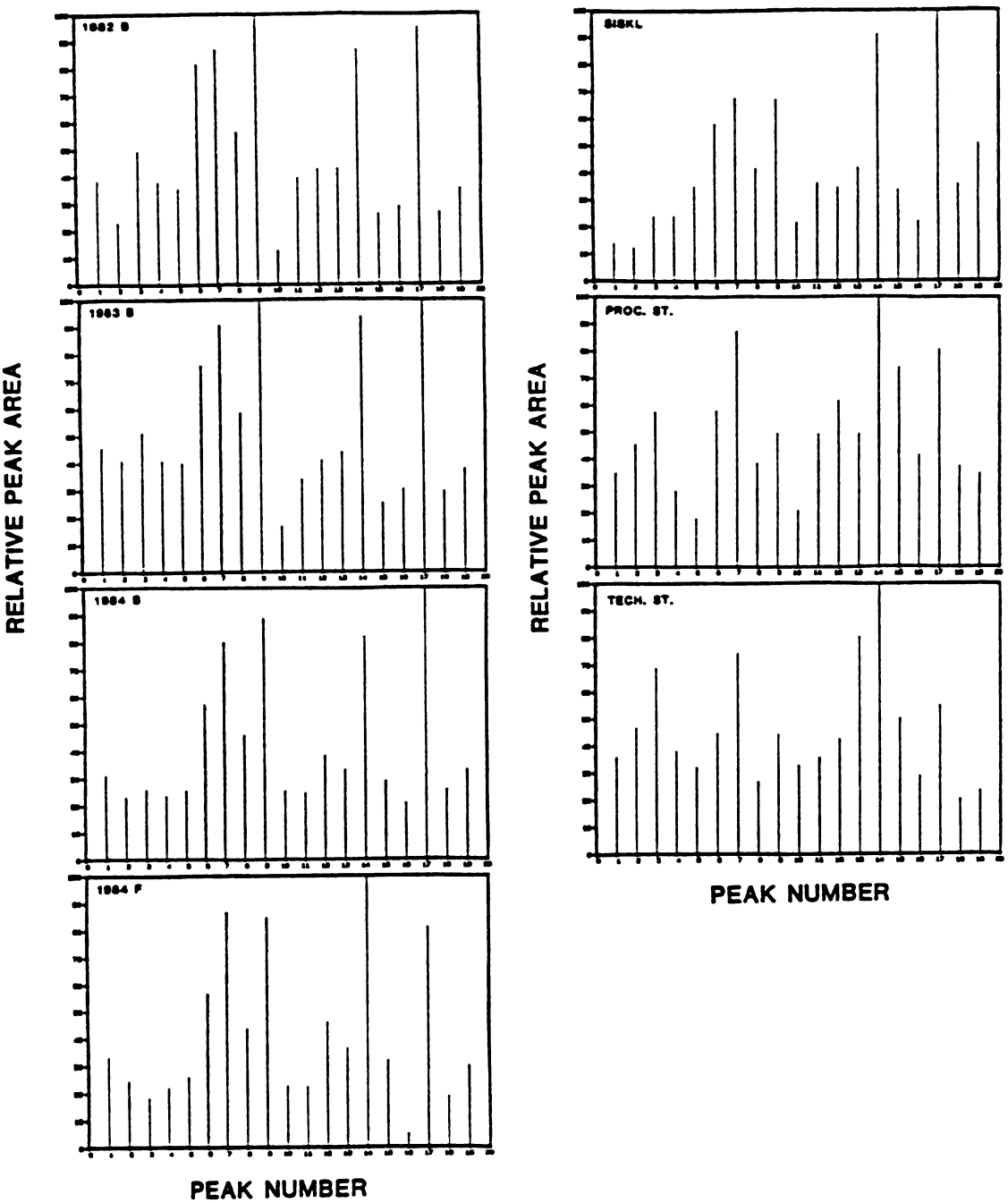

1982 B
1983 B
1984 B
1984 F
SISKL
PROC. ST.
TECH. ST.
RELATIVE PEAK AREA
RELATIVE PEAK AREA
PEAK NUMBER
PEAK NUMBER

Figure 7 leads to the conclusion that the purified residues isolated from lake trout are remarkably similar to both technical toxaphene and the toxaphene procedural spike. We emphasize purified since it is unlikely that this conclusion would be reached without using the purification procedures we have described. With the number of compounds present in Great Lakes fish, protocols designed for multiple residue quantitation do not provide adequate separation for detailed analysis of toxaphene residues. The additional steps we have applied are necessary to conduct more specific tests.

Of particular importance toxicologically, is the relative composition of toxicants A and B which are represented by peaks 7 and 3, respectively in the extracts from lake trout. We have demonstrated previously (Gooch and Matsumura, 1985) that these two constitutents, which are two of the most toxic, were present in less purified lake trout residues. Table 3 is a comparison of the composition of the residues and the standards with respect to these two components. These values only represent estimates, since the levels are derived from approximate standard curves made by taking fractional composition averages for toxicants A and B in technical material (4.8% and 3.4% respectively), multiplying by a known mass of material, and using the resulting mass/peak area relationship. In addition, the mass values obtained for A and B are then divided by the mass value obtained for total toxaphene, an estimate as well since we assume that each peak summed for quantitation is the same as that in the technical standard. The percentages for A and B in the standards have been determined the same way as the residue in order to make comparisons more direct. The fish residues have slightly more toxicant A and less B than the

Table 3: Percent composition estimates for toxicants A and B in residues and standards used in this study. Data are expressed as mass % (area %) where area % equals the integrated GC peak area of A or B/total peak area used in quantitation.

| | Toxicant A | Toxicant B | Total |
|---|---|---|---|
| 1982B[a] | 2.80 (9.20) | 1.21 (5.24) | 4.01 (14.44) |
| 1983B | 2.82 (9.12) | 1.19 (5.13) | 4.01 (14.25) |
| 1984B | 2.58 (10.21) | 1.19 (5.11) | 3.77 (15.32) |
| SiskL[b] | 2.44 (8.54) | 0.83 (3.17) | 3.27 (11.71) |
| Technical Standard | 2.39 (8.57) | 1.70 (8.09) | 4.12 (15.01) |
| Procedural Standard[c] | 2.77 (9.06) | 1.35 (5.95) | 4.09 (16.66) |

(a) B signifies adipose tissue rich "belly flap" and F denotes edible fillet.

(b) SiskL - Lake trout from Siskiwit Lake on Isle Royale in Lake Superior.

(c) Procedural standard is technical toxaphene isolated with the same procedure used for regular fish samples.

technical standard, the difference being due to the isolation procedure since the procedural toxaphene standard has a similar bias. The concentrations of toxicants A and B is highest for the standards.

The qualitative characteristics of the residues appear to be similar from year to year and from different tissues and locations. To date, their has been no systematic assessment of the changes that may be occurring with time in fish from the Laurentian Great Lakes. Since the most widely accepted hypothesis for how toxaphene enters the Great Lakes is primarily via atmospheric transport from the cotton-belt regions of the southern United States (Rice and Evans, 1984), and because toxaphene use decreased significantly after the late 1970's, levels and patterns of toxaphene residues may be expected to continuously change as biotic and abiotic environmental forces act on the mixture. Toxaphene is degraded by some microorganisms and mammalian species (Clark and Matsumura, 1979; Parr and Smith, 1976), while little is known of its metabolism in aquatic organisms. The patterns observed in fish, however, do not demonstrate dramatic change. The spectra in Figure 7 do not show any great changes in the residue from 1982 through 1984, an observation which also extends to toxicants A and B.

Recent studies suggest that current inputs of toxaphene are low (Strachan, 1985). Thus, toxaphene is apparently not being degraded in the Great Lakes system. If inputs are low and degradation is significant, we should expect to see a shift in the composition of the residue with time. Our data do not show this. A similar observation was made by fisheries biologists in the late 1950's and early 1960's when toxaphene was being tested as a possible piscicide. In many

cases, lakes poisoned with toxaphene remained toxic for a number of years (Johnson et al., 1966). We conclude that toxaphene will be present for a number of years and will probably degrade or dissipate in the biota very slowly.

**Toxicity**

Acute toxicity bioassays were conducted with mosquito larvae to demonstrate whether the toxaphene residues isolated from lake trout exhibited different toxicities than technical or procedural standards. At the time each test was conducted a positive control with technical toxaphene was performed in order to monitor any differences that might occur due to uncontrolled variables. After the data had been collected, a separate analysis of variance was conducted on the positive controls. Since there was no significant difference among tests (Tukey's HSD $\alpha$ = 0.10), the control data was pooled and used as a separate treatment. Statistical analysis of the overall data (Tukey's HSD $\alpha$ = 0.10) revealed that the residues from 1982 and 1984 were slightly less toxic than the technical standard (Table 4). There were, however, no differences among the different years when compared to each other or the procedural standard. This leads to the conclusion that the acute toxicity of toxaphene residues isolated from Lake Michigan lake trout is essentially equivalent to standard material. This supports our identification of toxicants A and B in these samples and suggests that though the residue appears quite different from the analytical standard, the biologic activity of the material is not greatly different. Though analysis of variance procedures are generally robust with respect to non-normality (Steel and Torrie, 1980), a

Table 4: Acute toxicity of toxaphene residues purified from Lake Michigan lake trout to mosquito larvae (Aedes egypti). Data are expressed as 24 hour $LC_{50}$ in µg/l ($\bar{X} \pm$ s.d. (n)).

| | |
|---|---|
| 1982B[a] | 761 ± 218 (3)*[b] |
| 1983B | 673 ± 135 (3) |
| 1984B | 733 ± 203 (3)* |
| Technical toxaphene | 441 ± 140 (8) |
| Procedural toxaphene | 603 ± 139 (6) |

(a) For description see Table 1.

(b) * = Significantly different from technical toxaphene ($p < 0.1$) Tukey's HSD.

nonparametric analysis of the data (Kruskal-Wallis) yielded similar results. The degree of variability we encountered in this test system was great and precluded any further comparisons from being statistically significant.

## $^{35}$S-TBPS Binding

Acute toxicity bioassays are integrated measures of the activity of all of the components in the test mixture and will reflect the summation of a number of factors including uptake, metabolism, excretion, partitioning to the active site, etc. Thus, mortality is a very nonspecific endpoint which measures the total activity of the test mixture irrespective of the mode of action. Since we measured residues from environmental samples, it is possible that some unknown factor, possibly toxic, could have been introduced by the procedure. Because our analytical endpoint is predominantly capillary GC with electron capture detection, anything not detected by this procedure is a potential interference.

When the mode of action of a class of chemicals is known and a putative target site has been identified, it is possible to conduct more specific tests in order to gain a greater degree of understanding about the toxicology of the test material. For cyclodiene type insecticides including toxaphene, the most plausible target site appears to be the GABA-chloride ionophore complex (Ghiasuddin and Matsumura, 1982; Matsumura and Ghiasuddin, 1983). GABA (gamma-aminobutyric acid) is the major inhibitory neurotransmitter of the central nervous system of nearly all vertebrates and functions by stimulating $Cl^-$flux across nerve membranes. Cyclodiene type

insecticides have been shown to inhibit this flux by binding at the picrotoxinin receptor. This receptor is proposed to be located at, or very near, the chloride channel (Matsumura and Ghiasuddin, 1983; Lawrence and Casida, 1984; Abalis et al., 1985). The result is an imbalance in excitatory and inhibitory signals and less stimulation causes an overproduction of excitatory activity.

Two major ligands, picrotoxinin and TBPS, have been found to bind to a site at or near the chloride channel (Ticku and Olsen, 1979; Squires et al., 1983; Ramanjaneyulu and Ticku, 1984). The ability of a compound to inhibit the binding of these ligands to the receptor site is highly correlated with toxicologic potency (Lawrence and Casida, 1984; Matsumura and Tanaka, 1984).

Inhibition curves for technical toxaphene and a residue isolated from the belly-flap region of fish collected in 1984 were compared (Figure 8). Both inhibitors yielded similar curves with $IC_{50}$ values of $5.0 \pm 3.8 \times 10^{-8}$ M for toxaphene and $2.9 \pm 2.1 \times 10^{-8}$ M for the lake trout residue (n=3 experiments). This similarity agrees with the toxicity data obtained earlier and the $IC_{50}$ values are similar to those obtained by Cole et al. (1984). $10^{-8}$ M was selected as an appropriate level to use for further point comparisons among residues.

Residues from different years, sources, and tissues inhibited the binding of $^{35}$-TBPS to the receptor site in rat brain membranes (Table 5). Extracts from belly-flap samples from 1982 and 1983 were the least potent and were not significantly different from one another. The 1982 sample was, however, different from the others. The 1983 sample was significantly different from the procedural standard. It is interesting to note that the samples from fillet tissue and from

Figure 8: Inhibition curve of toxaphene ( □ ) and the 1984B ( △ ) lake trout residue for [$^{35}$S]-TBPS binding to rat brain synaptic membranes. IC-50 values were 5.9 $\pm$ 3.8 X $10^{-8}$ M for toxaphene and 2.9 $\pm$ 2.1 X $10^{-8}$ M for the lake trout residue (n = 3 experiments).

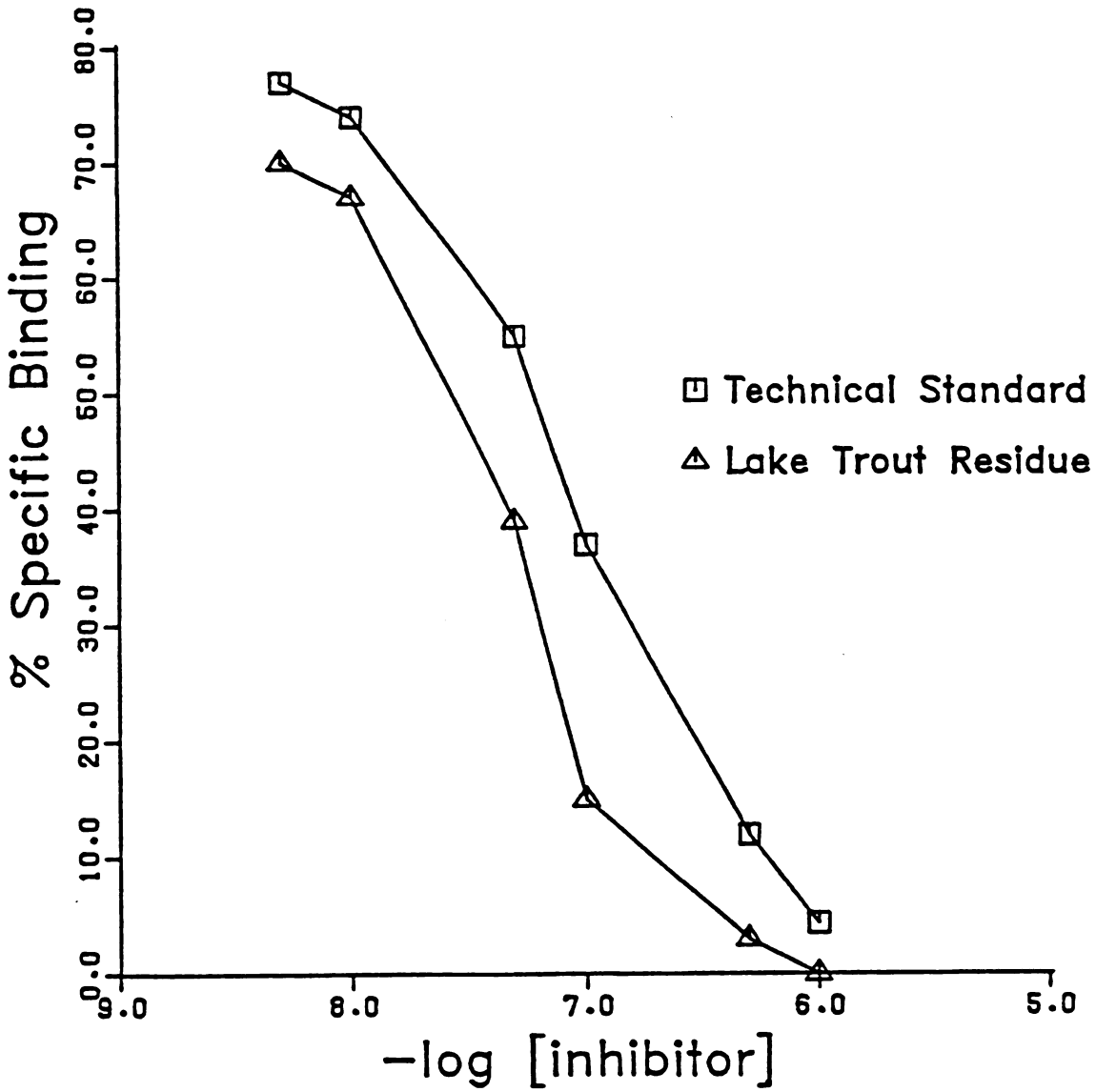

% Specific Binding
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
9.0
8.0
7.0
6.0
5.0
−log [inhibitor]
Technical Standard
Lake Trout Residue

Table 5: Effect of toxaphene residues from Lake Michigan lake trout on $^{35}$S-TBPS binding to rat brain synaptic membranes.[a]

| | TBP | PTX |
|---|---|---|
| 1982B | 31.4 (1)[b] | 31.3 |
| 1983B | 38.7 (1,2) | 38.7 |
| 1984F | 43.6 (2,3) | 43.5 |
| SiskL | 44.1 (2,3) | 44.0 |
| Technical standard | 40.8 (2,3) | 42.0 |
| Procedural standard | 49.5 (3) | 49.4 |

(a) Data are expressed as mean % inhibition from 3 experiments. [Inhibitor] = $1 \times 10^{-8}$ M. Specific binding was determined using both cold t-butylbicyclophosphate (TBP) and picrotoxinin (PTX) at $10^{-4}$ M as displacing ligands. The concentration of $^{35}$S-TBPS was approximately 2 nM. For further details see materials and methods.

(b) Means with the same number are not significantly different, Tukey's HSD $\alpha = 0.05$.

Siskiwit Lake on Isle Royale tended to be more inhibitory than the more fatty belly-flap samples and the technical standard, though the difference was not statistically significant. The most inhibitory sample was the procedural standard. If this data is compared to the relative composition estimates for the toxic constituents in Table 3, it is found that inhibitory potency only roughly follows the total level of these two constituents. This suggests that toxicity is mediated by components other than just toxicants A and B, an observation noted previously (Saleh et al., 1977).

By considering the acute toxicity data, the $^{35}$S-TBPS binding data, and the toxicant A and B composition estimates in concert we conclude: (1) Residues derived from toxaphene, isolated from Great Lake fish, possess toxicologic potency similar to technical material (i.e. toxaphene in the Great Lakes ecosystem has not been appreciably detoxified; (2) Composition estimates of toxicants A and B are not sufficient to predict toxicologic potency directly. Toxicity is probably attributed to many of the components of the toxaphene residue; (3) The spectrum of activity and the composition of the residues has changed very little from 1982 through 1985; (4) Edible portions of tissue (fillet) and fish from a remote island in Lake Superior, contain residues as toxic as standard toxaphene (though total levels are less than that found in fatty samples from lake trout from Lake Michigan) and (5) Neuroreceptor binding studies are useful for measuring activity of environmentally derived residues. These studies require much less material and provide more specificity for the target site.

## II. Inhibition of $^{35}$S-TBPS Binding to Lake Trout Brain Synaptic Membranes.

### MATERIALS AND METHODS

Lake trout were originally obtained as fingerlings from the Jordan River National Fish Hatchery, Elmira, Michigan. Fish were reared for two years on Biodiet pellets (Biodiet Products, Warrenton, Oregon) prior to use. Brains from two fish were removed (1.0 g total brain tissue), homogenized and processed as described for earlier studies with rat brain. All binding assays etc. were also conducted with the same methods as described previously.

### RESULTS AND DISCUSSION

Since only one report has appeared in the literature regarding TBPS binding in fish brain (Cole et al. 1984), preliminary experiments were conducted to determine the degree and nature of binding. These experiments demonstrated 140-168 fmoles/mg protein of 92-98% specific binding in the presence of 2 nM TBPS. Binding was 70% inhibited by $10^{-6}$ M GABA (Table 6), a behavior consistent with interaction at the GABA-chloride ionophore. The binding characteristics are also similar to those demonstrated for the blackfish (Orthodon microlepidotus) by Cole et al. (1984).

Table 6: Inhibition of $^{35}$S-TBPS binding to lake trout brain synaptosomes by residues isolated from Great Lakes lake trout. Data are expressed as % inhibition ($\bar{x} \pm$ s.d. (n)).

| Residue | [M] | % Inhibition[c] |
|---|---|---|
| 84B toxaphene[a] | $1 \times 10^{-7}$ | $74.6 \pm 10.9$ (3)[a,b] |
| 85B toxaphene | $1 \times 10^{-7}$ | $54.9 \pm 18.8$ (3)[a,b] |
| 84F toxaphene | $1 \times 10^{-7}$ | $87.3 \pm 7.3$ (3)[a] |
| 85F toxaphene | $1 \times 10^{-7}$ | $50.5 \pm 8.6$ (3)[b] |
| 85E toxaphene | $1 \times 10^{-7}$ | $70.6 \pm 7.8$ (3)[a,b] |
| L.Sisk | $1 \times 10^{-7}$ | $61.6 \pm 13.7$ (3)[a,b] |
| GABA | $1 \times 10^{-6}$ | $69.5 \pm 5.5$ (3)[a,b] |
| L.MI. $H_2O$[b] | 1 µl/50 µl | $36.0 \pm 9.1$ (4) |

[a]B, F, and E designations correspond to belly, fillet, and egg samples as previously described. Numbers are year designations, L. Sisk is fish from Siskiwit on Isle Royale.

[b]Lake Michigan water extract. For a more complete description see the results section.

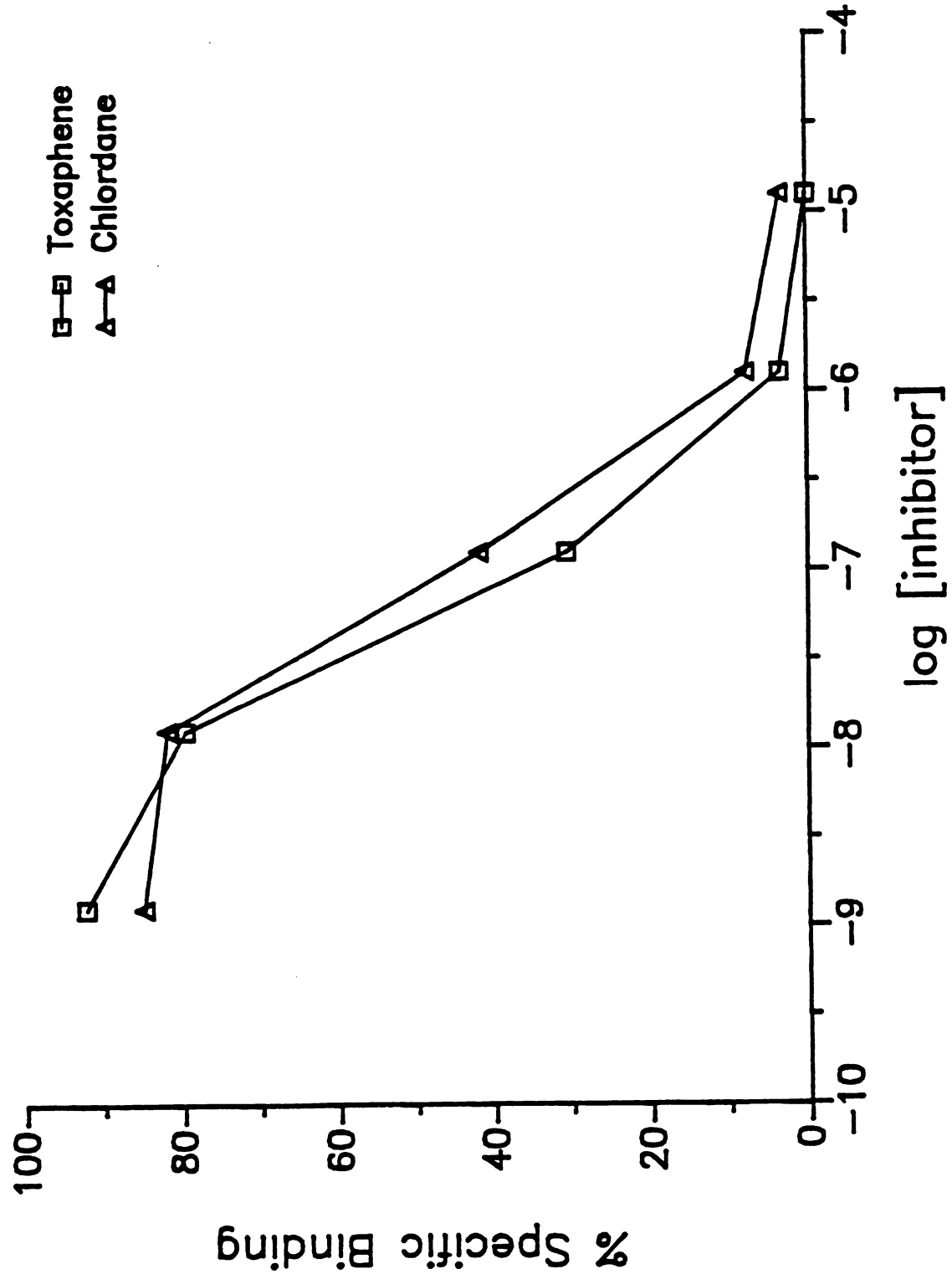
[c]Means with the same letter are not significantly different (Tukey's HSD $\alpha$=0.05). The minimum significant difference for any two means is 32.5%.

Inhibition curves were plotted for toxaphene and chlordane (Figure 9). Both of these complex mixture insecticides were found to inhibit $^{35}$S-TBPS binding to lake trout brain membranes in a sensitive concentration dependent fashion. Approximate $IC_{50}$ values of 6 and 8 x $10^{-8}$ M were determined graphically and correlate well with the sensitivity of fish to these two insecticides. Chlordane is slightly less toxic to salmonids (96 hr LC-50 23.5 $\pm$ 14.0 µg/l) than toxaphene (96 hr LC-50 7.3 $\pm$ 4.0 µg/l) (Johnson and Finley, 1980). The $IC_{50}$ values determined for lake trout are similar to values obtained for rat brain in this work and that of others (Cole et al., 1984; Abalis et al., 1985; Lawrence and Casida, 1984).

While fish are very sensitive to the effects of cyclodience insecticides (Podowski et al., 1979), there is no exceptional sensitivity at the putative target site demonstrated here or by Cole et al. (1984). This suggests that target site sensitivity is not a major reason for the high degree of toxicity associated with these compounds in fish.

The sensitivity of the lake trout GABA-chloride ionophore complex to various toxaphene residues isolated from Great Lakes fish is shown in Table 6. All of the toxaphene residues potently inhibited $^{35}$S-TBPS binding to lake trout brain membranes, a behavior consistent with the sensitivity of lake trout to this class of compounds. The only significant difference (at $\alpha$=0.05) noted among the residues was between fillet samples from 1984 and 1985. The reasons for this are not clear. The inhibition for both of the 1985 samples (belly and fillet) is less than was typically found. The relative amount of toxicants A and B (5.3% total for both samples) is similar to the samples reported in

Figure 9: Concentration - response curve for toxaphene and chlordane inhibition of $^{35}S$-TBPS binding to lake trout brain synaptic membranes.


Toxaphene
Chlordane
% Specific Binding
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
log [inhibitor]

Chapter 2 and therefore, a possible explanation for the difference is that there was an error in quantitating the 1985 test samples.

Since the in vitro binding assay is specific, sensitive and requires very little material, a preliminary experiment was conducted to determine if GABA-chloride ionophore complex specific neuroactive substances could be detected in Lake Michigan water. It requires 4.1 ng of toxaphene in a 1 ml test volume ($1 \times 10^{-8}$ M) to produce a readily detectable inhibition of $^{35}$S-TBPS binding. Assuming an extraction efficiency of 100% and a concentration factor of 40,000, a water concentration of 102 ng/l toxaphene equivalents should be easily detected. A concentration factor of 40,000 is fairly conservative, therefore, a concentration of 50 ng/l should be readily detectable. This assumes that the only specific neuroactive substance in the water is toxaphene and that there are no interferences in the assay.

When a 2 l sample of Lake Michigan water (open water, subsurface) was extracted with methylene chloride, fractionated with florisil, and concentrated as above, it produced 36% inhibition of binding. This corresponds to a toxaphene equivalent solution concentration of approximately $2 \times 10^{-8}$ M. With a concentration factor of 40,000, this corresponds to a water concentration of 205 ng/l. Reported toxaphene concentrations in Great Lakes waters are nearly 100 fold less than this (Sullivan and Armstrong, 1985). Since the Great Lakes are a repository for a large number of chlorinated cyclodienes, all of which interact at this binding site, the calculations and assumptions used here have little real meaning. It is interesting that a simple water extract possessed significant activity. Since no blank controls were done, this activity requires further verification. The specificity of the

system, however, in theory, should make it possible to take this approach for measuring the response of chloride channel antagonists in environmental samples. This experiment with one water sample and no blank controls is admittedly preliminary and hopefully, will serve as a stimulus for further investigation.

# CHAPTER 4

# SUMMARY OF RESIDUE LEVELS AND STATISTICAL PATTERNS ASSOCIATED WITH LAKE MICHIGAN LAKE TROUT RESIDUES FROM 1982-1985

## INTRODUCTION

Residues of toxaphene or toxaphene-like compounds have been measured in Great Lakes fish for the past several years. The data of Schmitt et al. (1981) from the National Pesticide Monitoring Program suggests that these residues have been detectable since the early 1970's. Subsequent reports (Schmitt et al., 1983; Schmitt et al., 1985) have shown an increase in concentrations and frequency of occurrence for samples from 1976-1981. Excellent summaries of this data can be found in Rice and Evans (1984) and Sullivan and Armstrong (1985).

Whole lake trout taken near Saugatuck, Michigan, from 1977-1979, contained a mean concentration near 7.0 µg/g (wet weight) (Sullivan and Armstrong, 1985). Schmitt et al. (1985) reported a downward trend at some stations in Lake Michigan and Lake Superior in 1980-81 (2-5 µg/g) compared to 1978-79 (5-10 µg/g). These data were generated using whole fish samples from a narrow size range (approximately 60-70 cm).

Trends presented by Schmitt et al. (1985) and Devault et al. (1986) suggest that many contaminant concentrations in Great Lakes, lake trout are decreasing. Since toxaphene has been banned for most uses in the United States (Federal Register, 1982), it is important to determine if concentrations of toxaphene are decreasing with time. In addition, it is important to study the differences, if any, that might be present in the profiles of the components of the toxaphene residue

from different years and from different tissues, particularly as these profiles might be related to toxicity.

Since the results reported earlier (Gooch and Matsumura, 1986) demonstrated that toxaphene residues are at least as toxic as technical material, it is important to determine whether or not any statistically identifiable differences exist in the various toxaphene residues that have been studied.

**Quantitative Results**

Toxaphene concentrations that were found in belly flap and fillet samples from lake trout analyzed in this study were summarized (Table 7). Appendix A contains all of the individual information for each fish and the results of replicate analyses of selected samples. Overall, values in belly flap samples ranged from 0.59-10.76 µg/g, while fillets ranged from 0.46 to 3.41 µg/g. The ratio of belly flap/fillet varied from 1.89 to 9.29. Lipid normalized residues may have been helpful in narrowing this range (Hughes, 1970). Concentrations found in fish collected from Siskiwit Lake on Isle Royale are similar to those found in the fillet samples from the Lake Michigan fish.

The data presented in Table 7 are the result of a number of factors regarding the type of samples that were analyzed. The most important is the range of the size of fish that were analyzed. No attempt was made in this study to segregate fish samples on the basis of size. Many of the largest fish caught on the day of sampling were intentionally selected in order to maximize the amount of residue obtained per unit weight of tissue for toxicology studies. This

Table 7: Summary statistics for toxaphene residues in Lake Michigan lake trout. All data are expressed as µg/g wet weight. $\bar{X} \pm$ s.d.(n) and range.

| | Belly | Fillet | Ratio B/F | Weight (kg) |
|---|---|---|---|---|
| 1982 | | | | |
| | 6.43 ± 2.10 (5)<br>(3.78 - 9.33) | 1.82 (2)<br>(1.67, 197) | 3.66 (2)<br>(3.48, 3.83) | - |
| 1983 | | | | |
| | 4.07 ± 2.17 (12)<br>(0.59 - 7.39) | 0.90 ± 0.70 (3)<br>(0.46 - 1.71) | 5.60 ± 1.50 (3)<br>(9.30 - 7.20) | 3.67 ± 0.90 (12)<br>(2.95 - 5.03) |
| 1984 | | | | |
| | 4.34 ± 1.41 (11)<br>(2.82 - 7.10) | 1.61 ± 0.96 (11)<br>(0.54 - 3.41) | 3.20 ± 1.10 (11)<br>(1.89 - 5.22) | 3.81 ± 0.68 (11)<br>(2.95 - 5.03) |
| 1985 | | | | |
| | 6.50 ± 2.81 (9)<br>(3.42 - 10.76) | 1.27 ± 0.33 (9)<br>(0.76 - 1.80) | 5.41 ± 2.60 (9)<br>(2.32 - 9.29) | 3.81 ± 1.25 (9)<br>(2.27 - 6.80) |
| Siskiwit Lake | | | | |
| | 1.17 ± 0.56 (7)<br>(0.14 - 1.70) | - | - | 1.28 ± 0.28 (7)<br>(0.71 - 1.60) |

sampling protocol, while not considered optimal for studying trends (Devault et al., 1986), will more realistically represent the degree of variability that is present in residues from larger fish. This data does not establish any apparent trend in the range of concentrations present in lake trout from 1982-1985. This suggests that, though the agricultural use of toxaphene has been banned and new inputs appear to be low (Strachan, 1985), toxaphene is not being rapidly eliminated in the Great Lakes or that the existing toxaphene reservoir in the Great Lakes ecosystem is so large that the cessation of toxaphene input not immediately impacted the residue levels.

## Pattern Recognition

**Background** - Each gas chromatographic profile for a sample can be considered a single observation defined by measures of several quantitative variables. As such, the values of the variables and their relationship to one another and to other samples defines that sample. In many cases, this relationship is similar for similar types of samples. Multivariate statistical pattern recognition techniques have been developed which are useful for defining or visualizing these types of relationships (Kowalski and Bender, 1972), and in recent years these techniques have been increasingly applied to complex chemical data (Derde and Massart, 1982). A full review of the philosophy and mathematics behind pattern recognition is beyond the scope of this work. In addition to the two works cited, excellent reviews and introductions can be found in Cooley and Lohnes (1971), Massart and Kaufman (1983), Delaney (1984), Wold and Sjostrom (1977) and Kowalski

and Bender (1973). Often, papers published where the authors have used a specific technique, will contain a parsimonious review of that particular technique. Examples of this include Dunn et al. (1984), Stalling et al. (1985), Carey et al. (1975), Weiner and Parcher (1973) and Pino et al. (1985). A recent American Chemical Society Symposium Volume (Breene and Robinson, 1985) contains numerous examples of these techniques applied to environmental chemical data.

As with any statistical technique, there are a number of assumptions behind the use of multivariate algorithms. In general, it is usually required that the variables be distributed as multivariate normal with common variance and covariance. This also applies between classification groups when they are present. To avoid chance patterns in the data, it is also required that the number of observations be greater than the number of variables being studied. As the simplest example, two points define a line whether there is any linear relationship among the two observations or not. Wold and Dunn (1983) contains an excellent discussion of this requirement.

The following is a brief discussion of each of the techniques used in this analysis. All of these techniques are available as part of the SAS statistical analysis package available on the IBM 4381 computer.

**PrinComp** - The Princomp procedure performs a principal components analysis and is used to examine relationships among several quantitative variables. It is often used for summarizing data and detecting linear relationships among variables that explain or help interpret some other classification. Because of the ability to explain the major portion of the variability in data by using linear combinations of the original variables, it is often used as a data

reduction technique prior to further statistical analysis.

Briefly, given p numeric variables, princomp constructs p principal components as linear uncorrelated combinations of the original variables. The first principal component, the first synthetic variable created from some combination of the original, explains the largest variance of any of the p principal components. Each successive principal component explains the next largest amount of variability via a combination of variables that is orthogonal to the rest. Generally, the first few principal components will explain the majority of the variance. The more principal components it takes to explain the data, the greater the likelihood of many unique observations in the data. Principal components can be generated from both correlation and covariance matrices. A plot of the first two principal components is often useful for visualizing patterns in the data since these two components generally explain a large portion of the variance. The eigenvectors generated from the matrix manipulations used to do this analysis are useful since they are the scoring (weighting) coefficients for the variables on the respective principal components. Inspection of these coefficients can often give insight into the major variables that are important in describing the variability in the data (i.e. which variables are most important for explaining differences).

It is important to emphasize that this technique assumes no underlying structure in the data. Principal components analysis is classified as an unsupervised pattern recognition technique, in contrast to other techniques to be described. It is therefore often useful for visualizing patterns in the data that are not readily evident.

**Fastclus** - The Fastclus procedure belongs to a family of techniques generally known as cluster analysis. Massart and Kaufman (1983) is an excellent review of the philosophy and mathematics behind these techniques. Specifically, the fastclus procedure performs a non-hierarchical (disjoint) cluster analysis of observations composed of several quantitative variables. It is most often used with large data sets and clusters observations based on a sorting algorithm which uses Euclidean distances between the quantitative variables for each observation. The Euclidean distance is a geometric construct described by the square root of the sum of all of the paired squared distances between corresponding variables among observations. The user generally designates the maximum number of clusters to be generated and how similar observations must be to be considered in the same cluster. Very simply, this means that observations with similar Euclidean distances will group together. Typically, several analyses are done using different numbers of clusters. The procedure generates several statistical parameters, which relate to the quality of the clustering.

**Candisc** - The Candisc procedure performs a canonical discriminant analysis on data characterized by a classification variable (in contrast to the other techniques) and several quantitative variables. This technique can also be considered a dimension reduction technique and is related to principal components analysis. In this case, we seek to derive canonical variables (similar to principal components) which are linear combinations of the original variables that summarize between-class variation in contrast to the total variation that princomp seeks to explain. The canonical variables (i.e. the synthetic one number variables) have the greatest multiple correlation

with the groups. The first linear combination of the variables that has the greatest multiple correlation with the groups is called the first canonical variable (component). The weighing coefficients for the variables are similar to the weighing coefficients of principal components analysis. Canonical variables are generated in descending order of their ability to explain multiple correlation with the groups in much the same way as principal components explains overall variability. A plot of the first two canonical variables is generally used to visualize the relationships between the classification variables.

This particular technique is often used if some preliminary treatment of the data has suggested a classification scheme, or if one was known a priori. It is also used as a method for visually displaying data generated by some other classification system.

**Standard Toxaphene Data** - In order to determine if any artificial patterns could potentially appear in later data analysis that were not due to meaningful differences, a set of 26 standards was chosen for preliminary pattern examination. Because these observations are all for the same material, this analysis should reveal any underlying patterns due to factors including, amount (ng injected), date, column conditions, etc. The standards were chosen to cover a broad range of conditions and are listed in Table 8. The peaks used are noted in Chapter 3, Figure 6.

Raw area data was entered for each of 20 peaks in the gas chromatographic profiles. All data was then scaled so that each peak was expressed as a fractional proportion of the total peak area for those twenty peaks. From this set of 20 peaks, a subset of 19 peaks

Table 8: Standard toxaphene data used for pattern recognition analysis.

| Std | Analysis Date | Quantity Injected (ng) | Total Peak Area |
|---|---|---|---|
| A | 4-26-85 | 9.0 | 217476 |
| B | 4-22-85 | 22.5 | 456645 |
| C | 5-2-85 | 9.0 | 218411 |
| D | 5-31-85 | 22.5 | 519793 |
| E | 7-25-85 | 22.5 | 200139 |
| F | 7-25-85 | 18.0 | 158688 |
| G | 8-21-85 | 22.5 | 210547 |
| H | 8-21-85 | 13.5 | 66460 |
| I | 9-21-85 | 24.0 | 265475 |
| J | 9-21-85 | 9.6 | 69313 |
| K | 10-14-85 | 24.0 | 247542 |
| L | 10-14-85 | 14.0 | 127388 |
| M | 10-17-85 | 24.0 | 611792 |
| N | 10-17-85 | 9.6 | 277760 |
| O | 10-28-85 | 24.0 | 633607 |
| P | 10-28-85 | 9.6 | 98452 |
| Q | 10-31-85 | 24.0 | 400626 |
| R | 10-31-85 | 9.6 | 198947 |
| S* | 11-7-85 | 24.0 | 216996 |
| T | 11-7-85 | 24.0 | 181192 |
| U | 11-7-85 | 9.6 | 84871 |
| V | 11-7-85 | 9.6 | 73225 |
| W | 1-13-86 | 9.6 | 132756 |
| X | 1-13-86 | 13.6 | 365750 |
| Y | 5-28-85 | 54.4 | 158755 |
| Z | 5-28-86 | 27.2 | 84635 |

*S, T, U, V, are replicate injections of the same standard.

with coefficients of variation of less than 25% was chosen for subsequent analysis. The peak that was removed was highly variable (c.v. = 41%) and was from a region of the chromatogram where resolution was poor and integrator performance highly variable.

A principal component analysis of the covariance matrix of this data, followed by a plot of the first two principal components, yielded the pattern shown in Figure 10. The first two principal components explained approximately 57% of the variance. The symbols plotted are the unique alpha character associated with each standard. The first principal component weighted heavily on peaks 3 and 7, which coincidently are toxicants A and B. The second component is weighted on peaks 7 and 14, again two of the more prominent peaks. A careful examination of the GC traces reveals a leading shoulder on peak 7, which may or may not be resolved and detected by the integrator on every run. This may explain why this peak is so important in explaining variability in the data. The principal component plot suggests approximately four different kinds of patterns in the data (outlined in Figure 10).

Since the first two principal components only explained 57% of the variability in the data, the same data was subjected to a disjoint cluster analysis, followed by a canonical discriminant analysis of the clusters. A preliminary screen using various numbers of clusters from 2 to 10 demonstrated that 4 clusters fit the data. The principal components analysis also suggested 4 groups. Figure 11 is a plot of the first two canonical variables for each sample. As with the principal components analysis, the disjoint cluster analysis produced 4 groups that are remarkably similar to the groupings suggested by

Figure 10: Plot of the 1st two principal components from analysis of the group of 26 standards outlined in Table 8. These two components explained 57% of the variance in the data.

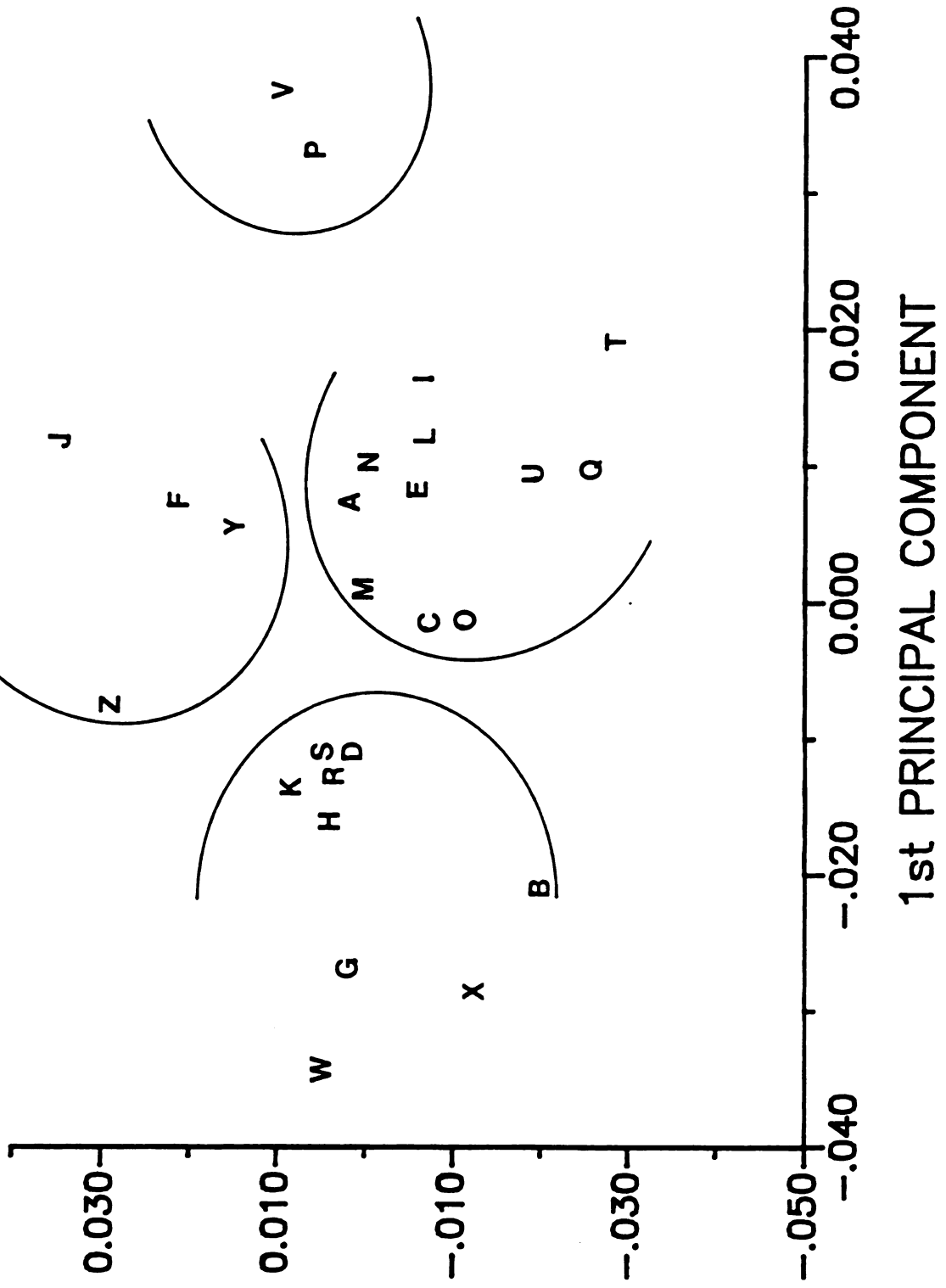
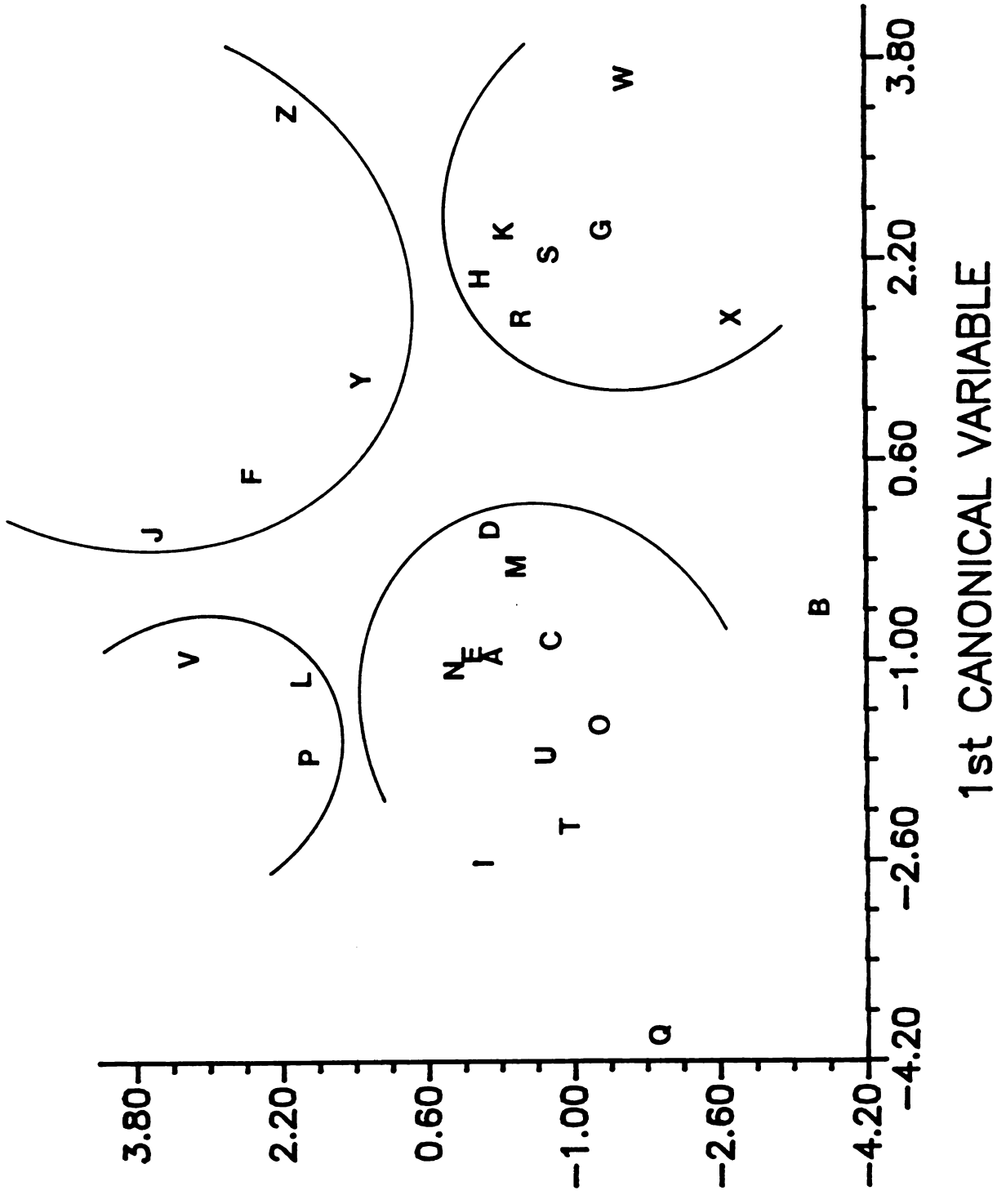

2nd PRINCIPAL COMPONENT
0.030
0.010
-.010
-.030
-.050
-.040
-.020
0.000
0.020
0.040
1st PRINCIPAL COMPONENT
J
F
Y
Z
V
P
K
H
R
S
D
M
A
N
E
L
I
C
O
U
Q
T
B
G
X
W

Figure 11: Canonical variate plot of the disjoint clusters produced from the analysis of technical standards. Disjoint clusters were produced from the proportional peak area data outlined in Table 8.


2nd CANONICAL VARIABLE
1st CANONICAL VARIABLE
3.80
2.20
0.60
-1.00
-2.60
-4.20
-4.20
-2.60
-1.00
0.60
2.20
3.80
V
L
P
J
F
Y
Z
N
E
A
D
M
C
I
T
U
O
Q
B
H
K
R
S
G
W
X

principal components analysis. Clustering of the first six principal components from the princomp procedure (89% of the variance explained) produced identical results, though the clusters were much tighter than those produced with the raw data (Figure 12). This suggests that there are approximately four distinctly different types of patterns in the analysis of the standards. Table 9 is a listing of the clusters and the associated sample identifications with analysis date, mass of material injected and total area. The structure of the clusters suggests that there may be some similarity in the way data was generated on the same date. However, injections S, T, U, and V are replicate injections of two different standards analyzed on the same day and these observations ended up in three different clusters widely separated, suggesting that no consistent bias due to date is present.

The conclusion from this analysis is that there are approximately four detectable types of injections, two major and two minor, associated with this GC, integrator system, and analyst. There does not appear to be any consistent bias due to amount injected, analysis date or total peak area of the chromatogram. It will be shown later that the variability demonstrated with the standards, is minor compared to the variability associated with different sample types.

**Data from Fish Analysis**

One of the goals of this research program was to determine whether or not there is any observable change in the residue pattern occurring with time. Since toxaphene is a complex mixture, and agricultural use has been banned, the atmosphere input function previously discussed should be decreasing. If the residues in the lakes are sensitive to

Figure 12: Canonical variate plot of disjoint clusters produced from analysis of standards using the first six principal components as input into the clustering procedure. The first six principal components explained approximately 90% of the variability in the original data and are by definition jointly uncorrelated.

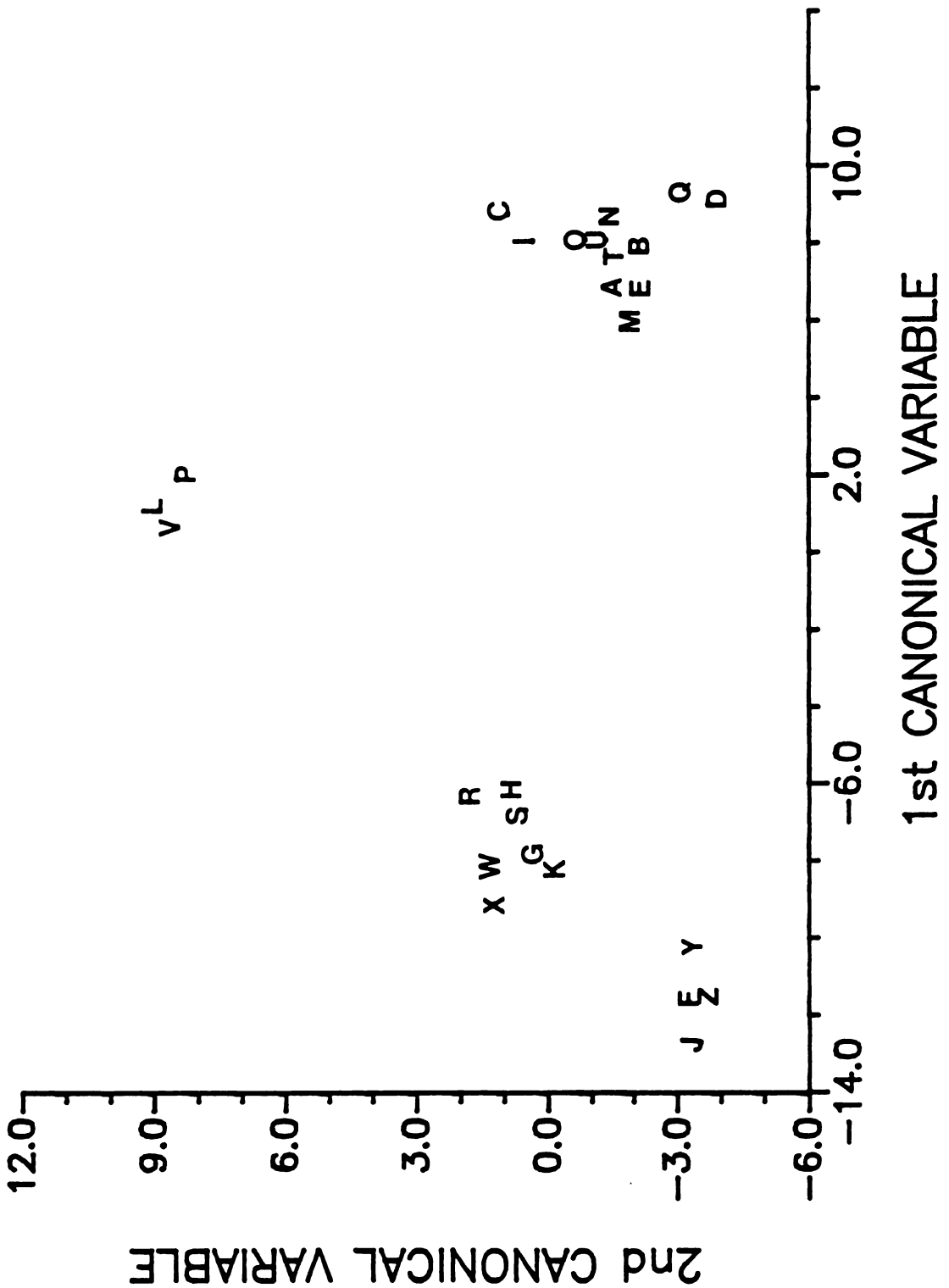

2nd CANONICAL VARIABLE
12.0
9.0
6.0
3.0
0.0
−3.0
−6.0
−14.0
−6.0
2.0
10.0
1st CANONICAL VARIABLE
V L P
X W R S H K G
J E Z Y
C I O N T A M E B
Q D

Table 9: Clusters produced by disjoint clustering and canonical discriminant analysis of 20 different standards.

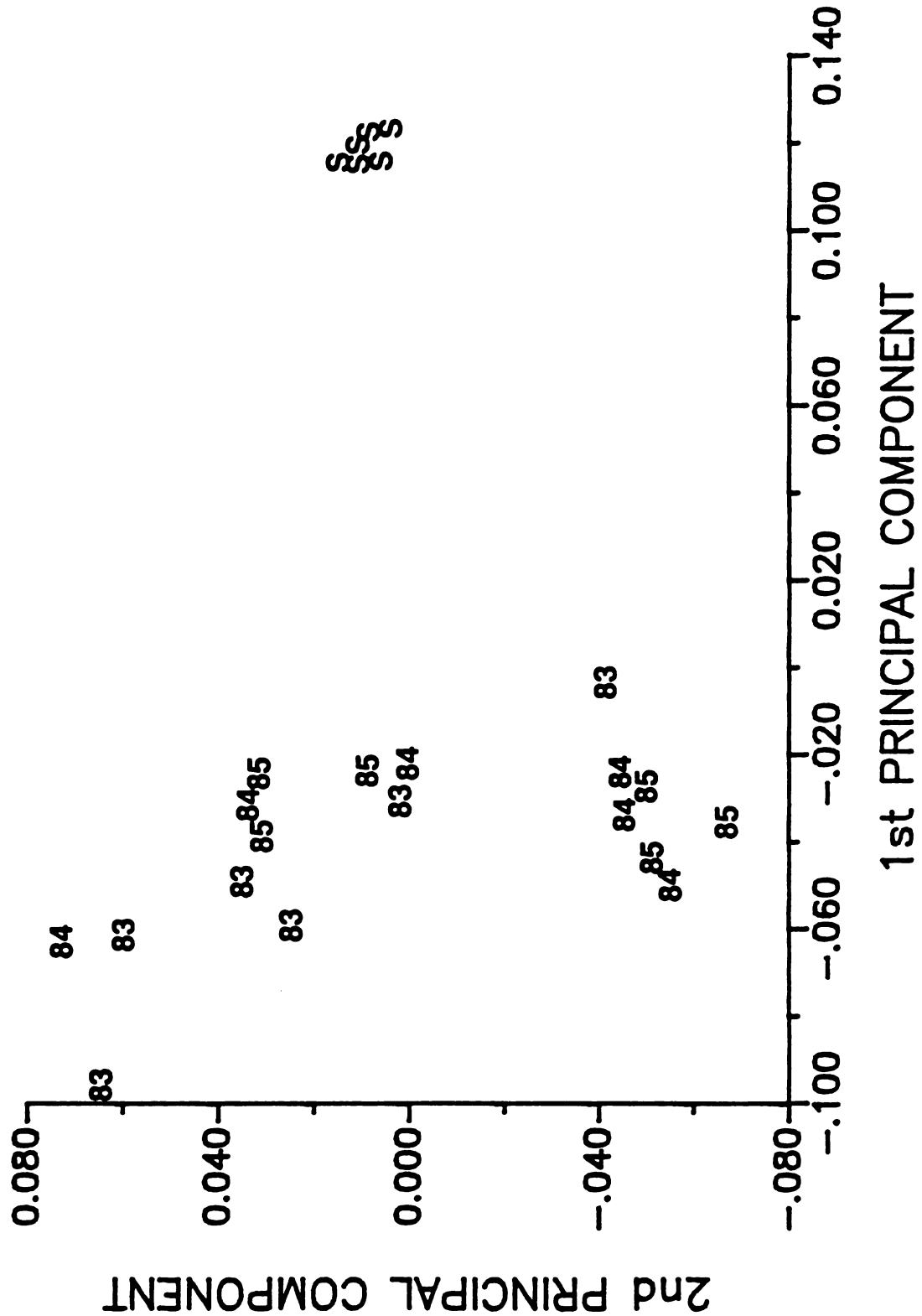
| | ID | Date | Mass Injected (ng) | Total Peak Area |
|---|---|---|---|---|
| Cluster 1 | R | 10-31-85 | 9.6 | 198947 |
| | G | 8-21-85 | 22.5 | 210547 |
| | K | 10-14-85 | 24.0 | 247542 |
| | S | 11-7-85 | 24.0 | 216996 |
| | H | 8-21-85 | 13.5 | 66460 |
| | X | 1-13-86 | 13.6 | 365750 |
| | W | 1-13-86 | 9.6 | 132756 |
| Cluster 2 | C | 5-2-85 | 9.0 | 218411 |
| | E | 7-25-85 | 22.5 | 200139 |
| | O | 10-28-85 | 24.0 | 633607 |
| | A | 4-26-85 | 9.0 | 217476 |
| | N | 10-17-85 | 9.6 | 277760 |
| | M | 10-17-85 | 24.0 | 611792 |
| | I | 9-21-85 | 24.0 | 265475 |
| | Q | 10-31-85 | 24.0 | 400626 |
| | U | 11-7-85 | 9.6 | 84871 |
| | D | 5-31-85 | 22.5 | 519793 |
| | B | 4-22-85 | 22.5 | 456645 |
| | T | 11-7-85 | 24.0 | 181192 |
| Cluster 3 | F | 7-25-85 | 18.0 | 158688 |
| | Y | 5-28-85 | 54.4 | 158755 |
| | Z | 5-28-86 | 27.2 | 84635 |
| | J | 9-21-85 | 9.6 | 69313 |
| Cluster 4 | V | 11-7-85 | 9.6 | 73225 |
| | P | 10-28-85 | 9.6 | 98452 |
| | L | 10-14-85 | 14.0 | 127388 |

degradative forces, the composition of the residue should shift with time. A lack of change in lake trout residues with time would suggest that the exposure variables (i.e. food and water) are not being altered substantially.

To address this question, 6 fish of similar size were selected from 1983, 1984, and 1985. The fish weighed 3.30 $\pm$ 0.31, 3.63 $\pm$ 0.26, and 3.48 $\pm$ 0.26 kg for each group respectively. The residue profile from the adipose tissue rich belly flap section was compared among the years and to 6 randomly selected standards. The 17 peaks used in this analysis are the same as those from the previous analysis with standards excluding the 4th, 7th, and 16th peaks.

Figure 13 is a plot of the first two principal components from an analysis of the covariance matrix for this data. Seventy-six percent of the variability in the data is contained in these first two components. The standards are evident as a tight cluster distinctly separate from the rest of the samples. None of the samples from different years consistently group together, though samples do tend to clump into 3 different groups. A review of the dates from the analyses suggests that for some cases, there may be patterns to the residue that are attributed to an analytical error term that encompasses operating conditions, purification procedure etc. and is unique to a particular analysis. Giesy et al. (1986) demonstrated a large analytical variability relative to other sources of variability using this same system. If this variability is large relative to differences present in residues, it would be very difficult to demonstrate consistent patterns with these techniques. Therefore, this data set does not suggest that there is any difference between the three years that is

Figure 13: Principal components plot from analysis of toxaphene residues from belly flap of Lake Michigan lake trout collected in 1983, 1984, and 1985. Technical standards were also included in the analysis. Seventy-six percent of the variability is contained in the first two components.


2nd PRINCIPAL COMPONENT
1st PRINCIPAL COMPONENT
0.080
0.040
0.000
-.040
-.080
-.100
-.060
-.020
0.020
0.060
0.100
0.140
83
84
83
83
85 84 85
83
85
83 84
83
84 84
85
84 85
85

large enough to outweigh analytical variability. This may mean that if there are indeed real changes occurring with time, they are not substantial.

**Large Data Set** - Residue profiles generated by this study were the result of analysis of several different years, different tissues and two different sources. In addition, many of the residues were analyzed at different stages of purification. Because a limited amount of material was obtained from each extraction, residues were pooled and reanalyzed before further toxicologic testing. Pattern recognition analysis of this data was conducted to determine if any statistically recognizable differences were evident among the different sample types.

The data consists of 101 observations from all of the possible types of traces that were generated during the course of this study. A general summary of the type of data present in the data set is presented in Table 10. It should be noted that the data contains chromatograms from each of the different tissues and purification steps for an individual fish, as well as a random sampling among all fish, tissues etc. The same 17 peaks were present in all of the samples analyzed. This should provide a common basis for meaningful interpretation of the results.

The first two principal components from an analysis of the covariance matrix of the data were plotted (Figure 14). Though only 56% of the total variance in the data was explained, several interesting observations can be made. In general, the standards and the spikes are very similar. This means that the procedure does not tend to create artificial similarities or dissimilarities between the samples and the standard. The samples from Siskiwit Lake on Isle

Table 10: Large data set used for overall pattern recognition analysis of toxaphene residues.

| Number of Observations | Sample Type | Comments |
|---|---|---|
| 24 | Belly flap (O) | Residue profile after silica gel column. i.e. this profile should be the most indicative of the native sample. Samples from 1982, 1983, 1984, and 1985 are represented. |
| 16 | Charcoal (□) | Residue profile after charcoal column purification to remove chlordane. May lose or decrease a proportion of early eluting compounds. Both fillet and belly samples (1984, 1985). |
| 12 | Fillet (⊕) | Same as belly flap, though less fatty dorsal muscle sample. (1982, 1983, 1984, 1985). |
| 12 | Lake Siskiwit (+) Fish | Fish from Isle Royale in Lake Superior. Two are pooled samples, one is a rainbow trout from Malone Bay, one is a repeat analysis (1984). |
| 8 | Nitrated (◇) | Individual fish residues after the entire purification procedure (1982, 1983). |
| 3 | Eggs (●) | Egg samples (1985). |
| 16 | Pool (✳) | Pooled samples after all manipulations. These samples were used for toxicity testing. Both B and F type samples (1982, 1983, 1984, 1985). |
| 4 | Spike (⧖) | Procedural spike taken through the purification procedure. Four replicate analyses of the same material. |
| 6 | Standards (△) | Technical standards |

Figure 14: Principal components plot of 101 data observations from various years, tissues, and stages of purification. For an explanation of sample types see Table 10. ○- Belly; ⊕- Fillet; ●- Eggs; □- Charcoal column; ✳ - Pooled sample; ◇- Nitrated final purification; + - Lake Siskiwit; △- Technical Standard; ⧖ - Procedural spike.

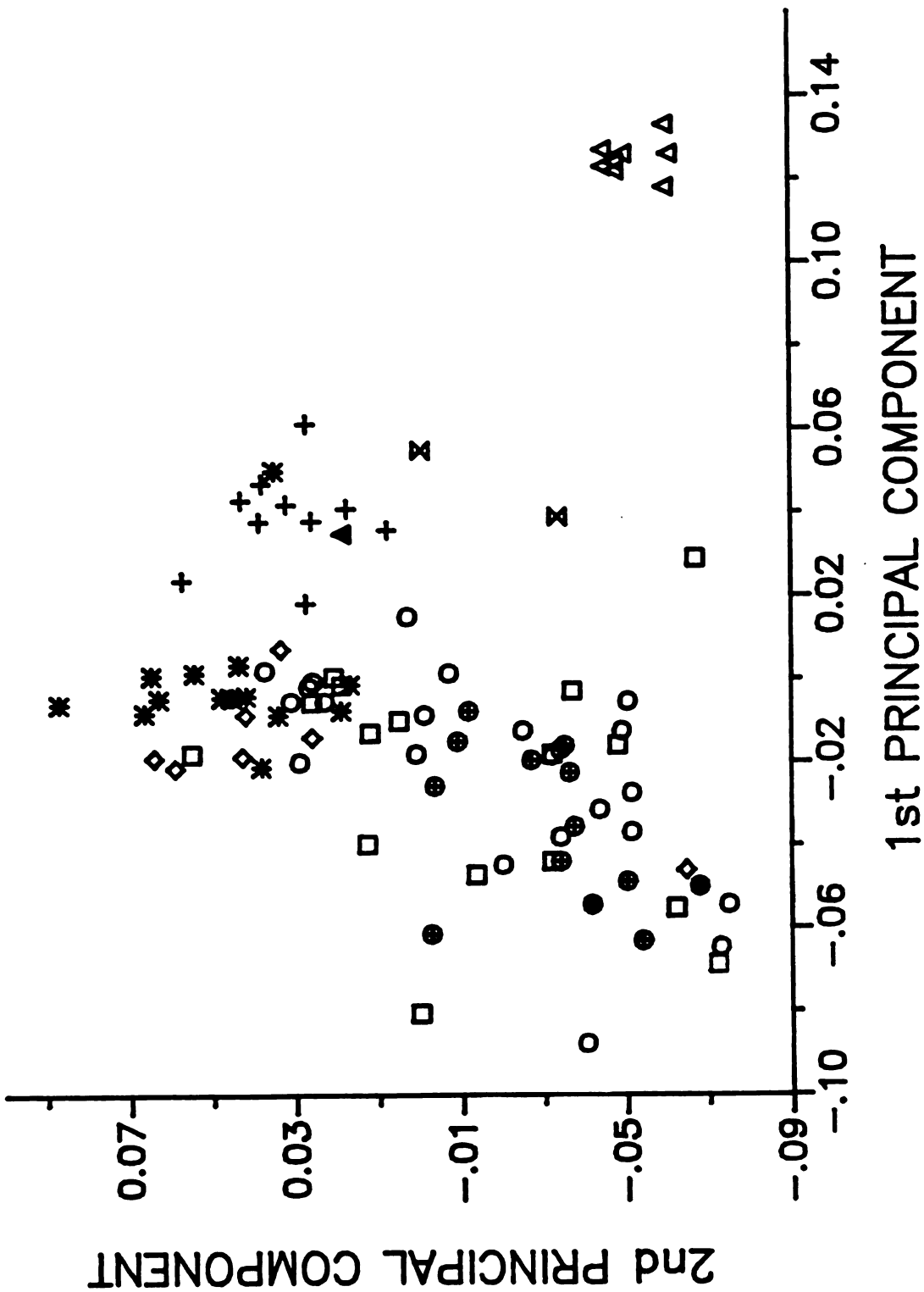
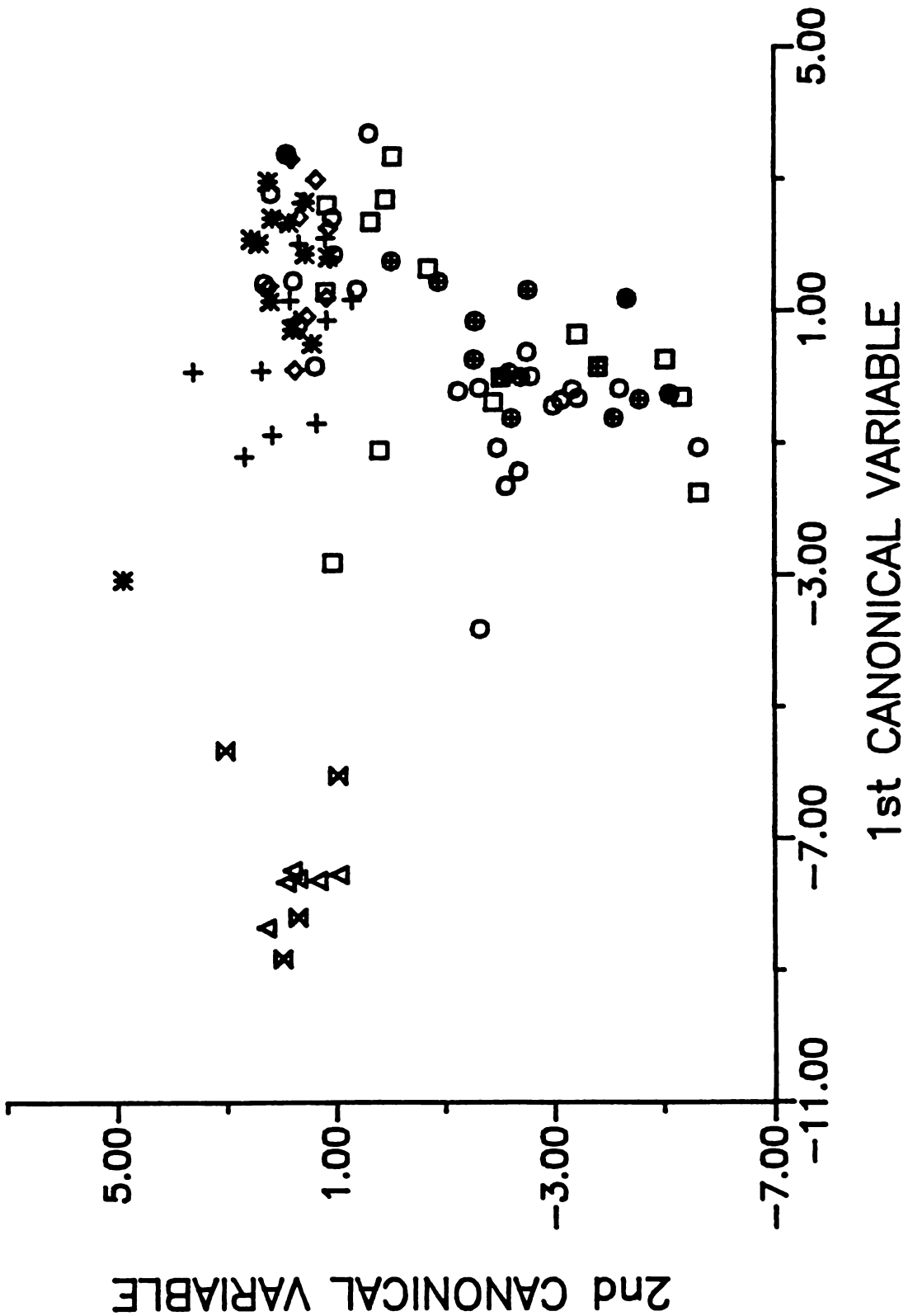

2nd PRINCIPAL COMPONENT
0.07
0.03
-.01
-.05
-.09
-.10
-.06
-.02
0.02
0.06
0.10
0.14
1st PRINCIPAL COMPONENT

Figure 15: Canonical variate plot from a cluster and discriminant analysis of the first six principal components obtained from the 101 observations in Figure 14. All symbols are the same as Figure 14.


2nd CANONICAL VARIABLE
5.00
1.00
-3.00
-7.00
-11.00
-7.00
-3.00
1.00
5.00
1st CANONICAL VARIABLE

Royale all tend to be very similar to one another as do samples that have been through the entire procedure. Samples from different years, tissues, silica gel, and charcoal column purification do not vary in any systematic fashion. Individual egg samples are similar to other tissues, while the pooled and purified samples are similar to the other samples of the same manipulation. All of the field samples are considerably different from the standards. The tentative conclusion from this analysis is that the samples are fairly hetenogeneous in the original state and that as more purification procedures are applied and samples are pooled for toxicologic testing, the residues become increasingly similar.

A second analysis was conducted using principal components, cluster and canonical discriminant analysis sequentially. Initially, a principal components analysis was done and the first six principal components, explaining 90% of the variation in the data, were entered into the clustering routine specifying a maximum of six clusters. A canonical discriminant analysis was done to maximize the between group correlations among the six clusters. Figure 15 is a plot of the first two canonical variables for each of the observations. The same observations that were made with the principal components analysis are evident here. The Lake Siskiwit samples are all similar and lie closest to the pooled test samples. Two of the observations are probable outliners from the rest. One is a pooled test sample of fillets from 1983 fish and one is an individual belly sample from a 1983 fish. Two charcoal column samples from fillets of 1984 fish also tend to be different from the rest.

It is interesting to note that both analyses provide essentially the same results. Many of these techniques can be sensitive to correlations among the variables so that using the first six principal components, which by definition are uncorrelated, for further analysis and obtaining the same results as with the original data, validates the assumption of non-significant correlation among the variables.

In summary, the statistical pattern recognition techniques applied to this data provides the following conclusions: 1) The analytical system used for this study has a statistically identifiable variability among replicate injections of the same material, the source of which was not identified. 2) No differences in peak patterns were evident in belly flap samples, standardized for size and tissue type, from 1983-1985. 3) Samples from different tissues and different years were indistinguishable. 4) As samples were purified and pooled, they became increasingly similar. 5) Samples from Lake Siskiwit on Isle Royale were all similar to one another and 6) All of the types of field samples were different from the standards.

# CHAPTER 5

# CAPILLARY GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF PURIFIED TOXAPHENE RESIDUES

## INTRODUCTION

Because of the complexity and dissimilarity between toxaphene and toxaphene like residues found in environmental samples, it has been recommended that all suspected toxaphene residues be confirmed by mass spectrometric methods (Ribick et al., 1982; Jansson and Wideqvist 1983). While there have been a number of published reports on the mass spectrometry of toxaphene (Saleh, 1983; Budde and Eichelberger, 1977; Holmstead et al., 1974), it is clear that methane enhanced chemical ionization with negative ion detecton is the most sensitive (Johnson and Wideqvist, 1983; Vaz and Blomkvist, 1985; Ribick et al., 1982). Under these conditions, toxaphene produces very simple spectra that are composed predominantly of ions and chlorine isotope clusters resulting from [M-Cl]- fragments. Swackhamer et al. (1986) and Jansson and Wideqvist (1983) have suggested using several specific ions related to toxaphene components with 5-10 chlorines as a sensitive, specific technique for detection of toxaphene. While this is a useful approach for confirmation and/or quantitation of residues, it suffers from a lack of fragmentation and thus provides little structural information. For isolation and identification of specific components, it is necessary to use the less sensitive, though fragmentation rich, electron impact mode.

This chapter examines the characteristics of the purified toxaphene residues that were generated and toxicologically tested during this study.

## MATERIALS AND METHODS

Capillary GC-MS was performed using a Nermag R-10-10C quadrupole GC-Mass spectrometer operated under the following conditions: Column - DB-1 (J and W Scientific) 30 m, 0.25 mm id, 0.25 μ film. Splitless injection, temperature programmed from 50°C (1 min) to 190°C at 30°/min followed by 2°/min up to 260°C. Spectra were acquired in the negative ion detection chemical ionization mode (Methane 0.3 torr, source temperature 150°C) at the rate of approximately 2.5 scans/second.

## RESULTS AND DISCUSSION

A comparison was made between the total ion current for technical toxaphene and a purified lake trout residue (Figure 16). With the exception of the peak at scan 867 due to cis-nonachlor (a chlordane component), there is a distinct similarity between the standard and the residue.

Figures 17 and 18 are the ion chromotograms and total ion current for the standard and the lake trout residue, respectively. Masses 343, 377, and 413 were chosen as representative of the major ion clusters resulting from toxaphene components with 7, 8, and 9 chlorines, respectively (See Table 11) (Swackhamer et al., 1986; Jansson and Wideqvist, 1983). The validity of this approach was verified by examining a mass spectrum of authentic toxicant A ($Cl_8$) available in

Figure 16: Chromatograms of total ion current for standard toxaphene (bottom) and a purified lake trout residue from 1985 (top).

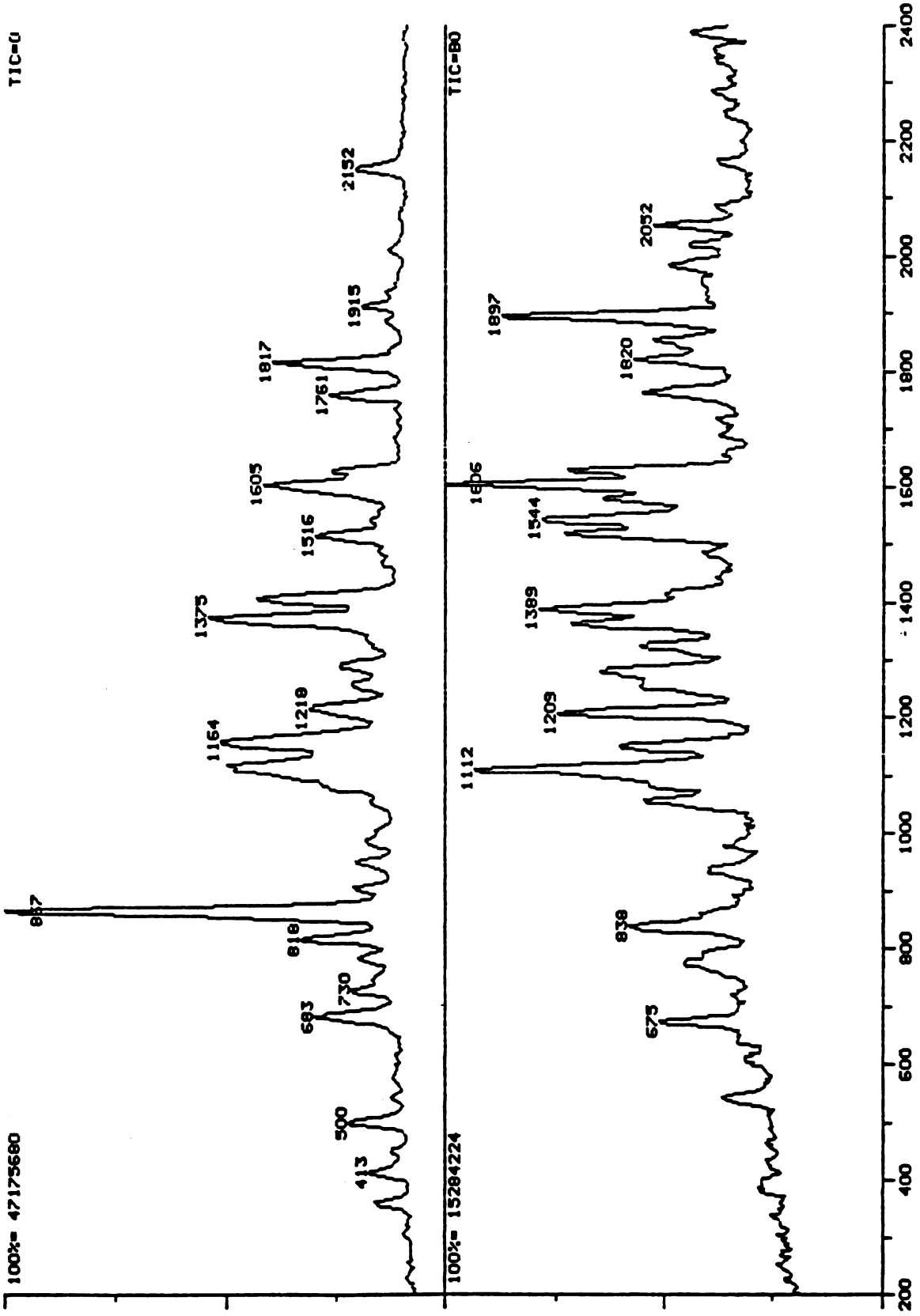

TIC=0
100%= 47175680
867
818
730
683
500
413
1164
1218
1373
1516
1605
1751
1817
1915
2152
TIC=80
100%= 15284224
1112
1209
1389
1544
1606
1820
1897
2052
838
675
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400

Figure 17: Ion current chromatograms from a toxaphene standard. TIC = total, RIC = 343, 377, and 413 for masses 343, 377, and 413 respectively. These masses correspond to the major ion in the chlorine isotope cluster from $[M-Cl]^-$ fragments from structures with 7, 8, and 9 chlorines respectively.

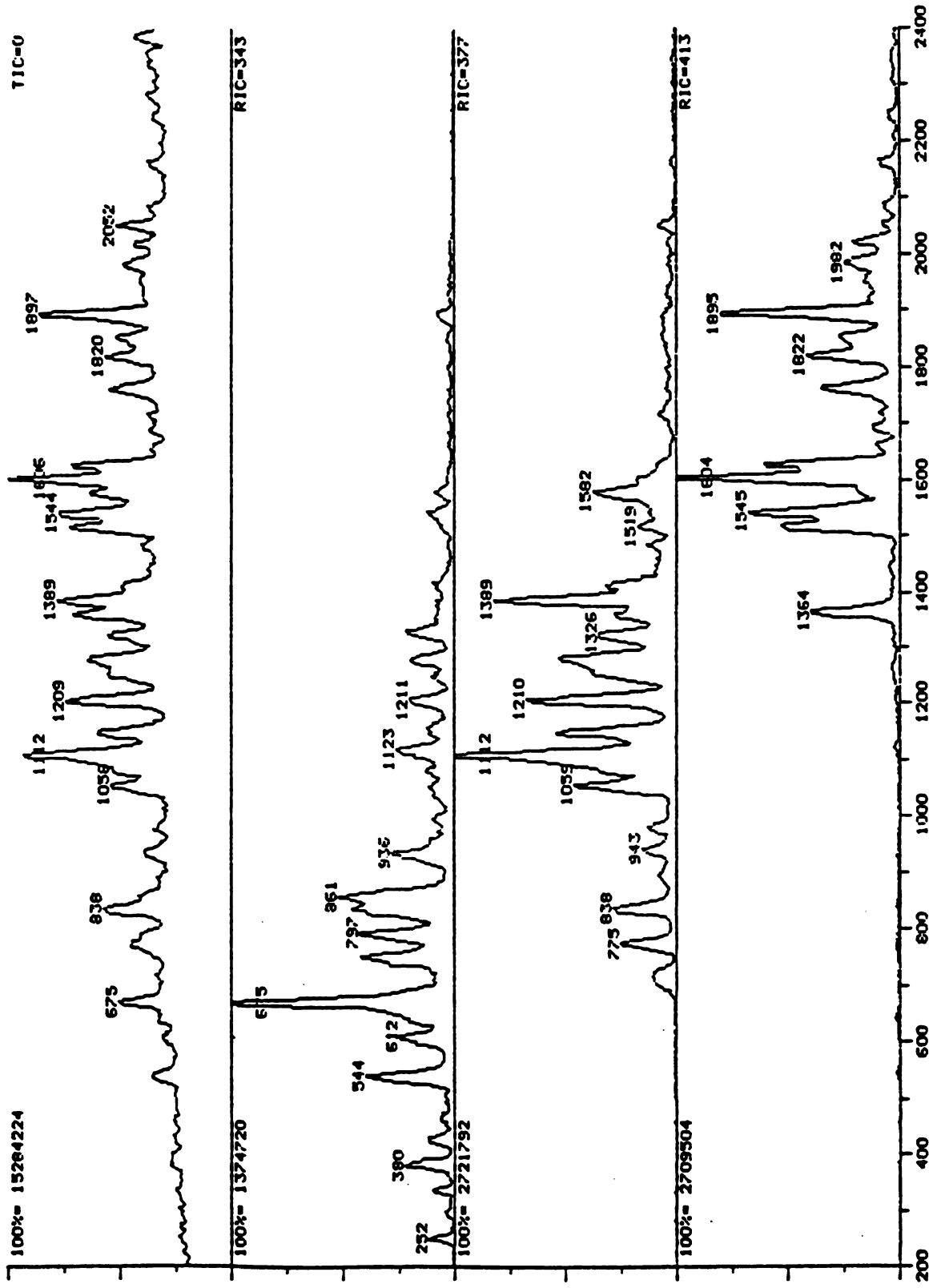

TIC=0
100%= 15284224
RIC=343
100%= 1374720
RIC=377
100%= 2721792
RIC=413
100%= 2709504
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400

Figure 18: Ion current chromatograms from a purified 1985 Lake Michigan lake trout residue. Profile descriptions are described in Figure 17.

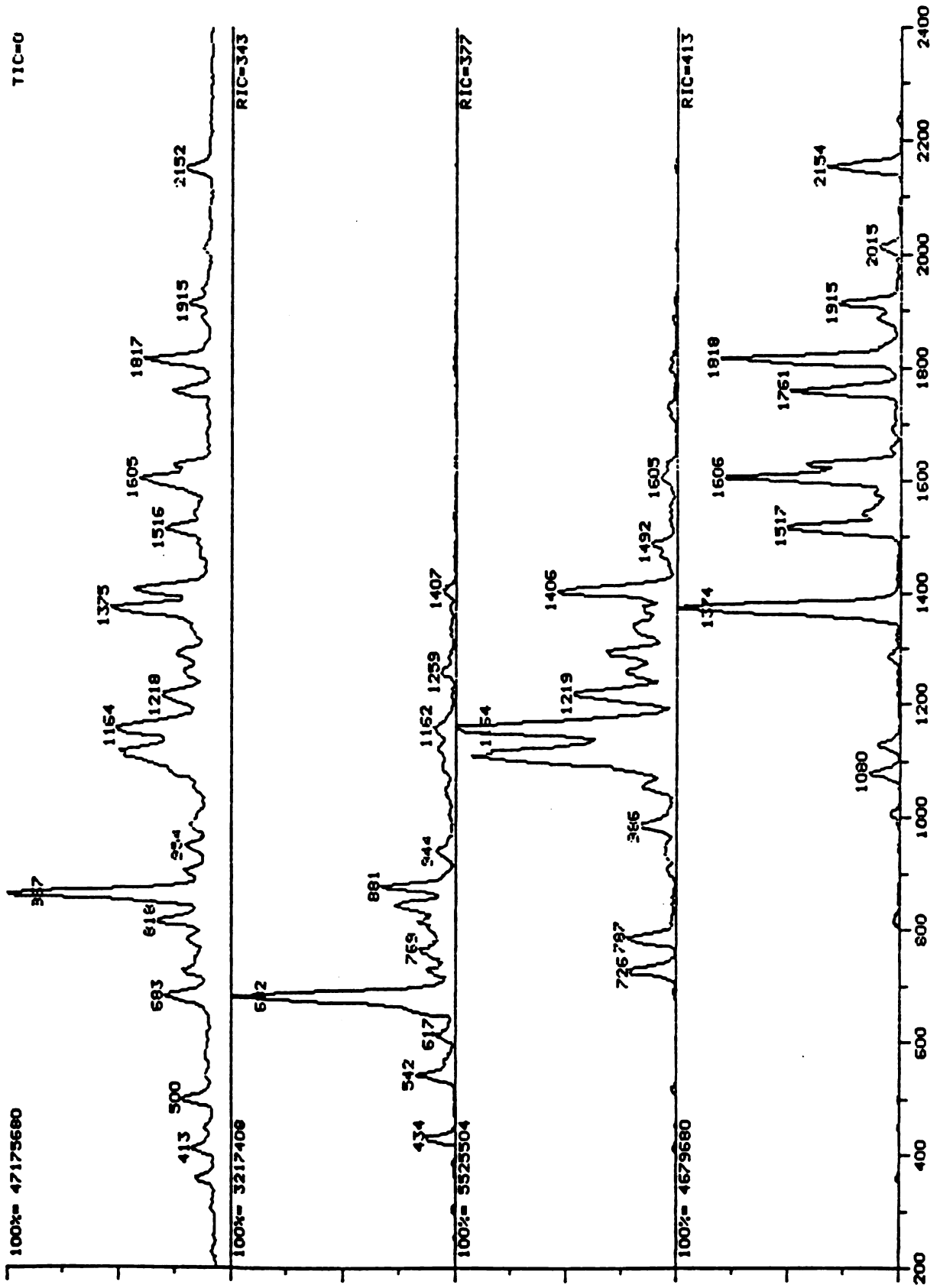

TIC=0
100%= 47175680
413
500
542
617
683
616
387
769
881
944
1164
1218
1373
1516
1605
1817
1915
2152
RIC=343
100%= 3217408
612
RIC=377
100%= 3525504
434
726
797
366
1162
1259
1407
1406
1492
1605
1219
1517
1606
1761
1818
1915
2015
2154
1080
RIC=413
100%= 4679680
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400

Table 11: Relative composition of standard toxaphene and purified lake trout toxaphene residue derived from ion chromotograms of the three major isotope clusters. The monitored ion represents the most intense ion in the spectrum from compounds with the indicated molecular formula.

| Fragment Ion | Molecular Formula $[M]^-$ | | $\Sigma$/TIC[a] | $\Sigma$/RIC[b] |
|---|---|---|---|---|
| 343 | $C_{10}H_{11}Cl_7$ | standard | 0.055 | 0.221 |
| | | residue | 0.034 | 0.126 |
| 377 | $C_{10}H_{10}Cl_8$ | standard | 0.102 | 0.406 |
| | | residue | 0.107 | 0.394 |
| 413 | $C_{10}H_9Cl_9$ | standard | 0.094 | 0.373 |
| | | residue | 0.161 | 0.593 |

a $\Sigma$/TIC equals the sum of the peak area counts from the ion divided by the total peak area counts for all ions.

b $\Sigma$/RIC equals the sum of the peak area counts from the ion divided by the sum of the peak area counts for masses 343, 377, and 413.

this laboratory (not shown).

In general, these traces show how the degree of chlorination changes with retention time as noted previously (Holmstead et al., 1974). They also demonstrate the incomplete resolution and overlap of the different constituents. In many cases, peaks are evident when a single ion is monitored that are less apparent in the total ion current.

Relative to the technical standard, the extracts from lake trout contain fewer components with seven chlorines, more with nine chlorines and approximately equal amounts of components with eight chlorines. Earlier results (see Chapter 3) indicated that the isolation procedure is biased towards later eluting compounds. While this may be a contributing factor in the differences seen here, it is probably not the sole cause since the relative composition of the eight chlorine components is the same. In addition, the peak at scan 1375 (Figure 18) in the extract from the lake trout is a major portion of the signal at mass 413 (32%) while it is much smaller in the standard (11.6%). This peak corresponds to the major unidentified component noted in Chapter 3. This peak was not used for quantitative analysis because of its disproportionate size under electron capture conditions. This data, and the corresponding full spectrum (not shown), indicates that this is a $C_{10}H_9Cl_9$ component of toxaphene.

The conclusion that purified lake trout extracts contain increased levels of components with nine chlorines, is surprising and unanticipated. Most of the literature regarding toxaphene degradation has indicated the greatest loss for the late eluting, highly chlornated, components (Clark and Matsumura, 1979; Williams and

Bidleman, 1978; Parr and Smith, 1976). The presence of the more highly chlorinated structures again suggests that toxaphene is not being degraded very rapidly in the Great Lakes. This observation is supported by the toxicity studies reported earlier.

Another factor contributing directly to this observation is the marked enhancement in sensitivity to the higher chlorinated structures shown by negative chemical ionization. Figure 19 is a comparison of the total ion current traces for toxaphene under electron impact and negative chemical ionization conditions. Note the increased sensitivity for the later eluting components. This difference is the major reason for the improvement in the detection of toxaphene residues (Ribick et al., 1982).

In summary, the capillary GC-MS analysis of the purified lake trout residue shows that the samples generated and tested for toxicity are composed mainly of toxaphene related components with a distribution of components similar, though not identical, to the technical standard. The major impurity is attributed to cis-nonachlor.

Figure 19: Electron impact and negative chemical ionization total ion chromatograms for technical toxaphene. Note the increased sensitivity for later eluting peaks.

TIC=0
100%= 236595
14106
TIC=80
100%= 10680064
16132
17111
18123
19129
19149
21115
21146
22148
25102
12100
13100
14100
15100
16100
17100
18100
19100
20100
21100
22100
23100
24100
25100

# CHAPTER 6

# SUMMARY

At the beginning of this research project there were a number of unanswered questions concerning toxaphene or "toxaphene-like" residues in Great Lakes fish. These included: 1) Are the chlorinated bornane residues detected in lake trout actually derived from the agricultural use of toxaphene? 2) If so, why are the profiles so different from the analytical standard? 3) Does the residue possess significant toxicologic activity? 4) Are there any differences in concentrations, profiles, or activity among different tissues? and 5) Are toxaphene concentrations decreasing or are profiles degrading with time? The experiments performed were designed to address aspects of all of these major questions.

Question 1 above was the first task to be addressed. Because this laboratory was involved with structural identification of one of the major toxic components of the toxaphene mixture, authentic samples were used to survey for the presence of chemical structures that were acknowledged as being related to toxaphene. It was reasoned that, if these structures were present in the "toxaphene-like" residue, it would be unlikely that they could be derived from any other source. The limited availability of these standards had precluded this approach by anyone else. The results of these experiments are presented in Chapter 2 and were published as Jay W. Gooch and Fumio Matsumura, (1985) "Evaluation of the toxic components of toxaphene in Lake Michigan lake trout." J. Agric. Food Chem. 33:844-848. The major conclusions were that toxicants A and B, two of the major toxic components, were present in lake trout extracts at relative compositions that were similar to the technical material. Various chromatographic techniques and mass

spectrometry were used to confirm the identities. The presence of these two components was offered as substantive proof of the toxaphene derivation of these residues.

The identification of these two toxic components in extracts reinforced the need to explore the toxicologic properties of these residues in fish. The major difficulty associated with attempting to address this question is related to the presence of other potent insecticidal residues present in the toxaphene fraction generated by available methods (See Figure 1, Chapter 2). Terryl Farrel from the chemistry laboratory of the Food and Drug Administration in Washington, D. C. improved a charcoal separation procedure for the separation of chlordane and toxaphene and it was this procedure that paved the way for further toxicologic testing of toxaphene derived residues. The details of the final purification procedure and the results of toxicity testing of extracts are described in Chapter 3. Using acute toxicity to mosquito larvae as a non-specific measure of activity and neuroreceptor target site binding affinity as a very specific measure, I have demonstrated that toxaphene residues in lake trout are as toxic as the technical material. Both measures of activity indicate that extracts from different tissues and years are essentially toxicologically equivalent. Lake trout taken from Isle Royale, where the only plausible source of toxaphene is atmospheric deposition, also contain residues that are equally toxic. Toxaphene from a 1985 lake trout egg sample also possessed activity as judged by the neuroreceptor binding assay. The data on acute toxicity and rat brain binding was submitted and has been accepted for publication in Archives of Environmental Contamination and Toxicology.

One of the primary reasons for the need to conduct this study was to determine whether or not toxaphene may be having an adverse impact in the Great Lakes ecosystem. The studies discussed above utilized mosquito larvae and rat brain as study materials and thus, the link to Great Lakes organisms is only suggestive. In order to investigate the correlation between salmonid acute sensitivity to toxaphene and sensitivity of the proposed neuromolecular target site, a series of experiments were conducted to determine if a GABA-Cl ionophore complex, sensitive to toxaphene, was present in the central nervous system in lake trout. These studies revealed that there was indeed a system with characteristics similar to the system in rat brain. The lake trout complex was shown to be sensitive to toxaphene and the toxaphene derived residues from various sources. This indicates that this system is probably involved in mediating toxicity in fish as in mammals and insects. Whether or not the levels of toxaphene present in various components of the Great Lakes is currently causing adverse effects on Great Lakes biota will require further study.

Chapter 4 addresses the question of temporal changes in the concentrations and profiles of toxaphene residues over the last four years. Since toxaphene usage began to decline in the late 1970's and was ultimately banned in 1982, it is hoped that the current high levels of toxaphene in Great Lakes fish will degrade or decline with time. The fact that questions regarding toxaphene in the Great Lakes were still a priority concern in the recent International Joint Commission Great Lakes Science Advisory and 1985 Annual Report, exemplifies the need to address the issue.

From the limited number and variable sizes of fish that were analyzed from 1982-1985, it is difficult to prove whether or not toxaphene levels are declining in Great Lakes lake trout. The samples that were analyzed reflect the difficulty in establishing trends, though they probably are a more accurate representation of the range of concentrations present in the larger fish of the population. There is no large decline in concentrations in either belly flap or fillet samples apparent in the samples studied in this investigation.

Statistical pattern recognition techniques were applied to the data in order to determine if changes were occurring in the profile of a selected group of 17 peaks used for quantitation in all of the samples. This analysis revealed that samples at the silica-gel stage of purification contained no identifiable groupings that could be related to different years or tissue types. Samples from Siskiwit Lake on Isle Royale tended to be very similar. As samples were pooled and/or further purified, the pattern among the peaks became increasingly similar so that ultimately, samples used for toxicity tests were more similar to one another than to any specific tissue or year. There was no readily identifiable degradation that could be related to a yearly time frame. Assuming current inputs are small, there should eventually be an identifiable change in the composition of the residue. It is difficult to predict the time frame over which these changes will occur, though the data presented here suggests that it will be long.

Capillary GC-MS data was generated for substantiating the purity of the material that was used in the toxicity studies and was presented in Chapter 5. This data revealed that the purified extracts were

ostensibly free of potential confounding interferents. The only notable exception was cis-nonachlor. The toxicity of nonachlors (cis or trans) to fish has not been determined. The data of Ivie et al. (1972) demonstrated that trans-nonachlor was much less toxic to mice than cis- or trans-chlordane, but was more toxic in insects. Data generated in this laboratory (Gooch and Matsumura, unpublished observation) showed that the toxicity of environmental chlordane residues could be explained by heptachlor epoxide, oxychlordane and cis- and trans-chlordane levels, even though a large amount of trans-nonachlor was present. These data suggest that nonachlors are not important interferences in the toxicity of the toxaphene residue.

The mass spectral data also provided evidence of the relative distribution of the variously chlorinated bornane structures. It was demonstrated that the extracts from fish contain more constituents with 9 chlorines than capillary GC-ECD had suggested. Based on the sum of ion currents for masses 343, 377, and 413, ions representative of structures with 7, 8, and 9 chlorines respectively, it was concluded that the toxaphene residue is composed of a greater concentration of structures with nine chlorines and a lower concentration of structures with 7 chlorines (relative to the total) than the technical standard. The relative concentration of structures with eight chlorines (corresponding to the average molecular weight) was similar for both. This data is further evidence that toxaphene is not being rapidly degraded in the Great Lakes since nearly all studies to date have shown that the strucures with a greater degree of chlorination are the most readily degraded by reductive and oxidative dechlorination.

Two appendices have been added at the end of this dissertation. Appendix A is a table with the relevant data for each individual fish that was analyzed. It provides the concentration numbers and the weights of each fish from the different sampling years.

Appendix B is a summary of experiments that were conducted in order to gain insight into possible reasons for the difference between the residue and the technical standard. It is now obvious that the residues found in organisms at high trophic levels in the Great Lakes are the result of a number of factors and processes. A complex series of events is further accentuated by the multicomponent nature of toxaphene. Each component of toxaphene is an entity in itself with its own vapor pressure, solubility, degradability, partition coefficient etc. and as such could be studied alone. Fortunately, or unfortunately depending on your perspective, most of the components of toxaphene have similar physico-chemical properties and thus they behave almost as one. What we are left with determining is the factors that are responsible for creating a residue of altered composition, though altered less than perhaps was originally anticipated. Data was available from atmospheric studies to suggest that the input source term for Great Lakes contamination was not very different from technical material. One group had suggested that atmospheric toxaphene was enriched in the early eluting, higher vapor pressure, GC peaks. Over longer time periods, however, it is probable that even the later eluting components volatilize, thereby producing a composition that is similar to the original toxaphene mixture. Ignoring alterations occurring during washout and dry deposition, this suggests that the aqueous exposure variable may not be the principal source of changes in toxaphene

composition in lake trout.

Preliminary experiments were performed on two of the possible mechanisms for changing the residue composition that are related to the fish itself, uptake and metabolism. A model exposure system was devised that was designed to take into account soulbility differences as well as uptake differences. These experiments were not designed to answer pharmacokinetic questions; rather, they were intended to demonstrate if any constituents were preferentially accumulated or degraded. These experiments revealed that the juvenile lake trout contained a residue that was apparently derived from toxaphene. Similar profiles in fish from control aquaria made it impossible to make any definite conclusions and suggested that background contamination, possibly from feed, was a confounding problem. Experiments conducted with $^{14}C$ labelled toxaphene gave reasonable estimates of exposure concentration and further showed that over half of the material placed into the system was lost in 10-20 days. Volatilization, driven by the aeration in the system, is the likely explanation for the loss.

Metabolism experiments were conducted with lake trout liver post-mitochondrial supernatant. No alterations that could unambigously be related to enzymatic degradation were detected. An iron redox couple could have catalyzed some of the apparent alterations in controls which received no enzyme source. Clearly, this is an area that requires further investigation.

In summary, the experiments described herein have addressed aspects of all five questions that were originally posed in the beginning of this summary. They have shown that the chlorinated

bornanes present in Great Lakes lake trout are derived from toxaphene, toxic, recalcitrant, and are probably generated by the full spectrum of environmental processes that act on persistent halogenated hydrocarbons.

As is all too often the case, the results of this study do not establish whether or not toxaphene in the Great Lakes is causing adverse effects on the biota. A few examples calculations may shed some light on the potential for harm.

The in vitro binding data demonstrates that $1 \times 10^{-8}$ M is a concentration near the target site that can produce a significant neurologic effect. This corresponds to a concentration of 4.1 µg/l (ppb). Mehrle and Mayer (1975) have shown that early life stages (fry) of fish tend to be the most sensitive and established an MATC of < 39 ng/l. This water concentration is 100 times less than the concentration necessary at or near the GABA-chloride ionophore complex to produce an effect. If we assume that the concentration in the plasma compartment of a fish is within an order of magnitude of what would be at the target site, toxaphene would have to be concentrated 100 to 1,000-fold in the plasma from the water in order to produce a concentration at the target site which may cause an effect. Since little or no studies have been done to correlate plasma and/or CNS concentrations with toxicity or chronic effects of toxaphene, it is difficult to determine if these calculations have any relevance or meaning. Water concentrations in the Great Lakes have been reported to be near 1 ng/l and are probably not high enough to have direct effects on fingerling or older fish.

Giesy et al. (1985) measured concentrations of toxaphene in chinook salmon eggs of 3.5 µg/g. I found concentrations in immature lake trout eggs of approximately 0.75 µg/g. This means that some compartment in the egg and developing embryo contains nearly a 100-fold greater concentration than that required to produce a demonstrable effect in vitro. Clearly, with this stage being the most sensitive to toxaphene effects, a 1000-fold partitioning decrease would still produce an active site concentration that may have some biological relevance. In addition, the added presence of chlordane and lindane components, both of which also interact potently at the GABA site, , may add to the potential effect. These calculations assume that any relevant effects are mediated entirely through the GABA-chloride ionophore complex, which of course, may not be the case.

In summary, the concentrations of toxaphene in Lake Michigan lake trout appear to be near some level that may be having an adverse impact. A field study directed specifically at looking for effects, most likely at the fry stage, known to be associated with toxaphene exposure would have to be done before more definitive conclusions could be reached.

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# APPENDIX A

## TOXAPHENE RESIDUE CONCENTRATIONS IN INDIVIDUAL FISH ANALYZED FROM 1982-1985

Table A-1: Summary of toxaphene concentrations in Lake Michigan lake trout collected in 1982 (µg/g, wet weight).

| 1982 | | | |
|---|---|---|---|
| Fish | [Belly] | [Fillet] | Ratio B/F |
| 1 | 5.81 | 1.67 | 3.48 |
| 2 | 5.69 | - | - |
| 3 | 3.78 | - | - |
| 4 | 9.33 | - | - |
| 5 | 7.55 | 1.97 | 3.66 |
| $\bar{x} \pm$ s.d. | 6.43 $\pm$ 2.10 | 1.82 | 3.66 |

Table A-2: Summary of toxaphene concentrations in Lake Michigan lake trout collected in 1983 (µg/g wet weight).

| 1983 Fish | [Belly] | [Fillet] | Ratio B/F | Fish Weight (kg) |
|---|---|---|---|---|
| 1 | 3.30 | 0.46 | 7.2 | 2.45 |
| 2 | 2.24 | - | - | 5.31 |
| 3 | 1.54 | - | - | 3.08 |
| 4 | 4.70 (4.60) | - | - | 3.95 |
| 5 | 2.79 (5.30) | 0.54 | 5.2 | 2.86 |
| 6 | 6.04 | - | - | 3.08 |
| 7 | 7.33 | - | - | 4.72 |
| 8 | 0.59 | - | - | 4.94 |
| 9 | 5.11 (6.36) | - | - | 3.31 |
| 10 | 7.39 (4.93) | 1.71 | 4.3 | 3.90 |
| 11 | 4.48 | - | - | 3.22 |
| 12 | 3.32 (2.31) | - | - | - |
| $\bar{x} \pm$ s.d. | 4.07 ± 2.17 | 0.90 ± 0.70 | 5.6 ± 1.5 | 3.67 ± 0.90 |

-Numbers in parenthesis are replicate analyses.

Table A-3: Summary of toxaphene concentrations in Lake Michigan lake trout from 1984 (µg/g wet weight).

| 1984 Fish | [Belly] | [Fillet] | Ratio B/F | Fish Weight (kg) |
|---|---|---|---|---|
| 1 | 3.85 | 0.91 | 4.23 | 3.63 |
| 2 | 4.20 | 1.04 | 4.04 | 2.95 |
| 3 | 3.61 | 0.95 | 3.80 | 3.22 |
| 4 | 4.08 | 1.95 (1.49) | 2.06 (2.74) | 4.54 |
| 5 | 3.94 | 1.07 | 3.79 | 5.03 |
| 6 | 2.82 | 0.54 (0.96) | 5.22 (2.94) | 2.99 |
| 7 | 3.63 | 1.44 | 2.52 | 3.49 |
| 8 | 6.90 | 3.00 (1.59) | 2.30 (4.34) | 3.63 |
| 9 | 4.60 (4.2) | 2.43 (0.98) | 1.89 (4.29) | 3.63 |
| 10 | 7.10 (4.3) | 3.41 (0.89) | 2.08 (4.83) | 3.90 |
| 11 | 3.00 (4.3) | 0.93 (0.93) | 3.23 (4.62) | 4.58 |
| $\bar{x} \pm$ s.d. | 4.34 ± 1.41 | 1.61 ± 0.96 | 3.20 ± 1.10 | 3.81 ± 0.68 |

-Numbers in parenthesis are replicate analyses.

Table A-4: Summary of toxaphene concentrations in Lake Michigan lake trout collected in 1985 (μg/g wet weight).

| 1985 Fish | [Belly] | [Fillet] | Ratio B/F | Fish Weight (kg) |
|---|---|---|---|---|
| 1 | 5.20 | 1.80 | 2.89 | 3.18 |
| 2 | 5.16 | 1.42 | 3.63 | 3.31 |
| 3 | 7.39 | 1.03 | 7.17 | 3.54 |
| 4 | 10.76 | 1.21 | 8.89 | 3.45 |
| 5 | 3.46 | 1.49 (0.67) | 2.32 | 3.95 |
| 6 | 8.83 | 0.95 (0.81) | 9.29 | 6.80 |
| 7 | 9.99 (6.09) | 1.59 | 6.28 (3.83) | 4.31 |
| 8 | 3.42 (2.23) | 0.76 | 4.50 (2.93) | 2.27 |
| 9 | 4.34 (3.77) | 1.18 | 3.68 (3.19) | 3.45 |
| 10 | 7.10 (4.3) | 3.41 (0.89) | 2.08 (4.83) | 3.90 |
| 11 | 3.00 (4.3) | 0.93 (0.93) | 3.23 (4.62) | 4.58 |
| $\bar{x} \pm$ s.d. | 6.50 ± 2.81 | 1.27 ± 0.33 | 5.41 ± 2.60 | 3.81 ± 1.25 |

-Numbers in parenthesis are replicate analyses.

Table A-5: Summary of toxaphene concentrations in Lake Siskiwit (Isle Royale) lake trout collected in 1984 (µg/g wet weight).

| **Lake Siskiwit** Fish | [Belly] | [Fillet] | Ratio B/F | Fish Weight (kg) |
|---|---|---|---|---|
| 1 | 0.14 | - | - | 0.71 |
| 2 | 1.70 (1.53) | - | - | 1.44 |
| 3 | 1.49 | - | - | 1.41 |
| 4 | 0.97 | - | - | 1.04 |
| 5 | - | - | - | 1.36 |
| 6 | 1.50 | - | - | 1.31 |
| 7 | 0.79 (0.70) | - | - | 1.60 |
| 8 | 1.59 (0.79) | - | - | 1.35 |
| 9 RT* | 0.73 | - | - | - |
| $x \pm$ s.d. | 1.17 $\pm$ 0.56 | - | - | 1.28 $\pm$ 0.28 |

-Numbers in parenthesis are replicate analyses.
*Rainbow trout from Malone Bay.

# APPENDIX B

# UPTAKE AND METABOLISM EXPERIMENTS

## APPENDIX B

During the course of this research program, a series of experiments was performed in an attempt to sort out some of the possible reasons for the pattern of toxaphene residues seen in the residues in Great Lakes lake trout. Because I considered the results equivocal for a number of reasons, a more complete series of experiments was never performed. I have elected to include this data as an appendix with the hope that someone may be able to benefit from the preliminary nature of the results and perhaps pursue the ideas in a more rigorous fashion. The two sets of experiments concern uptake in a model system and *in vitro* metabolism.

I. Uptake Experiments.

**Methods** - Uptake experiments were performed using a static circulating system. Briefly, toxaphene was sorbed to 600 g of quartz sand by solvent spike addition and the solvent was allowed to evaporate. Sand was placed in 18 l aquaria and covered by a mesh screen to isolate the sand from the fish. Aquaria were filled with charcoal filtered/dechlorinated tap water. A six inch horizontal air stone was placed in an isolated compartment at one end of the aquarium to pull water across the sand, drive flow up to the top and across a plexiglass deflector and back into the exposure chamber. This provided a gentle circulating current and fish were exposed to the chemical

profile that desorbed from the sand into the water column. A 24 h equilibration period was used before fish were exposed. Water was not monitored during the exposure. A photograph of the exposure system in operation is provided (Figure 1B). Juvenile lake trout (3-16 g) were obtained from the Jordan River National Fish Hatchery, Elmira, Michigan.

**Results** - Figure 2B is an example of the results that were obtained in this exposure system. Each aquarium contained 20-30 g of 2-3 fish. There are two main conclusions evident from this experiment: 1) The fish contained a residue from toxaphene, and 2) The pattern obtained in the system with no toxaphene added (control) was similar to the treatment tanks.

Careful examination of the profile of peaks in the fish and comparison with profiles obtained in environmental samples, suggests that the uptake system profiles are more similar to environmental samples than to the technical standard. Since the control samples contain residues similar to the treatment tanks, it is impossible to make any definitive conclusions. The residues may be the background contamination present in the fish due to contamination from feed. With the global distribution of toxaphene residues, it may be impossible to use a feed that contains fish meal, oil etc. for this type of study.

Because of the difficulty encountered with the above approach, two experiments were conducted with $^{14}C$-toxaphene. Ignoring profiles, $^{14}C$ activity was the only parameter measured in these experiments. In the first experiment fish were exposed for 10 days to sand spiked at 200 ng/g (ppb) and for 20 days at 100 ppb. A second experiment was conducted with 2 tanks spiked at 100 ppb for 10 days. The data in Table 1B summarizes the results of these experiments. Assuming

Figure 1B: Model uptake system used with juvenile lake trout.

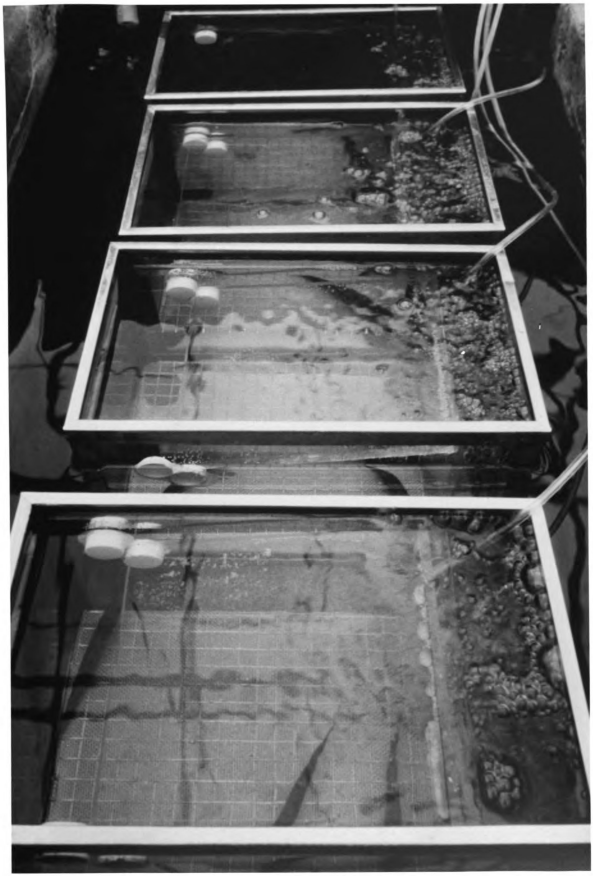


Figure 2B: Toxaphene residue in juvenile lake trout after exposure in a model uptake system. a) toxaphene standard; b) sand spiked at 100 ppb-exposure 10 days; c) 100 ppb - 15 days; d) 200 ppb - 10 days; and e) control.

Table 1B: Concentrations of $^{14}C$-toxaphene in different compartments of a model uptake system.

| [Sand] | Duration | [Water] | [Fish] | [$^{14}C$-lost] |
|---|---|---|---|---|
| 200 ng/g | 10 days | 0.30 µg/l | 0.10 µg/g | 68% |
| 100 ng/g | 20 days | 0.31 µg/l | 0.03 µg/g | 60% |
| 100 ng/g | 10 days | - | 0.36 µg/g | - |
| 100 ng/g | 10 days | - | 0.33 µg/g | - |

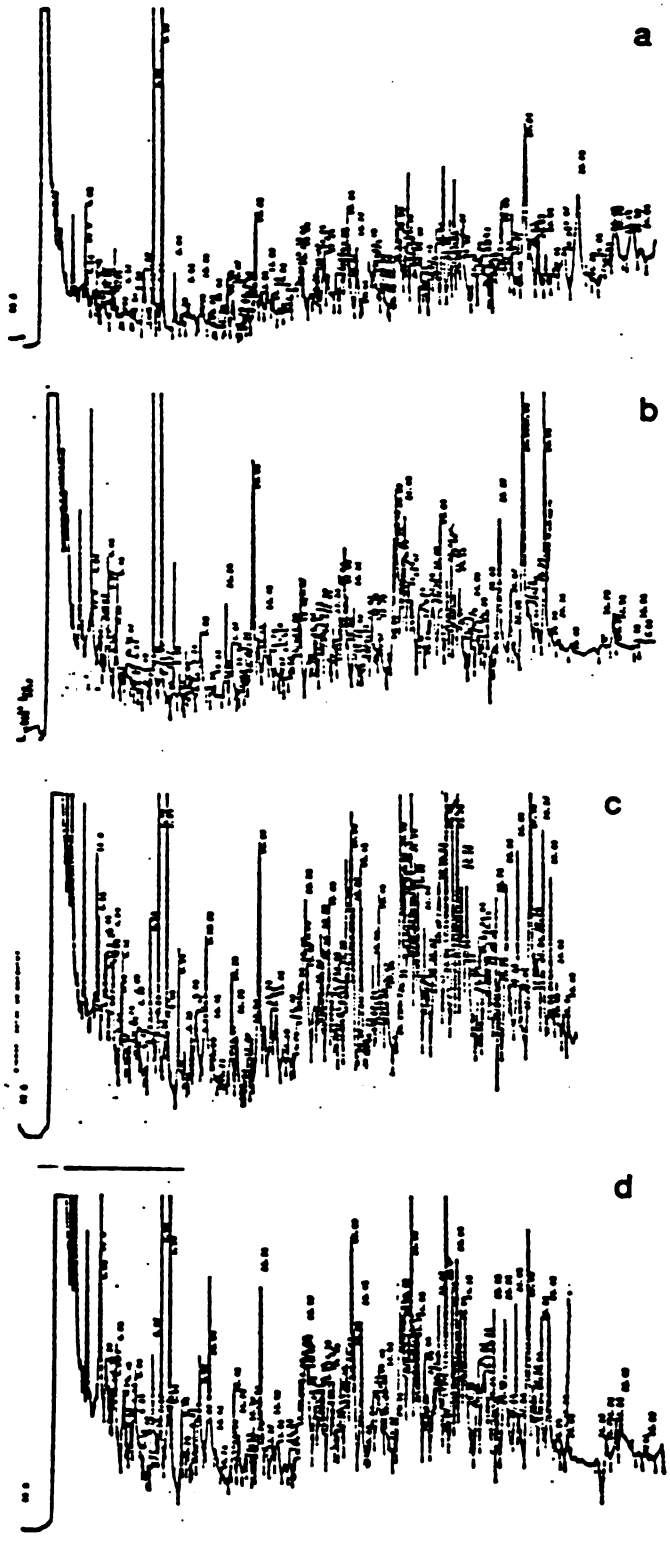
concentrations at the end of the experiment were present throughout the exposure, fish were being exposed to approximately 300 parts per trillion (µg/l) of toxaphene. Fish bioconcentrated toxaphene about 1000-fold. Mass balance of the $^{14}C$ label revealed that 60-68% of the toxaphene in the system was being lost by some mechanism. The most likely route of loss was volatilization enhanced by the airstone used to drive the current in the system. The fact that water concentrations were the same in tanks spiked at a 2-fold concentration difference, suggests that there may have been an equilibrium between volatilization losses, desorption from the sand and uptake by the fish. This experimental design requires further development before this approach can be considered very useful.

II. In vitro Metabolism

**Methods** - The liver from one hatchery lake trout was removed (2.4 g), rinsed with 0.15 M KCl, and homogenized in 5 volumes of 100 mM phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$ and 150 mM KCl. The homogenate was centrifuged at 9600 g for 20 min. One ml of the supernatant was incubated with and without 1 mg of NADPH and 6.3 x $10^{-5}$ M toxaphene at room temperature for 60 min. A boiled enzyme blank was also included. Tubes were extracted twice with 2.5 ml of dichloromethane, dried with anhydrous $Na_2SO_4$ and evaporated to 0.5 ml. Extracts were analyzed by capillary GC-ECD as described earlier.

**Results** - Figure 3B is a composite of the profiles obtained under different incubation conditions. There appears to be no consistent trend that can be unequivocally related to metabolism. While there does appear to be some indication of an alteration in the mixture (an

Figure 3B: Extracts from in vitro incubation of toxaphene with lake trout liver post mitochordrial supernatant. a) + NADPH, 24°C b) + NADPH, 4°C c) - NADPH and d) boiled enzyme.


a
b
c
d

increase in early eluting peaks) it is probably not related to enzymatic metabolism *per se*. It is possible that an iron redox couple functioning under these conditions was able to cause some transformation of the material (Saleh and Casida, 1978; Williams and Bidleman, 1978). In any case, no transformations are evident that could be related back to the residue seen in Great Lakes lake trout. This data suggests that lake trout have a limited capacity to metabolize toxaphene, though clearly, further studies need to be conducted to clarify this point.