THE DETECTION OF CONTAMINANTS IN SEDIMENTS: A COUPLED CHEMICAL FRACTIONATION/ BIOASSAY METHOD

by

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ABSTRACT

There is an urgent need in Canada for reliable chemical and biological screening methods for detecting and assessing the significance of the discharge of hazardous materials into the aquatic environment, particularly as it relates to biological communities; this thesis provided a unique opportunity to develop such a scheme in a natural environmental ecosystem.

Part 1 contains a comprehensive literature survey dealing with a) the analytical methodology required to isolate toxic chemicals from soil and sediment and b) the use of short term tests for screening toxic responses.

Part 2 describes the analytical and biological methods used to isolate and detect toxic organic compounds present in Tobin Lake sediment. Sediment samples were collected and provided by Dr. W.F. Warwick (National Water Research Institute) and Mr. J. Witteman (Environment Canada). A detailed description of the sampling surveys undertaken is provided.

Part 3 depicts the biological and analytical results obtained during this study. Both the Panagrellus redivivus assay and the Salmonella/microsomal assay (Ames test) indicated significant toxicity in extracts obtained from sampling Sites VII and VIII. The most toxic compounds found were those which were extracted under neutral pH with dichloromethane and readily eluted from a 5% water deactivated Florisil column with hexane:dichloromethane (1:1). Analysis of this fraction using gas chromatography-flame ionization detection (GC/FID) and gas chromatography- mass spectrometric detection (GC/MS) indicated a very complex extract with hundreds of compounds present. As a result of this

finding experiments were initiated to find a gas chromatographic column capable of resolving this mixture. A 50 m, glass, wall coated open tubular column coated with SE 54 was found to give adequate resolution of this complex mixture.

Fraction 2, from Sites VII and VIII was screened for the presence of organochlorine compounds using negative chemical ionization mass spectrometry (NCI/MS). No evidence was obtained using this technique to suggest that these compounds were present in sufficient quantity to allow detection.

Analysis of Fraction 2 from Sites VII and VIII using a 50 m fused silica column coated with SE 54 and a gas chromatographic-mass spectrometer-data system indicated the presence of aldehydes, ketones, alcohols, alkanes, benzenoids, alkane thiols, alkenes, and plant and animal sterols. These compounds were identified using a computerized deconvolution/library search and compound identification technique. Two mutagenic compounds, styrene and benzaldehyde were identified and confirmed.

Ortho-xylene, a compound with a high aquatic toxicity rating, was identified and confirmed. Two tumor promoting compounds, namely, o-and p-methyl benzaldehyde were identified but not confirmed. 2-Hexanethiol, an occupational hazard, was identified but not confirmed.

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INTRODUCTION

It is becoming increasingly apparent that our environment contains many hazardous substances of which we have been unaware. The significance of these components, either alone or in combination with other factors, is not readily apparent. We now recognize that cancer represents one of the most important consequences of environmental pollution. It is estimated that in highly industrialized countries, as much as 80% of cancer is of environmental origin (Commoner, 1977). Furthermore, laboratory and epidemiological studies indicate that the majority of known environmental carcinogens are organic compounds of synthetic origin - products of the huge increases in chemical production during the last 30 years.

Manufactured compounds enter the environment as a necessary outcome of their preparation and use. Many materials escape into the workplace environment, while others are disseminated into the environment as wastes, either intentionally or by accident, from industrial plants. In addition, combustion processes inadvertently disperse carcinogenically active compounds.

A recent study by the U.S. National Cancer Institute shows that cancer mortality is most pronounced in urban and industrialized areas, and particularly where the density of petrochemical processing is high (Hoover and Fraumeni, 1975). There is reason, therefore, to regard production and use of organic chemicals as a major source of environmental carcinogens.

Cancer derived from environmental contaminants confronts us with both a serious problem and an important opportunity. The problem, which has been given increasing recognition, is detecting, identifying, and tracing the origins of the compounds that must account for environmentally induced cancer. If we can succeed in this aim, we then have the opportunity to act to limit human exposure to these agents, and it should then be possible to reduce the incidence of cancer even as the effect of the rising dissemination of such agents develops.

To achieve this goal there is an initial, basic prerequisite: achieving a capability for analyzing environmental samples, each likely to contain a number of compounds, some of which are carcinogenic.

The conventional method by which agents producing risk in the environment are detected involves the analysis of environmental samples for the detection of specific chemicals from some "priority lists": to determine whether such chemicals are present, and whether they are present in concentration above some previously established "safe limit".

In the United States, reference is made to the Toxic Pollutant List or the EPA "Priority Pollutants" List (Keith and Telliard, 1979). In Canada, reference is made to the List of Priority Chemicals and Schedule to the Environmental Contaminants Act (Dept. of the Environment and Dept. of National Health and Welfare, 1980).

The conventional method for detecting agents producing risk in an aquatic system will de described in detail along with the shortcomings of this method. As a result, of these shortcomings, there is a need in Canada for reliable chemical and biological screening methods for detecting and assessing the significance of the discharge of hazardous materials into the aquatic environment, particularly as it relates to biological communities. Tobin Lake, Saskatchewan, provided a unique opportunity to develop such a scheme in a natural environmental ecosystem, since this reservoir acts as a primary sink for deposition of contaminants in the North and South Saskatchewan Rivers (Birkholz et al., 1980).

Using the conventional method to detect those agents producing risk in the environment is laborious and costly and does not address the synergistic and antagonistic interactions of chemicals. Furthermore, the literature that distinguishes between compounds that are toxic, and those that are not, is far from complete or unambiguous.

A more logical strategy to determine which components pose the greatest long and short term risk to biological systems is to apply short term biological screening assays to fractionated extracts of environmental samples.

Towards this objective, a method has been developed involving extraction and chemical fractionation of Tobin Lake sediment, coupled with biological testing of the fractions, as a means of establishing

which components of the contaminated system provide the greatest toxic potential. By applying this detection method to a fractionated sample extract, toxic fractions are located and subjected to analysis for the identification of the toxic components. Using this scheme, the primary objective is to establish which fractions pose the greatest risk and to identify those components present in these fractions with which the risk can then be associated. This new approach, which is the subject of this thesis, differs substantially from conventional procedures.

The objectives of this study were:

(1) to determine the presence of toxic substances in the sediments of Tobin Lake;

(2) to determine which fractions of Tobin Lake sediment extract pose the greatest risk to the biological systems in this lake. This is accomplished by testing the fractions for toxicity using the Salmonella/microsome assay and the P. redivivus assay; and

(3) to identify some of the toxic compounds responsible for this risk by advanced analytical techniques such as electron capture capillary gas chromatography, Hall electrolytic conductivity capillary gas chromatography, and capillary gas chromatography - mass spectrometry (GC/MS).

PART 1. The Conventional Method for Detecting Toxic Agents in the Environment

(1.1) INTRODUCTION

The conventional method for detecting toxic agents in the environment in the United States of America resulted from a lawsuit brought against the United States Environmental Protection Agency (EPA) by the National Resources Defense Council and the Environmental Defense Fund for not setting effluent limits as specified in the 1972 Clean Water Act (Keith and Telliard, 1979). In 1976 the suit ended in the "Flannery Decision", generally referred to as the consent decree. Under the consent decree, the EPA was committed to establishing effluent guidelines for 21 major industries covering 65 classes of toxic chemical compounds (ACS, 1978). The criteria employed to priorize and select specific representative compounds for each group were as follows:

(1) All compounds except those named in the Toxic Pollutant List were considered if they were found with a frequency of greater than or equal to 5% of the total known listings for that class of compounds in industrial effluents.

(2) Chemical production data was used as a guide for priorizing choices when they were available.

(3) Reference to a compendium of all known organic pollutants identified worldwide in water through June 1976 was used as a guide for priorizing choices.

(4) All compounds specifically named in the Toxic Pollutant List, as part of the consent decree, were automatically included.

(5) The availability of chemical standards for verification and quantification was considered a mandatory prerequisite to compound selection.

Previous experience with natural and drinking water samples had shown that many compounds in water could be identified and semi-quantified at the 1 part per billion (ppb) level using computer controlled GC/MS. Therefore, 10 ppb was suggested as a reasonable level to analyze for industrial effluents (Keith and Telliard, 1979).

In Canada, three sets of criteria that pertain to the hazards posed by a chemical substance to human health or the environment are applied to the selection of chemicals for the Priority Chemicals List (Dept. of the Environment and Dept. of National Health and Welfare, 1979):

(1) Toxic effects criteria. Evaluation of scientific data leads to the conclusion that the chemical substance could cause or causes adverse effects on human health or the environment.

(2) Persistence criteria. Evaluation of scientific data leads to the conclusion that the chemical substance accumulates or could accumulate to significant concentrations in air, water, soil, sediment or tissue.

(3) Quantity and use criteria. Evaluation of available data on the importation, manufacture or processing of a chemical substance leads to the conclusion that the substance could enter or has entered the environment in significant quantities.

For many substances, no release regulations are in effect, thereby making it difficult to curb industrial discharges of these chemicals (Dept. of the Environment and Dept. of National Health and Welfare, 1979).

The conventional method described for detecting toxic agents in the environment suffers from a number of serious shortcomings as follows:

(1) Risk is associated purely with the presence or absence of chemicals in environmental samples as defined on some priorities list.

(2) Toxic chemicals present in the environment, but not incorporated into a priorities list are not addressed.

(3) The conventional method requires the complete analysis of all samples taken for the presence of all the listed priority chemicals. This is cumbersome, slow, and costly as exemplified by the analysis for the EPA priority pollutants in environmental samples (Finnigan et al. , 1979).

The conventional approach is valid for a system which has a limited exposure to one particular type of contamination. However, many real ecosystems are exposed to a wide range of environmental contaminants from a wide range of sources.

The Saskatchewan River system, which extends through the provinces of Alberta, Saskatchewan and into Manitoba is exposed to contamination from a number of sources. These include agricultural runoff, industrial waste discharges, municipal waste discharges and combustion processes. The two main industrial waste discharges include those from the petrochemical industry and the pulp and paper industry.

The Saskatchewan River system, as depicted in Figure 1, flows into Tobin Lake and is impounded behind the Squaw Rapids Dam.

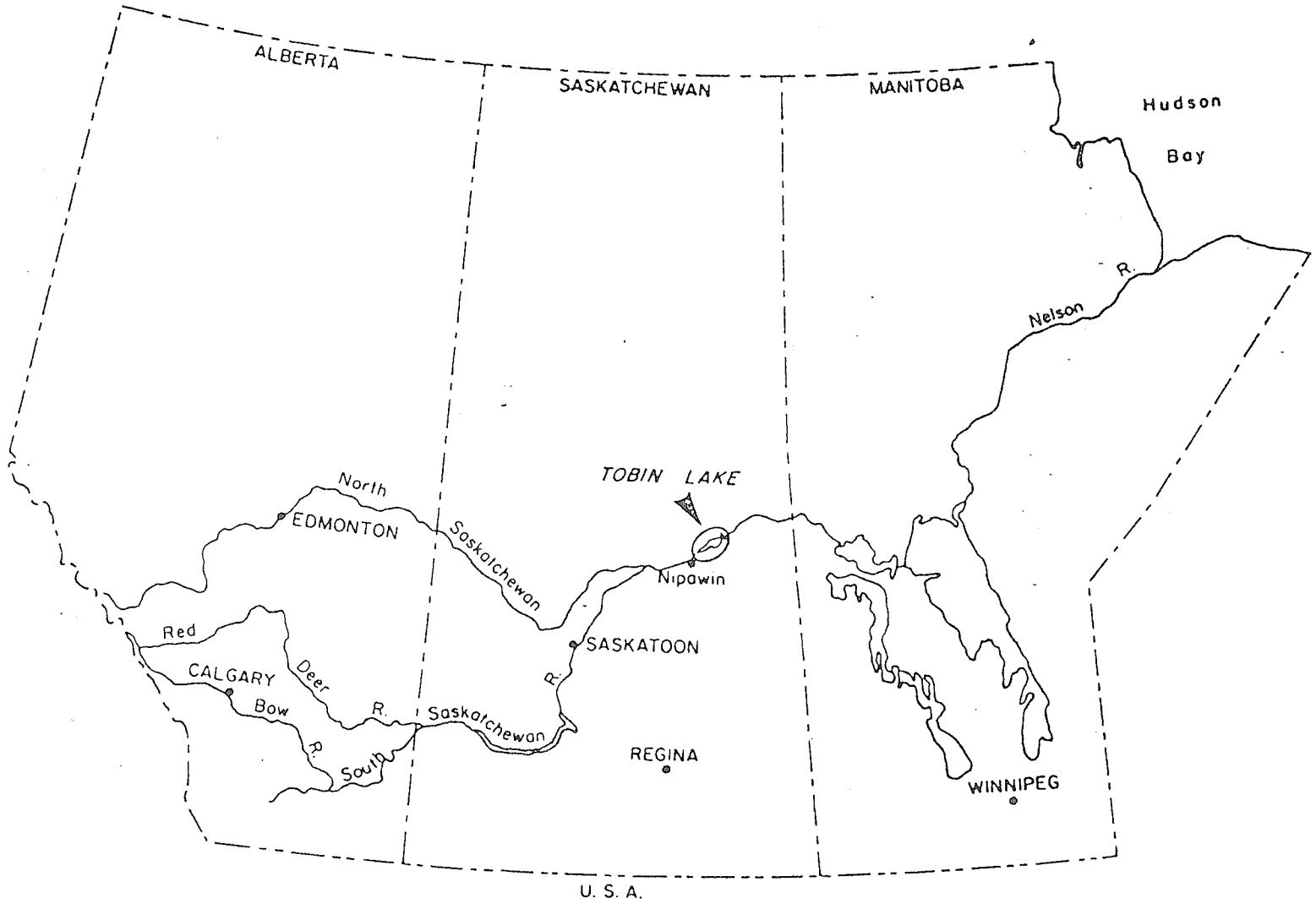


FIGURE 1. Tobin Lake acts as a Settling Basin for much of the Saskatchewan River Watershed (from Birkholz et al., 1980).

Since many toxic substances are relatively insoluble in water, and when released to a water body, associate with suspended matter (Alford, 1977), the sediment present in Tobin Lake represents a sink for contaminants discharged into the Saskatchewan River system. We would expect to find associated with this sediment a degree of toxicity or risk to the benthic populations associated with Tobin Lake. Studies by Warwick (Birkholz et al., 1980) have demonstrated that the Chironomid population in Tobin Lake carry morphological deformations and developmental deformations similar to those that are seen in systems which have suffered extreme exposure to toxic chemicals, (Warwick, 1980).

The question we are faced with is the determination of the causative agents of toxicity in the Tobin Lake system.

Since many chemicals are suspected to be present in Tobin Lake sediment, synergistic and antagonistic interactions between chemicals are a real possibility. Furthermore, very little is known about the metabolic conversions of many contaminants in the ecosystem. Rather than analyze for toxic chemicals present on a list, such as the EPA Priority Pollutants List, a new approach was needed in an attempt to isolate and identify chemicals with which actual toxicity could be associated.

(1.2) BIOLOGICAL TESTING FOR ENVIRONMENTAL CONTAMINANTS

(1.2.1) TESTING FOR LONG AND SHORT TERM EFFECTS

Detecting environmental chemical hazards which cause immediate (or acute) toxic effects is a relatively straightforward task. For

example, Leach and Thakore (1973) used juvenile coho salmon (Oncorhynchus kistuch) to detect acutely toxic nonvolatile constituents in a kraft pulping effluent. Using column adsorption techniques followed by partition chromatography these authors were able to isolate acutely toxic agents which eventually lead to the identification of the toxic diterpene resin acids.

Rogers and Mahood (1974) also isolated toxic chemicals from a kraft mill effluent using column adsorption followed by partition chromatography. Using juvenile sockeye salmon (Oncorhynchus nerka) they were able to isolate toxic fractions; they identified the toxic agents using gas chromatography - mass spectroscopy (GC/MS). Toxicity to sockeye salmon smolts was associated with diterpene resin acids and diterpene aldehydes and ketones closely related to the resin acids.

Rogers and Keith (1976) isolated and identified two toxic organochlorine compounds from kraft bleaching effluent using chemical fractionation and bioassay. Using column adsorption techniques followed by partition chromatography, these authors observed extreme toxicity with acidic compounds. They speculated that the toxicity was due to the presence of chlorinated acids and/or chlorinated phenols. After extensive investigation these workers identified conclusively the presence of 4,5,6-trichloroguiacol and 3,4,5,6-tetrachloroguiacol, both of which were subsequently shown to be toxic.

Detecting the long-term (or chronic) toxic effects associated with environmental biohazards is much more difficult. Chronic effects such as cancer, birth defects, and genetic disease characteristically appear several years or decades after the initial chemical exposure has occurred, and long-term studies using live animals must be conducted in order to detect these latent effects. Such studies are expensive and time-consuming, and require the use of highly specialized facilities and personnel. A single test of this sort for a chemical's carcinogenicity (cancer-causing ability) may take as long as 3 years and cost $250,000 or more.

In response to this situation, short-term tests have been developed to serve as rapid and relatively inexpensive predictors of a chemical's potential to cause chronic effects. These tests employ bacteria, yeast, plants, insects, isolated mammalian cells and whole animals. Short-term tests can detect a chemical's genotoxicity, that is, its ability to alter a cell's genetic material, namely, deoxyribonucleic acid (DNA). An increasing amount of evidence exists to indicate that latent diseases such as cancer, birth defects, and genetic disease may be initiated by alterations in the DNA (United States Environmental Protection Agency, 1979b).

(1.2.2) SHORT-TERM TESTS FOR CARCINOGENS, MUTAGENS AND OTHER GENOTOXIC AGENTS

Because of their rapid and inexpensive nature, short-term tests are extremely useful for screening and identifying toxic substances. According to the EPA (United States Environmental Protection Agency, 1979b), five different types of biological activity related to genotoxicity can be studied in short-terms tests; namely,

1) DNA damage and repair

2) Gene mutation

3) Chromosome alterations

4) Cancer-like (oncogenic) cell transformation

5) Tumor formation

1) DNA damage and repair

Whenever a cell's DNA is disturbed or damaged, cell mechanisms come into action to repair the damaged parts. DNA damage and repair tests take advantage of this fact in searching for evidence of genotoxic effects. These tests look either for direct evidence of alterations in the DNA, or for evidence that DNA repair mechanisms are in action. If the repair mechanisms can be demonstrated to be operating above the normal level, then damage to the DNA is indicated. DNA damage and repair tests are available using bacteria, yeast, mammalian cells, and whole animals.

2) Gene Mutations

Gene (or point) mutations are submicroscopic DNA alterations occurring in a single gene and leading to an altered gene product. Most gene products are proteins. Since proteins are involved in all the chemical reactions taking place in a cell or organism, the biological consequences of even a small change can be severe. For instance, a minute change in an enzyme (a type or protein) can interfere with a cell's normal functioning by making a key chemical reaction impossible.

Gene mutations are most easily detected by looking for altered gene products, such as enzymes. An enzyme deficiency can manifest itself in any number of ways that can be conveniently measured. In test systems using bacteria, yeast, or mammalian cells in culture, a cell's requirement for a certain nutrient may change, or its tolerance of a chemical poison may be altered. In short-term tests involving whole organisms such as fruit flies or plants, specific changes in the test organism's features (i.e., color or shape) can be observed as evidence of mutation.

3) Chromosome Aberrations

Chromosome alterations or aberrations are microscopically visible disturbances in chromosomes. They can include the loss or gain of entire chromosomes, chromosome breaks, and faulty assembly processes such as nondisjunctions and translocations. Chromosomal aberrations are a major cause of heritable human disease, and their occurrence is often associated with cancer. They are detected either by searching for microscopically evident alterations or by examining tissues or organisms for traits known to result from such alterations. Cells from insects or mammals are frequently used.

4) Oncogenic Transformation

Oncogenic transformation is the chemically induced conversion of normal cultured mammalian cells into malignant-like cells. Whether or not transformed cells are actually malignant (or cancerous) can be ascertained by injecting them into whole animals to see if they give

rise to tumors. Most frequently, transformed cells are distinguished in culture by abnormal growth patterns that are visible under the light microscope. Syrian hamster embryo cells or cells from the mouse cell line BALB/C3T3 and C3H10T1/2 are frequently used.

5) Tumor Formation

Tumor formation in rodents is a definitive indicator of a chemical's carcinogenicity. Short-term tests measuring tumor formation use special strains of mice and rats that develop tumors especially rapidly - within 10 to 26 weeks of chemical treatment.

In short-term tests for tumor formation, the number of tumors appearing in treated animals is compared to the number that have appeared spontaneously in an untreated control group of animals. A higher number of tumors in the treated animals indicates potential carcinogenicity. Non malignant tumors may be counted when their presence correlates with the later appearance of malignant tumors.

Two short-term tests which can be used to screen for the presence of potentially hazardous substances include the Salmonella/microsome mutagenicity assay (Ames test) developed by Dr. Bruce Ames and co-workers at the University of California, Berkely (Ames et al., 1975a) and the Panagrellus redivivus assay developed by Dr. Martin Samoiloff and co-workers at the University of Manitoba (Samoiloff et al., 1980). The Ames test detects gene mutations whereas the P. redivivus assay detects gene mutations as well as chromosome alterations.

(1.2.3) THE SALMONELLA/MICROSOME MUTAGENICITY TEST (AMES TEST)

The Ames test detects chemicals by means of their mutagenicity (ability to damage DNA, the genetic material) and is about 90% accurate in detecting carcinogens as mutagens (Hushon et al. , 1979). During the past decade the somatic-cell mutation theory of carcinogenesis has gained widespread acceptance. This theory states that alteration of genetic material in a somatic cell upon exposure to an external agent or substance may result in changes in the cell's growth pattern that transform it to the malignant state of uncontrolled growth. This theory has gained credence as most carcinogens also show positive results in mutagenicity tests (Hushon et al. , 1979).

The Ames test uses the bacterium Salmonella typhimurium as the target and measures the mutagenic capacity of test substances. The test is based on the well substantiated belief that chemicals which cause cancer do so by inflicting mutations of various types (Lewin, 1976). Unlike most animal tests, the Ames system is, therefore, not measuring carcinogenicity directly, but, instead is detecting mutagenicity. The concept behind the Ames test is to exploit bacteria that are unable to grow normally because they have a mutation in the gene group responsible for the manufacture of the amino acid histidine. If the bacteria are exposed to mutagens there is a probability that the original histidine mutation will be repaired accidentally; i.e., that it will revert. Since there are about one billion bacteria on a single test

dish, there is a high probability for this to happen. Once a single bacterium has reverted it can grow and establish a colony against a background of still dormant mutants.

By themselves the bacteria in Ames's system are not a good model for human carcinogenesis; however, by adding to the bacteria an extract from rat or human liver, many of the metabolic conversions encountered by chemicals entering the body are simulated (Lewin, 1976).

There are several standard bacterial tester strains, containing different types of histidine mutations which can be used for mutagenesis testing. One strain (TA1535) can be used to detect mutagens causing base-pair substitutions and two (TA1537 and TA1538) to detect various kinds of frameshift mutagens. Frameshift mutations occur by shifted pairing in repetitive sequences of DNA and frameshift mutagens can be very specific for the particular sequences they mutate. TA 1538 has a repetitive -C-G-C-G-C-G-C-G- (Cytosine-Guanine) sequence near the site of the histidine mutation, and is reverted particularly well by many carcinogens, such as 2-nitrosofluorene. The other frameshift tester strain, TA1537, appears to have a run of C's at the site of mutation and, is reverted well by carcinogens such as 9-aminoacridine. In addition to the histidine mutation, each tester strain contains two additional mutations that greatly increase its sensitivity to mutagens. One causes loss of the excision repair system, and the other, loss of the lipopolysaccharide barrier that coats the surface of the bacteria. By transferring a resistance transfer factor (R factor) to the tester

strains TA1535 and TA1538, two new strains were developed, namely TA100 and TA98. These strains are extremely sensitive to a number of carcinogens not detected with parent strains. The polycyclic-hydrocarbons are an example.

For screening purposes the following tester strains are recommended: TA1535, TA1537, TA100 and TA98 (Ames et al., 1975a). The sample to be tested along with approximately 1 billion bacteria of a particular tester strain and rat (or human) liver homogenate are combined on a petri dish. Following incubation at 37°C for 2 days the number of bacterial colonies present in the dish are scored.

Not only does the Ames system give an answer as to whether a test substance is mutagenic or not, it can also measure the degree of mutagenicity: potent mutagens revert more bacteria, giving a higher number of growing colonies.

This kind of dose-response information which is very difficult to obtain with animal trials, is particularly important. When screening for environmental carcinogens, it is necessary to determine degree of risk based on factors such as amount of chemical involved in exposure, and the carcinogenic potency (Lewin, 1976).

(1.2.4) TESTING FOR ENVIRONMENTAL HAZARDS USING THE AMES TEST

While still in its infancy, the Ames test was used primarily as a prescreen for determining the genetic and potential carcinogenic hazard associated with "pure" compounds. Up until 1976 about 300

carcinogens and non-carcinogens of a wide variety of chemical types were tested. The results of these tests were published in two papers (McCann et al. , 1975 and McCann and Ames, 1976).

Recently, the Ames test has been used as a screen for the presence of hazardous compounds in the environment and in commercial products. Talcott and Wei (1977) assayed particulate air borne pollutants, collected in Buffalo, New York, and Berkeley, California for mutagenic activity using the Ames Salmonella typhimurium test system. The positive activity of the samples was attributed to the presence of polynuclear aromatic hydrocarbon derivatives (PAH) due to the nature of the response; and the presence of PAH was confirmed through the use of thin-layer chromatography (TLC).

The Ames test has also proven useful in a detailed study that has been made of the mutagenic activity of cigarette smoke condensate and 12 standard smoke condensate fractions. An analysis of fractions of smoke condensate revealed that the detected mutagenic activity was distributed in several of the fractions. Most of the activity of the whole condensate was in basic fractions and in weakly acidic fractions, (Kier et al. , 1974).

Ames et al. (1975b) demonstrated that 89% of commercial oxidative (hydrogen peroxide) hair dye formulations were mutagenic with the Salmonella typhimurium assay. Of the 18 components of these dyes, nine showed various degrees of mutagenicity. Three hair dye components (p-phenylenediamine, 2,5-diaminotoluene and 2,5-diaminoanisole) became strongly mutagenic after oxidation by H_2O_2. Another hair dye

component, 2,4-diaminotoluene was also shown to be mutagenic.

Commoner (1977) systematically analyzed for the occurrence of mutagenically active substances in air particulate samples. Standard high-volume air sample filter papers were extracted with benzene/hexane and the residues taken up in dimethyl sulfoxide (DMSO) and applied to Salmonella test plates. He demonstrated that it was possible to fractionate such samples and recover the original activity in the separate fractions. Fractionation was accomplished using thin layer chromatography.

Epler et al. (1978) fractionated crude products from coal - conversion processes and natural crude oils into primary classes (acids, bases, and neutrals) by liquid - liquid extraction, and then further fractionated by column chromatography using Florosil. Fractions and/ or control compounds to be tested were suspended in DMSO and assayed for mutagenicity with the Salmonella/microsomal activation system (Ames test). The neutral fractions and certain basic fractions accounted for most of the activity. Polynuclear aromatic hydrocarbons were assumed to be present in the neutral fraction and believed responsible for the activity whereas heterocyclic nitrogen compounds are believed to be responsible for the activity in the basic fraction.

Rubin et al.(1976) fractionated products from two coal liquefaction processes and tested the various fractions from the liquid fuel samples using the Salmonella typhimurium assay developed by Ames. Four strains of bacteria were used which allowed for the

detection of mutagens that induce base-pair substitutions and frameshift mutations. Several fractions showed mutagenic effects. Some overlapping of compound types in successive fractions eluted from the Florosil column (eg. PAH's) occurred. Tumor promoting activity was observed mainly in the ether soluble weak acid fraction which contained mostly phenolic compounds. Mutagenic activity was also noted in the ether soluble base fraction.

Rao et al. (1979) used the Ames Salmonella histidine reversion test to assay the mutagenic potential of chemically fractionated crude shale oil, product water from shale oil process and leachates and extracts from raw and spent shale. Two fractionation schemes were compared.

The shale oil contained significant activity in the neutral fractions and in other fractions, particularly in the basic fraction. The sum of activities from the neutral subfractions corresponds to the value obtained from the unfractionated neutral material.

The product water contained significant activity primarily in the basic fractions although the overall contribution of the contaminated organic portion appeared to be low.

The results obtained with the extracted and chromatographically separated materials from spent shale demonstrated the real utility of the fractionation process. The total benzene extract from spent shale masked any mutagenic effects through its extreme toxicity; however, the individual chemical fractions obtained via TLC demonstrated mutagenic activity.

Mutagenicity was detected in both neutral and polar fractions. Correlating the biological activity to compounds that were either identified or predicted to occur in these materials suggested the presence of polycyclic aromatic hydrocarbons and nitrogenous polycondensed species such as acridine, dibenzacridines, along with some acids, phenols and high molecular weight aromatic amines.

Tabor and Loper (1980) used analytical fractionation coupled with the Salmonella microsome mutagenicity assay to isolate toxic components from organic residues of drinking water. The method essentially consisted of 1) a semi-solid/liquid extraction 2) reverse phase high pressure liquid chromatography of the neutral fraction 3) Sep-pak concentration and solvent exchange of HPLC subfractions for chemical and biological characterization. A 125-g archival sample of carbon/chloroform extracted organics prepared from 190,000 L of finished Cincinnati drinking water in 1962 was used as a model. Three mutagenic HPLC subfractions were isolated, accounting for the bulk of the activity of this complex neutral fraction. Preliminary GC/MS results indicate the mutagenic activity is due to isomeric chlorinated aliphatic ethers.

This data demonstrated that drinking water contained a large number of compounds (perhaps thousands, most of which are non-mutagenic) only a small number of which are highly mutagenic. Analytical fractionation and coupled bioassay has been shown to be a logical way to isolate and identify these toxic agents.

(1.2.5) THE PANAGRELLUS REDIVIVUS ASSAY

The free-living nematode Panagrellus redivivus has been shown to be a good bioassay organism for the detection of mutagens and carcinogens in aquatic environments (Samoiloff et al. , 1980). It has several biological characteristics that make it a good test organism for the detection of environmental contaminants (Samoiloff, 1981):

1. It has a short life span, going from the earliest free-swimming stage to an adult in 4 days.
2. It contains specific digestive, excretory, integumentary, muscular, nervous and reproductive systems, but contains only 520 somatic cells.
3. The postembryonic development of Panagrellus redivivus has been well defined, and the effects of mutagens, inhibitors of macromolecular biosynthesis, radiation, carcinogens, nutritional stress, and neuroinhibitors on this development have been presented.
4. Panagrellus redivivus thrive in a liquid medium or on a solid substrate, nutritional requirements are simple and synchronous cultures can be easily established.
5. Panagrellus redivivus is a hardy organism, approximately 1000 times less sensitive to toxic chemicals than mammalian systems.
6. Panagrellus redivivus has been utilized to assay for mutagens and carcinogens, giving a sensitivity similar to those reported for bacterial and mammalian assays.

Panagrellus redivivus is ovoviviparous. Under standard conditions, embryogenesis takes approximately 20 hours, producing a first stage juvenile (L1) within the egg shell. The L1 undergoes a moult giving rise to a second stage juvenile (L2), which emerges from the egg shell and is actively expelled by the female from the oviduct via the vulva. L2 is the first free-swimming stage, readily isolated from mass cultures.

L2 animals held in a salt solution will not grow or develop. In the presence of nutrients, the L2 grows and moults to the third juvenile stage (L3). The L3 grows and moults to the fourth juvenile stage (L4) which, under appropriate conditions, grows and moults to the adult stage. The moults are entrained to environmental stimuli and require bursts of RNA and protein biosynthesis.

The major criterion for determination of each stage is length. The L2 to L3 moult occurs at a length of approximately 350 microns, which represents the upper limit for the length of the L2 stage. The L3 to L4 and L4 to adult moults occur at approximately 550 and 850 microns respectively. Adults may reach lengths of 1800 microns. During post-embryonic development the number of somatic nuclei remains nearly constant, with approximately 520 somatic cells in the organism. The duration of postembryonic development, from L2 to adult, varies with culture conditions. For purposes of biological assay, conditions were established such that 50% of wild-type (C-15) L2 animals will reach the adult stage in a 96 h growth period. Growth for longer than this period results in the

reproduction of those animals that have reached the adult stage.

The developmental assay utilizes the standard "wild-type" strain C-15 of Panagrellus redivivus obtained by two rounds of 15 generations of sib-sib matings. To obtain synchronous L2 animals, gravid females are transferred to a drop of M9 buffer on an agar surface. For each developmental assay 150 to 300 gravid females are transferred to M9 buffer, and the L2 progeny harvested after 14-16 hours.

The agent to be tested is mixed in autoclaved M9-yeast-cholesterol medium. Ten second stage juveniles are placed in each of a series of 2.5 mL autoanalyzer cups containing 0.5 mL M9-yeast-cholesterol medium with or without a known concentration of the agent tested. Each experiment consists of 10 such cultures for each agent tested. Each experiment is replicated 3 times, using freshly prepared media each time. Test animals are grown for 96 hours at 22°C. After the growth period, the number of survivors in each cup are recorded and the worms transferred from the cups to a microscope slide.

The slide is heated to dryness at 50°C to heat kill and relax the nematodes which are stained with cotton blue lacto-phenol and measured. In this manner, the size distribution of each experimental population is determined. From this size distribution can be calculated a probabilistic measure of developmental success. For example, if a, b, c and d represents

the number of L2, L3, L4 and adult animals respectively, in the experimental population, the frequencies of successful completion of the three moults can be calculated by:

$$P_1 = \frac{b + c + d}{a + b + c + d}$$

$$P_2 = \frac{c + d}{b + c + d}$$

$$P_3 = \frac{d}{c + d}$$

The values P_1, P_2, and P_3 represent the frequency of successful development through the L2 to L3, L3 to L4, and L4 to adult moults, respectively (Samoiloff et al., 1980).

For statistical analysis of the distribution of stages following the 96-h growth period, each group of animals exposed to a sample extract is compared to a control population which has been exposed to a reagent blank. Comparisons are made using a 2 X 4 contingency analysis or Chi-square test (Wagner et al., 1980). For example, if a, b, c and d represents the number of L2, L3, L4 and adult animals observed in a population of nematodes exposed to a sample extract and e, f, g and h represents the number of L2, L3, L4 and adult animals observed in a population of nematodes exposed to a reagent blank, χ^2 is determined as follows:

$$\frac{(a-e)^2}{e} + \frac{(b-f)^2}{f} + \frac{(c-g)^2}{g} + \frac{(d-h)^2}{h} = \chi^2$$

An example should clarify these calculations

Sample	Size Distribution			
	L2	L3	L4	Adult
Test Sample	17	43	31	2
Control	41	49	5	0

$$\chi^2 = \frac{(17-41)^2}{41} + \frac{(43-49)^2}{49} + \frac{(31-5)^2}{5} + \frac{(2-0)^2}{0}$$

$$= 14 + 0.73 + 135 + 0$$

$$= 150$$

P_1 - Test Sample = $(43 + 31 + 2)/(17 + 43 + 31 + 2) = 0.82$

P_2 - Test Sample = $(31 + 2)/(43 + 31 + 2) = 0.43$

P_3 - Test Sample = $2/(31 + 2) = 0.06$

P_1 - Control = $(49 + 5 + 0)/(41 + 49 + 5 + 0) = 0.57$

P_2 - Control = $(5 + 0)/(49 + 5 + 0) = 0.09$

P_3 - Control = $0/(5 + 0) = 0$

These calculations can be summarized as follows:

Sample	P_1	P_2	P_3	CHI-SQUARE
TEST	0.82	0.43	0.06	150
CONTROL	0.57	0.09	0.00	

A decreased ability to complete the L4 to adult moult, as determined by the reduction in P_3, is the most sensitive indicator of toxicity, while survival is the least sensitive. If failure to complete

a moult represents the effects of either mutagenesis or abnormal information processing, one would expect that P_3 would be the most sensitive indicator, since the final moult requires the most gene activity (Samoiloff et al. , 1980). In other words, an observed decrease in P_3 for a population of worms exposed to a sample extract relative to an observed P_3 for a control population exposed to a reagent blank is indicative of mutagenesis. A decrease in P_1 and P_2 values for a population of worms exposed to a sample extract relative to a control population is indicative of toxicity. This may manifest itself as mortality or developmental inhibition. The Chi Square value tells us whether or not the observed toxicity is significant. A χ^2 value of greater than 15 is indicative of significant toxicity (Samoiloff, 1982).

1.3 CONVENTIONAL ANALYTICAL METHODS

(1.3.1) SAMPLE CONTAINERS AND CLEANING PROCEDURES

Environment Canada (1979) recommends the use of an all-glass system or metal container for the collection of sediment samples. Lining the cap of the sample bottle with cleaned aluminum foil to prevent the sample from contacting the glue lining of the bottle top is also suggested. Aluminum cans are recommended as an alternative to the all glass system. This is supported by the United States Environmental Protection Agency (1980a) which recommends the use of quart or 2-quart size Mason jars for the collection of environmental samples. One layer of industrial gauge aluminum foil (0.001 in.) or two layers of regular household grade foil should be used as cap liner.

Chau (1972) recommends the following procedure for the cleaning of glassware including sample containers:

(1) Use soap and water and wash vigorously or rinse several times with agitation.

(2) Rinse well with hot water.

(3) Rinse with distilled water.

(4) Wash twice with washing acetone.

(5) Without drying, shake vigorously with pesticide grade ethyl acetate. Repeat.

(6) Without drying, shake vigorously with pesticide grade hexane.

(7) Dry in oven at 300°C until ready for use.

The United States Environmental Protection Agency (1980a) recommends the following basic cleaning steps:

(1) Removal of surface residuals immediately after use.

(2) Hot soak to loosen and flotate most of soil.

(3) Hot water rinse to flush away flotated soil.

(4) Soak with deep penetrant or oxidizing agent to destroy traces of organic soil.

(5) Hot water rinse to flush away materials loosened by deep penetrant soak.

(6) Distilled water rinse to remove metallic deposits from the tap water.

(7) Acetone rinse to flush off any final traces of organic material.

(8) A preliminary flush of the glassware just before using with the same solvent to be used in the analysis.

(1.3.2) SAMPLE STORAGE CONDITIONS

Environment Canada (1979) recommends that sediment samples be frozen immediately after collection in an all glass system or metal container as a preservation step prior to analysis for pesticides and polychlorinated biphenyls (PCBs).

The United States Geological Survey (1972) recommends that sediment samples taken for chlorinated hydrocarbon insecticide analysis be refrigerated and protected from light prior to analysis. In the case of sediment samples taken for the analysis of chlorinated phenoxy acid herbicides they recommend freezing prior to analysis.

The United States Environmental Protection Agency (1980a) recommends that agricultural or environmental samples that are to be analyzed for organophosphates be placed in tight containers and stored in a deep freeze as soon as possible after sampling unless sample preparation is to be conducted within a few hours.

(1.3.3) SAMPLE PREPARATION

Giger and Schaffner (1978) froze aliquots of wet sediment via a cooled isopropyl alcohol bath under continuous rotation. Subsequently the samples were evaporated and freeze-dried. Lyophilization times varied between 4 and 14h at a pressure of approximately 0.05 Torr.

Freeze drying of the samples was chosen because it allows a much cleaner sample storage and handling. No significant losses of PAH and alkane reference compounds were observed.

Coburn and Comba (1981) freeze dried sediments collected from several small craft harbors on the Great Lakes prior to analysis for chlorinated hydrocarbon pesticides and polychlorinated biphenyls (PCB). Polychlorinated diphenyl ethers were isolated and subsequently identified using this technique.

Hom et al. (1974) used freeze drying as a sample preparation method to study the deposition of DDE and PCBs in dated sediment.

(1.3.4) EXTRACTION METHODS FOR SOIL AND SEDIMENT

Bellar and Lichtenberg (1975) evaluated several methods for the extraction of polychlorinated biphenyls from fortified sediments. The results obtained demonstrated that air-dried sediment with 10 percent moisture added prior to Soxhlet extraction with hexane: acetone (9:1) provided significantly greater extraction efficiency than any of the other methods tested.

Pionke et al. (1968) found the Soxhlet system with n-hexane: acetone (41:59) for 12 hours using 200 mL of the solvent mixture with 100 g of soil to be the most efficient method for the extraction of organochlorine insecticides.

Williams (1968) compared four extraction methods and obtained

the greatest recovery of organochlorine insecticides from Fraser River silt loam when water was added to the air dried soil (5-40% w/w) before Soxhlet extraction with hexane:acetone (41:59).

Woolson and Kearney (1969) in a report of a collaborative test of 12 laboratories for the extraction and detection of 11 chlorinated hydrocarbon insecticides in three soil types, noted that wetting the soil and Soxhlet extraction with hexane:acetone (1:1) gave the highest recoveries.

Seidl and Ballschmiter (1976) compared five extraction methods and obtained the greatest recovery for ^{14}C-labelled Clophen A-30 (a mixture of di-, tri- and tetrachlorobiphenyls) from dry forest soil using Soxhlet extraction with acetone or acetonitrile.

(1.3.5) SAMPLE CLEANUP

Following extraction of sediment samples with acetone: hexane (1:3) for chlorinated insecticides, Goerlitz and Law (1974) partitioned the extract (320 mL) with distilled water (500 mL). Following separation of the solvent, the water was extracted with hexane, and the extracts were combined and washed with fresh distilled water. These steps were followed primarily to remove acetone which could interfere with any column chromatography. The extract was dried by addition of sodium sulfate, preconcentrated using a Kuderna-Danish apparatus and chromatographed on an alumina column. Two

fractions were obtained by eluting with 20 mL of hexane and continuing to 35 mL.

The United States Environmental Protection Agency (1978) recommends the following cleanup and separation procedures for the determination of organochlorine pesticides in industrial effluents. Following extraction of the effluent with 15% methylene chloride in hexane, the extract is carried through an acetonitrile partition. This procedure is used to isolate fats and oils from the sample extracts. Further cleanup is achieved using Florisil column adsorption chromatography. Four fractions are obtained by eluting with 6% ethyl ether in petroleum ether, 15% ethyl ether in petroleum ether, 50% ethyl ether in petroleum ether and 100% ethyl ether.

In determining concentrations of priority pollutants in industrial effluents that are associated with the consent decree and are solvent extractable and amenable to gas chromatography, the United States Environmental Protection Agency (1979a) recommends the following scheme. Base/neutral compounds are separated by adjusting the pH of the sample with 6N NaOH to pH 11 or greater. Multirange pH paper is used for the measurements. Extraction is effected by using methylene chloride. Acidic compounds are separated by adjusting the pH of the water sample previously extracted for base-neutrals with 6N H_2SO_4 to pH 2 or below. Extraction is again carried out using methylene chloride.

Recently, a list of voluntary reference compounds was formulated that could be widely used to compare improvements in new or modified techniques with existing methodology (Keith, 1979). This list has been designated as consenus voluntary reference compounds (CVRC) and has been approved by the Executive Committee of the ACS Division of Environmental Chemistry and has been endorsed by the ACS Joint Board Council Committee on Environmental Improvement. Known water pollutants were the prime candidates for reference compounds: the choice, however, was not restricted to water pollutants for it was more important to obtain a wide variety of representative compounds. Of the fifteen classifications of organic compounds depicted in the CVRC, ten classifications are depicted in the United States Environmental Protection Agency's analytical protocol. The extraction and separation schemes outlined by the U.S. EPA for the detection of priority pollutants and pesticides is large enough in scope that most of the CVRC's are included.

Leoni (1971) used a silica gel microcolumn to separate fifty pesticides and related compounds into four groups. Using a 5% water deactivated silica gel (w/w), elution was carried out using the following solvents: n-hexane, 60% benzene in n-hexane, benzene, and 50% ethyl acetate in benzene.

Following extraction of sediments from the Niagara River with isopropyl alcohol, Hites et al. (1980) chromatographed the extract on a Florisil column. This was accomplished by pre-concentrating the extract and exchanging it into dichloromethane (DCM).

The DCM extract was transferred to the head of the column and allowed to dry. Elution of compounds was carried out using hexane followed by hexane-DCM (10% v/v), hexane-DCM (50% v/v), DCM and finally methanol. The extracts were subjected to analysis by GC/MS in an attempt to identify anthropogenic compounds responsible for the high incidence of fish tumors in the western end of Lake Ontario and the eastern end of Lake Erie.

Tabor and Loper (1980) developed an analytical fractionation method for the isolation of mutagenic components from organic residues of drinking water. Starting with a 125 g archival sample of carbon/chloroform extracted organics prepared from 190,000 L of finished Cincinnati drinking water, these workers separated neutral, acidic and basic compounds by successive extractions with hexane under neutral, acidic (pH 2-1.5) and basic (pH 12) conditions. The remaining residue was neutralized and extracted into 1-butanol. Further separation of the neutral fraction was conducted by reverse phase octadecylsilane HPLC. Using a Waters radial compression module unit containing an 8 mm by 10 cm column packed with 10 μm silica particles bonded with octadecylsilane, these workers found that 20 mg samples could be routinely chromatographed. Following exchange of the neutral extract into acetonitrile, chromatography was initiated by applying a series of linear gradients from water to acetonitrile. A 254 nm absorbance detector was used to monitor the effluent and serve as a guide to the fraction collection. The elution procedure is depicted in Figure 2.

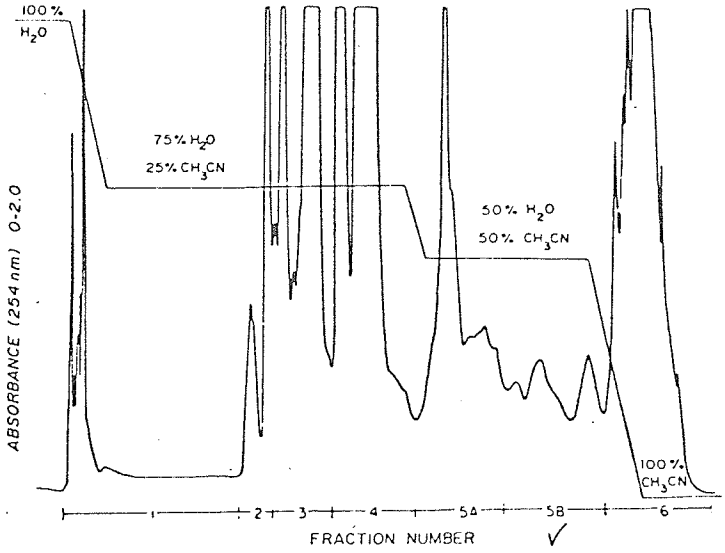


FIGURE 2 RCM/HPLC reverse phase HPLC of 20 mg sample of neutral fraction for mutagen isolation. Subfraction 5B containing activity for TA100 is indicated by a check, ✓.

(from Tabor and Loper, 1980)

After collection each fraction was diluted 3 fold with water. The diluted sample was then loaded and concentrated on a Sep-pak C_{18} cartridge. The compounds were eluted with a small volume of methylene chloride. To avoid taking the solutes to dryness, the methylene chloride was partially removed by evaporation and a small amount of DMSO was added. The remaining methylene chloride was then evaporated and portions of the resultant solution were plated for mutagenicity assay using the Ames test.

(1.3.6) REMOVAL OF SULPHUR INTERFERENCE

Elemental sulfur is encountered in most sediment samples, marine algae and some industrial wastes. The solubility of sulfur in various solvents is very similar to the organochlorine and organophosphate pesticides; therefore, the sulfur interference follows the pesticides through the normal extraction and cleanup techniques. The sulfur will be quite evident in gas chromatograms obtained from electron capture detectors, flame photometric detectors operated in the sulfur or phosphorus mode, and Coulson electrolytic conductivity detectors (United States Environmental Protection Agency, 1980a). In a GC/MS system this interference manifests itself in terms of S_8 molecules (Ahling and Jensen, 1970).

The United States Environmental Protection Agency (1980a) suggests removal of sulfur by reacting 1.0 mL of sample extract with bright copper powder. The amount of copper required is contingent upon the levels of sulfur present. After vortexing this mixture for 30 seconds, copper sulfide precipitates from solution and one can decant the supernatant and subject this to analysis.

Goerlitz and Law (1971) recommend shaking a small drop (100 to 200 microliters) of metallic mercury with the concentrated extract to remove sulfur and sulfur-organic compounds by precipitation. Elemental sulfur is removed very rapidly, within 1 or 2 minutes whereas other sulfur compounds react more slowly with the metallic mercury.

Jensen et al. (1977) described an efficient, rapid, nondestructive technique to remove sulfur according to the reaction:

$$2TBAHSO_4 + Na_2SO_3 + S_{(s)} \longrightarrow (TBA)_2S_2O_3 + 2NaHSO_4$$

where TBA = tetrabutylammonium ion

This was accomplished by taking the extract up in trimethylpentane (2mL) and shaking it with 1 mL of tetrabutylammonium hydrogen sulfate-sodium sulfite reagent for at least 1 min. If the precipitated sodium sulfite disappears, more is added in 100 mg portions until a solid residue remains after repeated shaking. Water (5 mL) is added and the test tube is shaken for another minute, followed by centrifugation, and the trimethylpentane is transferred to a test tube. The trimethylpentane phase extract is carefully shaken with an equal volume of sulfuric acid monohydrate. After centrifugation, the trimethylpentane phase is removed for analysis.

PART 2. Experimental

(2.1) INTRODUCTION

During the fall of 1979 through to the spring of 1980, a cooperative study was conducted at Tobin Lake, near Nipawin, Saskatchewan with participants from Environmental Protection Service, the University of Manitoba and the National Water Research Institute. Sediment samples were collected during four field trips in September and October of 1979 and in February and June of 1980. Field work was spearheaded by Dr. W. F. Warwick (National Water Research Institute) and Mr. J. Witteman (Environment Canada). Other participants in the field work included technical staff from the National Water Research Institute, Environment Canada, Parks Canada, Energy, Mines and Resources Canada, and Dr. G.R.B. Webster (Pesticide Research Laboratory, University of Manitoba). Sample preparation, extraction, fractionation, solvent exchange into biologically suitable solvents, and analysis was conducted by the author. Bioassay analysis of sediment extracts was performed by Dr. M. Samoiloff (Dept. of Zoology, University of Manitoba) and Mr. J. Bell (Environment Canada).

(2.2) MATERIALS AND METHODS

Pesticide grade organic solvents were obtained from Caledon Laboratories Inc. Anhydrous sodium sulfate was obtained from Fisher Scientific Co. Prior to use, this material was Soxhlet extracted with acetone:hexane (1:1, v/v) overnight and dried in a vacuum desiccator. It was stored in an oven maintained at 130°C until use. Florisil-PR grade (60/100 mesh)

was obtained from Floridin Company, Berkeley Springs, W. VA. This adsorbent was Soxhlet extracted with acetone:hexane (1:1, v/v) and dried in a vacuum desiccator. It was stored in an oven at 130°C until use. Pyrex glass wool was obtained from Fisher Scientific Co., and was Soxhlet extracted with acetone:hexane (1:1, v/v) for 4h. It was then heat treated at 300°C for 4h, allowed to cool, and stored in an oven maintained at 130°C until use. Cellulose extraction thimbles (33mm i.d. X 80mm) were obtained from Fisher Scientific Co. These thimbles were Soxhlet extracted overnight with acetone:hexane (1:1, v/v) prior to use.

Organic free water was prepared by passing tap water through a prepared column of XAD-7 resin. The column was 51 cm long by 3 cm i.d. and was equipped with a 500 mL reservoir and teflon stopcock. XAD-7 resin, ca. 80 g (Rohm and Haas), was Soxhlet extracted for 48h with distilled in glass methanol. The column was slurry packed with HPLC grade water to a height of 14 cm. The column was backflushed with 1 L of HPLC grade water prior to use. After passage of ca. 1.5 L of water through the column, the water was extracted with 3 X 100 mL DCM in a 2 L separatory funnel and the DCM layer drained and discarded. Sufficient water was processed each time to fill an empty Caledon solvent bottle (4 L).

A Florisil chromatography column was prepared by wet packing a column (1 cm i.d. X 50 cm) with 10 g of Florisil (5% water deactivated) and hexane. The column was equipped with a 50 mL reservoir and a removable teflon stopcock. The stopcock was attached

to the column by means of a ₹14/23 ground glass joint and 1/2 inch stainless steel springs. The removable stopcock allowed for easy cleaning of the column after use. After the Florisil had settled, ca. 1 g of Na_2SO_4 was added and the solvent drained to the Na_2SO_4 layer. The column was rinsed with 2 X 20 mL of hexane prior to use.

Sodium sulfate drying columns were prepared by adding 1 g of Na_2SO_4 to a Pasteur pipette (5mm i.d. X 150 mm). The column was rinsed with 3 X 2 mL DCM prior to use.

6N NaOH was prepared by adding 240 g of NaOH (ACS grade) to 1 L of organic free water. This reagent solution was extracted with 3 X 100 mL of DCM in a 2 L separatory funnel. The DCM was drained and discarded and the reagent solution transferred to a rotary evaporator maintained at 70°C and under reduced pressure to remove all traces of DCM.

6N H_2SO_4 was prepared by adding 167 mL of concentrated H_2SO_4 (ACS grade) to 1 L of organic free water. This reagent solution was extracted with 3 X 100 mL of DCM in a 2 L separatory funnel. The DCM layer was discarded and the solution transferred to a rotary evaporator maintained at 70°C and under reduced pressure to remove any residual DCM.

Pear shaped flasks, 300 mL, were obtained from Tiffany's Glass Place, Winnipeg. These flasks were calibrated and etched at the 10 mL level and were equipped with a female ₹24/29 glass joint.

Forty mL pear shaped flasks, obtained from the same supplier, were calibrated at the 2 mL level and also contained a female ₹ 24/29 glass joint.

(2.2.1) PREPARATION OF SAMPLE CONTAINERS

Tin plated cans (one quart size) were obtained from Kay's Containers, Winnipeg. Upon receipt, the cans and lids were washed with detergent in a laboratory dishwasher. They were subsequently rinsed with distilled water followed by pesticide grade acetone and hexane. The cans and lids were allowed to air dry and then placed in a muffle furnace and heat treated at 400°C for 4 h. Upon cooling, the lids were placed onto the cans with the aid of a rubber mallet.

(2.2.2) SAMPLING SURVEYS

Preliminary sediment samples from Sites I-IV (Figure 3) were collected by Mr. J. Witteman (Environment Canada) in September, 1979, and submitted to Dr. W. F. Warwick (National Water Research Institute) to determine if morphological deformities were present among the larvae of the chironomid community and to establish the validity of the 'contaminants in Tobin Lake' concept. Since

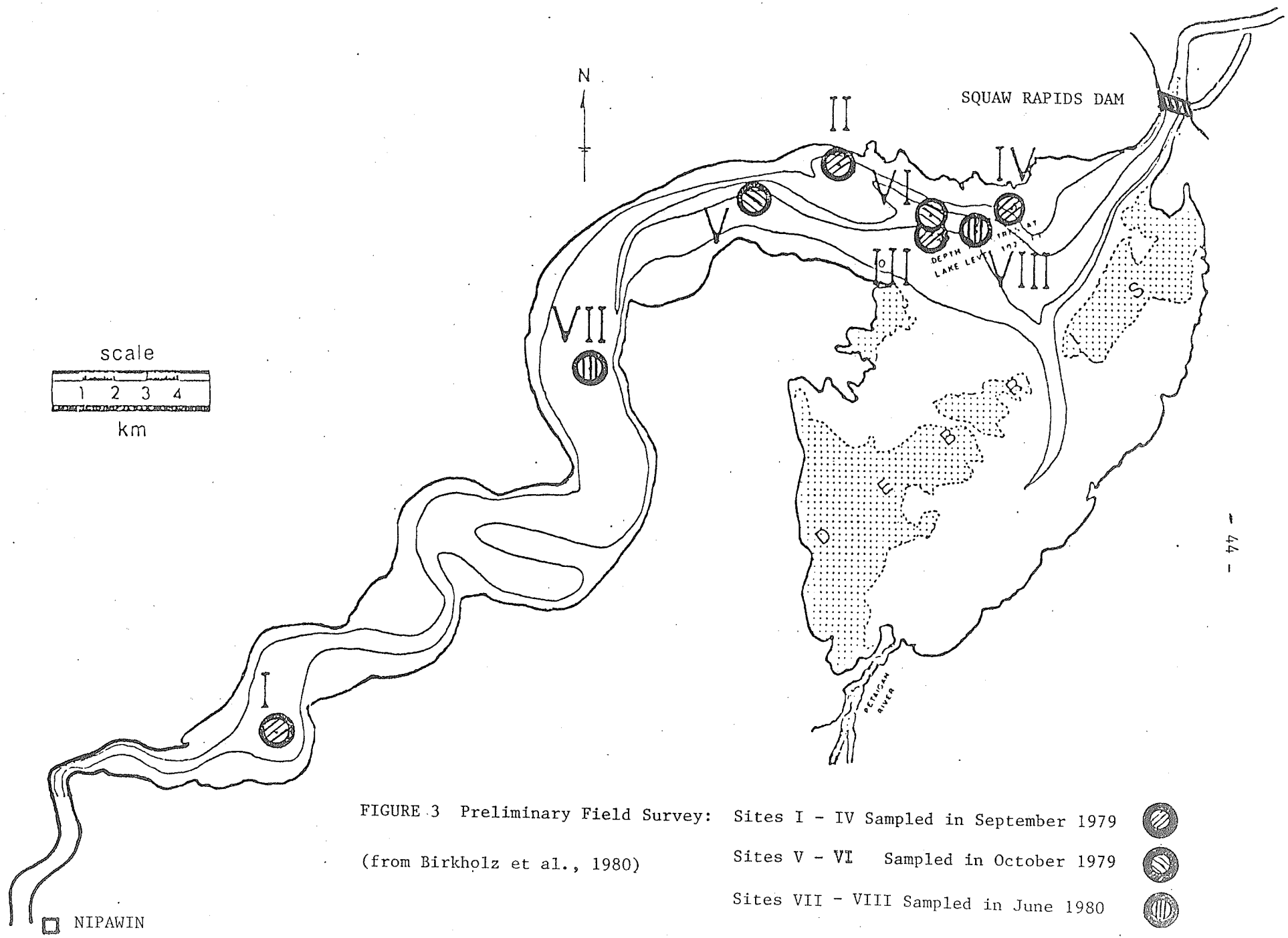


FIGURE 3 Preliminary Field Survey: Sites I - IV Sampled in September 1979

(from Birkholz et al., 1980) Sites V - VI Sampled in October 1979

Sites VII - VIII Sampled in June 1980

the September samples constituted only the partial contents of a single Ekman dredge another field trip was scheduled for October, 1979.

At this time, six bulk sediment samples from Sites V and VI (Figure 3) each containing the contents of 7-8 Ekman dredges were collected. To accomodate such a large sample size, five gallon plastic containers were used to contain the samples. A five gallon sample from Site V was made available to the author for method development purposes. This sample was designated as the October Bulk sample.

Based on the biological results of the October survey, the decision was made to proceed with the Tobin Lake project. A comprehensive field sampling program was drawn up by Dr. W. F. Warwick. The survey design took into account previous field surveys conducted in the reservoir, evidence from the preliminary survey, known and inferred hydrologic characteristics of the basin and the comparative basin morphometries.

The survey design essentially divided the reservoir into two basic units - the old Saskatchewan River channel and the area along the Petaigan River flooded by water backed up from the primary reservoir. The two units, as depicted in Figure 4 are essentially separate basins.

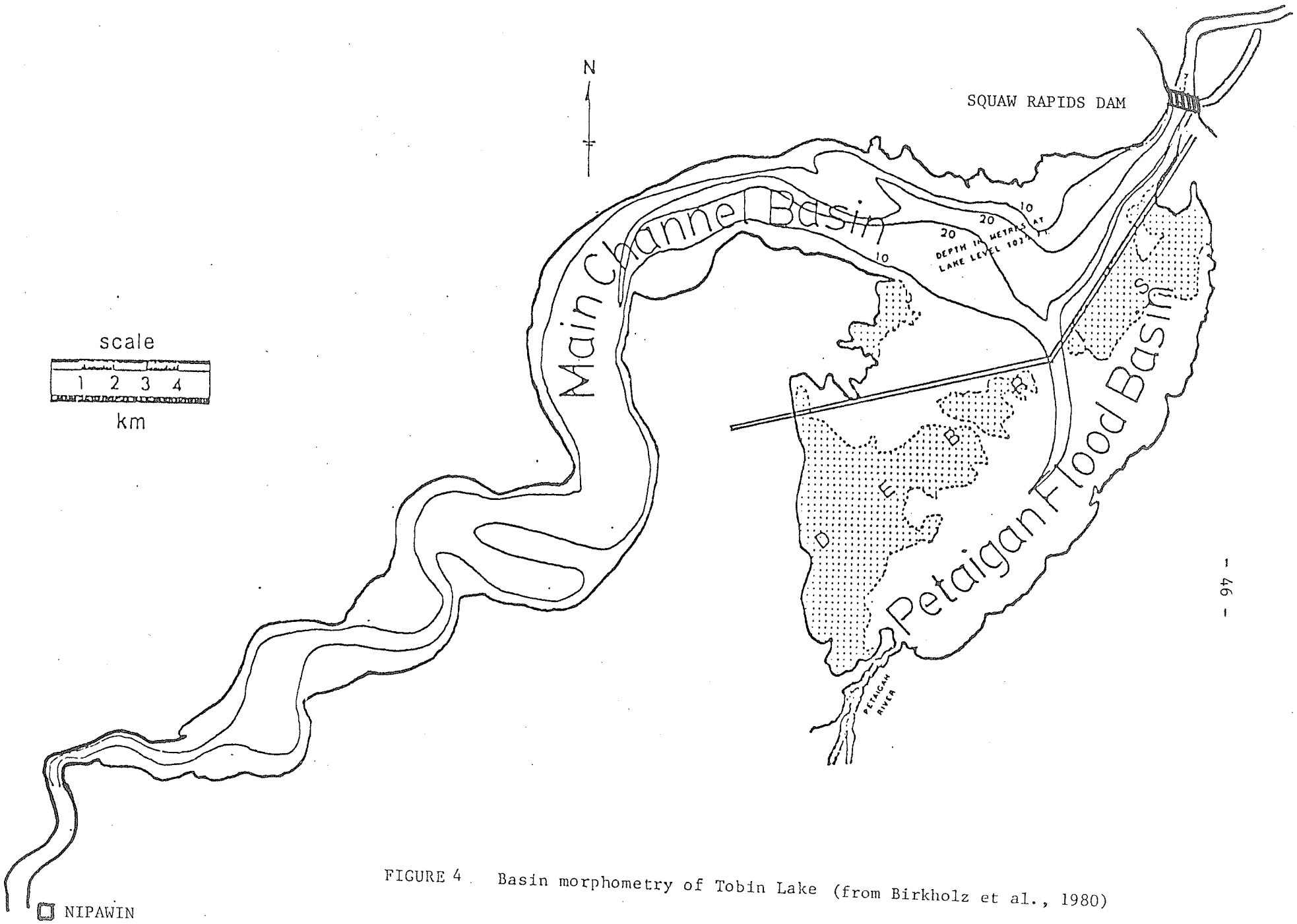


FIGURE 4 Basin morphometry of Tobin Lake (from Birkholz et al., 1980)

Since the entrance of the Petaigan River into the Saskatchewan River is restricted to a relatively narrow passage, the direct interchange of water, and particularly water-borne materials, is probably minimal (Birkholz et al., 1980). For this reason, sampling sites were established along the old Petaigan River channel to act as controls for the sampling sites in the main channel.

Fifteen major sampling sites (Sites 1-15) were established to study the longitudinal deposition of contaminant-bearing materials along the old Saskatchewan River channel (Figure 5). The bed and bank configurations of the reservoir indicate a succession of baffles for the hydraulic energy of the Saskatchewan River. These features of the basin morphometry coupled with the increasing water depths, suggest a stepwise decrease in hydraulic energy which would govern the deposition patterns of water-borne contaminant bearing particulate materials (Birkholz et al., 1980). Major sampling sites, beginning with Site 1 in the active river channel above the upper end of the reservoir, were established in succession to monitor each stage in the dissipation of the system's hydraulic energy and, hence, load-carrying capacity for contaminant bearing materials. The majority of the sites were concentrated at the lower end of the reservoir because contaminant materials are generally believed to be borne bound on suspended organic materials and, since these materials are light, they will only settle out in an environment of very low hydraulic energy. The high incidence of deformities among the chironomidae from this area

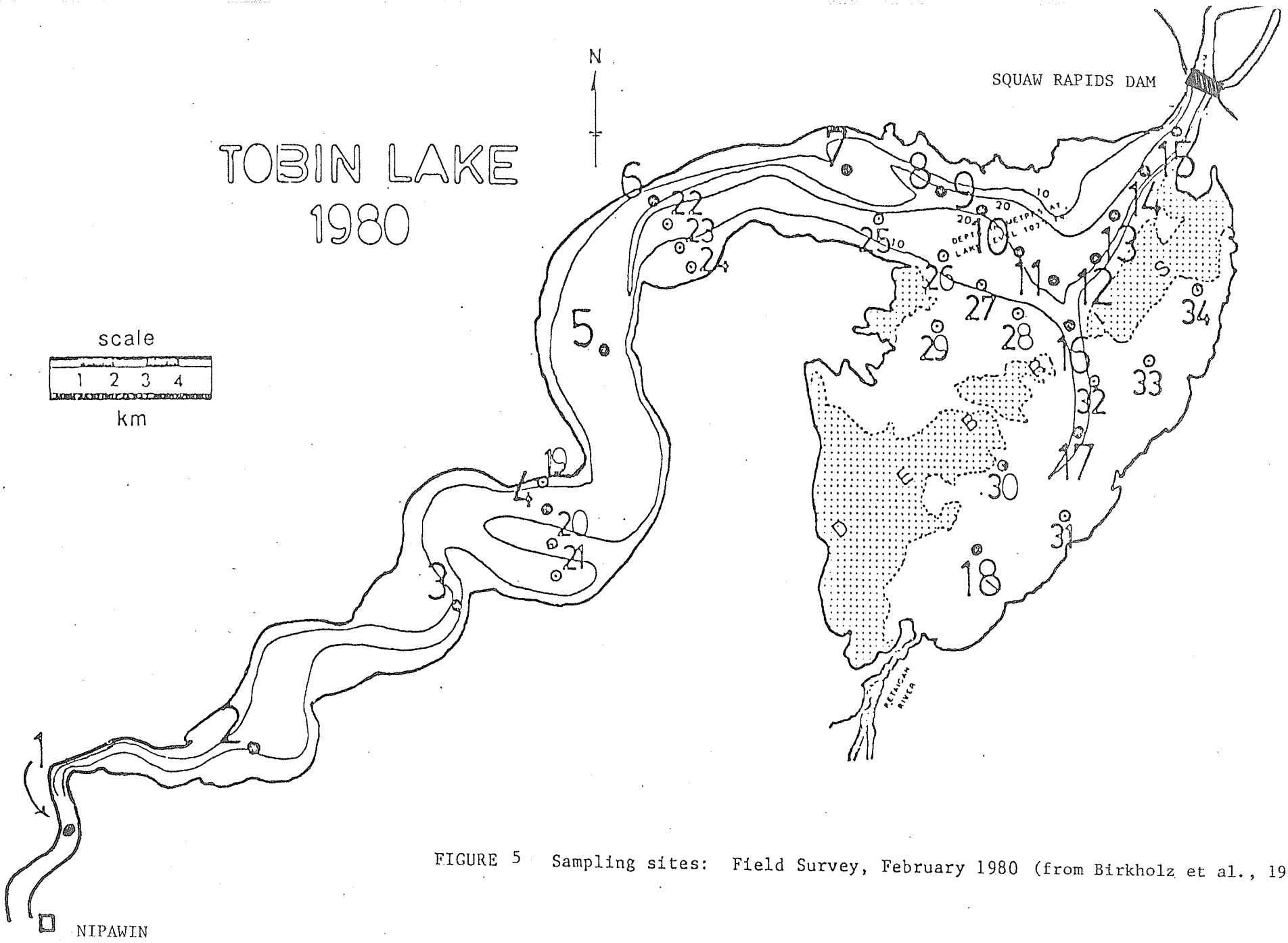


FIGURE 5 Sampling sites: Field Survey, February 1980 (from Birkholz et al., 1980)

also suggest it is an area where contaminants are actively accumulating (Birkholz et al., 1980).

Three major sampling sites (Sites 16-18, Figure 5) were established along the flooded Petaigan River channel to act as controls for the site along the main river channel. Although it is difficult to estimate the degree of interchange of water between the two basins, the reduction in hydraulic energy at this stage suggests most of the particulate materials bearing contaminants will have settled or begun to settle, the morphometric separation of the basins indicates minimal direct interchange and the inflow from the Petaigan River would counterbalance any direct interchange and dilute any Saskatchewan River water that did penetrate into the basin (Birkholz et al., 1980).

The location and marking of the sampling sites was undertaken in January, 1980 by Mr. Witteman (Environment Canada), Mr. Bouck (Energy Mines and Resources Canada) and their respective staff.

Sampling operations were conducted in February, 1980 by staff from the National Water Research Institute and were supported by personnel from Environment Canada, the University of Manitoba, and Parks Canada.

Duplicate sediment samples were collected at all stations with the aid of an Ekman dredge. The sediment samples were transported back to base camp in sealed plastic containers and subsampled the

same evening into metal cans to minimize contact with plastic surfaces.

The sediment samples were frozen then stored at -40°C to await analysis.

Great cost and effort had been expended to generate these samples. To this end the project committee wanted the analytical methodology to be well developed before processing any of the actual survey samples.

The use of the bulk sample from Site V (Figure 3) for method development purposes was questioned because this sample was contained in a 5 gallon plastic container. Di(2-ethylhexyl)phthalate (DEHP) is a very common plasticizer which is used in a variety of plastic products (United States Environmental Protection Agency, 1980b). The U.S. National Cancer Institute has tested DEHP and determined that the compound is carcinogenic both in rats and mice (United States Environmental Protection Agency, 1980b). Therefore it was reasonable to suspect that the sediment sample from Site V could be contaminated with DEHP and therefore obscure the toxicity date. Consequently, a fourth field trip was undertaken in June 1980 by Mr. J. Witteman (Environment Canada) at which time new sediment samples were taken from the vicinity of Site VII and VIII (Figure 3) for method development purposes. These sites were approximated since no survey equipment was used to locate the sites as had been the case in the February survey. Sufficient sample was taken to fill five quart size tin cans from each site.

(2.2.3) CLEANING OF LABORATORY GLASSWARE

All glassware used was washed by hand using a test tube brush, detergent and hot water. The glassware was then rinsed with hot water and allowed to soak overnight in a tank containing Chem Solv 2157 (Mallinckrodt Chem. Co.) which is an oxidizing detergent recommended by the United States Environmental Protection Agency (1980a). A hot water rinse was used to remove the oxidizing detergent and this was followed by several rinsings with pesticide grade acetone. The glassware was allowed to air dry, placed in a large convection oven, and heat treated at 300^{o}C for 4h. Upon cooling the glassware was covered with aluminum foil and placed in a clean drawer until needed. Prior to use the glassware was rinsed with appropriate pesticide grade solvent.

(2.2.4) SAMPLE PREPARATION

Sediment samples from Sites V, VII, VIII, 9, 14 and 15 were allowed to thaw in their containers. The sediment samples were then transferred to clean glass freeze drying flasks with the aid of a clean spatula and refrozen.

Subsequently the samples were evaporated and freeze-dried. Lyophilization times varied between 1 and 2 days at a pressure of approximately 0.10 Torr.

The entire sample from each site was processed in this manner and then sieved through a 2 mm mesh screen in order to remove sticks and other debris. The sample was then transferred to a clean, labelled, wide mouth (900 mL) jar fitted with a teflon lined lid. Several clean clay balls were added, the jar capped, and placed on a soil roller for several hours to ensure homogeneous mixing. The samples were refrigerated at $4^{o}C$ until required. Samples from Sites 14 and 15 were combined because of insufficient sample size and because the sites were in reasonably close proximity. This combined sample shall be referred to as 14/15.

(2.2.5) SAMPLE EXTRACTION AND FRACTIONATION

The method of Goerlitz and Law (1974) was employed to extract anthropogenic substances from the sediment, with minor modifications. Fractionation of the extract was accomplished using a modified version of the methods proposed by the United States Environmental Protection Agency (1979a) and Hites et al. (1980). The entire scheme is depicted in Figure 6.

A preextracted cellulose thimble (33 mm i.d. X 80 mm) was weighed on a clean top loading balance and the weight recorded. Prepared sediment was transferred to this thimble to fill it to 80% of its capacity and the thimble and its contents were weighed. The amount of sediment present in the thimble was calculated and sufficient organic free water was added to raise the moisture content to 25%. Clean glass wool was added to the thimble to contain

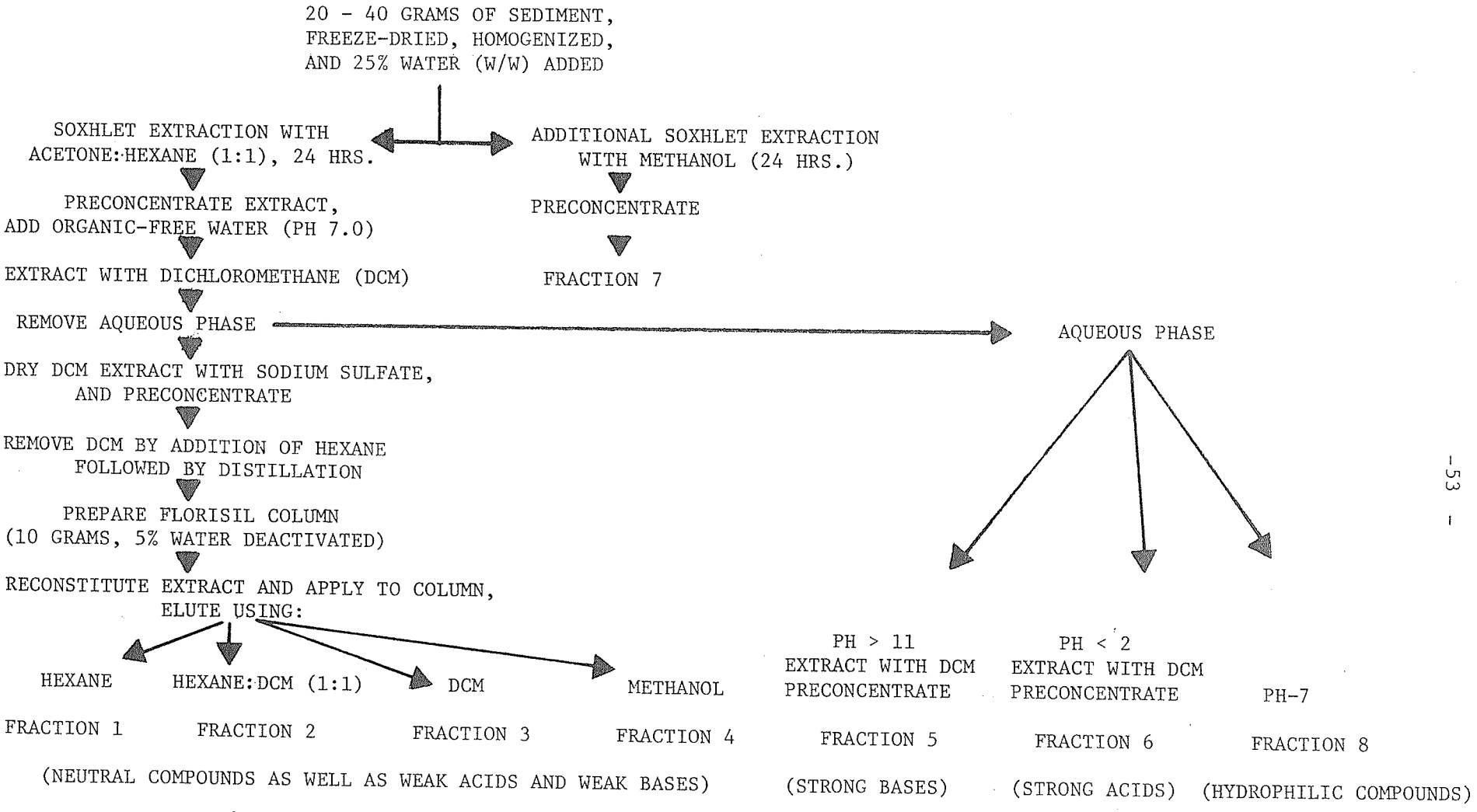


FIGURE 6: EXTRACTION AND FRACTIONATION SCHEME

the sediment during extraction. The amount of freeze dried sediment extracted ranged from 20 to 40 g depending upon sample site.

The thimble was transferred to a 'Hot' Soxhlet Extractor (Kontes K-586100-0022) equipped with a Friedrich condenser (Kontes K-456250-0022). To a 250 mL flat bottom flask (Kontes K-601500-0324) was added 150 mL of distilled in glass acetone:hexane (1:1, v/v) along with 3 or 4 clean glass beads. The flask was attached to the Soxhlet Extractor by means of a ₹24/40 joint and the entire apparatus was mounted in an Electrothermal Extraction Assembly (Fisher Scientific-28-4278). The heater was turned on and sufficient heat was applied to insure a minimum of 20 cycles per h. After 24 h of extraction the heater was turned off and the apparatus was allowed to cool. The flask was removed and replaced with a clean one containing 150 mL of distilled in glass methanol and a few clean glass beads. Heat was applied and extraction was continued for an additional 24 h. The heater was then turned off, and the apparatus was allowed to cool. The flask was removed, stoppered and labelled Fraction 7.

The acetone-hexane extract was preconcentrated to approximately 20 mL using a rotary evaporator with the flask immersed in a 70^{o}C water bath. The pressure of the system was reduced with a tap aspirator. This extract was transferred to a 500 mL separatory funnel with the aid of 2 X 20 mL of dichloromethane (DCM). Two

hundred and fifty mL of organic free water was added (neutral pH) and the contents were thoroughly mixed. Neutral compounds, weak acids and bases were extracted with 100, 50, and 50 mL of DCM. The DCM was drained and collected in a 300 mL pear shaped flask. The flask was stoppered and labelled 'neutral compounds'.

The pH of the water phase was adjusted to greater than 11 (as detected by multirange pH paper) by addition of 6N sodium hydroxide. Strongly basic compounds were extracted by the addition of 100, 50 and 50 mL of DCM. The DCM was drained into a 300 mL pear shaped flask, stoppered, and labelled Fraction 5.

The pH of the water was again adjusted, this time to pH less than 2. Strong acids were extracted by the addition of 100, 50 and 50 mL of DCM. The DCM was collected in a 300 mL pear shaped flask, stoppered, and labelled Fraction 6.

Finally, the pH was adjusted to neutral pH by the addition of 6N NaOH. The water was drained into a 300 mL pear shaped flask and labelled Fraction 8. This fraction was expected to contain hydrophilic compounds.

The extract containing the 'neutral compounds' was preconcentrated to ca. 10 mL using a rotary evaporator with the flask immersed in a 70°C water bath. The extract was passed through a micro sodium sulfate column, with the aid of 3 X 5 mL of DCM, and collected in a 40 mL pear shaped flask. The DCM was preconcentrated to near dryness (50 - 100 µL) using a rotary evaporator with the flask

immersed in a 70°C water bath. Hexane (10 mL) was added and the mixture was preconcentrated to 2 mL using the rotary evaporator. This step was used to remove all traces of DCM through distillation. At this point, hexane insoluble material was observed to precipitate out of solution, however, despite this observation the extract was quantitatively transferred to a chromatographic column (1 cm i.d. X 50 cm), containing 10 g of Florisil (5% water deactivated) and ca. 1 g of sodium sulfate, with the aid of 4 x 10 mL hexane. The material applied to the column was then eluted with 160 mL of hexane and the eluant collected in a 300 mL pear shaped flask, stoppered and labelled Fraction 1.

The 40 mL pear shaped flask, which contained the 'neutral compounds', was then rinsed with 4 x 10 mL of hexane:DCM (1:1) in order to dissolve remaining hexane insoluble material. These rinsings were quantitatively transferred to the same chromatographic column and the applied material eluted with 160 mL of hexane:DCM (1:1). The eluant was collected in a 300 mL pear shaped flask, stoppered and labelled Fraction 2.

The chromatographic column was then eluted with 200 mL of DCM (10 bed volumes); the eluant collected in a 300 mL pear shaped flask, stoppered, and labelled Fraction 3.

A final elution of the column was effected using 200 mL of methanol. The eluant was collected in a 300 mL pear shaped flask, stoppered, and labelled Fraction 4.

(2.2.6) SOLVENT EXCHANGE MECHANISM PRIOR TO BIOASSAY

Prior to biological assay, an aliquot of each fraction was removed and saved for chemical analysis. The remaining material in fractions labelled 2, 3, 4, 5, 6 and 7 was exchanged into dimethyl sulfoxide (DMSO). Fraction 1 was exchanged into mineral oil, while Fraction 8, being an aqueous solution, was assayed as is. These steps are summarized in Figure 7 and in the following text.

Fraction 1 was preconcentrated to ca. 10 mL using a rotary evaporator and water bath maintained at 70°C. This fraction was transferred to a 40 mL pear shaped flask with the aid of 3 X 5 mL of hexane and preconcentrated to 2.00 mL using the rotary evaporator. One mL of this material was removed and transferred to a labelled 1.5 mL amber screw cap vial (Pierce 13080) fitted with teflon-laminated rubber septum (Pierce 12408). This material was saved for analytical purposes. The remaining 1 mL of material was transferred to another labelled 1.5 mL amber vial and was submitted for bioassay. This material was diluted into mineral oil by the bioassay laboratories (J. Bell and M. Samoiloff).

Fractions 2 and 3 were preconcentrated to ca. 10 mL using a rotary evaporator and were transferred to a 40 mL pear shaped flask with the aid of hexane:DCM (1:1), and DCM respectively. After preconcentrarion to 2.00 mL, 1.00 mL from each fraction was removed and transferred to a labelled 1.5 mL amber screw cap vial for analytical purposes. To the remaining material in the flask was

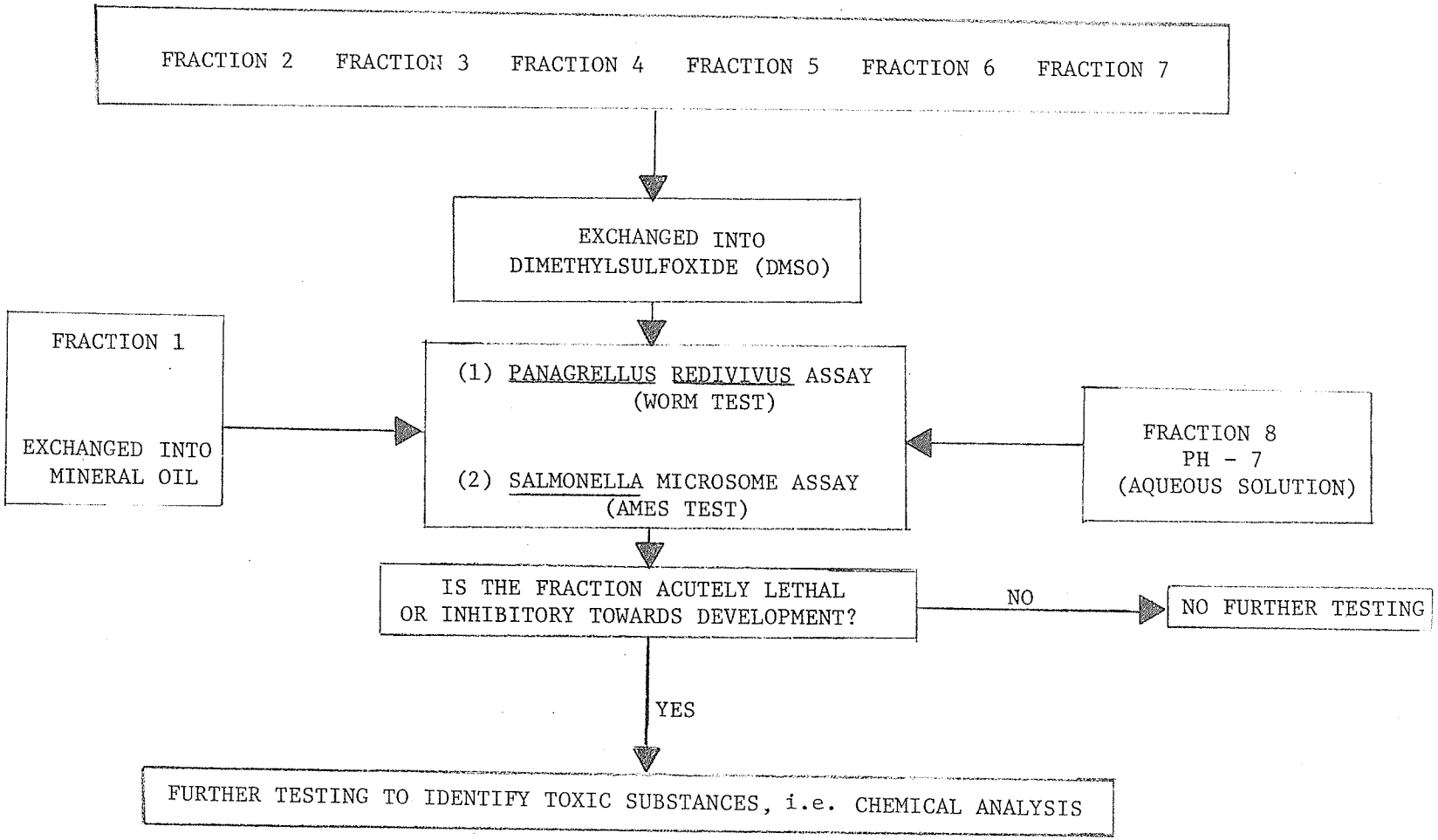


FIGURE 7: SOLVENT EXCHANGE MECHANISM PRIOR TO BIOASSAY

added 1.00 mL of DMSO. Quantitative exchange into DMSO is accomplished through the use of a rotary evaporator under reduced pressure. This material was transferred to labelled 1.5 mL amber screw cap vials and was submitted to bioassay.

Fractions 4 and 7 were preconcentrated to ca. 10 mL using a rotary evaporator. Five mL of each fraction was removed and transferred to labelled 7 mL amber screw cap septum vials (Pierce 13084) fitted with teflon-silicone discs (Pierce 12713) for analytical purposes. To the remaining extract was added 5.00 mL of DMSO. Exchange into DMSO was accomplished using the rotary evaporator under reduced pressure. This material was transferred to labelled 7 mL amber vials and submitted for bioassay.

Fractions 5 and 6 were preconcentrated to ca. 10 mL using rotary evaporation. The extracts were passed through micro sodium sulfate drying columns with the aid of 3 X 5 mL DCM and collected in 40 mL pear shaped flasks. The fractions were preconcentrated to 2.00 mL using rotary evaporation. One mL from each fraction was removed and transferred to labelled 1.5 mL amber screw cap vials for analysis. To the remaining material, 1.00 mL of DMSO was added and quantitative exchange was accomplished using a rotary evaporator under reduced pressure. The fractions were transferred to labelled 1.5 mL amber screw cap vials and submitted for bioassay.

Fraction 8 was transferred to two labelled 40 mL amber screw cap vials (Pierce 13090) filled with teflon-silicone discs (Pierce 12722). Excess material was discarded. One vial was retained for analysis and the other

was submitted for bioassay.

This entire procedure was applied to samples from each site five times. Five analytical controls were also processed using this procedure for each site. The controls simulated the samples in that the entire procedure was followed, including extraction of thimbles filled with glass wool.

For fractions limited to 1 mL of extract for bioassay, 200 µL were submitted to Dr. M. Samoiloff (Dept. of Zoology, University of Manitoba) and 800 µL were submitted to Mr. J. Bell (Environment Canada). For fractions limited to 5 mL, 1 mL was submitted to Dr. Samoiloff and 4 mL were submitted to Mr. Bell.

(2.2.7) STATISTICAL TREATMENT OF BIOLOGICAL RESULTS

Due to the large amount of data generated, the biological results from each site were pooled. In a similar fashion, control data from each site were also pooled. Chi-square determinations were then made to determine whether significant toxicity was apparent in the samples relative to the controls (reagent blanks).

(2.2.8) GAS-LIQUID CHROMATOGRAPHY

Fractions which demonstrated a toxic response with the Salmonella/microsome mutagenicity assay and the P. redivivus assay were subjected to analysis using capillary gas-liquid chromatography (GLC) equipped with either a flame ionization detector (FID) or an electron capture detector (ECD).

Prior to use, the ends of glass capillary columns were straightened using a butane micro torch (Supelco 2-2969) and deactivated using a solution containing 8.8 mg/mL Carbowax 20 m in DCM as recommended by Jennings, (1980).

(2.2.8.1) Gas Liquid Chromatography-Electron Capture Detection (GLC/ECD)

Throughout this study, GLC/ECD conditions used were as follows: a Hewlett-Packard model 5830 GLC equipped with a glass 30 m X 0.241 mm wall coated open tubular (WCOT) column coated with SP 2100 was used (J. & W. Scientific, Inc.).

Temperatures (^{o}C): inlet, 250; column, 40 for 0.1 min , 30/min to 150, 3.4 min hold, 2.4/min to 230, 60 min hold; detector, 300.

Carrier gas, He, linear velocity, 22 cm/sec Make-up gas, Ar-methane (95:5) at 29 mL/min.

Injection mode, splitless, purge time, 0.5 min. Injection volume, 3 µL.

Chart speed, 1cm/min.

(2.2.8.2) Gas Liquid Chromatography-Flame Ionization Detection (GLC/FID)

Throughout this study, GLC/FID conditions used were as follows:

Method A

- a Hewlett Packard model 5880 GLC equipped with a glass 30 m X 0.241 mm WCOT column coated with SP2100 (J. & W. Scientific, Inc.).

Temperatures (^{o}C): inlet, 250; column, 40 for 2 min , 10/min to 250, 60 min hold; detector, 300.

Carrier gas, He, linear velocity, 24.5 cm/sec. Make-up gas, N_2 at 30 ml/min.

Injection mode, splitless, purge time, 0.5 min. Injection volume, 3 µL.

Chart speed, 1 cm/min.

Method B

- a Hewlett Packard model 5830 GLC equipped with a glass 60 m X 0.25 mm WCOT column coated with SE 54 (J. & W. Scientific, Inc.).

Temperatures (^{o}C): inlet, 250; column, 40 for 2 min, 4/min to 280, 120 min hold; detector, 300.

Carrier gas, He, linear velocity, 24 cm/sec. Make-up gas, N_2 at 30 ml/min.

Injection mode, splitless, purge time, 0.5 min. Injection volume, 3 µL.

Chart speed, 1 cm/min.

Method C

- a Hewlett Packard model 5880 GLC equipped with a 50 m X 0.31 mm fused silica column chemically bonded with SE 54 (Hewlett Packard).

Temperatures (^{o}C); inlet, 250; column, 40 for 2 min , 10/min to 280, 10 min hold; detector, 300.

Carrier gas, He, linear velocity, 20 cm/sec. Make-up gas, N_2 at 30 ml/min.

Injection mode, splitless, purge time, 0.5 min. Injection volume, 3 µL.

Chart speed, 1 cm/min.

(2.2.9) Gas-Chromatography - Mass Spectrometry (GC/MS)

Two GC/MS instruments were used during this study, namely, a Hewlett-Packard model 5985 GC/MS and a Finnigan model 4021 GC/MS. Both instruments were equipped with capillary columns and computerized data systems.

Throughout this study, GC/MS conditions used were as follows:

Method A

- a Hewlett Packard model 5985 GC/MS equipped with a 12 m X 0.21 mm fused silica column coated with SP2100 (Hewlett Packard).

Temperatures (^{o}C): inlet, 275; column, 40 for 2 min , 5/min to 250, 60 min hold; ion source, 200.

Carrier gas, He, linear velocity, 20 cm/sec.

Injection mode, splitless, purge time, 0.5 min. Injection volume, 3 µL.

Ionization mode, electron impact (70 eV); masses scanned, 40 to 700 amu.

Source pressure, ca. 10^{-6} Torr.

Method B

- a Finnigan model 4021 GC/MS equipped with a 50 m X 0.21 mm fused silica column coated with SP2100 (Hewlett Packard).

Temperatures (^{o}C): inlet, 250; column, 65 for 5 min , 2.5/min to 280, 25 min hold; ion source, 200.

Carrier gas, He, linear velocity, 20 cm/sec.

Injection mode, splitless, purge time, 0.5 min. Injection volume, 3 µL.

Ionization mode, electron impact (70 eV); masses scanned, 50 to 500 amu. Source pressure, ca. 10^{-6} Torr.

Method C

- a Finnigan model 4021 GC/MS equipped with a 50 m X 0.31 mm fused silica column coated with SE 54 (Hewlett Packard).

Temperatures (^{o}C): inlet, 250; column 40 for 5 min , 2.5/min to 80, 10/min to 280, 20 min hold; ion source, 200.

Carrier gas, He, linear velocity, 35 cm/sec.

Injection mode, splitless, purge time, 0.8 min. Injection volume, 3 µL.

Ionization mode, electron impact (70 eV); masses scanned, 50 to 400 amu. Source pressure, ca. 10^{-6} Torr.

Method D

- a Finnigan model 4021 GC/MS equipped with a 50 m X 0.31 mm fused silica column coated with SE 54 (Hewlett Packard).

Temperatures (^{o}C): inlet, 250; column, 40 for 5 min, 2.5/min to 80, 10/min to 280, 20 min hold; ion source, 200.

Carrier gas, He, linear velocity 35 cm/sec.

Injection mode, splitless, purge time, 0.8 min. Injection volume, 3 µL.

Ionization mode, negative ion chemical ionization (NCI); reagent gas, methane; source pressure, ca. 0.3 Torr; masses scanned, 5 to 500 amu.

Method E

- a Finnigan model 4021 GC/MS equipped with a 12 m X 0.21 mm fused silica column coated with OV-1 (Hewlett Packard).

Temperatures (^{o}C): inlet, 250; column, 40 for 0.6 min , 20/min to 280, 20 min hold; ion source, 200.

Carrier gas, He, linear velocity 20 cm/sec.

Injection mode, splitless, purge time, 0.5 min. Injection volume, 2 µL.

Ionization mode, NCI; reagent gas, methane; source pressure, ca. 0.3 Torr; masses scanned, 5 to 500 amu.

PART 3.

Results and Discussion

(3.1) BIOLOGICAL RESULTS

(3.1.1) Panagrellus Redivivus Assay

Table 1 depicts the biological results obtained for Fractions 1 - 8 from Sites V, VII, VIII, 9 and 14/15 using this assay.

Four biological effects were noted with this test organism, namely, lethality, sublethality, toxicity and mutagenicity. These effects were defined in the following terms:

Lethality - all animals exposed to a test sample were found to be dead within 96 hours.

Sublethality - a significant decrease in survival of test animals, relative to both the mean survival of all other test animals run at the same time and the survival of the specific control sample (reagent blank) for the test population.

Toxicity - a significant decrease in the frequency of completion of both the first and second molts, relative to the means of all other tests performed at the same time, with a similar decrease observed relative to the reagent blanks. A positive test for toxicity precludes testing for mutagenicity.

Mutagenicity - A significant decrease in the frequency of completion of the final molt, relative to the other test populations run at the same time, with a similar decrease observed relative to the blank.

From this data one can rank the degree of observed toxicity among the various fractions within a specific sample site using the following guidelines:

TABLE 1 PANAGRELLUS REDIVIVUS ASSAY OF FRACTIONATED SEDIMENT EXTRACTS

SAMPLE SITE	BIOLOGICAL EFFECT WITH CORRESPONDING X^2							
	FRACTION 1	FRACTION 2	FRACTION 3	FRACTION 4	FRACTION 5	FRACTION 6	FRACTION 7	FRACTION 8
SITE V (October bulk sample)	NE	SL (145)	MUT. (53)	TOX. (85)	NA	NA	NE	NA
SITE VII (Jackfish Point)	NE	SL (100)	TOX. (99)	TOX. (39)	NE	NE	MUT. (98)	TOX. (153)
SITE VIII (Channel)	NE	TOX. (234)	TOX. (68)	NE	MUT. (28)	NE	TOX. (46)	TOX. (111)
SITE 9	TOX. (20.5)	TOX. (84)	NE	NE	TOX. (16.4)	MUT. (12.8)	NA	NA
SITE 14-15	MUT. (19.0)	NE	TOX. (27.3)	TOX. (48.8)	TOX. (41.5)	TOX. (86.6)	NA	NE

SL = sub-lethal, TOX = toxic, MUT = mutagenic, NE = no effect and NA = not available

(1) a lethal effect is more significant than a sublethal effect which in turn is more significant than a toxic effect which in turn is more significant that a mutagenic effect; i.e., lethal > sub-lethal > toxic > mutagenic.

(2) X^2 values can be compared within a sample site since the biological tests were conducted during the same day. They cannot be compared to other sample sites since testing was performed on different days. Had these tests been conducted the same day, site comparisons could have been made.

The ranking of this data is depicted in Table 2.

TABLE 2 PANAGRELLUS REDIVIVUS ASSAY: RANKING OF OBSERVED BIOLOGICAL EFFECTS

SAMPLE SITE	RANKING OF BIOLOGICAL EFFECTS FROM MOST SIGNIFICANT TO LEAST SIGNIFICANT
SITE V	Fraction 2 > Fraction 4 > Fraction 3
SITE VII	Fraction 2 > Fraction 8 > Fraction 3 > Fraction 4 > Fraction 7
SITE VIII	Fraction 2 > Fraction 8 > Fraction 3 > Fraction 7 > Fraction 5
SITE 9	Fraction 2 > Fraction 1 > Fraction 5 > Fraction 6
SITE 14 - 15	Fraction 6 > Fraction 4 > Fraction 5 > Fraction 3 > Fraction 1

From Table 2 a number of empirical observations can be made:

(1) the most significant biological effect is observed with Fraction 2 in all sites except 14 - 15.

(2) Sites VII and VIII show a similar pattern of biological effects indicating that perhaps similar chemicals are being deposited at these sites.

(3) chemical fractionation is a standard way of separating a complex mixture of compounds into ordered classes, eg. acids, bases, and neutral compounds. The observation that similar fractions have demonstrated a toxic response among the various sites, is indicative of the presence of similar compounds or the presence of compounds with similar polarity. On the other hand, the observation of different fractions giving rise to toxic responses among the various sites is indicative of different classes of compounds, or compounds of differing polarity being deposited at the various sites.

The observation that toxicity is associated with certain fractions can give clues as to sources of contamination. For example an observed toxic response for Fraction 6 may be due to the presence of diterpene resin acids which are discharged by pulp mills. An observed toxic response for Fraction 1 may be due to the presence of pesticides which may be present in agricultural runoff.

(3.1.2) SALMONELLA/MICROSOME ASSAY (AMES TEST)

Five strains of Salmonella typhimurium were used in this assay, each strain being exposed to fractionated sediment extract with and without the presence of liver homogenate. The liver homogenate consisted of Aroclor induced rat liver (S9). Due to the large amount of work involved in assaying these extracts, sufficient time was only available to allow for the assay of fractionated extracts from Sites VII and VIII.

Table 3 depicts the biological results obtained for Fractions 1 - 8 from Site VII using this assay. Two types of effects were noted with this assay, namely, toxicity and mutagenesis. These effects were defined in the following terms:

Toxic - a significant decrease in the mean number of test colonies, observed relative to the mean number of colonies observed for the spontaneous control and the specific control sample (reagent blank) for the test populations.

Mutagenic - a significant increase in the mean number of test colonies observed, relative to the mean number of colonies observed for the spontaneous control and the specific control sample (reagent blank) for the test populations.

For some fractions both toxicity and mutagenicity were observed. This observation can be rationalized as follows: fractions were tested at varying dilutions, and in some cases, toxicity was observed at the higher concentration and mutagenicity at the lower concentration.

TABLE 3 SALMONELLA/MICROSOME ASSAY OF FRACTIONATED SEDIMENT EXTRACT FROM SITE VII (JACKFISH)

STRAIN	FRACTION 1	FRACTION 2	FRACTION 3	FRACTION 4	FRACTION 5	FRACTION 6	FRACTION 7	FRACTION 8
1535	NE	TOX & MUT	NE	NE	NE	NE	NE	NE
1535 + S9	NE	MUT	NE	NE	NE	NE	NE	NE
1537	NE	NE	NE	NE	NE	NE	MUT	NE
1537 + S9	NE	NE	NE	NE	NE	NE	NE	NE
1538	NE	TOX & MUT	NE	NE	NE	NE	NE	NE
1538 + S9	NE	TOX & MUT	NE	NE	NE	NE	MUT	MUT & TOX
98	NE	TOX	NE	NE	NE	NE	NE	NE
98 + S9	NE	TOX & MUT	NE	NE	NE	NE	NE	NE
100	NE	TOX & MUT	NE	NE	NE	NE	NE	NE
100 + S9	NE	TOX	NE	NE	NE	NE	NE	NE

TOX = toxic response, MUT = mutagenic response,

NE = no observed effect, S9 = Aroclor induced rat liver homogenate

By referring to Table 3, the following observations can be made with respect to Site VII:

(1) the most significant biological activity is associated with Fraction 2;

(2) toxicity and mutagenesis are evident in this fraction;

(3) mutagenesis as a result of base-pair substitutions is evident in Fraction 2 since strains TA1535 and TA100 demonstrated a positive response with this fraction. The presence of frameshift mutagens is also evident in Fraction 2 since strains TA1538 and TA98 demonstrated a positive response with this fraction. This may indicate that more than one chemical is responsible for the observed effect.

(5) for strains TA1535, TA1538, TA98 and TA100, the addition of liver homogenate (S9) to Fraction 2 is not a prerequisite for a mutagenic or toxic response. Furthermore, for strain TA1537, the addition of liver homogenate (S9) to Fraction 7 is not a prerequisite for a mutagenic response. These observations are indicative of the presence of direct acting chemical(s) in Fractions 2 and 7.

(6) for strain TA1538, the addition of liver homogenate (S9) to Fractions 7 and 8 is a prerequisite for a mutagenic and toxic response. This strain does not respond to these fractions directly; however, upon the addition of liver homogenate (S9), which contains the necessary microsomes for metabolic conversion, mutagenesis and/or toxicity is observed. The resultant metabolite(s) cause frameshift mutations as exemplified by response with this particular strain.

Table 4 depicts the biological results obtained for Fractions 1 - 8 from Site VIII using the Ames Test. Results from Site VIII (Table 4) lead to the following observations:

(1) the most significant biological activity is associated with Fraction 2.

(2) toxic and mutagenic effects are observed with this fraction.

(3) mutagenesis as a result of base-pair substitutions is evident in Fraction 2 since strain TA100 demonstrated a positive response with this fraction. The presence of frameshift mutagenesis is also evident in this fraction since strains TA1537, TA1538 and TA98 demonstrated a positive response with this fraction. This may indicate once more that more than one chemical is responsible for the observed effect.

(4) for strains TA1535, TA1537, TA1538, TA98 and TA100, the addition of liver homogenate (S9) to Fraction 2 is not a prerequisite for a mutagenic or toxic response; indicative once more of the presence of direct acting chemical(s) in this fraction.

(5) strains TA1535, TA1537, TA1538, TA98 and TA100 demonstrated a toxic and/or mutagenic response when subjected to Fraction 2 directly; however, upon the addition of liver homogenate (S9), no effect was observed with these strains when subjected to this fraction, strain TA98 being the exception. This observed detoxification of Fraction 2 upon the addition of liver homogenate (S9), may be the result of metabolism of the chemical(s) to less or non-toxic

TABLE 4 SALMONELLA/MICROSOME ASSAY OF FRACTIONATED SEDIMENT EXTRACT FROM SITE VIII (CHANNEL)

STRAIN	BIOLOGICAL EFFECT							
	FRACTION 1	FRACTION 2	FRACTION 3	FRACTION 4	FRACTION 5	FRACTION 6	FRACTION 7	FRACTION 8
1535	NE	TOX	NE	NE	NE	NE	NE	NE
1535 + S9	NE	NE	NE	NE	NE	NE	NE	NE
1537	NE	TOX & MUT	NE	NE	NE	NE	NE	NE
1537 + S9	NE	NE	NE	NE	NE	NE	NE	NE
1538	NE	TOX & MUT	NE	NE	NE	NE	NE	NE
1538 + S9	NE	NE	MUT	NE	NE	NE	NE	TOX & MUT
98	NE	TOX & MUT	NE	NE	NE	NE	NE	NE
98 + S9	NE	TOX & MUT	NE	NE	NE	NE	NE	NE
100	NE	TOX & MUT	NE	NE	NE	NE	NE	NE
100 + S9	NE	NE	NE	NE	NE	NE	NE	NE

TOX = toxic response, MUT = mutagenic response, NE = no observed effect

S9 = Aroclor induced rat liver homogenate

forms.

(6) for strain TA1538, the addition of liver homogenate (S9) to Fractions 3 and 8 is a prerequisite for a mutagenic and toxic response. This strain does not respond to these fractions directly; however, upon the addition of liver homogenates (S9), mutagenesis and/or toxicity is observed. Again, the presence of metabolites causing base-pair substitutions in DNA is evident.

Comparing the biological effects observed for Fraction 2 from Site VII to those obtained from Site VIII, it is apparent that the observed toxicity associated with Site VII is more pronounced than that associated with Site VIII. This is evident from the observation that the addition of liver homogenate (S9) to Fraction 2 - Site VII (Table 3) does not detoxify this extract as is generally the case for Fraction 2 - Site VIII (Table 4).

The presence of mutagenic metabolites is evident in in Fraction 8 from Sites VII and VIII using strain TA1538. The deposition of similar compound(s) at these sites may be responsible for this observation.

Mutagenicity is observed with strain TA1537 in Fraction 7- Site VII. Toxicity and mutagenicity is observed with this strain in Fraction 2- Site VIII. Mutagenicity is observed with strain TA1538, in the presence of liver homogenate (S9), in Fraction 7 - Site VII and in Fraction 3 - Site VIII. These observations demonstrate that very polar compound(s) (Fraction 7) which are mutagenic are present exclusively in samples from Site VII, or are present in sufficient concentration for detection. These compound(s) are either not present in samples from Site VIII or are not present in sufficient concentration to allow detection. Furthermore, polar neutral compound(s) (Fraction 3) which are mutagenic are observed in samples from Site VIII but are not detected in samples from Site VII. What we may be seeing here is selective deposition of toxic chemicals at specific sites. This selective deposition may be a function of the varying hydraulic energy of the Tobin Lake system.

(3.1.3) COMPARISON OF THE P. REDIVIVUS ASSAY TO THE SALMONELLA/ MICROSOME ASSAY

In comparing the data obtained from Site VII and VIII using the P. redivivus assay to that obtained using the Salmonella/ microsome assay one can see evidence of similarity. Some general conclusions can be made with reference to Tables 1, 2, 3 and 4: (1) both assays indicate that the most pronounced biological effect is associated with Fraction 2 from both sites.

(2) both assays show evidence for mutagenic agent(s) being present in Fraction 7 - Site VII, although the Salmonella/microsome assay evidence is weaker.

(3) the P. redivivus assay indicates the presence of toxic agent(s) in Fraction 8 from Sites VII and VIII. This is supported by the Salmonella/microsome assay although the evidence is weaker since response was restricted to only one strain.

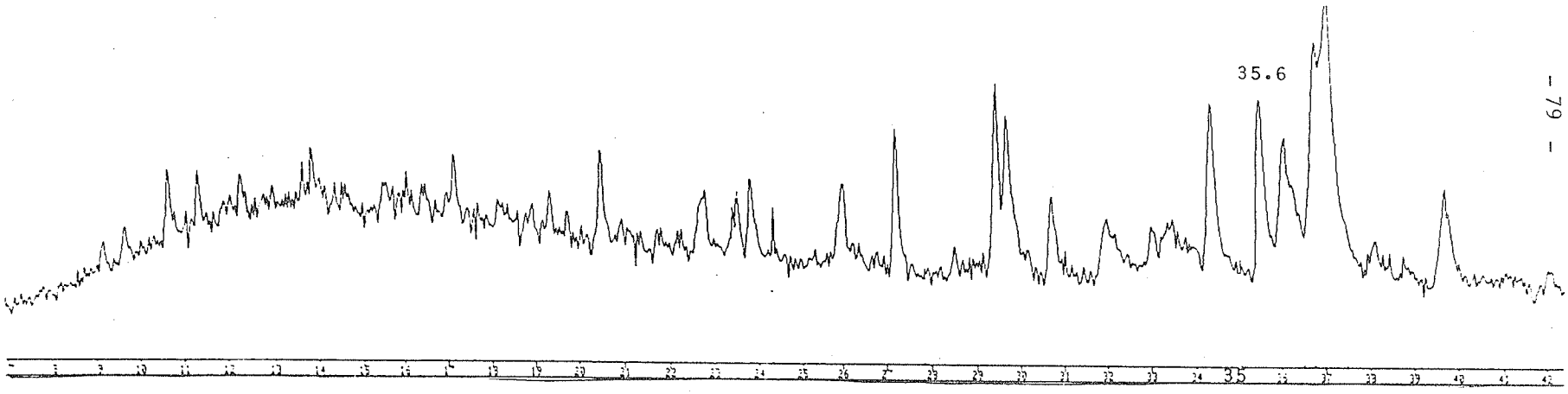
(4) the P. redivivus assay indicates the presence of toxic agent(s) in many more fractions than does the Salmonella/microsome assay.

The differences in the test results obtained using these two assays is not unexpected since they represent two independent biological tests with differing sensitivities to toxic chemicals. It is, however, noteworthy that both assays isolated Fraction 2 as being the most toxic fraction from Site VII and VIII. For this reason attention was focused on the analysis of this fraction for the purposes of isolating and identifying the toxic agents.

(3.2) ANALYTICAL RESULTS

Figure 8 represents a total ion chromatogram of Fraction 2 - Site VIII. This chromatogram was obtained using the Hewlett Packard model 5985 GC/MS system and Method A (Section 2.2.9). From this chromatogram the complexity of this fraction is evidenced, giving rise to an excess of one hundred discernible gas chromatographic peaks.

Figure 9 represents a mass spectrum of an unknown compound eluting at a retention time of 35.6 minutes. The top portion represents a mass spectrum taken at the apex of the peak (spectrum 792). The bottom portion represents a background subtracted spectrum



SCAN TIME

Figure 8 Total Ion Chromatogram of Fraction 2-Site VIII - 12 m capillary column

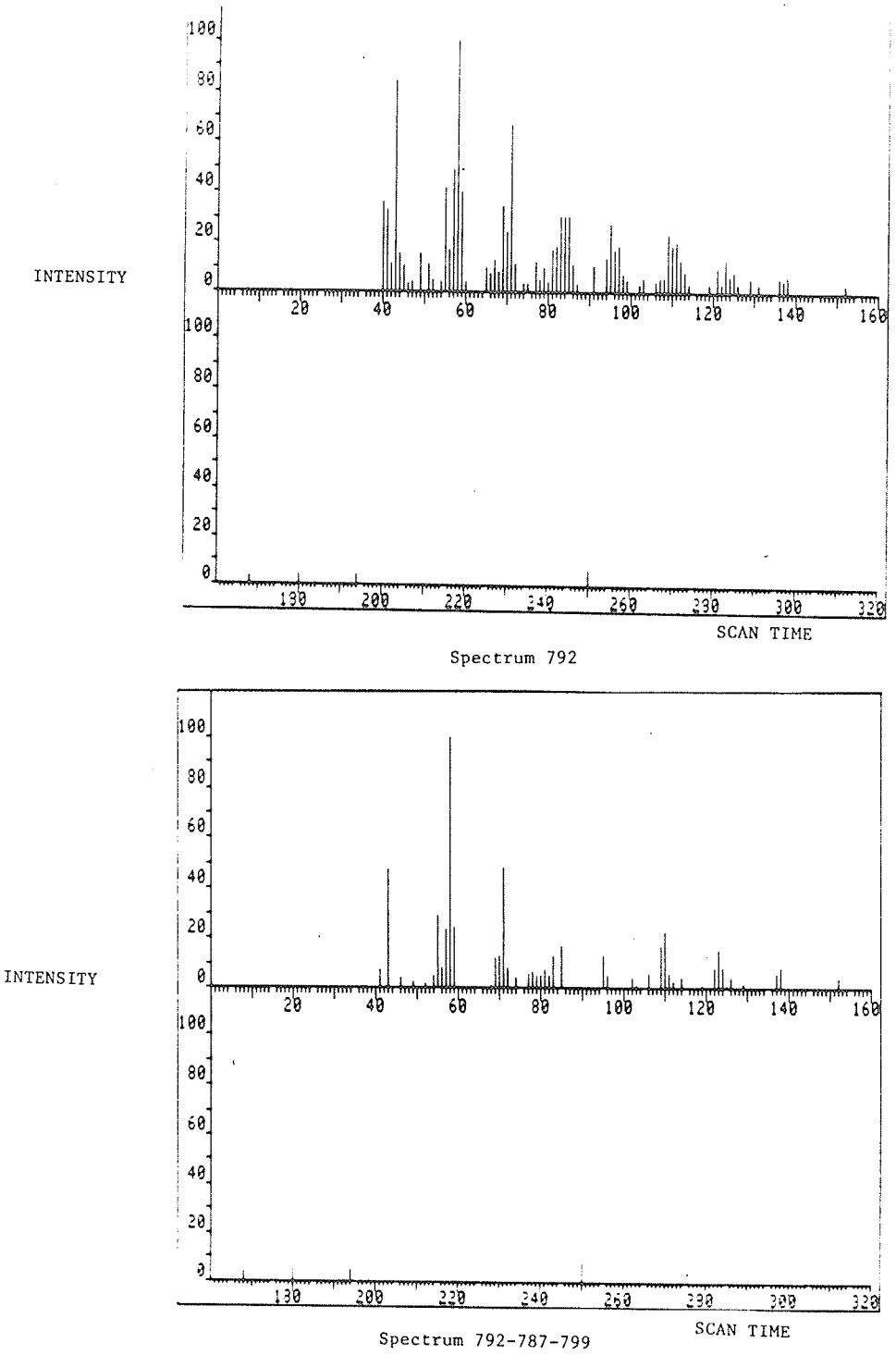


Figure 9: Spectrum 792 with and without background subtraction

(792-787-799). The difference in the two spectra is indicative of a high level of background, which may be caused by contributions from unresolved neighboring components during partial separation on the analytical column, i.e., the gas chromatography column did not adequately separate all the components in this sample.

The inadequate resolution of the analytical column can be illustrated by reference to Figure 10, which depicts a reconstructed ion chromatogram of m/z's 305, 228, and 208 over scan numbers 700 to 900. These ions were common to the many spectra taken and as such were thought to be useful in discerning the number of components responsible for a GC peak. By referring to Figure 10, it is evident that masses 305 and 208 do not maximize at the same retention time; this is indicative of the presence of two differing compounds. These masses do, however, fall in the retention window of the gas chromatographic peak eluting at 35.6 min. A mass spectrum taken at the apex of this peak (Figure 9 - top portion), shows that neither mass 305 or 208 is present in the spectrum. This evidence suggests that at least three compounds are responsible for the gas chromatographic peak appearing at retention time 35.6 min., indicating that the analytical column used does not adequately resolve this complex fraction into discernible individual components. In light of this information, it was not surprising to see a computerized deconvolution/library search and compound identification came up with only one good fit, namely, that of octadecyl alcohol for spectrum 763 (retention time = 34.4 min). This was probably the result of inadequate resolution of this complex mixture.

As a result of this information, experiments were initiated to determine the type of analytical column needed to best resolve the complex mixture present in Fraction 2.

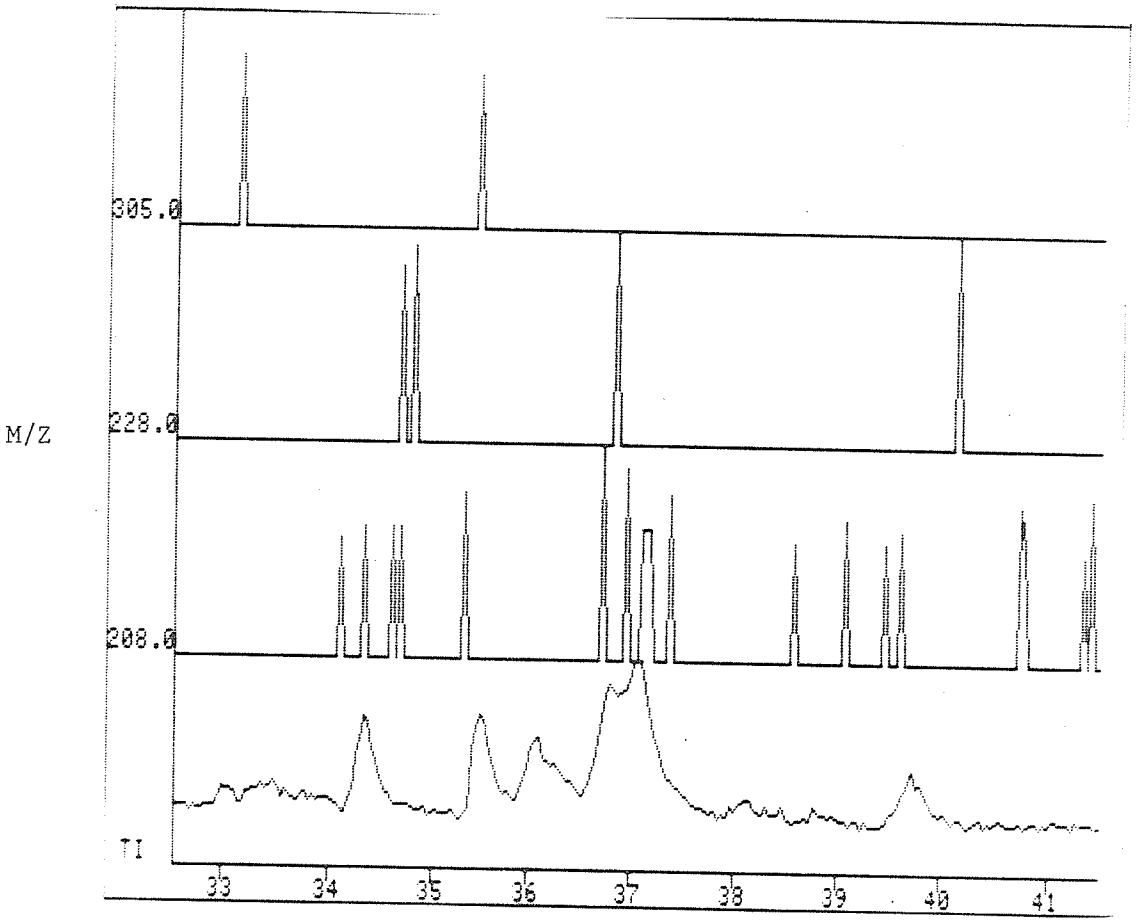


Figure 10 Mass Chromatogram of m/z 305, 228, and 208

Figure 11 depicts a GC/FID chromatogram obtained using a 30 m capillary for Fraction 2 - Site VIII. The experimental conditions are described in Method A Section 2.2.8.2. Comparison of this chromatogram to that of Figure 8, obtained on a 12 m capillary column, demonstrates that much better resolution is obtained with the 30 m column; however, the lack of baseline resolution for many components indicates that co-elution is still a problem.

The tentative identification of octadecyl alcohol in Fraction 2-Site VIII, indicated the utility of a more polar stationary phase. Since compounds were still eluting off the capillary well after a temperature of 250°C had been obtained, it was desirable to use a stationary phase stable to high temperatures. The stationary phases available which met the criteria of high temperature stability and greater polarity than SP2100 were SP2250 and SE54, according to the Supelco Capillary Catalogue. According to Supelco these stationary phases are stable to 320°C.

Evaluation of glass WCOT columns, 0.20 mm i.d. X 60 m indicated that SE54 was much more stable at high temperatures than either SP2100 or SP2250. The SP2100 and SP2250 coated capillary columns demonstrated much more bleed at 280°C than did the SE54 coated column and it is for this reason that the SE54 column was chosen to attempt resolution of the complex mixture present in Fraction 2-Site VIII.

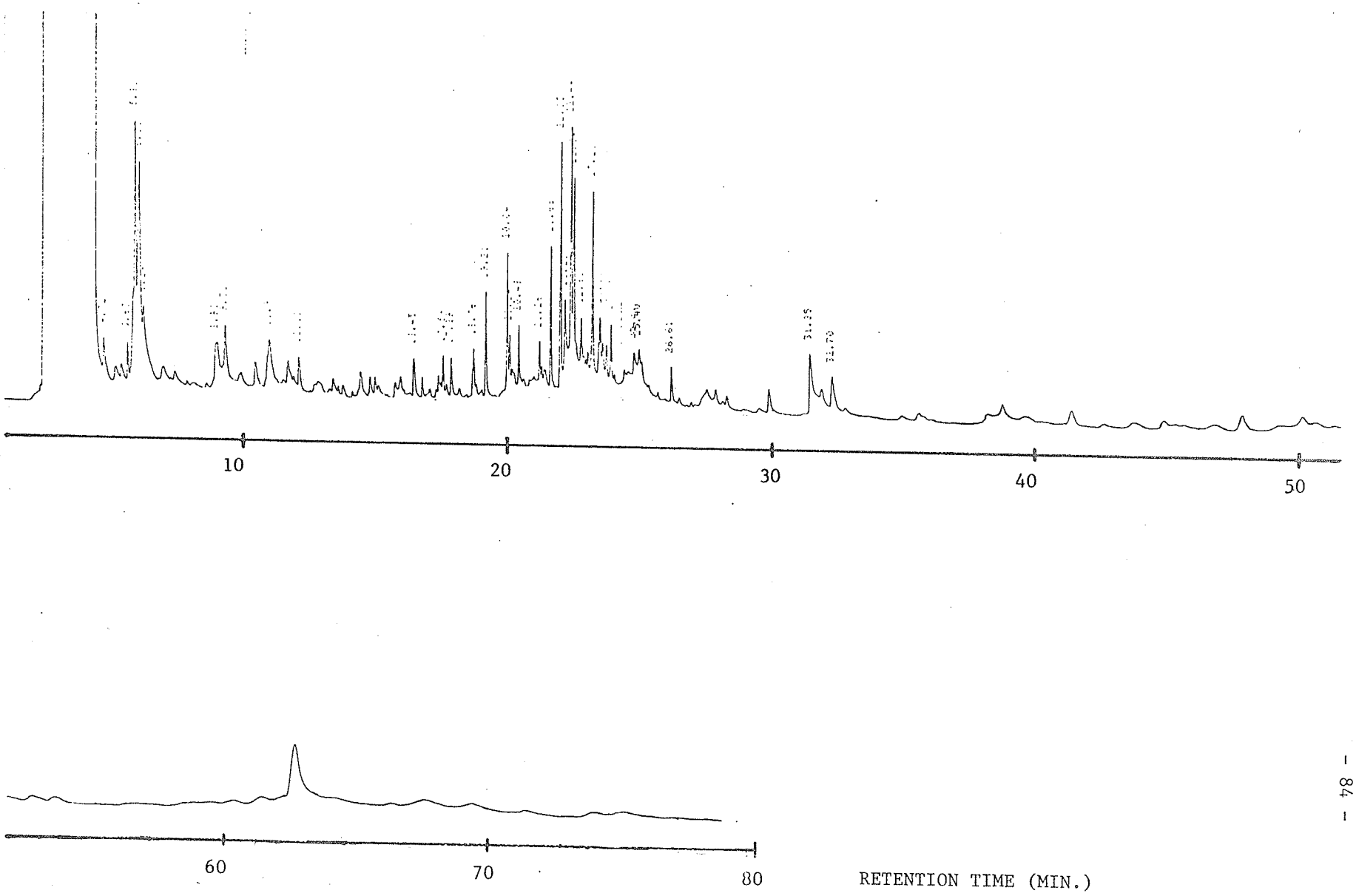


Figure 11 GC-FID chromatogram of Fraction 2-Site VIII - 30 m capillary column

Figure 12 depicts a GC/FID chromatogram of Fraction 2-Site VIII run on a 60 m column (Method B, Section 2.2.8.2). The superior resolution of this column compared to the 30 m SP2100 column is evident. Baseline resolution of most of the components was readily achieved. From this chromatogram the complexity of this fraction is readily apparent indicating the presence of many low and high boiling compounds. A run time of 3 h was required to elute all of the compounds.

Because of the complexity of this fraction it was decided to try and simplify the task of identifying those compounds responsible for the observed toxicity.

A major generalization which has developed in the past few years is that the ultimate carcinogenic forms of chemical carcinogens are usually, if not always, electrophilic reactants. This generalization is based in part on studies with carcinogenic alkylating agents (Miller and Miller, 1976).

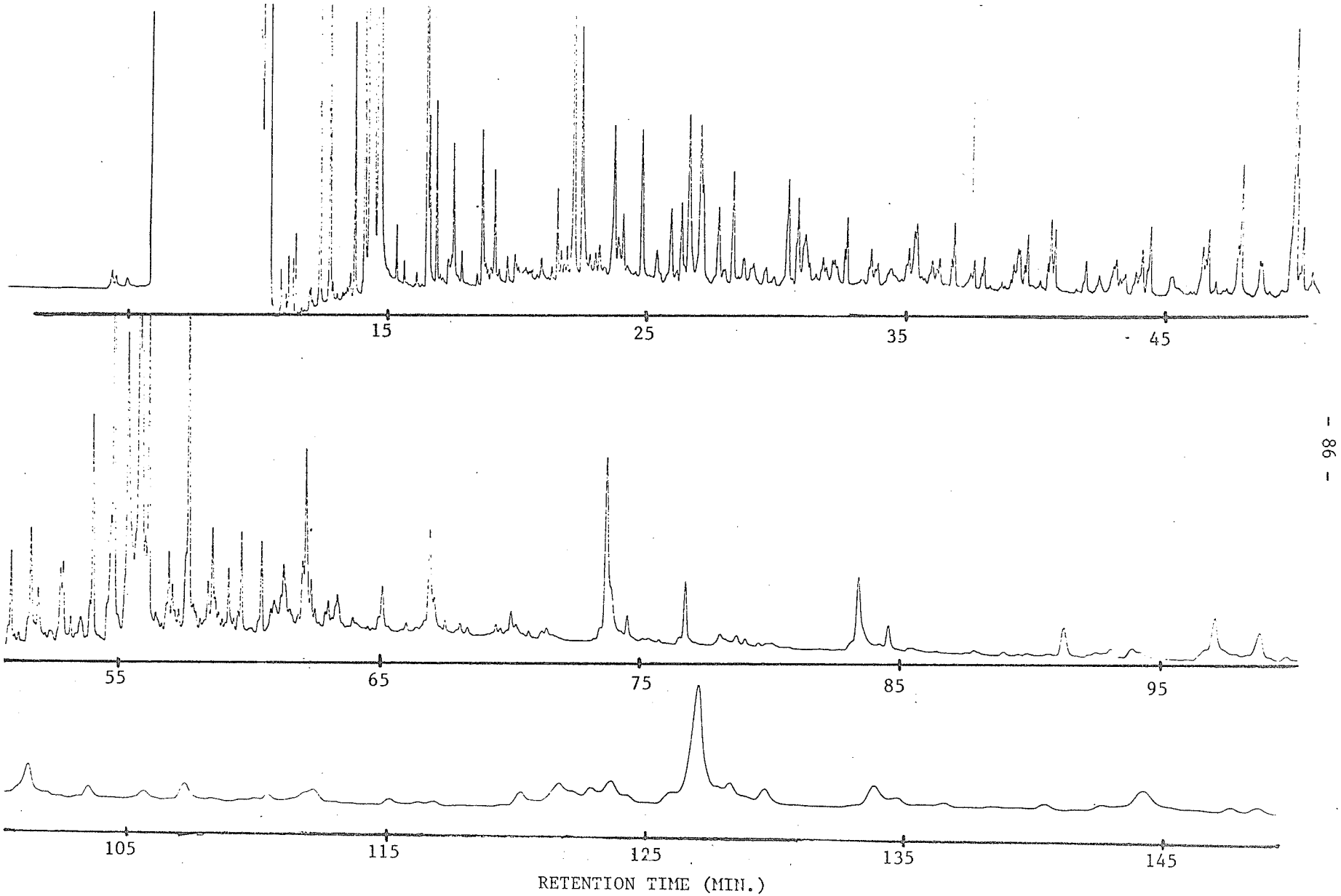


Figure 12 GC/FID chromatogram of Fraction 2-Site VIII - 60 m capillary column

Since an electron capture detector is sensitive to electron capturing (electrophilic) compounds, it was decided to look for the presence of these compounds in Fraction 2 by using a capillary column and an electron capture detector. The extract from Site VII was shown to be the most toxic using the short term bioassays, therefore the extract from this site was chosen for study.

Figure 13 depicts a GC/ECD chromatogram of Fraction 2 - Site VII using a 30 m column (Section 2.2.8.1). Comparison of this chromatogram to that obtained for the reagent blank (Figure 14) demonstrates that many electrophilic compounds are present in this fraction.

Since GC/MS, operated under electon impact (EI) ionization conditions, is commonly used for the identification of gas chromatographable organic compounds (Keith, 1976), it was considered desirable to apply this technique to identify the electrophilic compounds depicted in Figure 13. In this manner, possible carcinogens (electrophiles) present in Fraction 2 - Site VII could be identified by interpretation of the EI mass spectra from first principles.

Unfortunately, the limits of detection between EI/MS and ECD differ by as much as 2-3 orders of magnitude (Pellizzari et al., 1981). Furthermore, EI/MS is not selective towards electrophilic substances as is ECD. EI/MS responds to all compounds which readily ionize upon electron bombardment. Therefore, EI/MS is not a good method to selectively detect and identify electrophilic compounds.

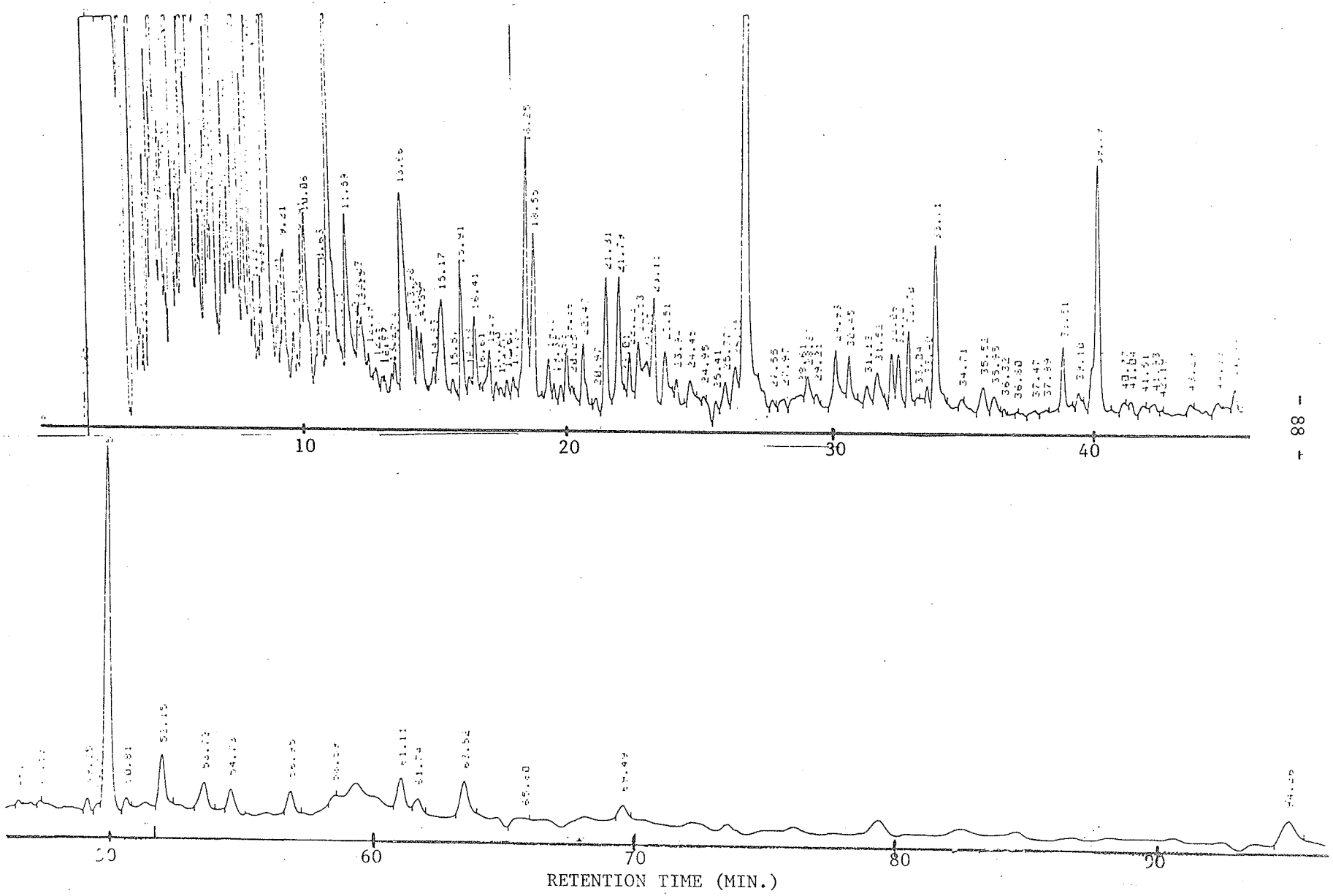


Figure 13 GC/ECD chromatogram of Fraction 2-Site VII - 30 m capillary column

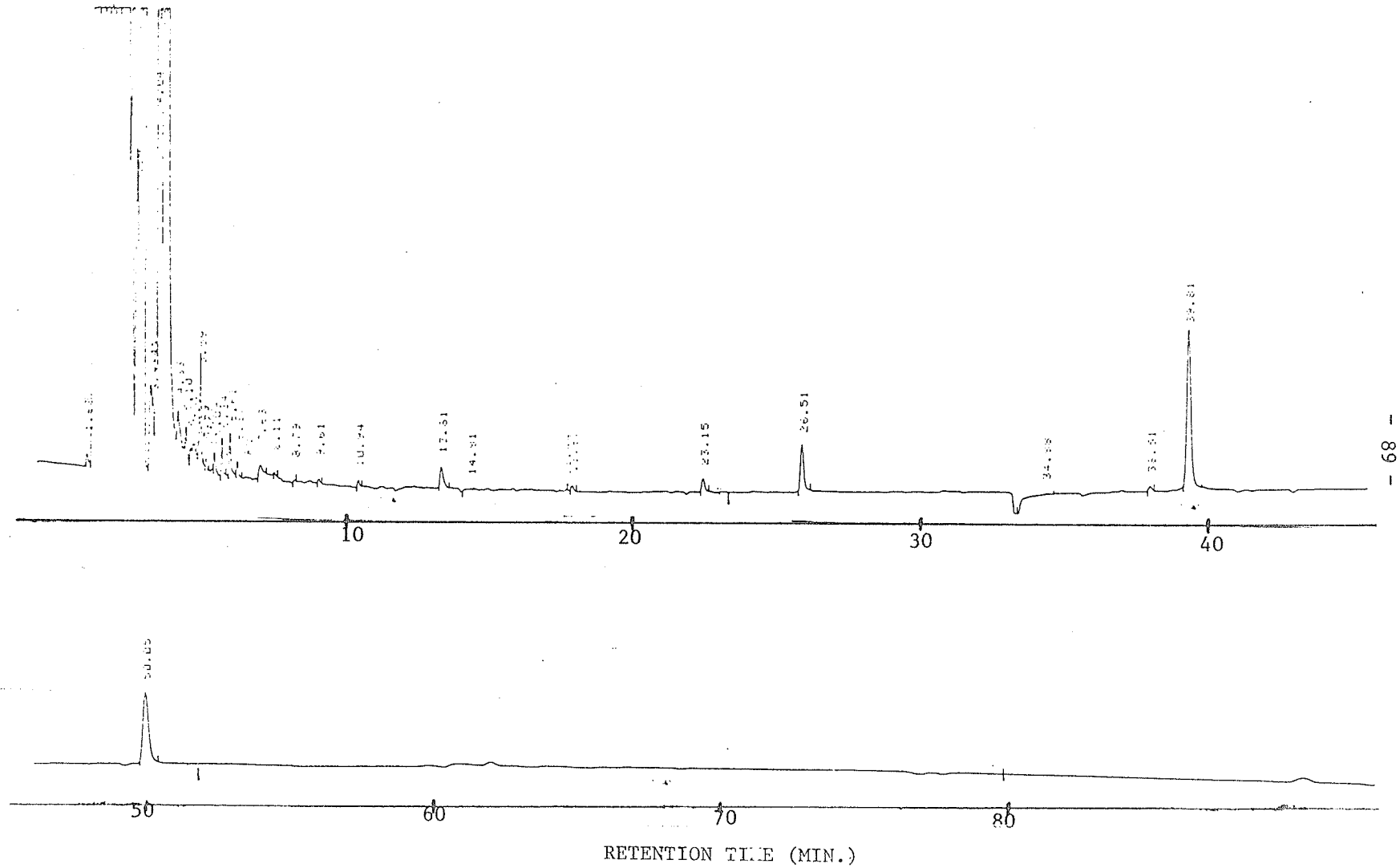


Figure 14 GC/ECD chromatogram of reagent blank

Negative chemical ionization (NCI) mass spectrometry, on the other hand, is highly responsive to electrophiles. The analytical application of NCI mass spectrometry is broader but quite similar to that of gas chromatography with electron-capture detection. Compounds that give intense electron-capture responses will virtually always be sensitive to NCI mass spectrometry (Kuehl and Dougherty, 1980).

NCI mass spectra are obtained by operating a chemical ionization mass spectrometer (ion source pressure ca. 0.75 to 1.1 Torr) in the negative ion mode. At 0 to 10 eV ionizing voltage, the major negative ion forming reactions involve resonance capture of low energy electrons. With a wide spectrum of low energy electrons in the source, negative ion formation is highly probable and the resultant ion beams are often several times as intense as the positive ion beams obtained under identical conditions (Dougherty et al., 1972).

NCI spectra show very little structural information as opposed to EI spectra. Due to the large population of thermal electrons in the ion source, resonance electron capture is the dominant mechanism for the formation of negative ions under CI conditions. As a result, spectra recorded under NCI conditions exhibit abundant molecular anions ($M^{\cdot -}$) for many types of molecules (Hunt and Sethi, 1978).

Because NCI spectra are dominated by molecular anions ($M^{\cdot -}$), and show little fragmentation, which is desirable for discerning the structure of unknown compounds, this technique has been primarily

applied to the identification of toxic organochlorine compounds. These compounds produce NCI mass spectra which are usually dominated by a chlorine-containing isotope cluster (Dougherty and Piotrowska, 1976). The characteristic isotopic distribution of chlorine atoms (i.e. 75.8% ^{35}Cl and 24.2% ^{37}Cl) permits easy recognition of compounds which contain one or more of these atoms. The chlorine-containing isotope cluster is recognized from the characteristic isotopic ratio of the ions separated by two mass units (McLafferty, 1980). The characteristic isotopic ratios of ions containing from one to five chlorine atoms are depicted in Table 5.

Table 5 Natural Abundances of combinations of chlorine (from McLafferty, 1980)

Number of Chlorine Atoms	Mass	Relative Abundance	Number of Chlorine Atoms	Mass	Relative Abundance
1	A	100.0	4	A	76.9
	A + 2	32.5		A + 2	100.0
				A + 4	48.7
2	A	100.0		A + 6	10.5
	A + 2	65.0		A + 8	0.9
	A + 4	10.6			
			5	A	61.5
3	A	100.0		A + 2	100.0
	A + 2	97.5		A + 4	65.0
	A + 4	31.7		A + 6	21.1
	A + 6	3.4		A + 8	3.4
				A + 10	0.2

The mass "A" in this table refers to the first ion of the cluster. When a major chlorine-containing isotope cluster is observed in the NCI spectrum of a mixture, the number of chlorines present is estimated by isotope abundance and the compound is identified by searching available spectra whose molecular weight is related to the mass of the first ion of the cluster as (1) exact, (2) minus 35 daltons, (3) plus one dalton, and (4) minus 19 daltons, respectively. The identity of the compound can then be confirmed by examining the NCI spectra and gas chromatography mass spectra of the unknown and an authentic sample (Dougherty and Piontrowska, 1976). NCI mass spectra have been obtained for polychlorinated pesticides (Dougherty et al. , 1973), polycyclic chlorinated insecticides (Dougherty et al. , 1972), aromatic chlorinated pesticides (Dougherty et al. , 1975), polychlorinated-p-dioxins (Haas et al. , 1978), polychlorinated diphenyl ethers (Busch et al. , 1979), polychlorinated 2-phenoxy phenols (Busch et al. , 1980), polychlorinated biphenyls (Pellizzari et al. , 1981) and other chlorinated xenobiotic chemicals (Dougherty et al. , 1980).

Since biomolecules do not in general attach thermal electrons or gas phase nucleophiles, NCI mass spectrometry has great potential for selective detection of toxic substances, like organochlorine compounds, in complex sample extracts (Dougherty, et al. , 1980).

In light of these considerations, NCI mass spectrometry was used to determine whether or not the electrophilic compounds depicted in Figure 13 contained chlorine, i.e. were organochlorine compounds. Using this method, selective detection and identification of toxic organochlorine compounds in a complex sample is possible.

Figure 15 depicts a total NCI chromatogram of Fraction 2 - Site VII obtained using a 50 m column (Method D, Section 2.2.9). This chromatogram depicts the presence of several electrophilic compounds well resolved from one another. The mass spectra are displayed in Figures 16 through 20. Careful study of the spectra reveals that no chlorine containing compounds are present, i.e., no ions two mass units apart and having isotopic ratios similar to those depicted in Table 5 are evident. Furthermore, the spectra show little structural information making identification of the compounds difficult if not impossible. In order to identify the electrophilic compounds presented in Figure 15, additional structural information is required. This additional information is usually provided by obtaining an electron impact (EI) ionization mass spectrum of the compounds of interest. Unfortunately, compounds detected by NCI mass spectrometry are not always detected by EI mass spectrometry due to sensitivity differences. The sensitivity associated with ion formation by electron capture in the CI source can be 100 to 1000 times greater than that available by any positive ion (such as EI) methodology (Hunt and Sethi, 1978). This can be illustrated by reference to Figure 21. The upper portion depicts a total NCI chromatogram obtained for Fraction 2 - Site VII using Method D, Section 2.2.9. The bottom portion depicts a total EI chromatogram for the same sample using Method C, Section 2.2.9. The GC conditions for both methods were the same. The two most electrophilic compounds (spectrum 1262 and 1399) detected under NCI conditions are barely

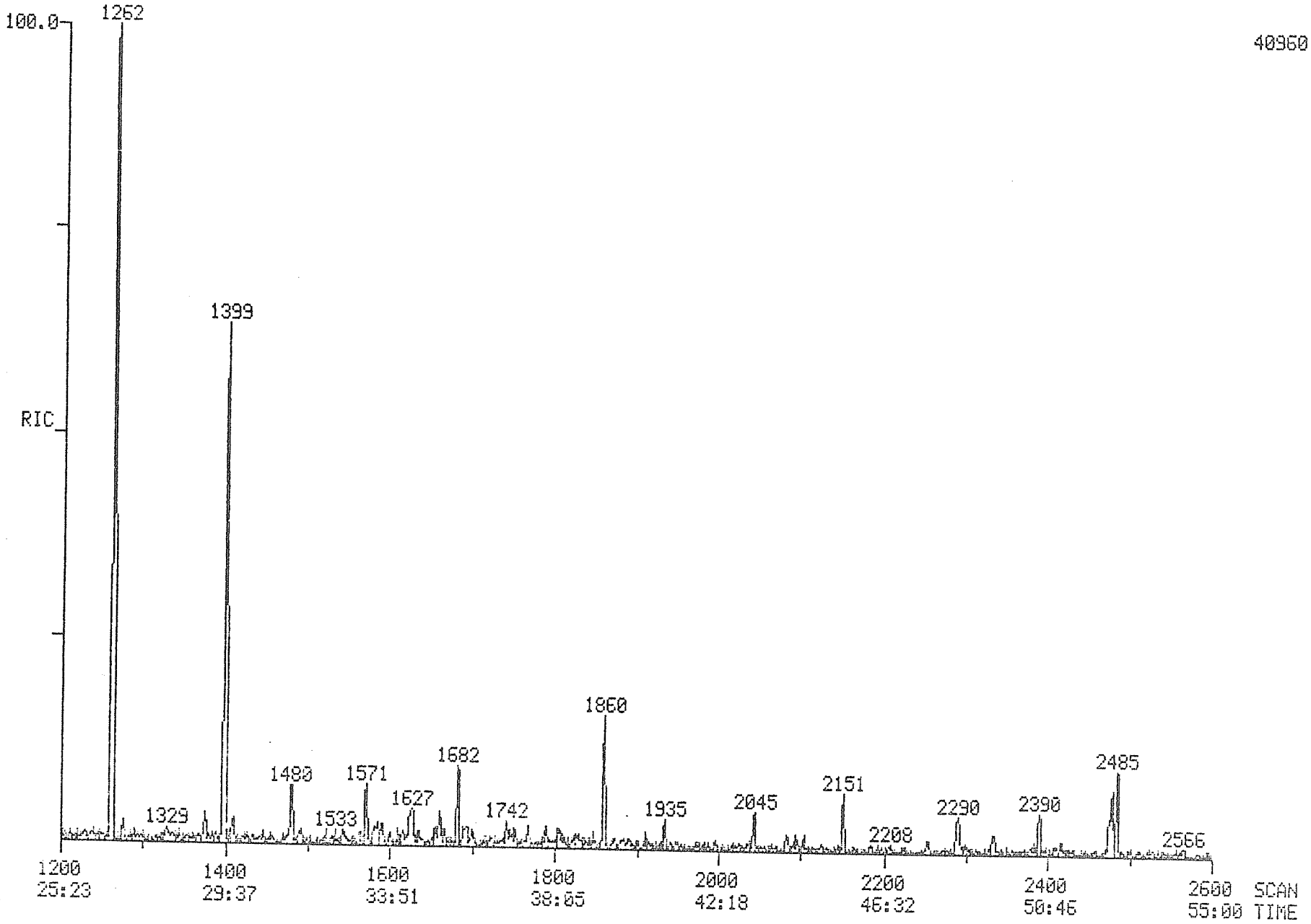


Figure 15 Total ion chromatogram, Fraction 2-Site VII, negative chemical ionization, 50 m column

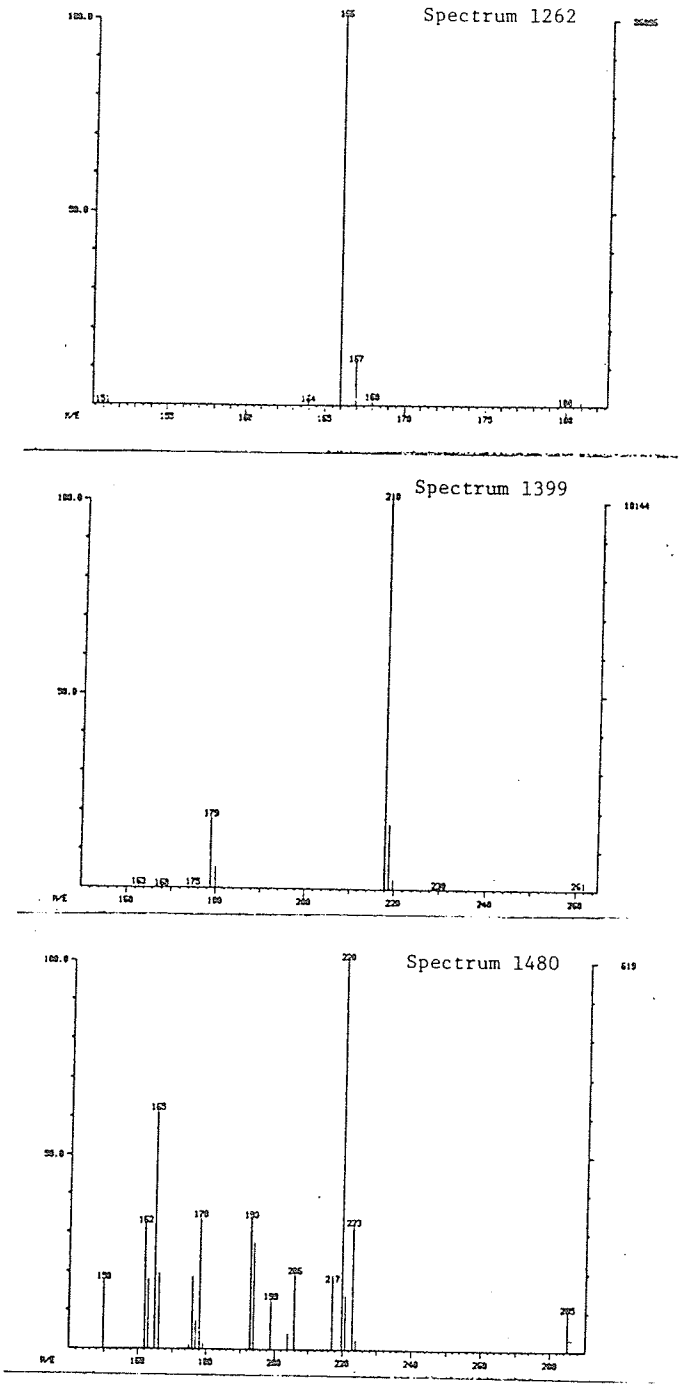


Figure 16 NCI Spectra 1262, 1399 and 1480

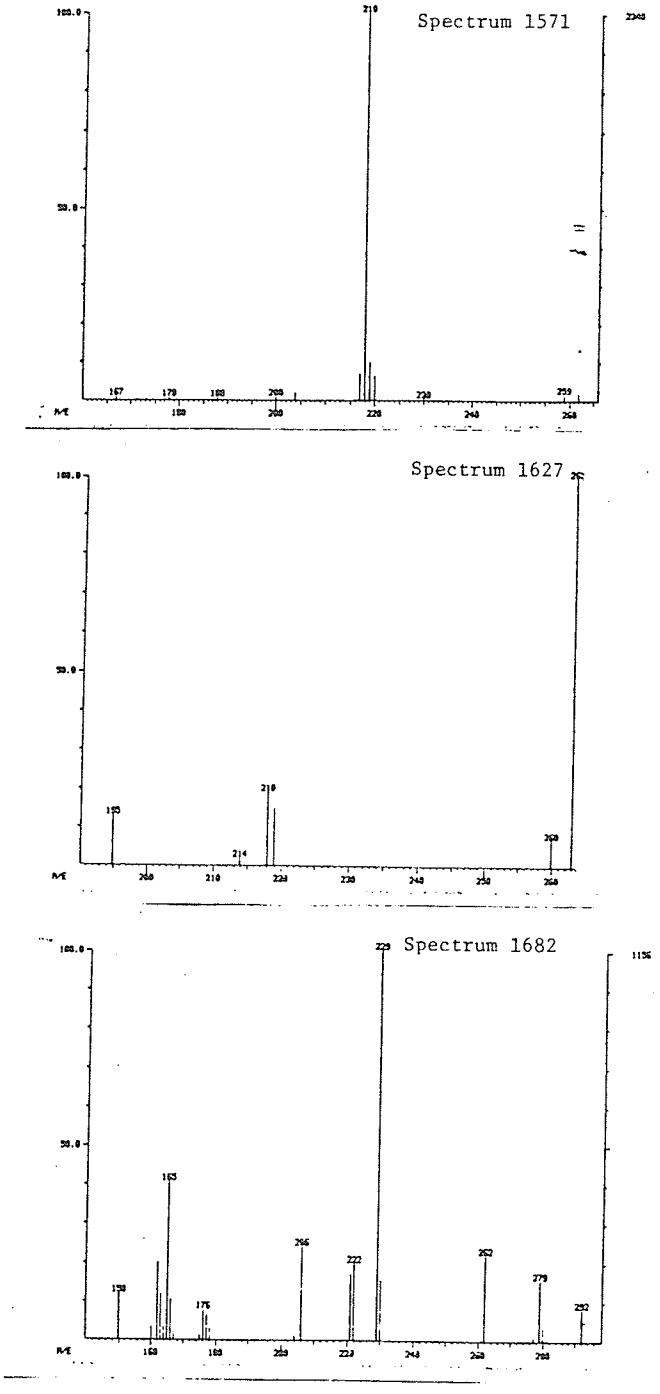


Figure 17 NCI Spectra 1571, 1627 and 1682

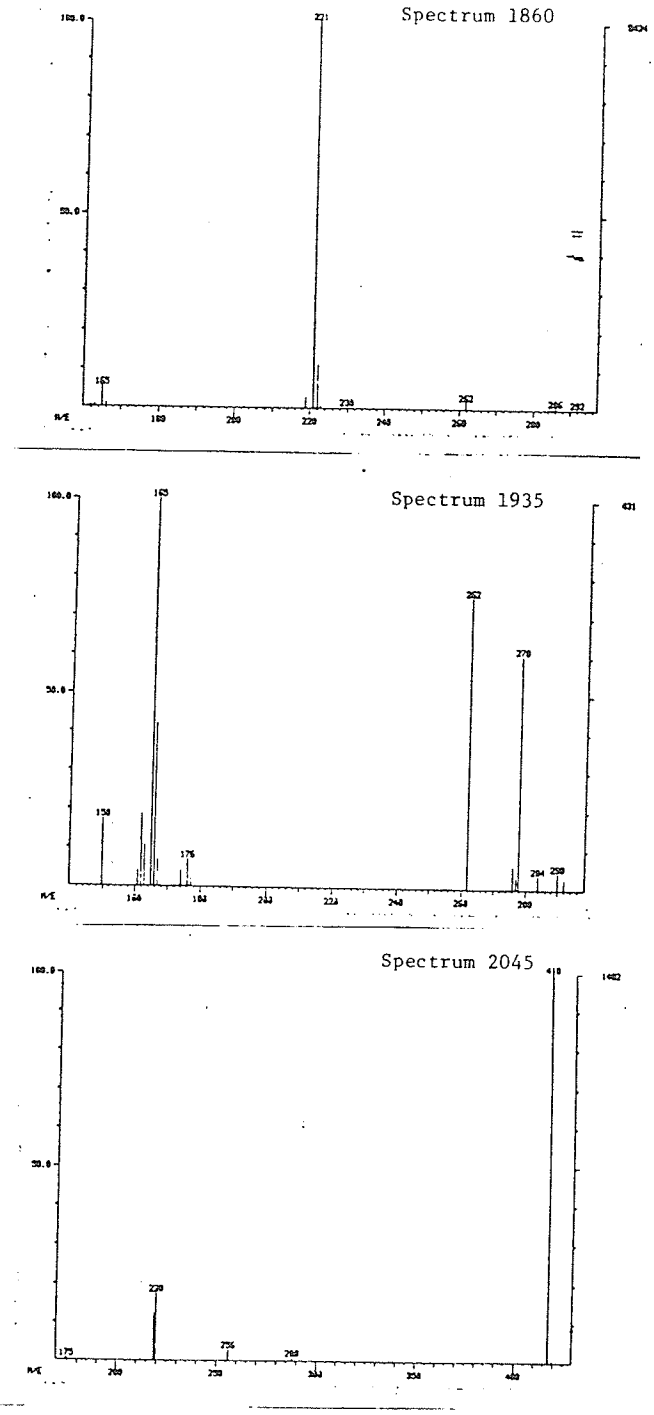


Figure 18 NCI Spectra 1860, 1935 and 2045

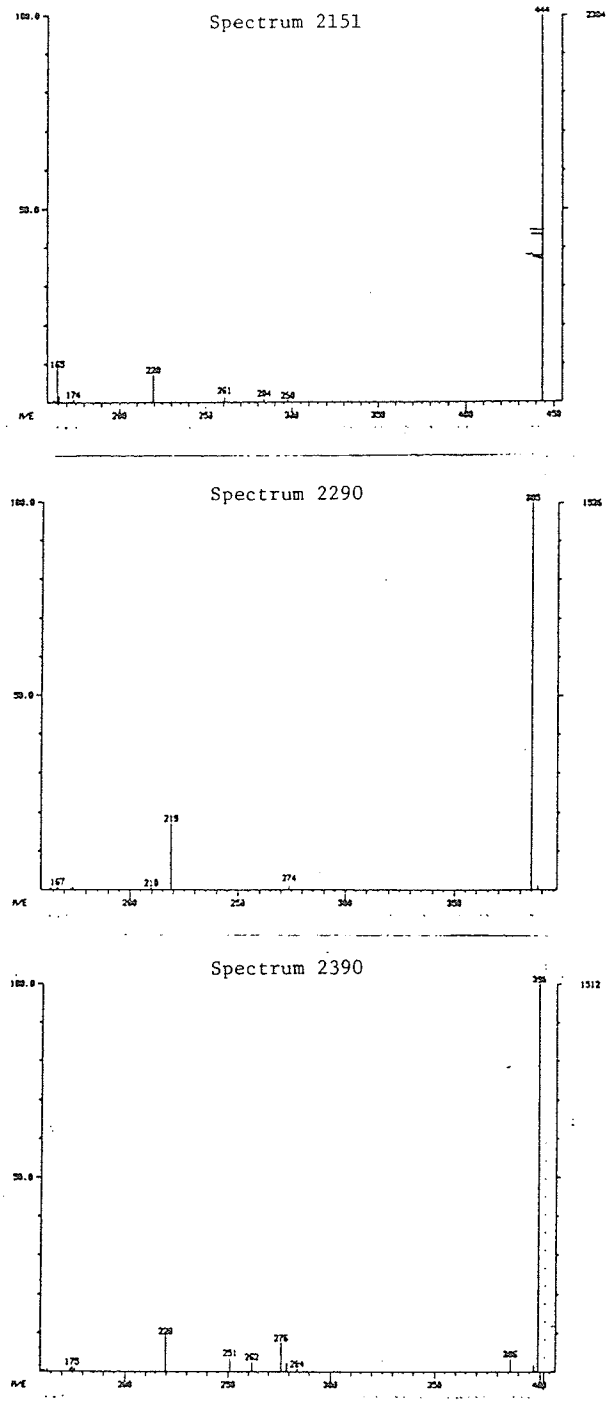


Figure 19 NCI Spectra 2151, 2290 and 2390

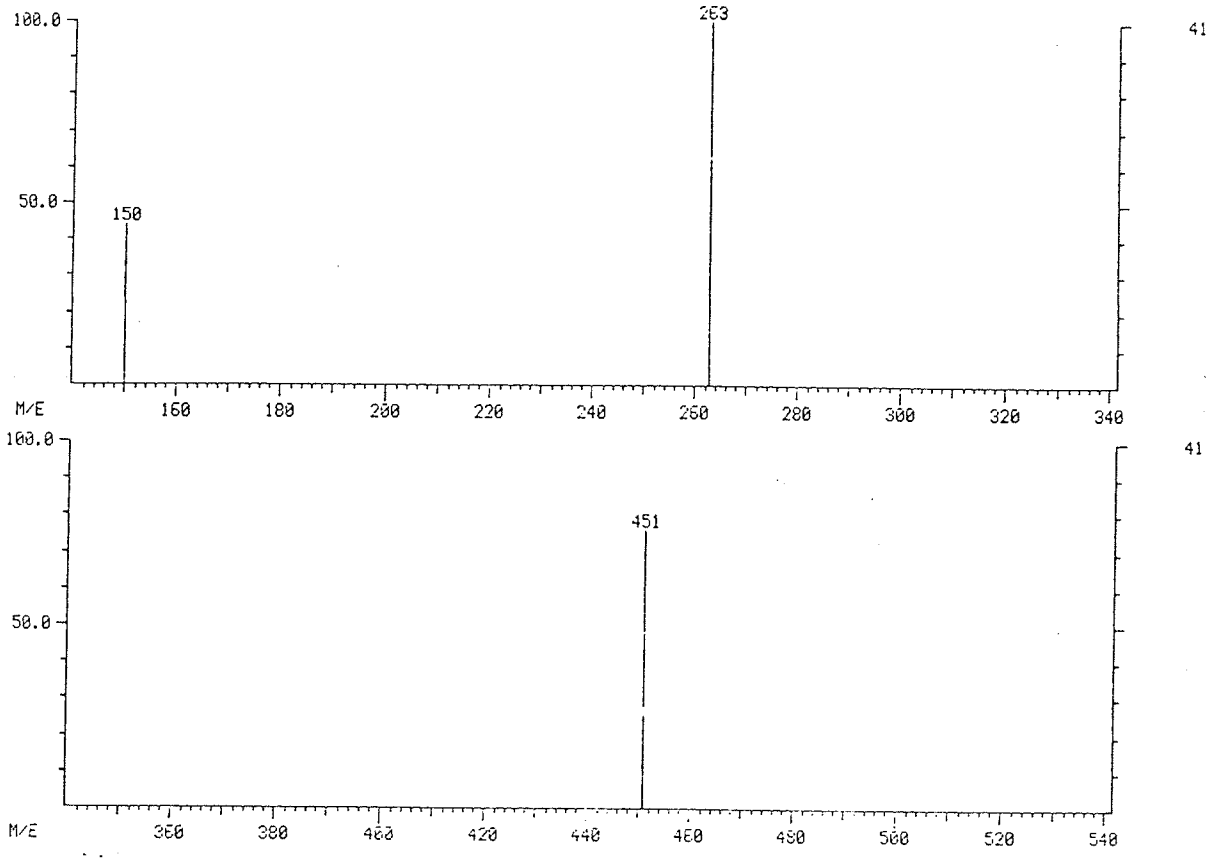


Figure 20 NCI Spectrum 2495

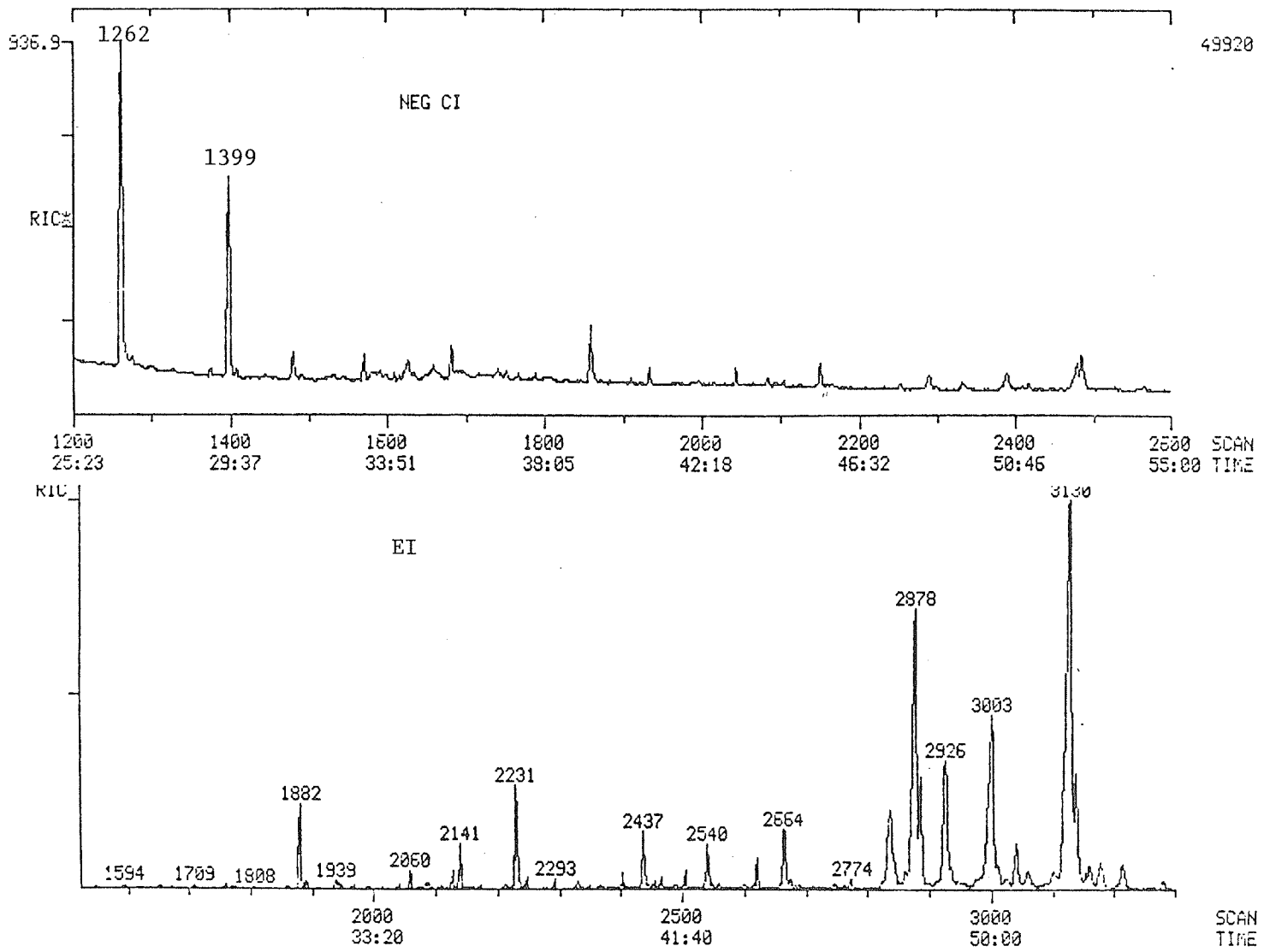


Figure 21 Comparison of NCI total ion chromatogram to EI total ion chromatogram, Fraction 2-Site VII.

detectable under EI conditions, thereby showing the sensitivity differences between the two methods. Figure 22 depicts the EI spectrum of the most electrophilic compound present in this sample (NCI spectrum #1262). Comparison of this spectrum to that of the NCI spectrum (Figure 16) shows the presence of m/z 166 in both spectra, which is consistent with the same compound being present. This is supported by the observation of similar retention times since NCI spectrum 1262 (Figure 21) had a retention time of 26.51 min and EI spectrum 1594 (Figure 21) had a retention time of 26.54 min under the same GC conditions.

EI spectrum 1594 (Figure 22) was searched in the Eight Peak Index of Mass Spectra (1974), a reference library of mass spectra. No reference spectrum could be found to match this spectrum. This is not surprising since this compound was barely detectable under EI ionization; only 59 ion counts were registered for this spectrum. It is possible that only the most abundant ions of this compound were detected under EI conditions due to insufficient material. As a result, other less abundant ions characteristic of this compound would not be detected and portrayed in the spectrum, making interpretation difficult. This hypothesis can be validated, if one compares the NCI spectrum (#1262, Figure 16) to the EI spectrum (#1594, Figure 22). Spectrum 1262 shows the presence of an m/z 167 ion which is the ^{13}C isotope of m/z 166. The relative abundance of m/z 167 to m/z 166 is 11.2%. Since the abundance ratio of ^{13}C / ^{12}C is 1.08% (McLafferty, 1980), m/z 166 could contain from 9 to 11 carbon atoms if one allows for a 10% experimental error as recommended by McLafferty (1980). The carbon number is calculated as follows: $(11.2 \pm 1.12)/1.08 = 10 \pm 1$. Since spectrum 1594 (Figure 22) is the EI spectrum of the same compound depicted in NCI spectrum 1262 (Figure 16), it should also show a ^{13}C isotope for m/z 166, i.e., the presence of a m/z 167 ion with an

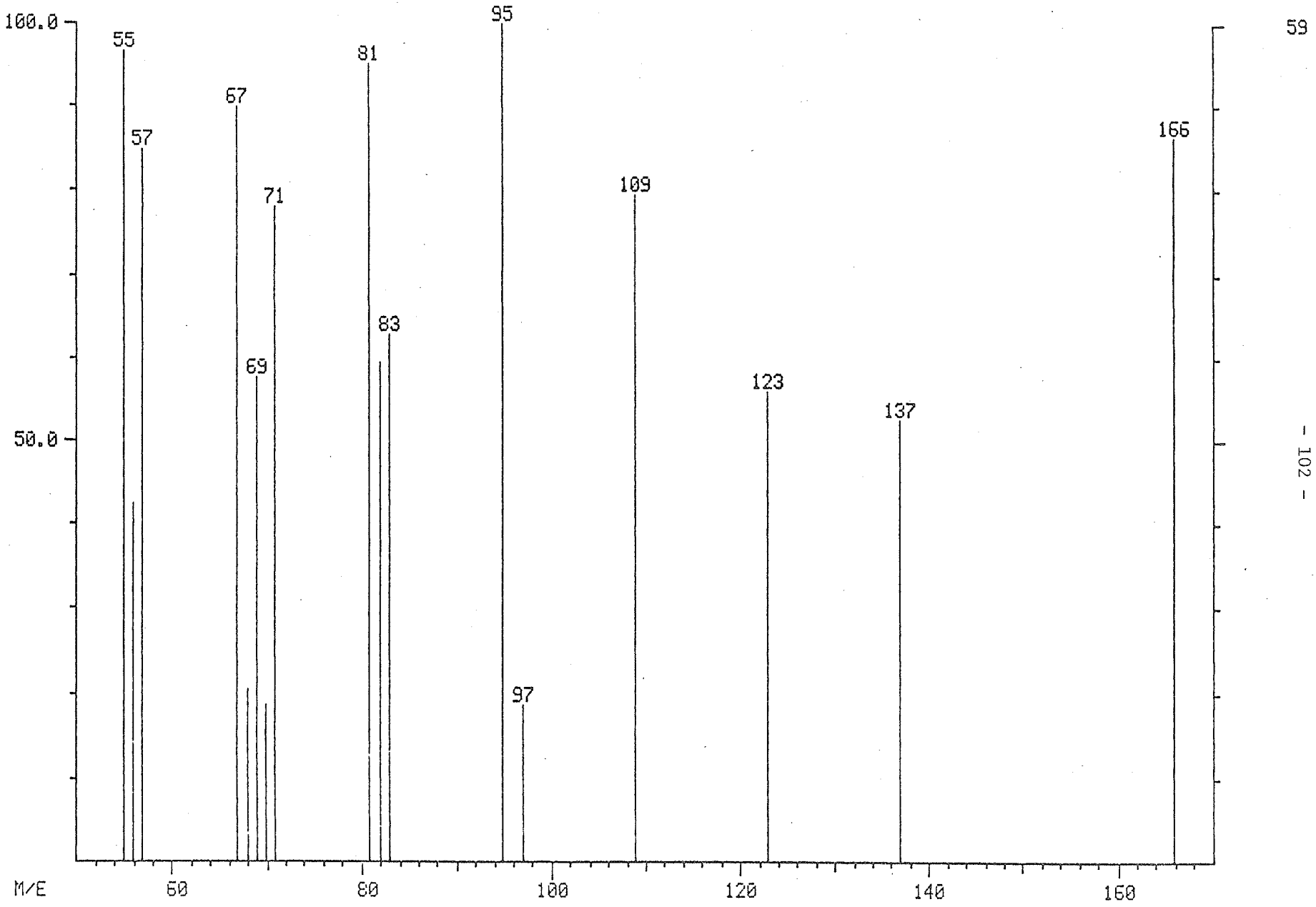


Figure 22

Spectrum 1594

abundance of 11.2% relative to the intensity of m/z 166 should be evident. The observation that a m/z 167 ion is absent in this spectrum (Figure 22) is evidence that there is insufficient material present under EI conditions to obtain a good spectrum, and that the spectrum was obtained below the instrument's detection limit.

Reference to Figure 15 shows the presence of another strongly electrophilic compound eluting at a retention time of 29.4 min. The NCI mass spectrum for this compound is depicted in Figure 16 (spectrum 1399). This spectrum is dominated by m/z 218 and its ^{13}C isotope m/z 219. Using EI ionization, analysis of this sample did not detect any compound eluting at a retention time of 29.4 min. Had this electrophilic compound been detected under EI ionization, a GC peak should have appeared around scan number 1766 (Figure 15). Furthermore, an ion chromatogram for m/z 218 and m/z 219 generated in the retention window of 28 to 31 min. indicated the complete absence of any compound containing these ions under EI ionization.

These results demonstrate that NCI mass spectrometry is very capable of detecting low concentrations of electrophilic compounds; they also show that NCI mass spectrometry is limited in terms of providing sufficient structural information to allow for the identification of these compounds. Furthermore, it is not always possible to obtain an EI spectrum for a compound detected under NCI conditions because NCI/MS is often more sensitive towards electrophiles that is EI/MS. An EI mass spectrum would certainly provide the necessary structural information to allow for the identification of an unkown electrophile. It is perhaps for these reasons that NCI/MS has been used primarily to detect and identify organochlorine compounds, since these compounds yield spectra

which are more informative than NCI spectra of unchlorinated compounds.

Since NCI mass spectrometry is sensitive and selective towards organochlorine compounds, which are generally toxic (Dougherty et al., 1980), this method should be applied first as a screen for these compounds in complex extracts which are known to be toxic. Although chlorinated pesticides were not in general put through the fractionation scheme portrayed in Figure 6, in order to determine which chlorinated pesticides associated with the various fractions, it was determined experimentally that when methoxychlor was applied to a 5% water deactivated Florisil column, 98% was recovered by elution with hexane: DCM (1:1). There is reason, therefore, to use NCI/MS to screen Fraction 2 extracts for organochlorine compounds, since chlorinated compounds with polarities similar to methoxychlor would be recovered in Fraction 2. Furthermore, methoxychlor has been used in each of the north and south branches of the Saskatchewan River for over a decade as a black fly larvicide (Fredeen, 1979). Since methoxychlor is rapidly adsorbed onto silt particles carried by the water (Fredeen et al., 1975), and since Tobin Lake acts as a sink for these two river systems (Birkholz et al., 1980), NCI mass spectrometry is a good method to check for the presence of this compound in Fraction 2 extracts from Tobin Lake.

In light of these considerations, it was decided to look for the presence of organochlorine compounds in Fraction 2-Site VIII using NCI mass spectrometry even though no evidence for their existence was found in Fraction 2-Site VII using this method.

Figure 23 depicts a total NCI ion chromatogram of Fraction 2-Site VIII. The analytical conditions are described in Section 2.2.9 Method E. Careful study of the spectra did not reveal the presence of any chlorinated compounds. Some typical spectra are depicted in Figure 24.

Since little success was encountered in identifying electrophilic compounds present in Fraction 2-Site VII and VIII, using NCI/MS directly another method needed to be applied to allow for the identification of these compounds. These compounds are good candidates for chemical carcinogens (Miller and Miller, 1976) and therefore should be identified if at all possible. Furthermore, the NCI total ion chromatograms for these fractions (Figure 15 and 23) are less complex than the corresponding EI chromatograms (Figure 25 and 46) thereby increasing the probability of identification of these compounds under NCI since selective detection reduces the confusion created by coelution.

One technique that could be applied for the identification of these compounds is mass spectrometry/mass spectrometry (MS/MS). In its simplest form, MS/MS involves the selection, from a mixture, of one ionic species formed by an initial operation (ionization in a source), followed by an energetic collision of the ion with a neutral target gas in a field-free region, followed by mass analysis of the daughter ions using a second mass analyzer (Cooks

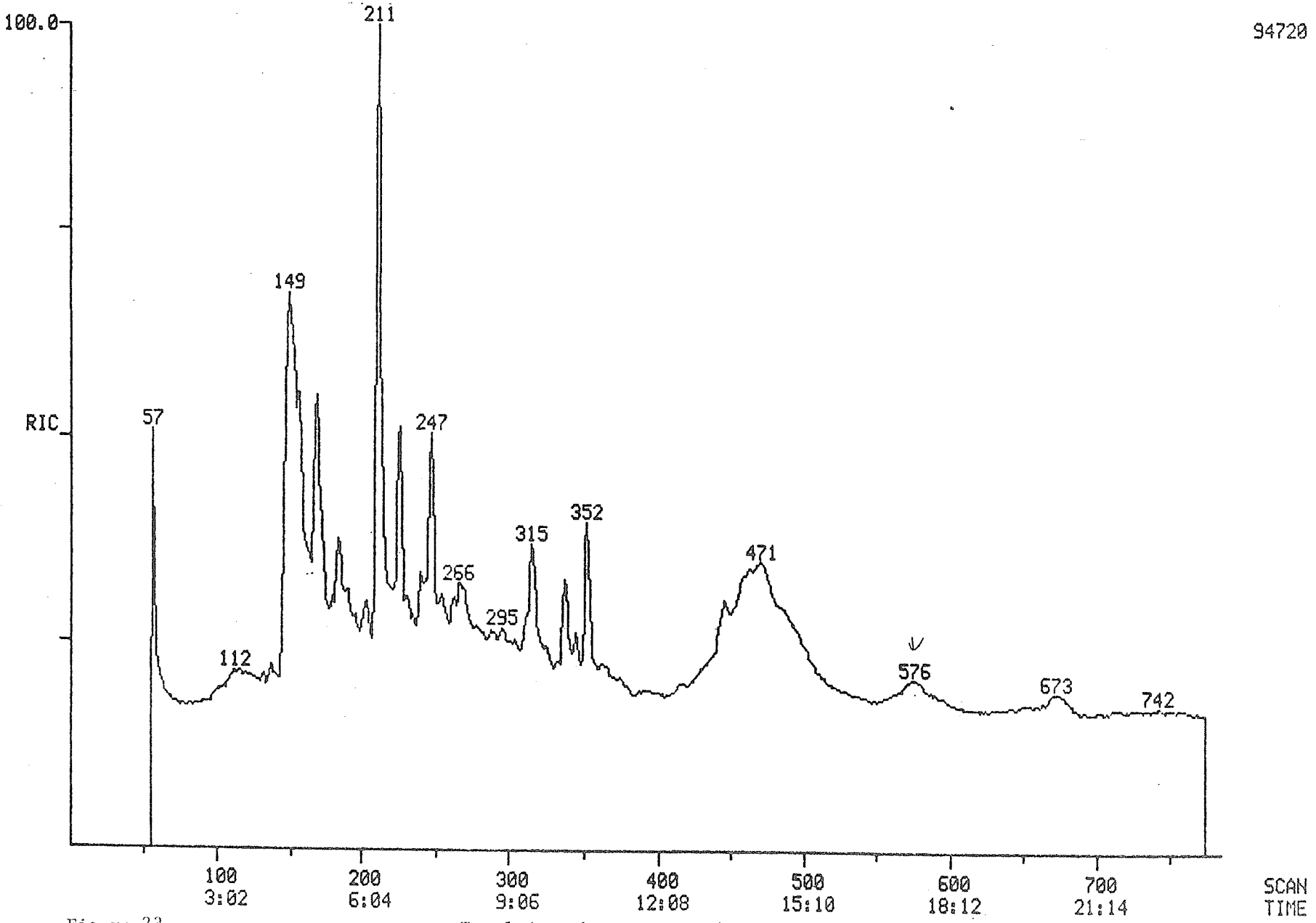


Figure 23 Total ion chromatogram (NCI) Fraction 2 Site VIII

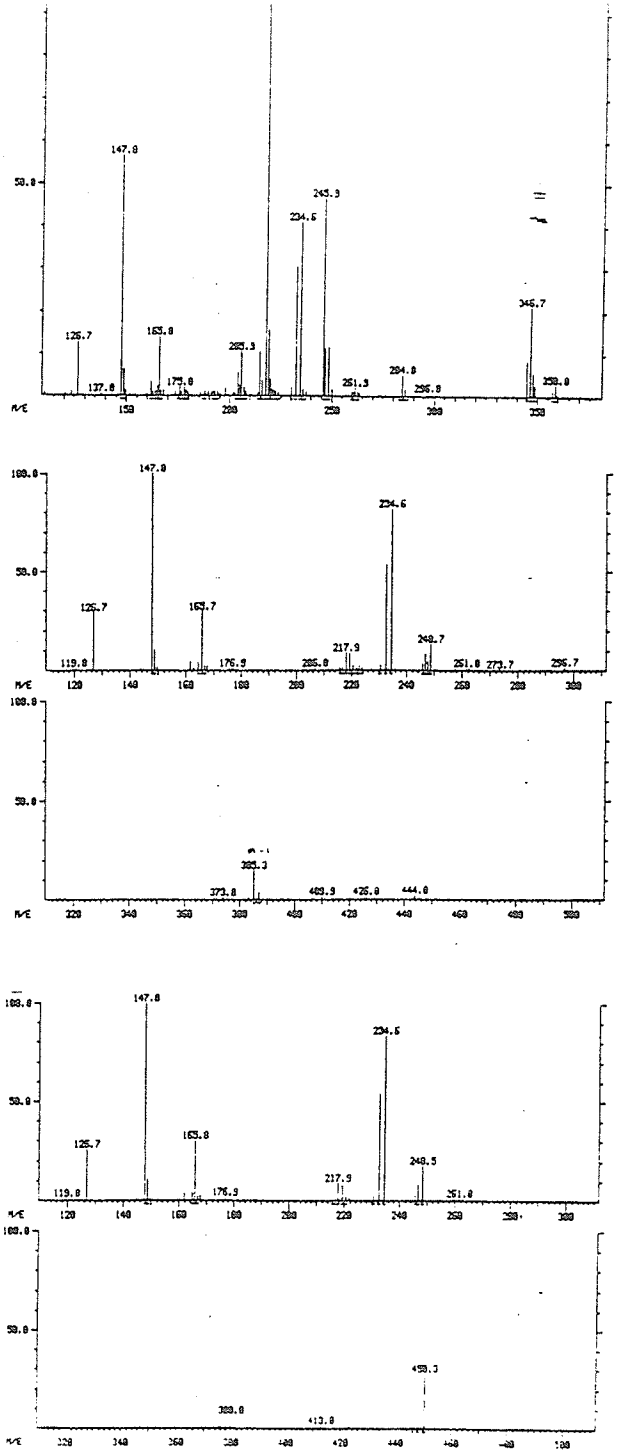


Figure 24 NCI Spectra 211, 576 and 673

and Glish, 1981). As an example of the use of this technique, the identification of the electrophile appearing at scan number 1262 (Figure 15) in Fraction 2 - Site VII could be effected by ionizing the extract in the source of the instrument using NCI. The ion with m/z of 166 (assumed parent ion of scan 1262, Figure 16) would be selected by the first mass analyzer and subjected to an energetic collision with a neutral target gas in the field free region of the instrument. The resulting daughter ions (cations) produced by this collision would then be scanned using a second mass analyzer and from this spectrum identification of the compound could be effected by interpretation of the MS/MS spectrum from first principles.

Unfortunately, such instrumentation was not readily available to the author, however, there are some universities such as Purdue and Cornell and private companies such as SCIEX where such instrumentation is available and analysis of samples can be accommodated on a cost recovery basis. Due to the cost involved, MS/MS analysis of Fraction 2 extracts was not pursued, however, GC/NCI/MS followed by MS/MS might be a very cost effective tool for assessing the presence and identification of potential chemical carcinogens for Environment Canada.

Since MS/MS analysis of the Fraction 2 extracts was not feasible a more conventional method, namely EI/MS was used in an attempt to identify the toxic components present in Fraction 2 - Site VII and VIII.

Three replicate samples of Fraction 2 - Site VII were analyzed using GC/MS under EI ionization. The analytical conditions are described in Section 2.2.9 Method C. Figure 25 depicts a typical total ion chromatogram obtained from this sample. All mass spectra obtained were subjected to a computerized deconvolution/library search and compound identification routine using the NBS mass spectral data file. Table 6 depicts those compounds for which good matches were obtained. A search of the computerized NIH/EPA Chemical Information System file NIOSH-Registry of Toxic Effects of Chemical Substances indicated that methylbenzene (toluene), 2-hexanethiol, ethenylbenzene (styrene), benzaldehyde and 1,2-dimethylbenzene (o-xylene) were toxic. This data file indicated that styrene and benzaldehyde were both mutagens, that o-xylene had a high aquatic toxicity rating (TLm96: 100-10 ppm) and that n-alkane thiols were toxic upon inhalation; a threshold limit value of 0.5 ppm in air was recommended. In light of this information, it was decided to have a closer look at the spectra of these compounds, compare them with reference spectra and determine experimentally if the retention times of the unknowns matched reference standards.

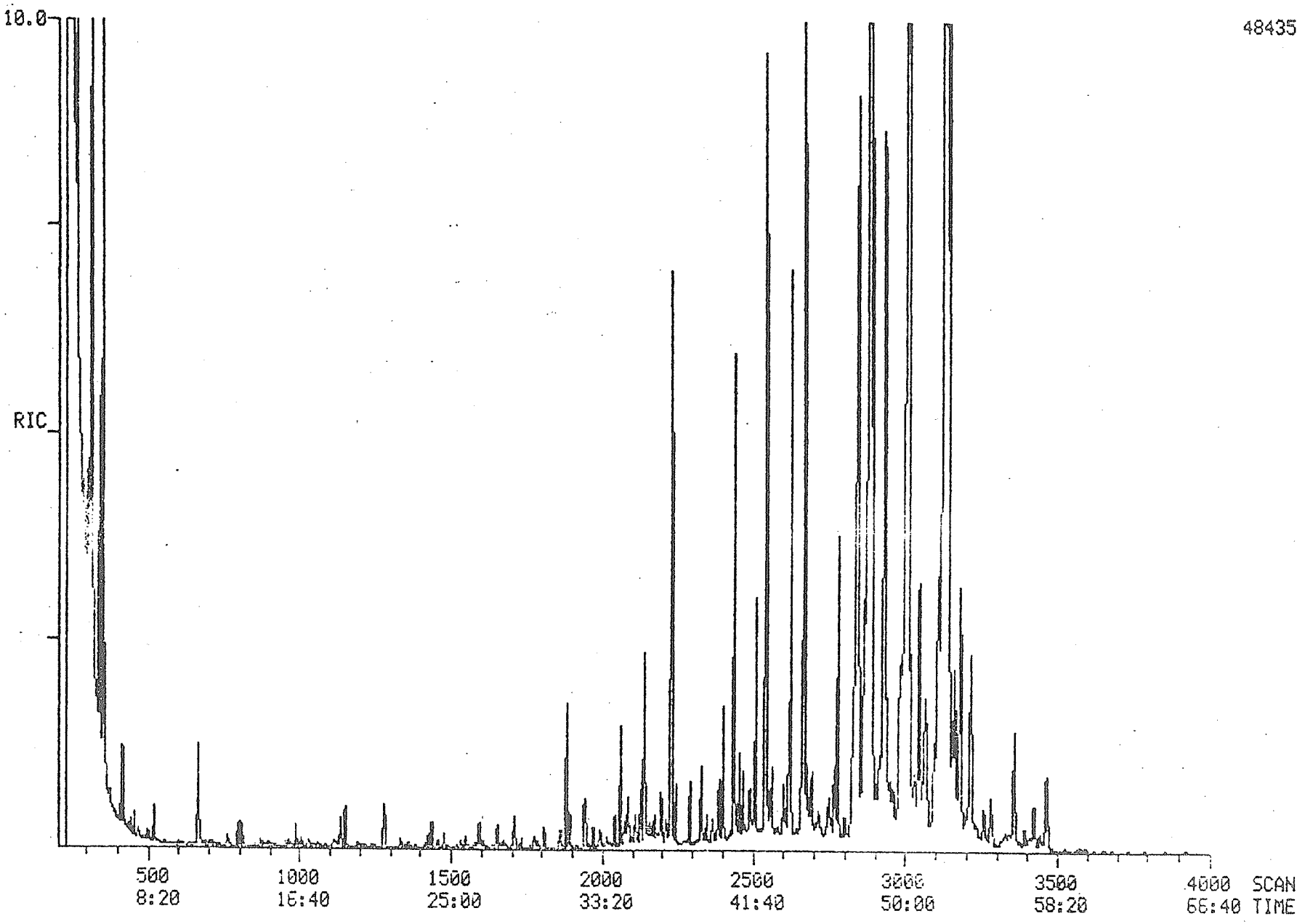


Figure 25 Total ion chromatogram (EI) Fraction 2 Site VII

Table 6 Computerized deconvolution/library search and compound identification, Fraction 2- Site VII

Name of compound	Toxic property	Reference
cis-1,2-diethylcyclobutane		
methylbenzene	occupational hazard	NIOSH
2,3,3-trimethyl-1-butene		
2-methyl-3-pentanol		
trans-2-methyl-cyclopentanol		
2-hexanethiol	occupational hazard	NIOSH
ethenylbenzene	mutagenic	NIOSH
benzaldehyde	mutagenic	NIOSH
diethyl phthalate		
o-neo-isomenthol		
6,10,14-trimethyl-2-pentadecanone		
citronellyl propionate		
13-octadecanal		
7-butyl-bicyclo (4.1.0)heptane		
1-doctriacontanol		
17-octadecanal		
1,2-dimethylbenzene	high aquatic toxicity	NIOSH
1-hexene-3-ol		
4-methylhexanal		
6-methyl-2-heptanone		
3,3-dimethylhexane		
1-hexyl-3- methylcyclopentane		
2-nonenal		
2,2,3,4-tetramethylpentane		
tetradecanal		
nonadecanol		
1,12-tridecadiene		
hexadecanal		
ergost-5-en-3β-ol		
3-methyl-2-cyclohexene-1-one		
1,9-nonanediol		

Figure 26 depicts an enhanced total ion chromatogram of early eluting compounds obtained from Fraction 2 -Site VII. Spectra of toxic substances, as identified by the NIOSH-Registry of Toxic Effects of Chemical Substances, were retrieved and compared to reference spectra. Spectrum number 326 is depicted in Figure 27. Comparison of this spectrum to that of the NBS spectrum for toluene, depicted in Figure 28, shows a very good fit. Spectrum number 399 is depicted in Figure 29. Comparison of this spectrum to that for the NBS spectrum of 2-hexanethiol, depicted in Figure 30, shows a very close resemblance. Spectrum number 541 is presented in Figure 31. Comparison of this spectrum to that of the NBS spectrum for styrene (Figure 32) shows a remarkably good fit. Spectrum number 659 is presented in Figure 33. Comparison of this spectrum to that of the NBS spectrum for benzaldehyde (Figure 34) again shows good similarity. In an effort to confirm these findings, another replicate sample of Fraction 2 -Site VII was analyzed using the method outlined in Section 2.2.9 Method C. Figure 35 depicts an enhanced total ion chromatogram of early eluting compounds. Again, toluene, 2-hexanethiol and benzaldehyde were found to match spectra 319, 444 and 666 respectively. Spectrum 455 of this repeat run is presented in Figure 36. This spectrum is very similar to that of the NBS library spectrum

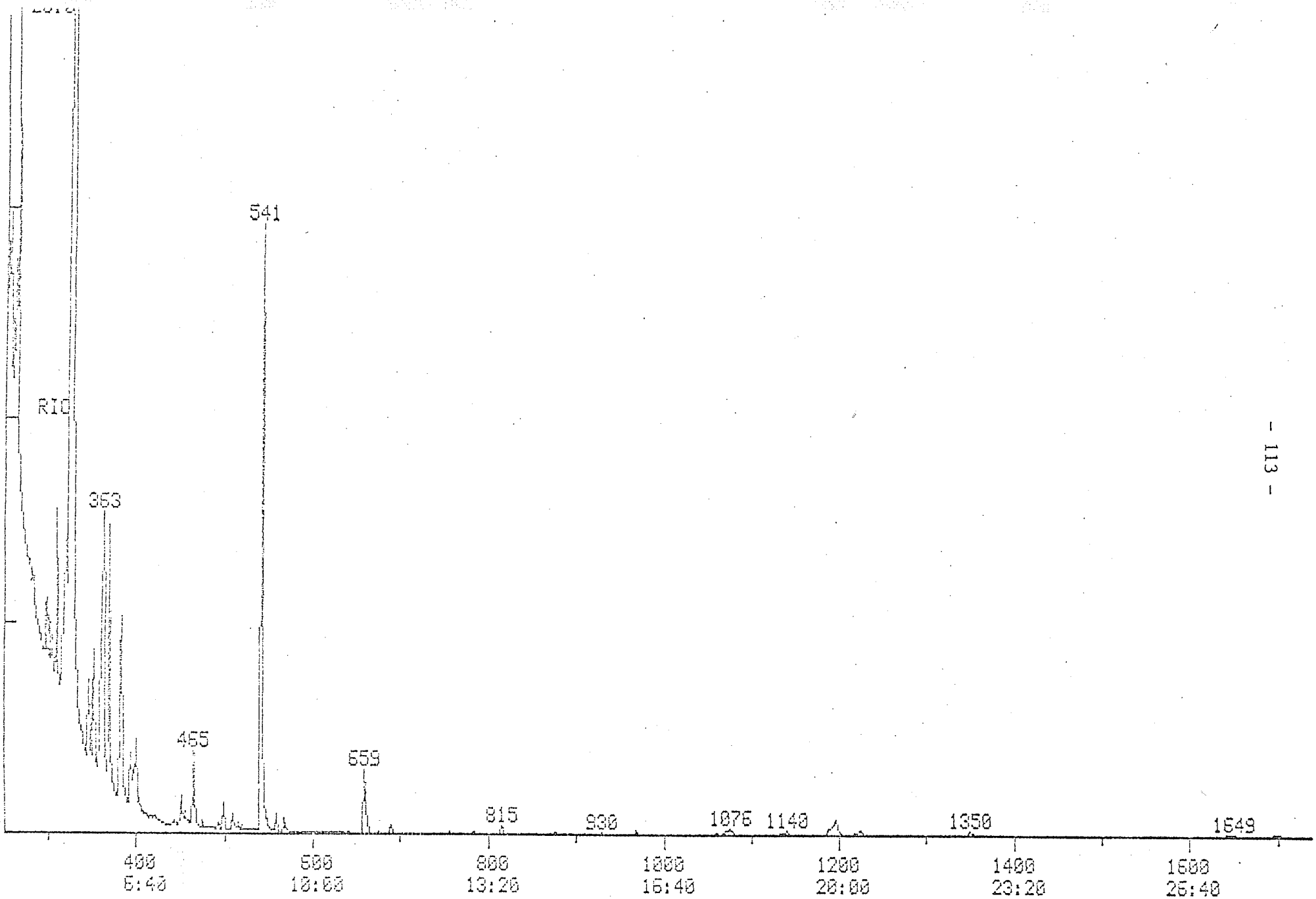


Figure 26 Enhanced total ion chromatogram (EI) Fraction 2 - Site VII, replicate 1

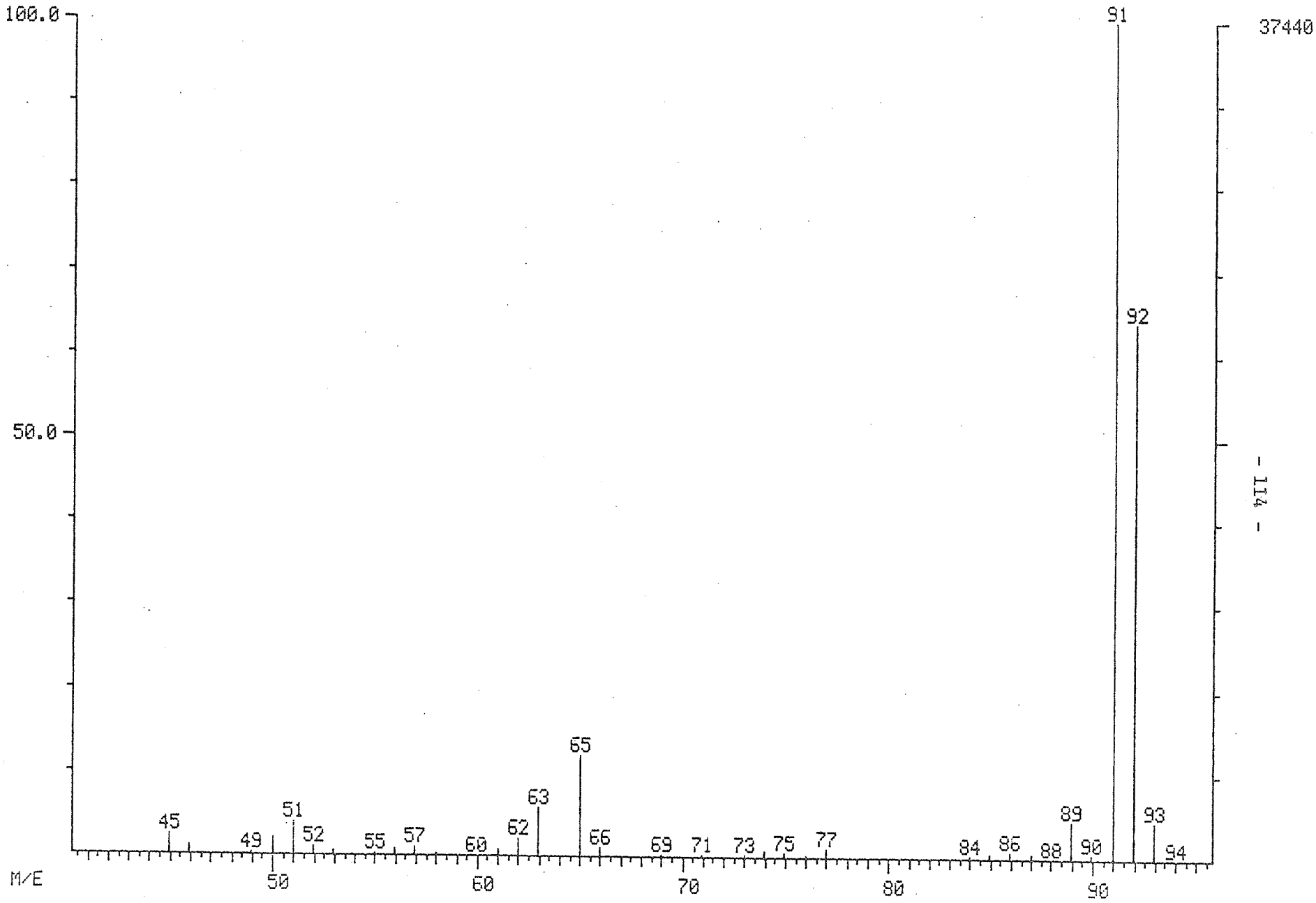


Figure 27

Spectrum 326 (EI)

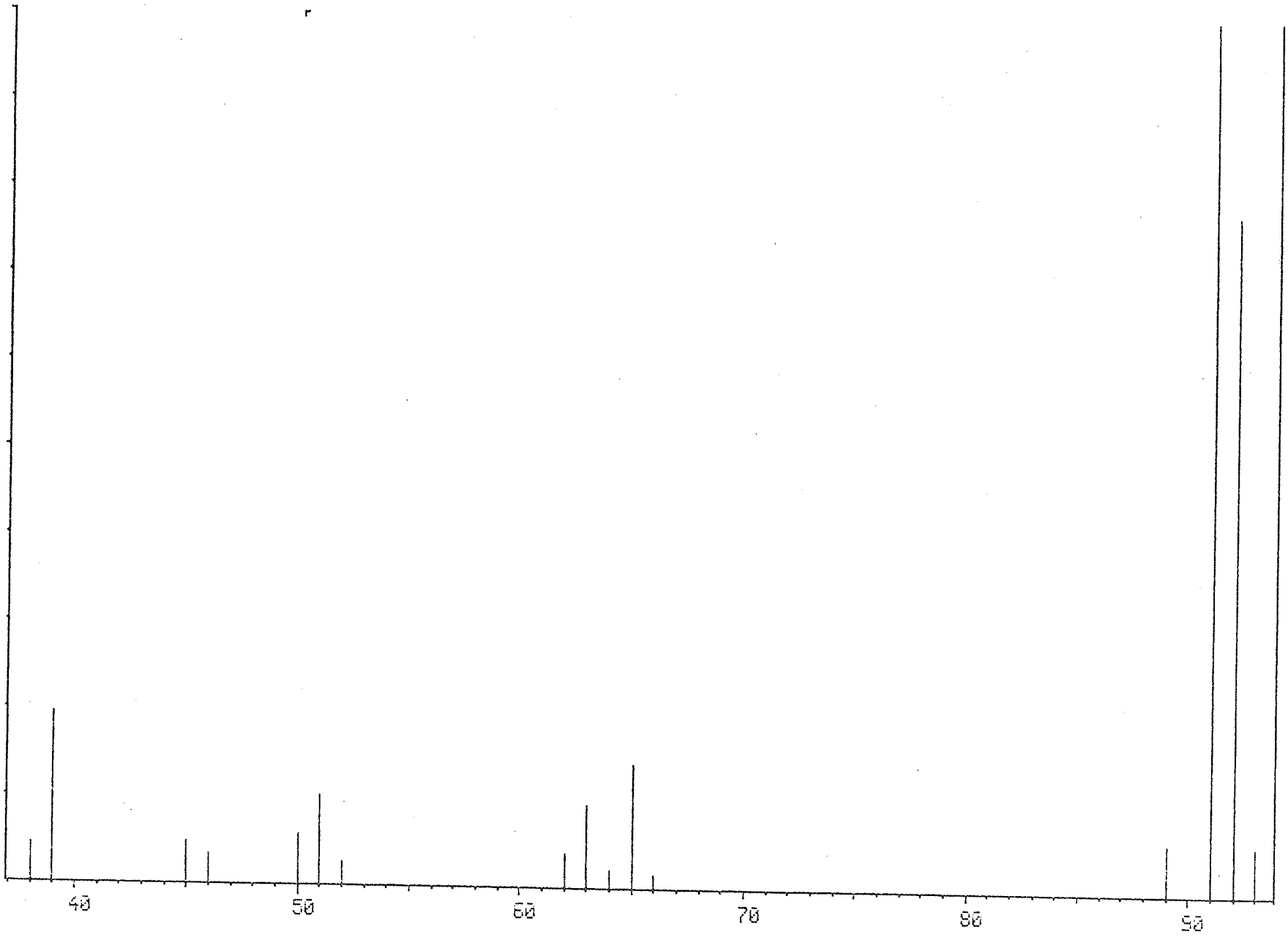


Figure 28 NBS mass spectrum (EI) for toluene

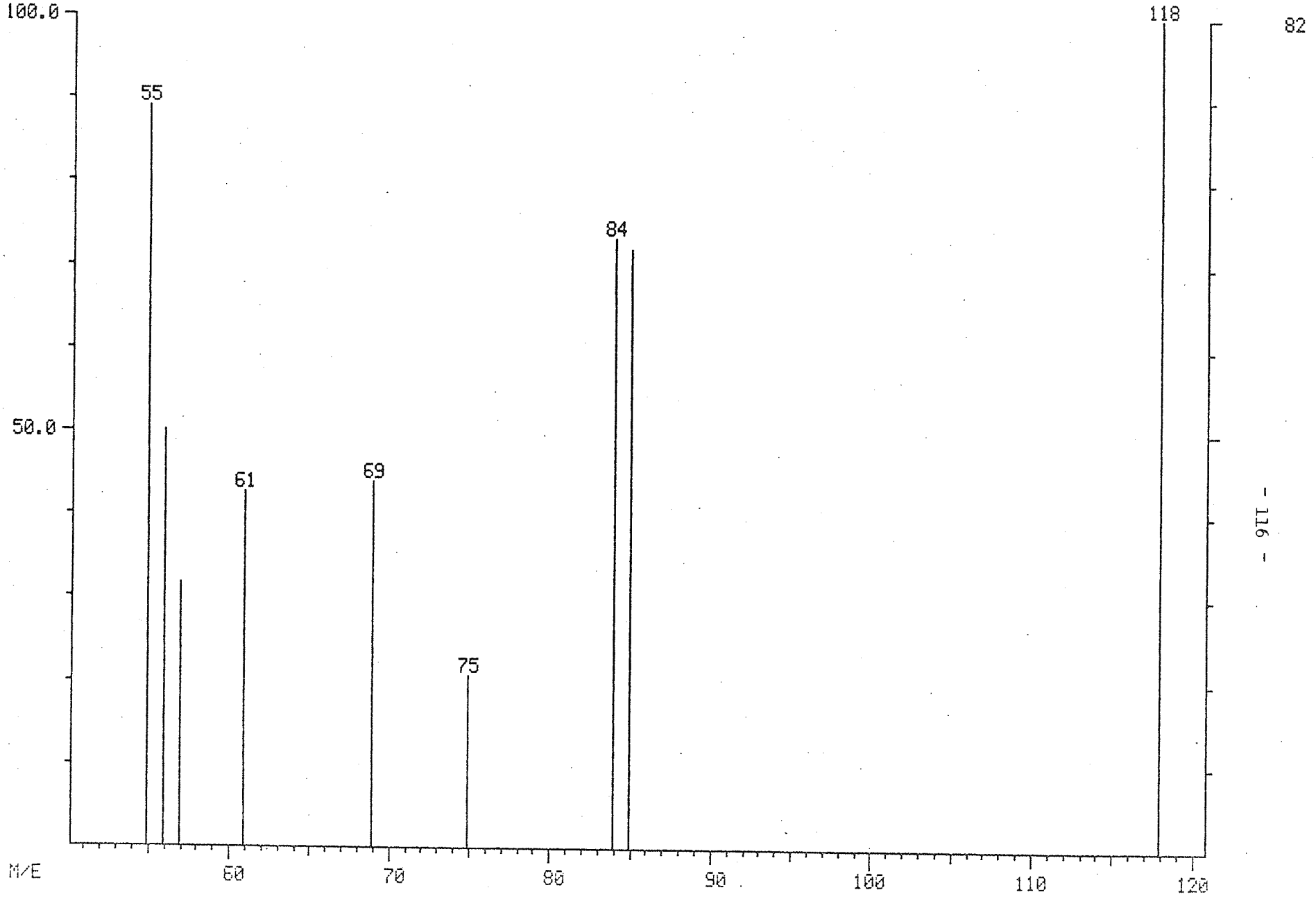


Figure 29

Spectrum 399 (EI)

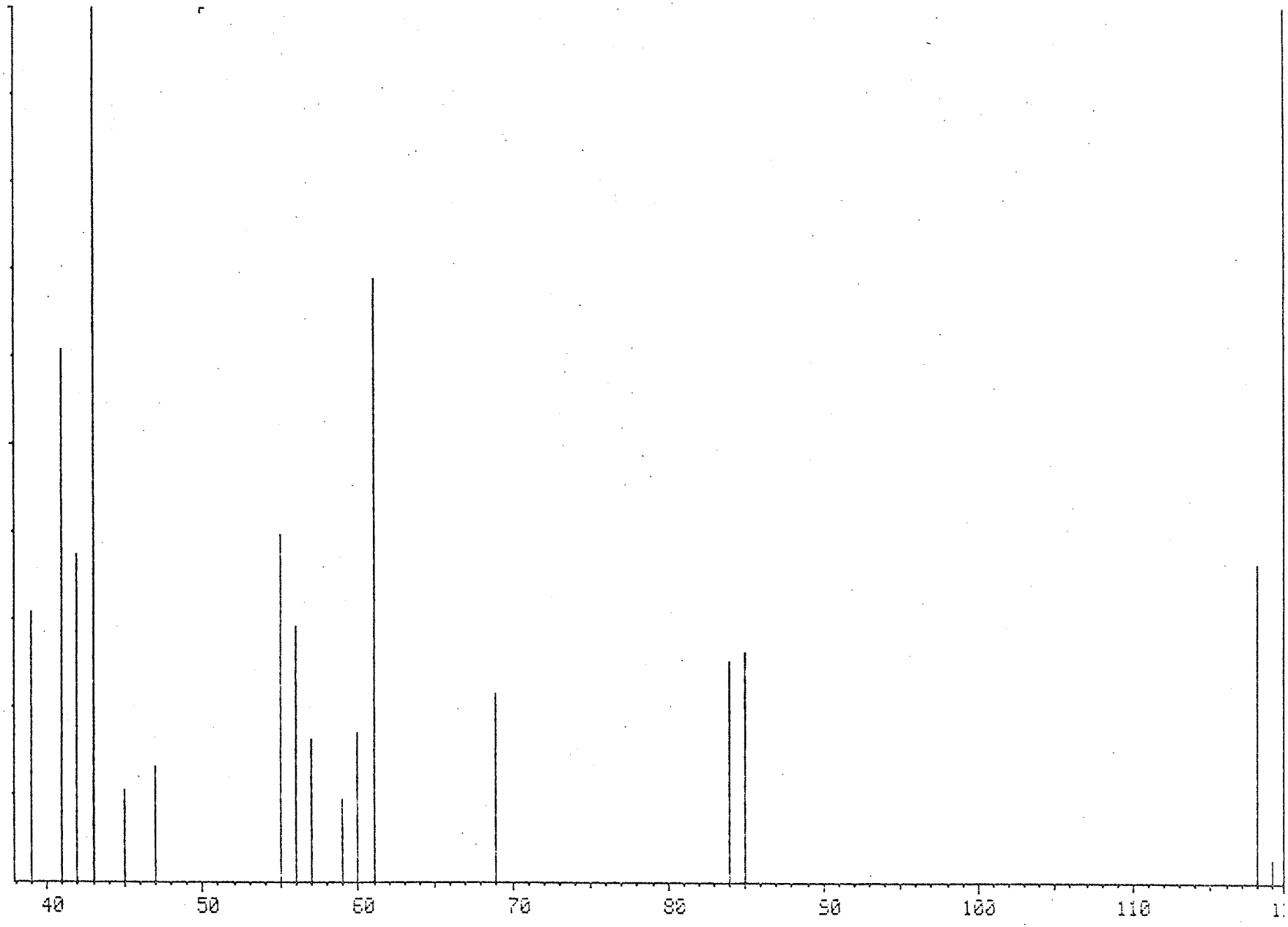


Figure 30 NBS mass spectrum (EI) for 2-hexanethiol

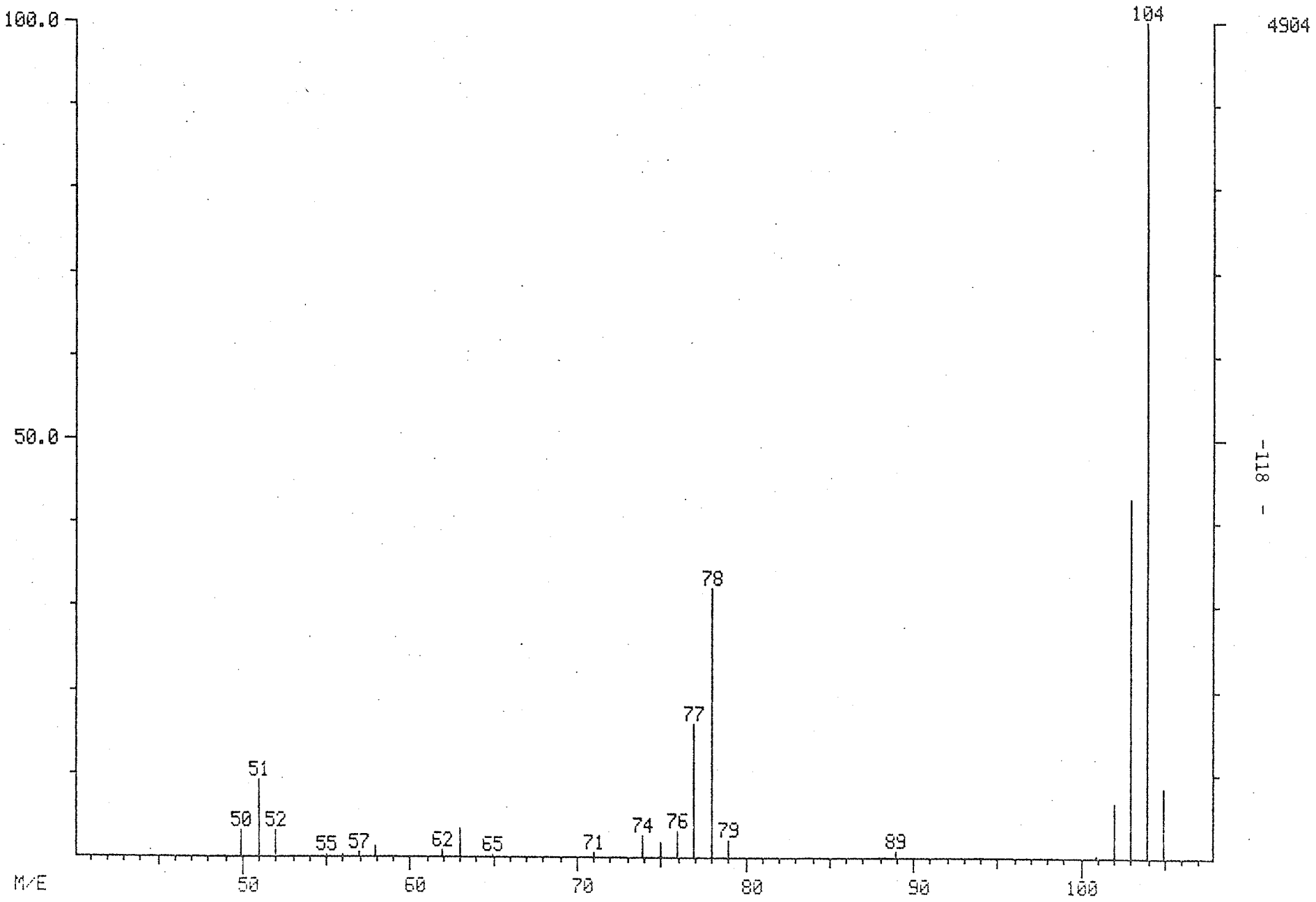


Figure 31

Spectrum 541 (EI)

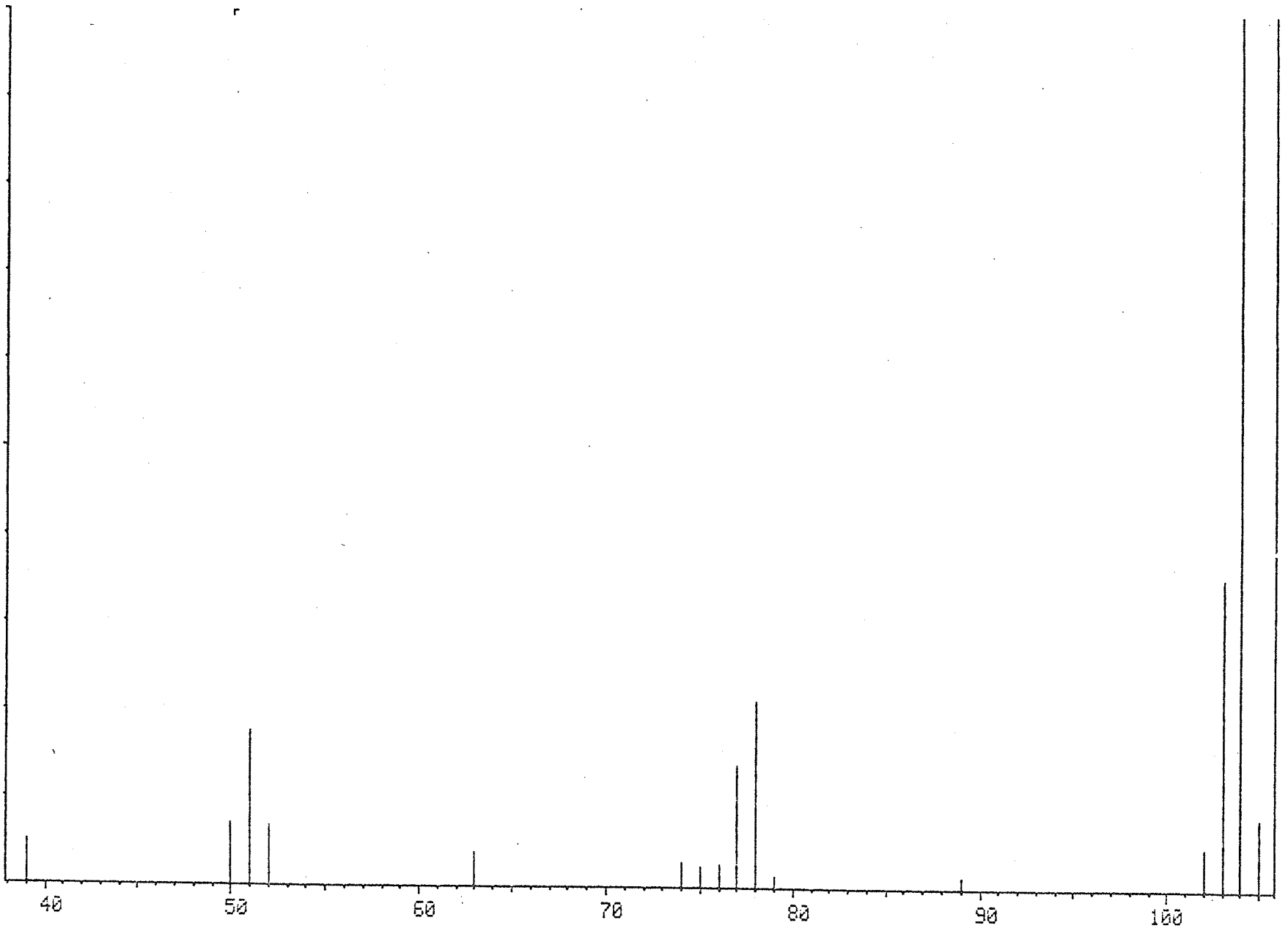


Figure 32 NBS mass spectrum (EI) for styrene

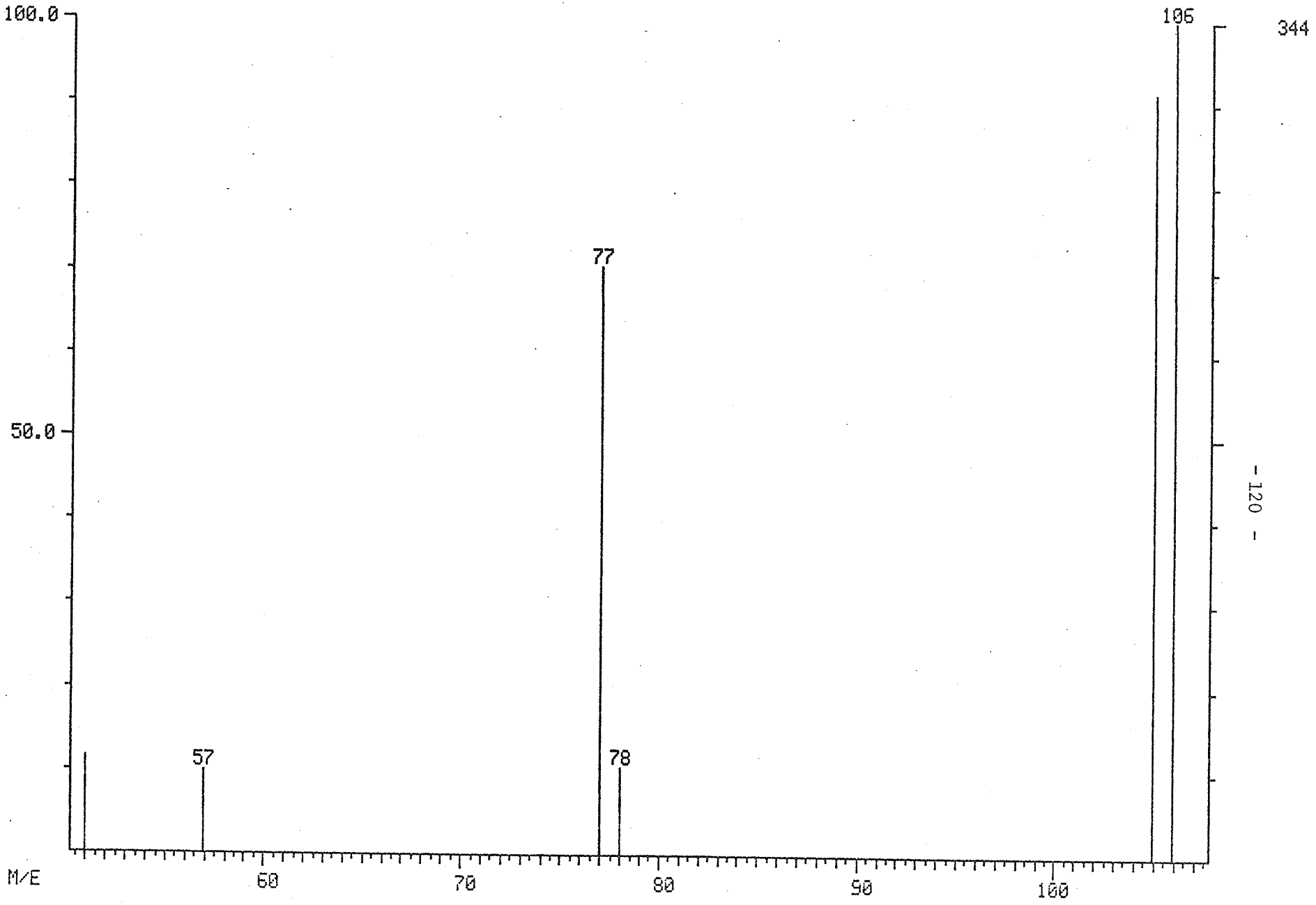


Figure 33

Spectrum 659 (EI)

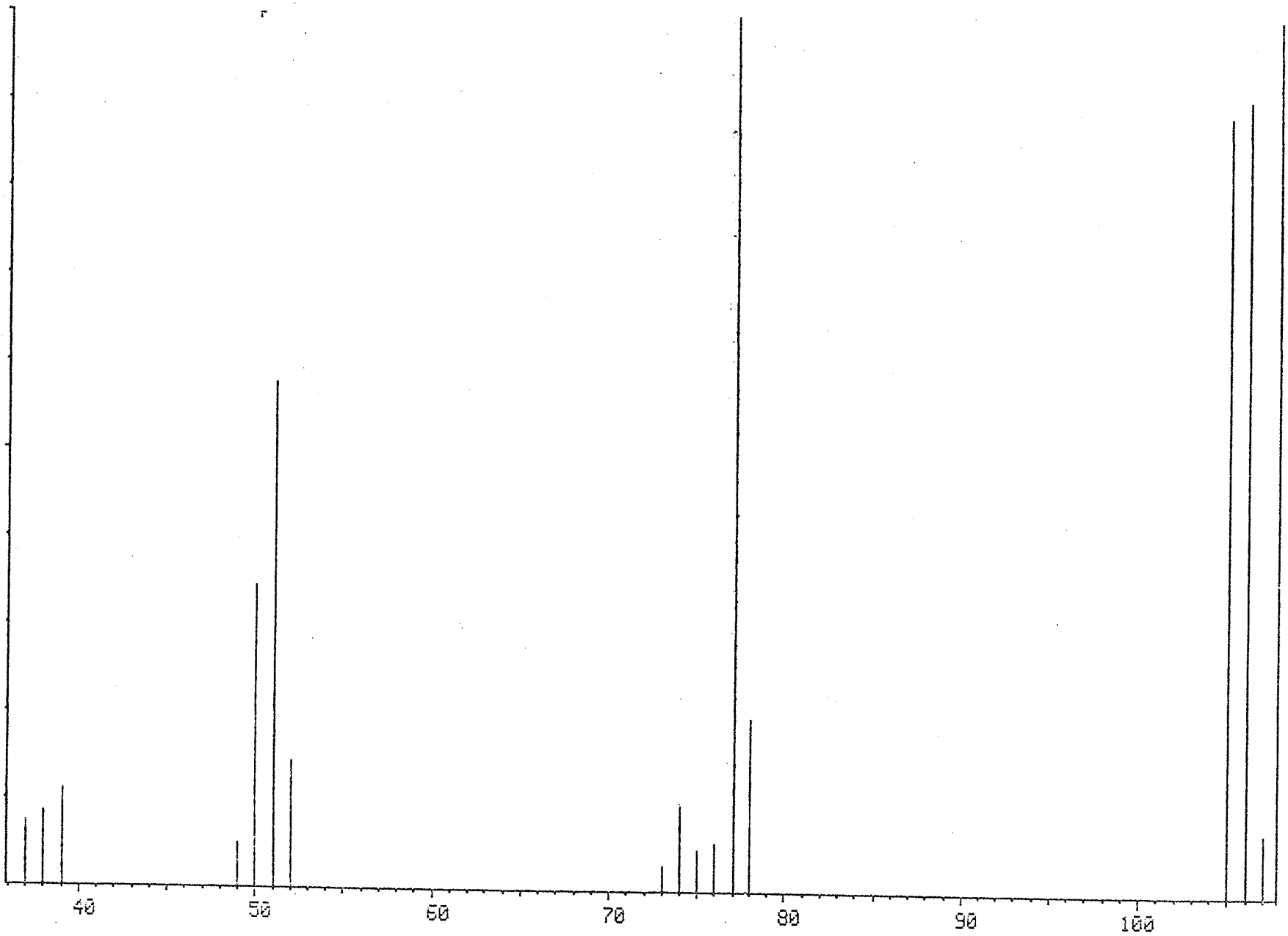


Figure 34

NBS mass spectrum (EI) for benzaldehyde

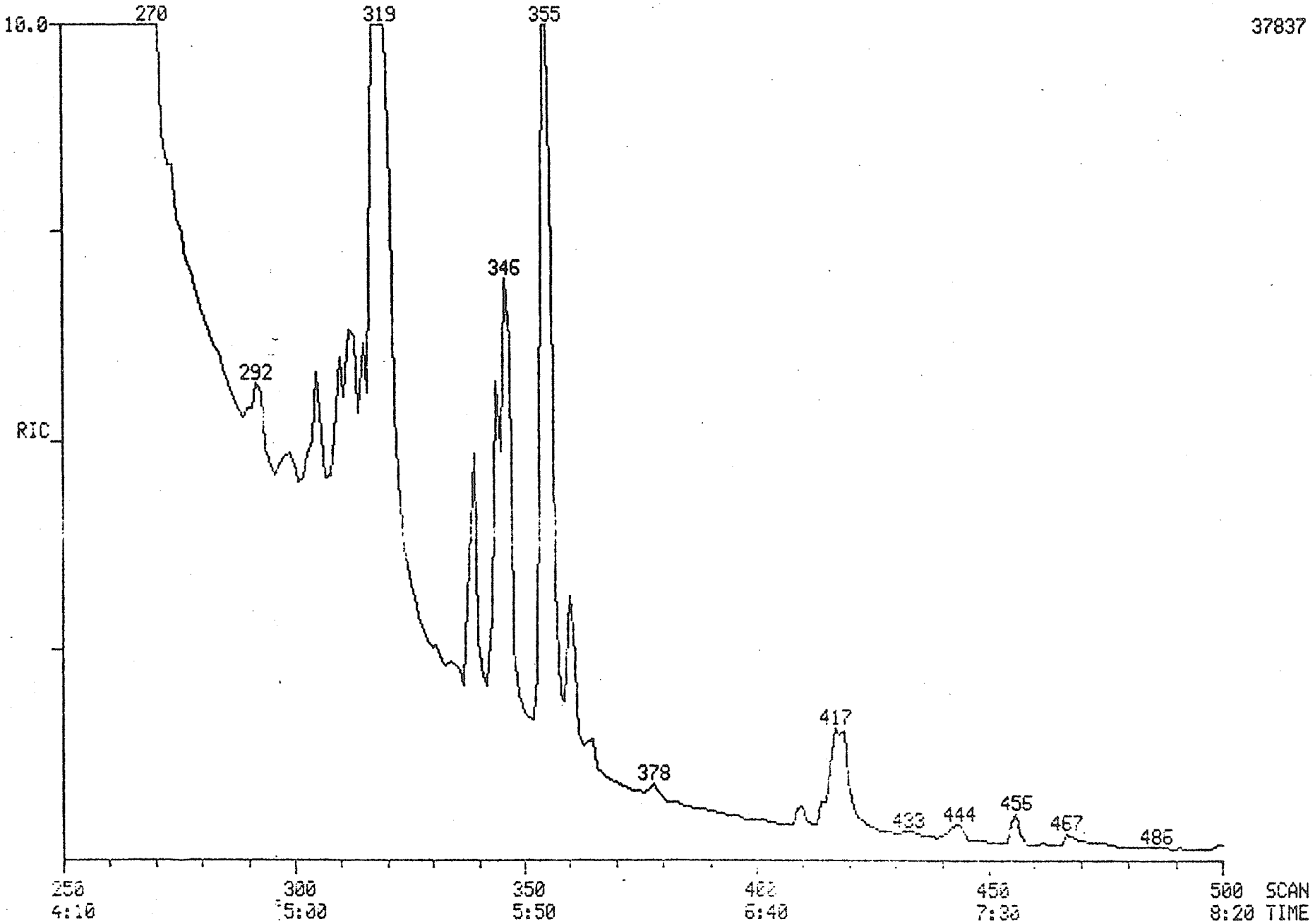


Figure 35 Enhanced total ion chromatogram (EI) for Fraction 2-Site VII, replicate 2

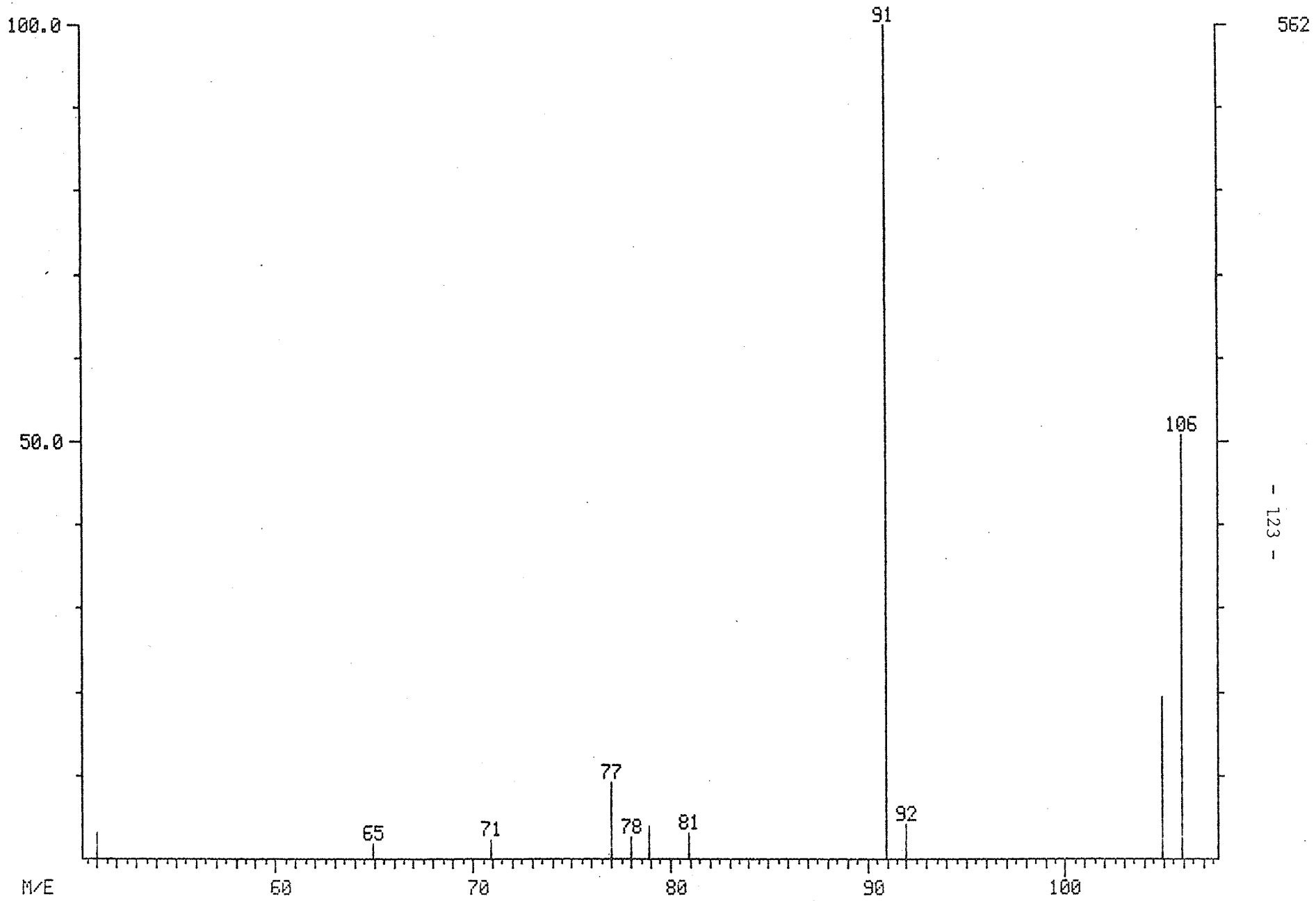


Figure 36

Spectrum 455 (EI)

of o-xylene, which is depicted in Figure 37.

As another means of confirming the presence of these compounds these samples were analyzed using capillary gas chromatography with flame ionization detection. The analytical method is described in Section 2.2.8.2 **Method C.** The retention times of the eluting sample components were compared to the retention times of a standard mixture containing toluene, o-xylene, m-xylene, p-xylene, styrene and benzaldehyde.

Figure 38 depicts a high resolution GC/FID chromatogram of the same sample, Fraction 2-Site VII. Compounds are observed to elute at retention times of 9.36, 11.26, 11.46, 11.91, 11.97 and 13.42 min. Figure 39 depicts a high resolution GC/FID chromatogram of a solution containing toluene, p-xylene, m-xylene, styrene, o-xylene and benzaldehyde. The observed retention times of these compounds were 9.36, 11.29, 11.44, 11.91, 11.98 and 13.39 min. respectively. This finding confirms the presence of these compounds since the observed elution times of the sample peaks match the retention times of the standard mixture within experimental error. According to the NIOSH-Registry of Toxic Effects of Chemical Substances, styrene has been shown to be mutagenic using the Salmonella/microsome assay at applications of one micromole per plate. This compound was only found in one replicate sample analyzed using GC/MS and gave a good spectrum with a total ion count of 4904. To confirm the presence of styrene in this replicate, this sample was also analyzed by high

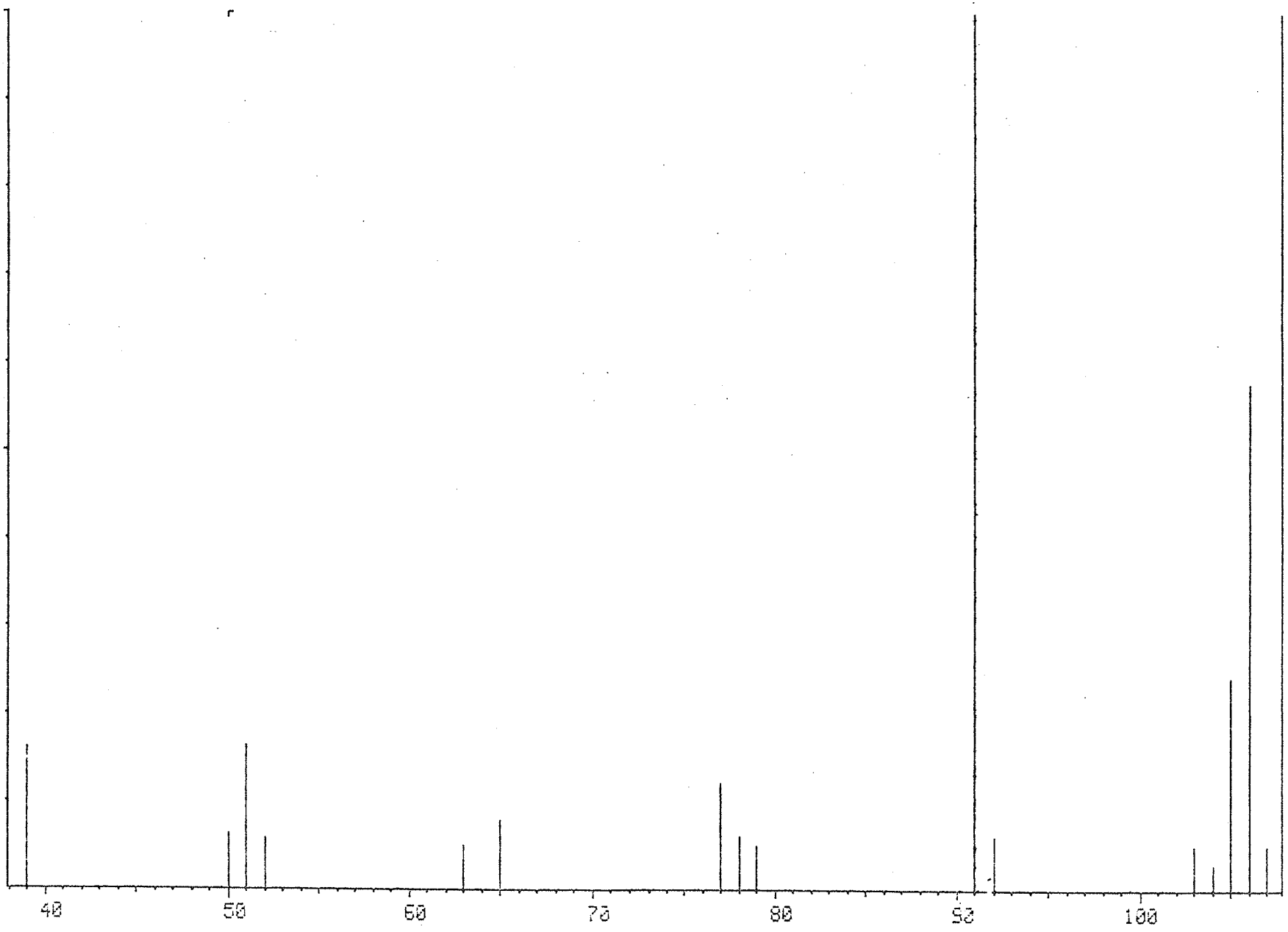


Figure 37

NBS mass spectrum (EI) for o-xylene

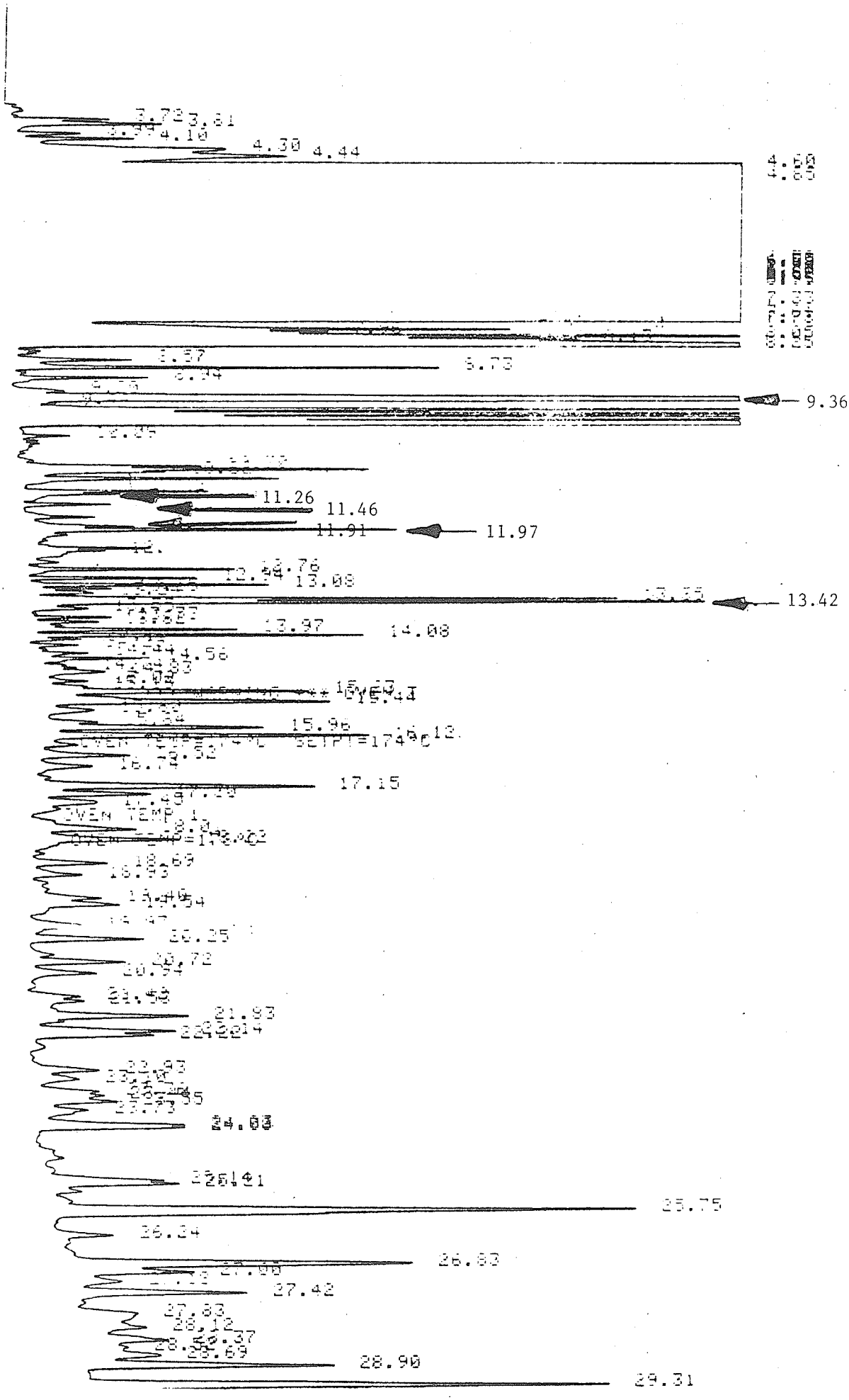


Figure 38 High resolution GC-FID chromatogram of Fraction 2-Site VII, replicate 1

Retention time 9.36 = toluene

Retention time 11.29 = p-xylene

Retention time 11.44 = m-xylene

Retention time 11.91 = styrene

Retention time 11.98 = o-xylene

Retention time 13.39 = benzaldehyde

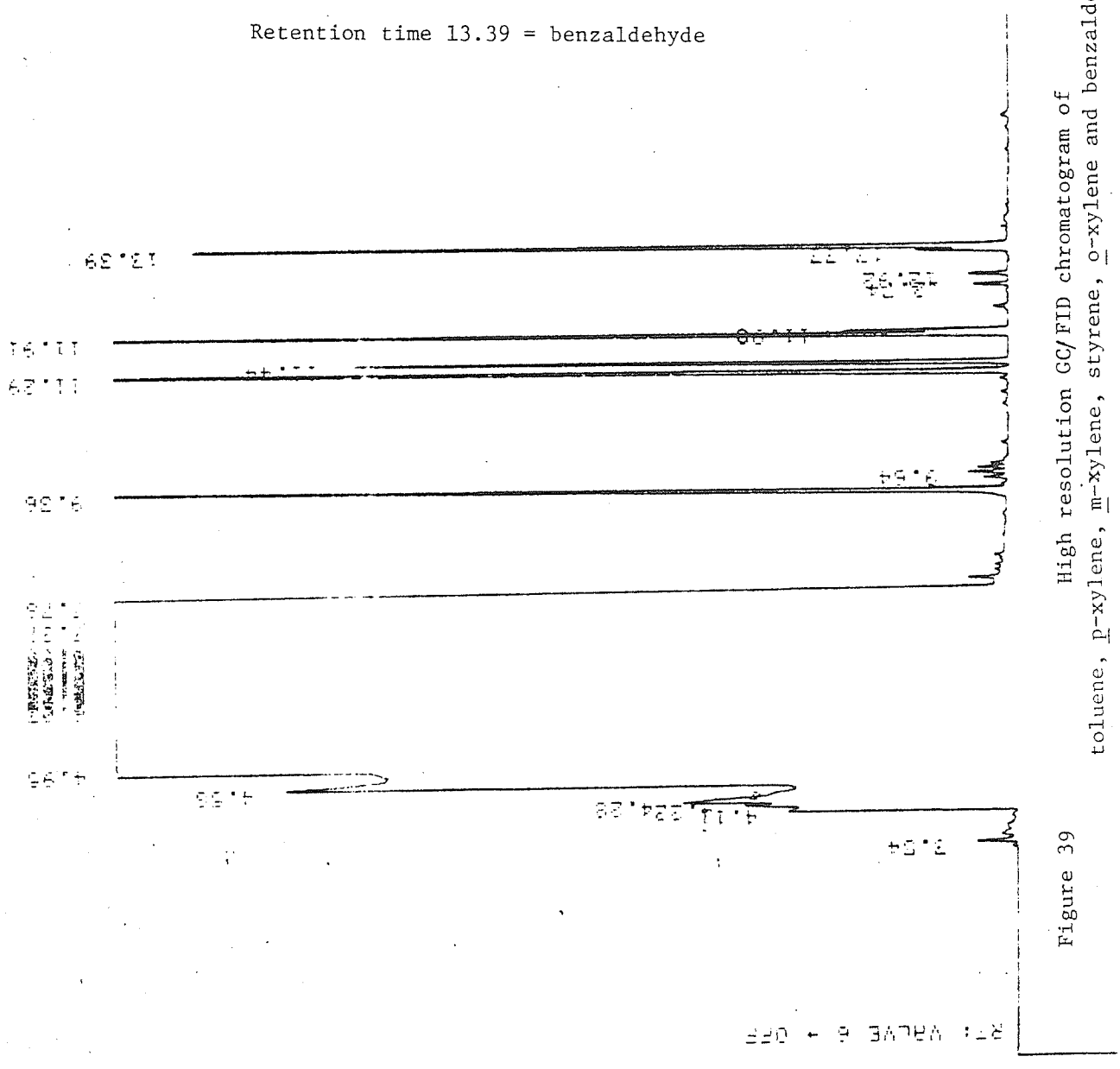


Figure 39 High resolution GC/FID chromatogram of toluene, p-xylene, m-xylene, styrene, o-xylene and benzaldehyde

resolution gas chromatography using flame ionization. A 25 m X 0.31 mm ID fused silica column coated with SE 54 was used. The GC conditions outlined in Section 2.2.8.2 Method C were used except that a final temperature program rate of 20°C/min. was used.

Figure 40 depicts the GC/FID chromatogram obtained for this replicate, of Fraction 2 - Site VII, suspected of containing styrene. Comparison of this chromatogram to that obtained for pure styrene (Figure 41) confirms that styrene is present in this sample. Its concentration was determined to be 18.1 µg per mL of sample extract.

Figure 42 portrays a GC/FID chromatogram for the analytical control sample (reagent blank) for Fraction 2 - Site VII. The analytical conditions are described in Section 2.2.8.2 Method C. Comparison of this chromatogram to that of Figure 39 indicates that toluene (retention time 9.32 min) and benzaldehyde (retention time 13.42) are present. To confirm the presence of these compounds, this same control was analyzed by GC/MS using the analytical conditions described in Section 2.2.9 Method C. Figure 43 depicts a total ion chromatogram (EI) for this control. To check for the presence of toluene, 2-hexanethiol, benzaldehyde, styrene, and o-xylene a reconstructed ion chromatogram of m/z's, 77, 84, 91, 104 and 106 was displayed (Figure 44). Toluene is present in the reagent blank as evidenced by an early eluting ion chromatogram for m/z 91. It appears around scan number 245 as opposed to scan number 326 for the sample. This is because the Finnigan GC/MS system has difficulty maintaining temperatures at 40°C without using the cryogenic option.

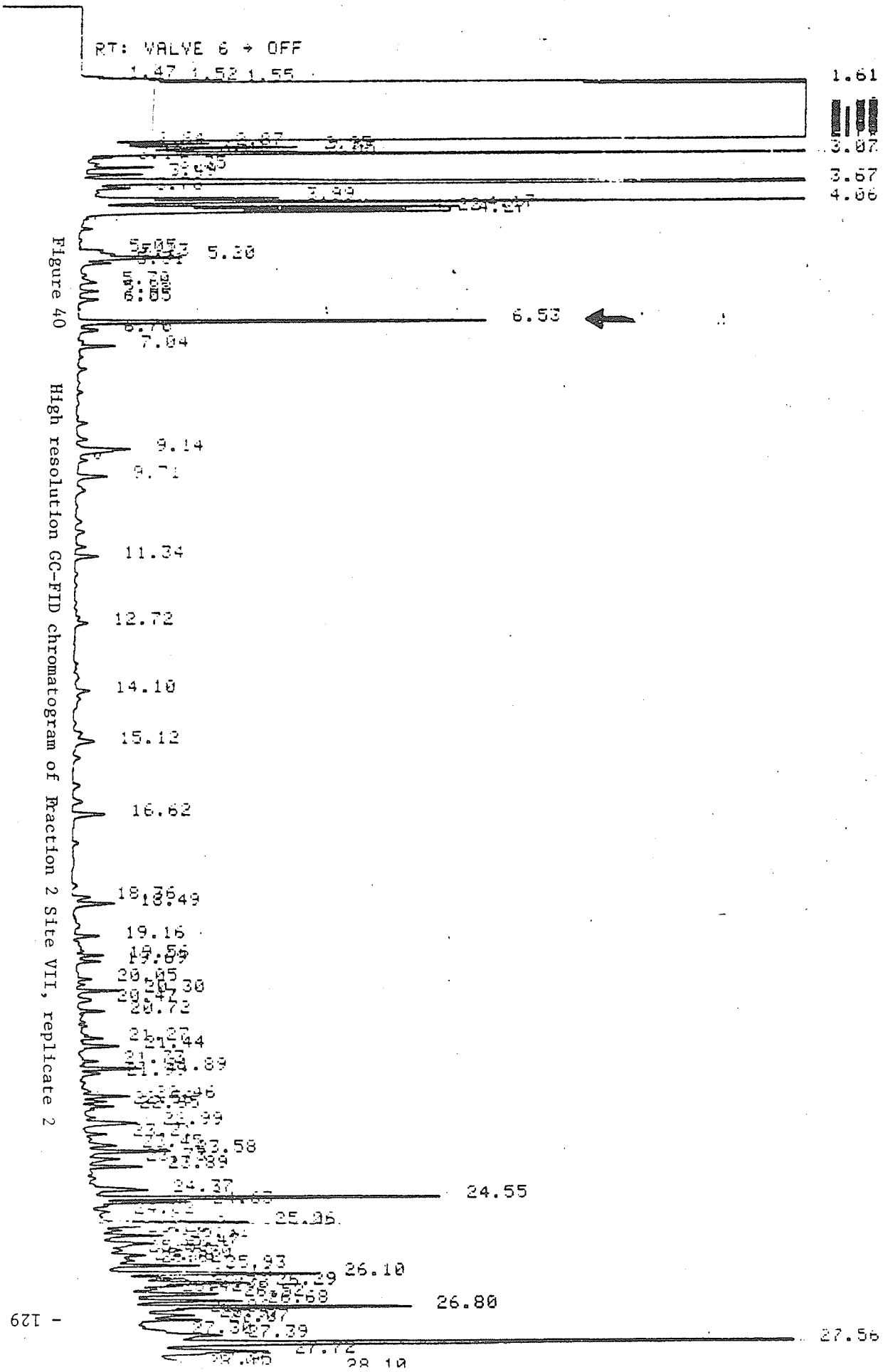


Figure 40 High resolution GC-FID chromatogram of Fraction 2 Site VII, replicate 2

ATTN 2↑2

********** STYRENE 9.06 NG **********
VALVE 6 ON

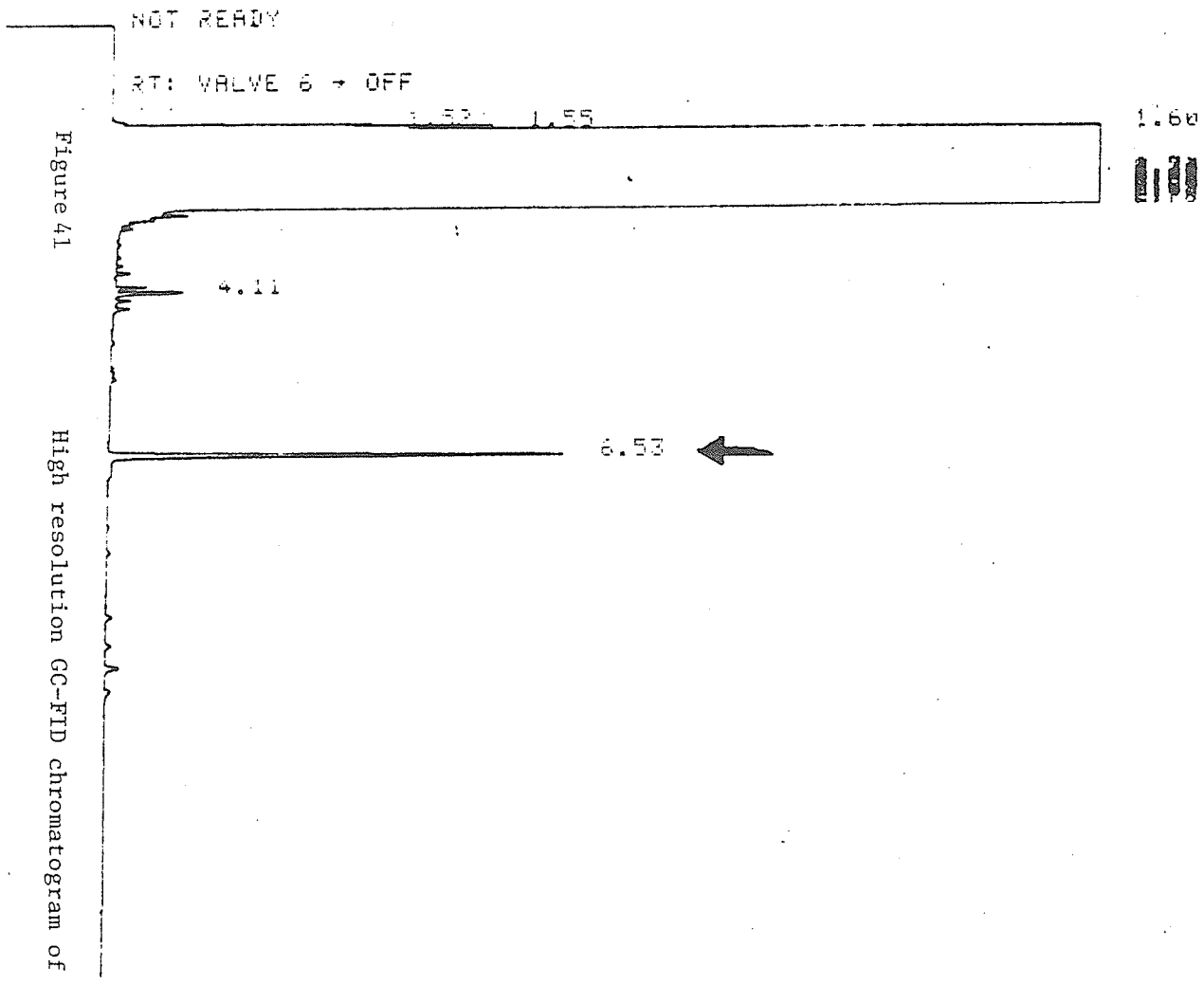


Figure 41 High resolution GC-FID chromatogram of styrene

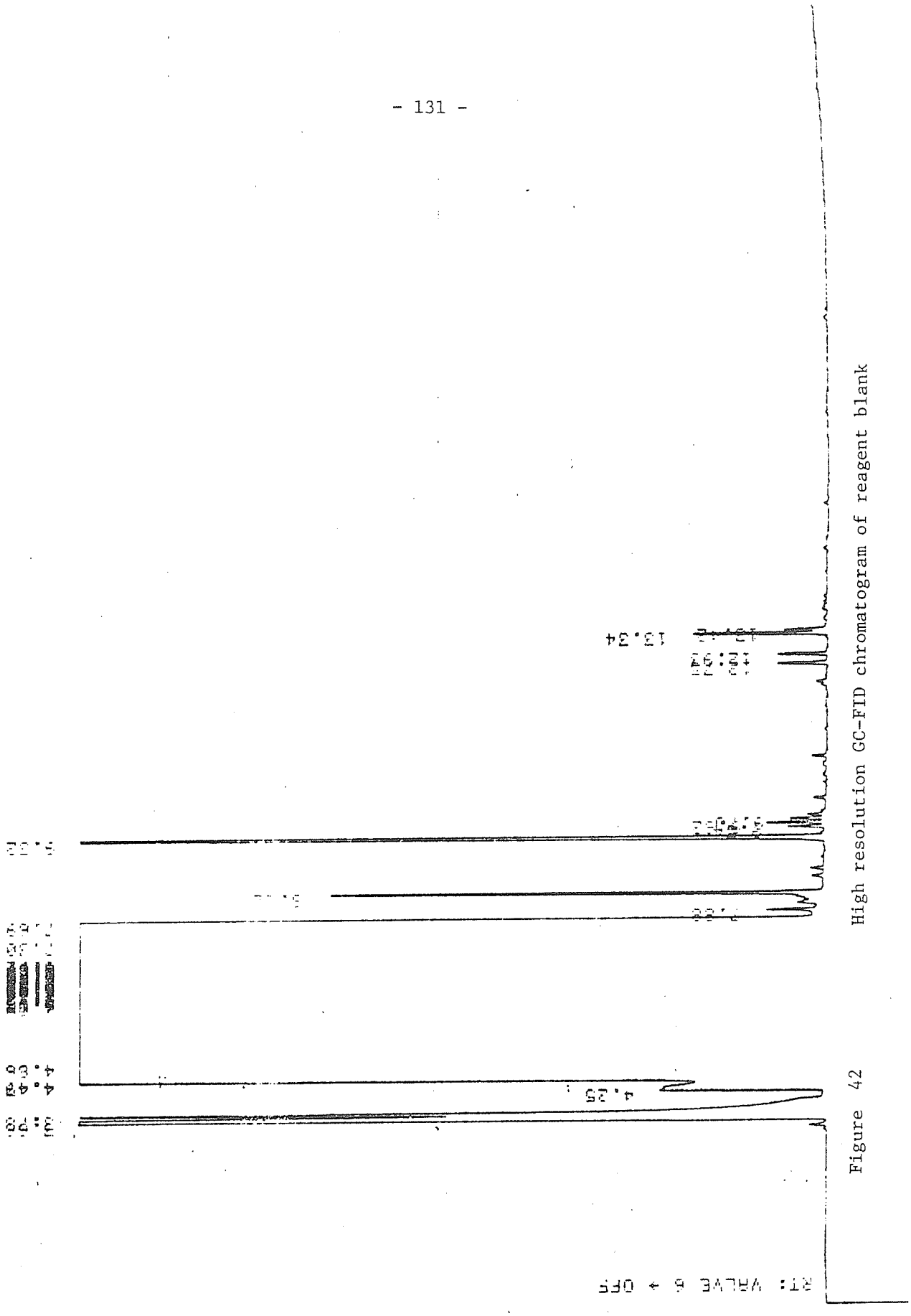


Figure 42 High resolution GC-FID chromatogram of reagent blank

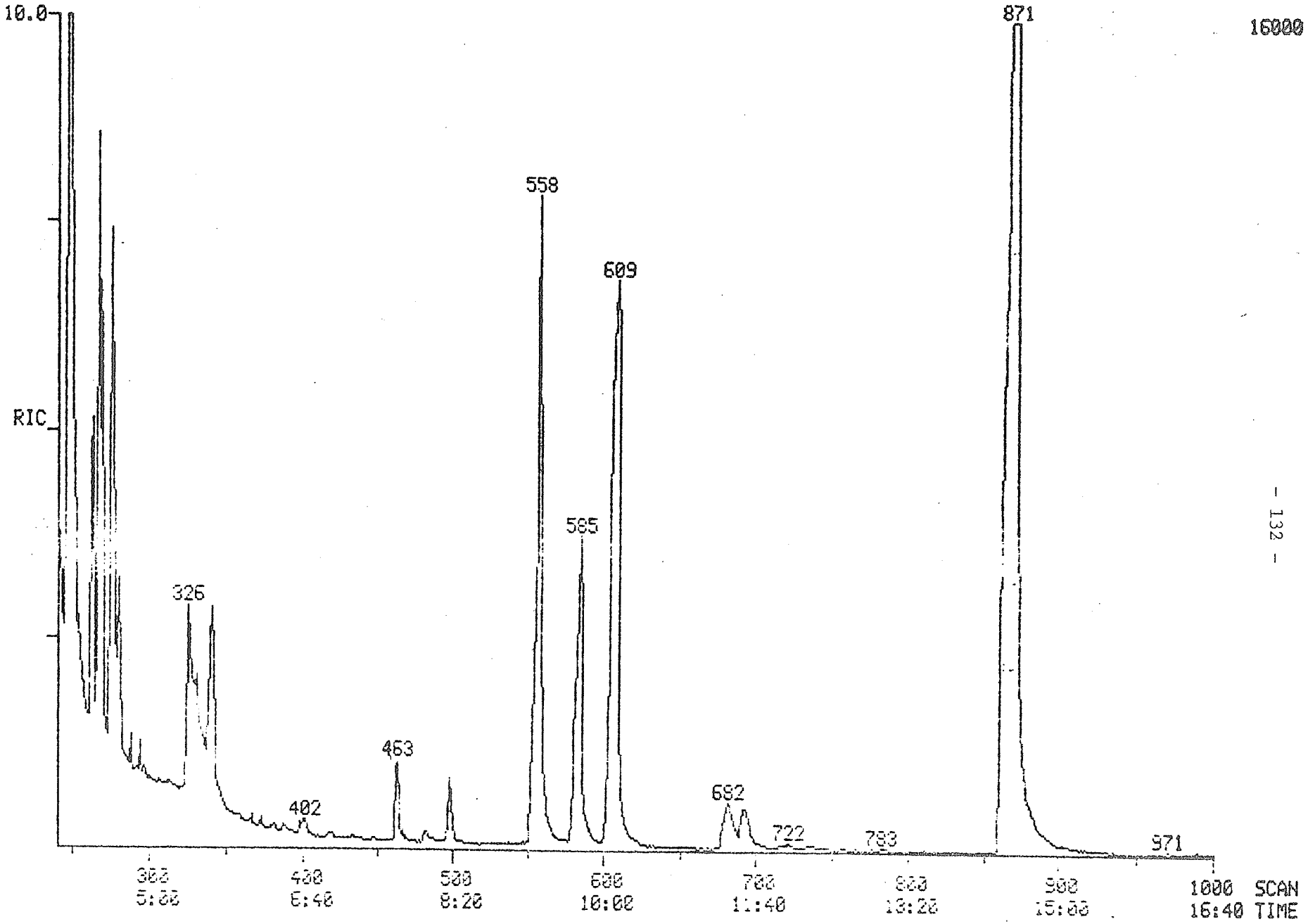


Figure 43

Total ion chromatogram (EI), Fraction 2 - Site VII, control

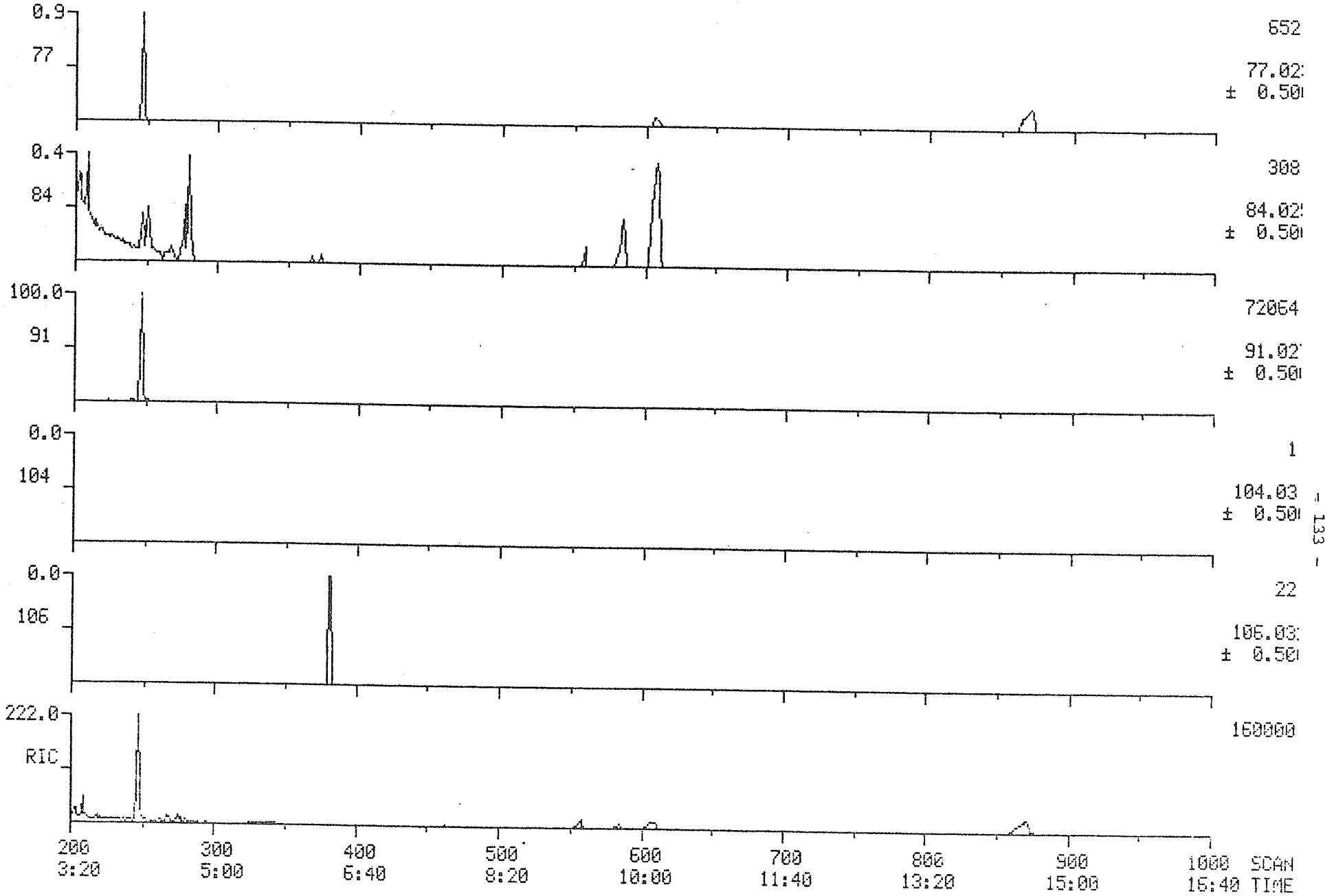


Figure 44 Reconstructed ion chromatogram for m/z's 77, 84, 91, 104 and 106. Reagent blank, Fraction 2

To overcome this problem the oven door was left open for a set period of time and then closed allowing the temperature program to take over. As a result reproducible retention times were difficult to obtain. Reference to Figure 44 shows a peak for m/z 84 appears at scan 555. Since 2-hexanethiol appears at scan 499 in our sample this can be ruled out as being a thiol. In fact these compounds are alkenes as a result of the large abundance of $C_nH_{2n-1}^+$ peaks (McLafferty, 1980). Figure 45 depicts spectrum number 608. This spectrum has a m/z 84. Masses 55 and 83 are due to C_4H_7 and C_6H_{11}. The lack of a m/z 69 (C_5H_9) is indicative of a branched chain alkene. Mass 71 is due to C_5H_{11} (RCH_2^+) which is indicative of branched chain alkenes (McLafferty, 1980).

The absence of an ion chromatogram for m/z 104 is indicative of no styrene being present in the control. If benzaldehyde were present in the blank, an ion chromatogram for m/z 77 and 106 would show peaks at the scan number for benzaldehyde. If xylene were present, an ion chromatogram for m/z 91 and 106 would show peaks at the scan number for xylene. Since neither of these events was observed (Figure 45) these compounds can be assumed to be absent.

Since Fraction 2-Site VIII was also shown to be toxic using the P. redivivus assay and the Salmonella/microsome assay, this sample was analyzed using GC/MS under EI conditions. Two replicate samples were analyzed using the analytical conditions described in Methods B and C, Section 2.2.9. A typical total ion chromatogram is

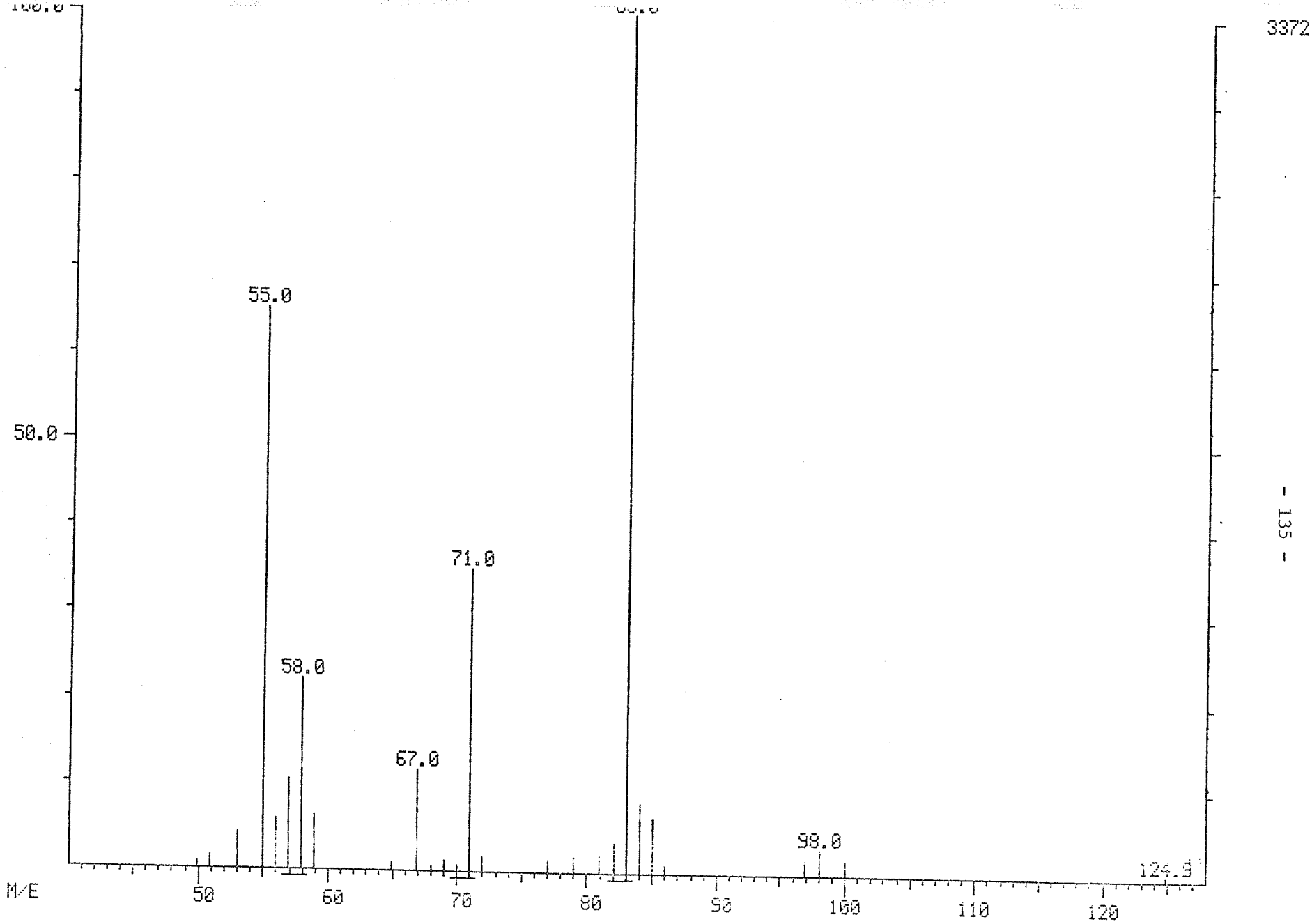


Figure 45

Spectrum 608, Fraction 2 control

presented in Figure 46. All mass spectra obtained were subjected to a computerixed deconvolution/library search and compound identification routine using the NBS mass spectral data file. Table 7 depicts those compounds for which good matches were obtained.

A search of the computerized NIH/EPA Chemical Information System, file NIOSH-Registry of Toxic Effects of Chemical Substances indicated that 2-hydroxy-1,2-diphenylethanone (benzoin), and methylbenzaldehyde (irrespective of isomer) were tumor promoting (oncogenic) compounds. Therefore spectra of these compounds along with the spectra of other toxic compounds, which were identified earlier in Fraction 2-Site VII, were retrieved and compared to NBS reference spectra in order to verify their identification.

Figure 47 depicts an enhanced total ion chromatogram of a minor region of the total ion chromatogram shown in Figure 46. This chromatogram for Fraction 2-Site VIII was obtained using the analytical method described in Section 2.2.9 Method C.

Spectrum 710 is depicted in Figure 48. Comparison of this spectrum to that of Figure 32 shows a close similarity to benzaldehyde. Furthermore, the similar retention times lend support for this identification.

Spectrum 1025 is portrayed in Figure 49. This spectrum is very similar to the NBS mass spectrum of 2-hydroxy-1,2-diphenyl-ethanone (benzoin) which is presented in Figure 50, however there

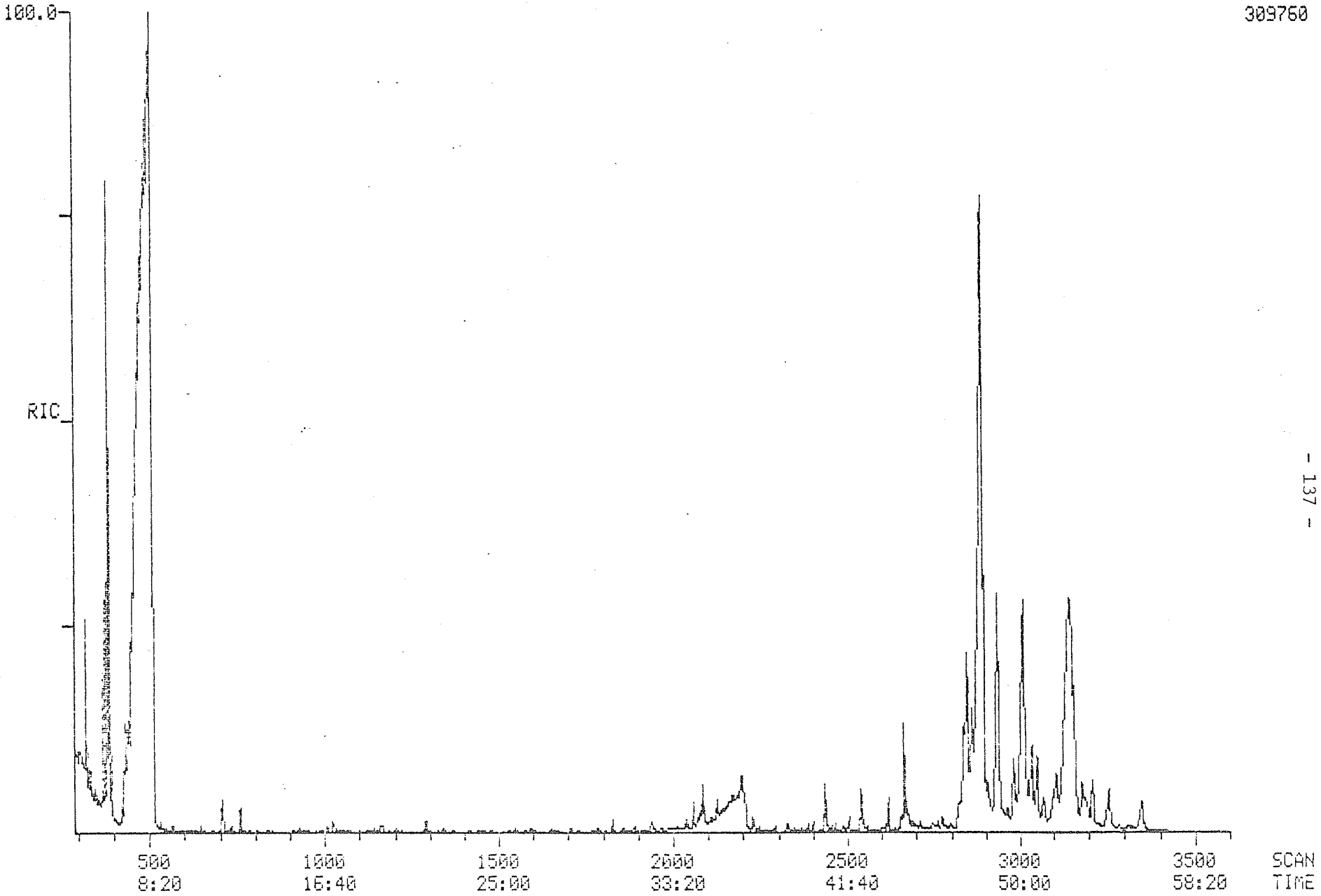


Figure 46 Total ion chromatogram (EI) Fraction 2-Site VIII

TABLE 7 COMPUTERIZED DECONVOLUTION/LIBRARY SEARCH AND COMPOUND IDENTIFICATION, FRACTION 2 SITE VIII

Name of compound	Toxic Property	Reference
4-hydroxy-4-methyl-2-pentanone		
4-hydroxy-5-methyl-2-hexanone		
methylbenzene		
1-ethylcylopentanol		
benzaldehyde		
1-phenylethanone		
1-ethoxy-2-heptanone		
dihydro-5,5-dimethyl-2(3H)-furanone		
2-ethyl-1-hexanol		
3,3-dimethylhexanal		
1-hexyl-3-methylcyclopentane		
2-hydroxy-1,2-diphenylethanone	oncogenic	NIOSH
2-methylbenzaldehyde	oncogenic	NIOSH
3 or 4 methylbenzaldehyde	oncogenic	NIOSH
3-propoxy-1-propene		
3,5 dimethyl-2-cyclohexen-1-one		
2-nonenal		
4-decanone		
1,1,3-trimethylcyclopentane		
isooctanol		
1,9-nonanediol		
cyclododecane		
3,7-dimethyl-6-octen-1-ol		
2-methyl-1-dodecanol		
tetradecanal		
5-octadecanal		
cholest-5-en-3β-ol		
stigmast-5-en-3β-ol		

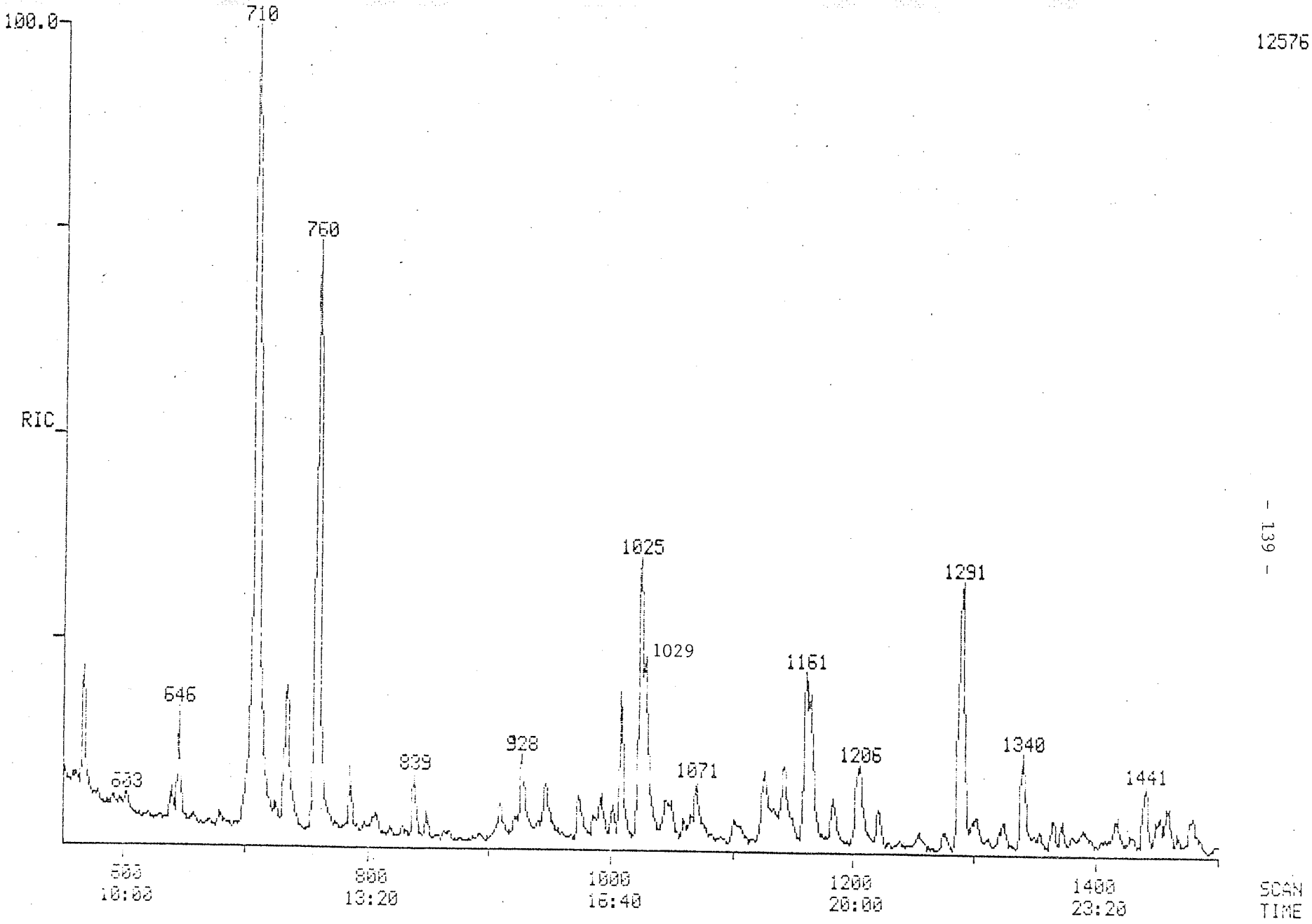


Figure 47 Enhanced total ion chromatogram Fraction 2-Site VIII

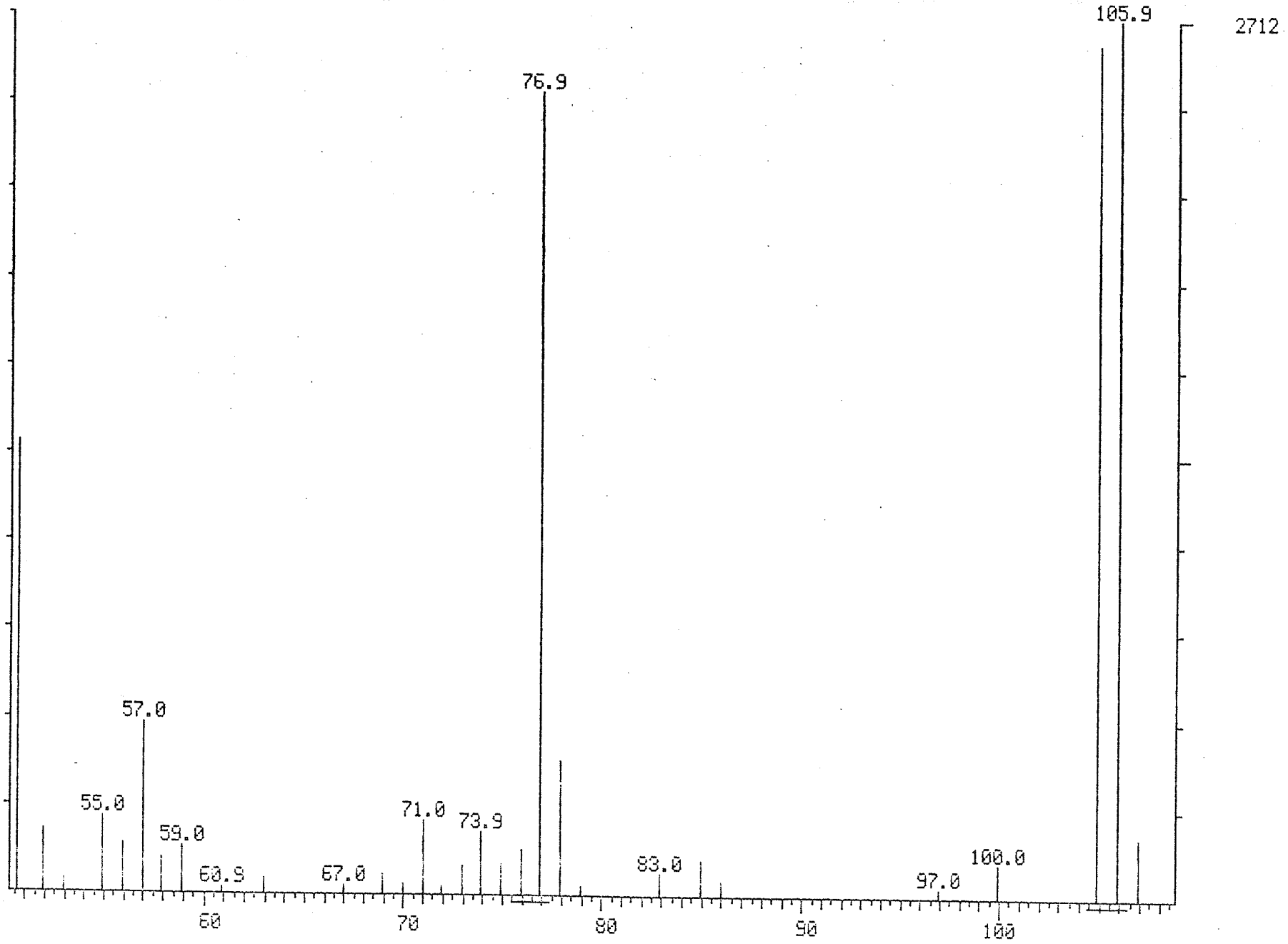


Figure 48

Spectrum 710, Fraction 2-Site VIII

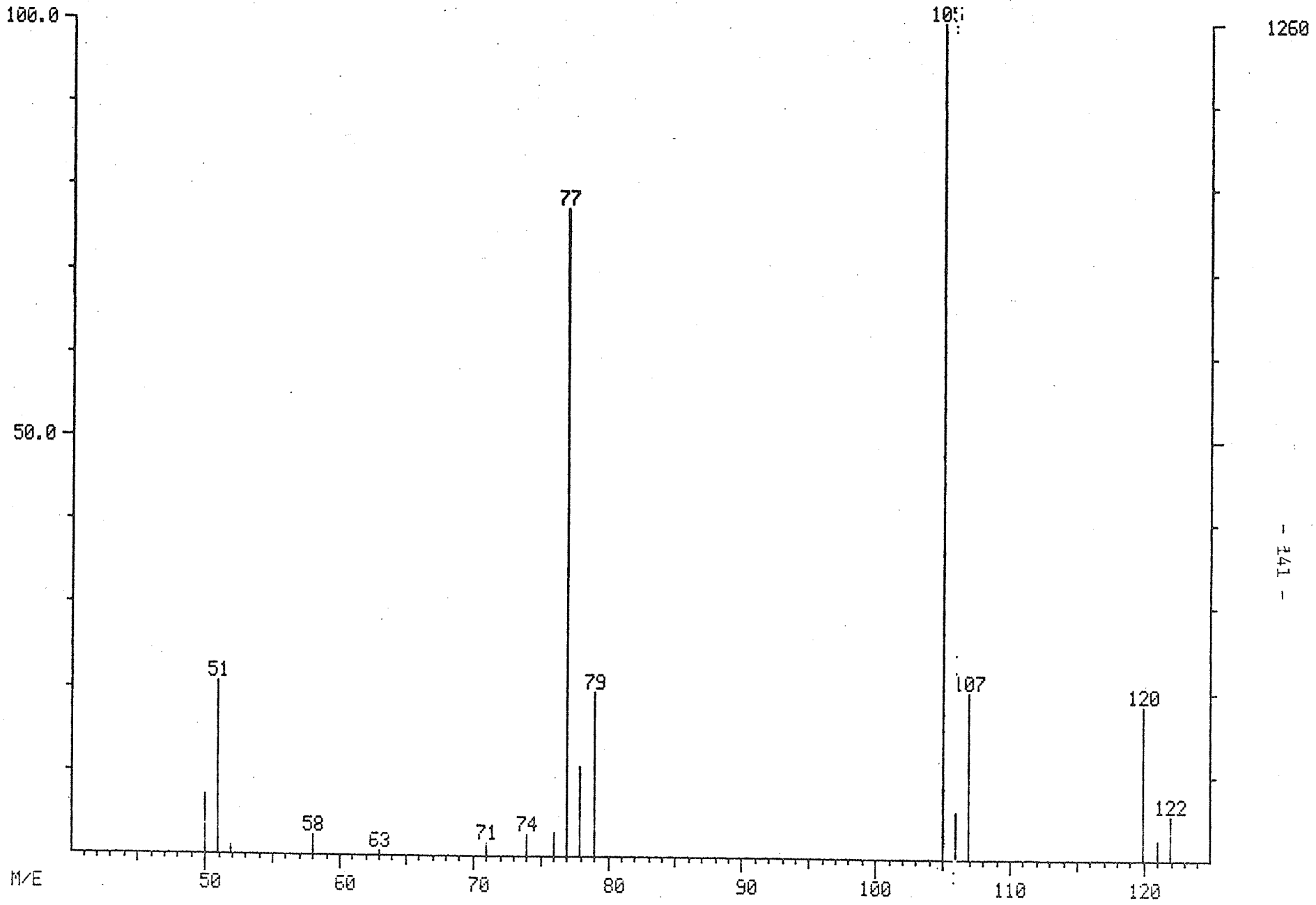


Figure 49 Spectrum 1025, Fraction 2-Site VIII

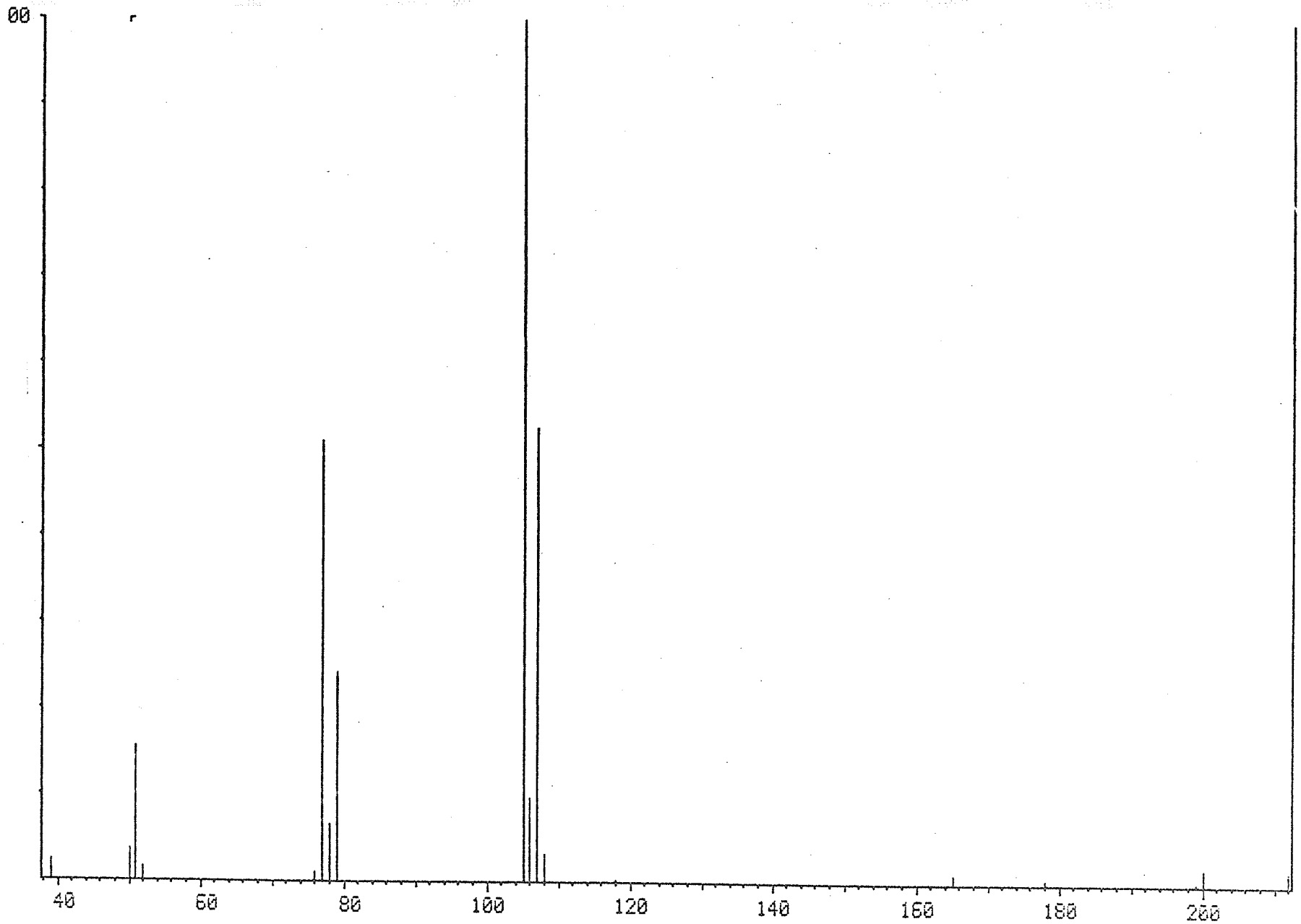


Figure 50

NBS mass spectrum for ethanone, 2-hydroxy-1,2-diphenylethanone

are differences in these spectra. The NBS mass spectrum for benzoin (Figure 50) shows the presence of a weak molecular ion (m/z 212), which corresponds to the molecular weight of this compound. This ion was observed to be absent in spectrum 1025 (Figure 49) since the presence of a vertical axis at m/z 125 is indicative of no additional ions being detected above this m/z on the Finnigan system. Furthermore, examination of spectrum 1025 (Figure 49) shows the presence of two ions which are absent in the NBS mass spectrum for benzoin (Figure 50), namely, m/z 120 and m/z 122. A manual search of spectrum 1025 in the Eight Peak Index of Mass Spectra (1974) revealed that this spectrum was similar to acetophenone and benzoin. Table 8 depicts the eight most abundant ions, and their relative abundances, for benzoin, acetophenone and spectrum 1025. The data for benzoin and acetophenone was obtained from the Eight Peak Index of Mass Spectra (1974).

TABLE 8 Eight Most Abundant Ions, and Their Relative Abundances for Benzoin, Acetophenone and Spectrum 1025

	Benzoin	Acetophenone	Spectrum 1025
m/z	RA	RA	RA
120	-	23	18
107	60	-	33
106	11	-	-
105	100	100	100
79	50	-	20
78	12	8	11
77	75	81	78
51	26	29	21
50	7	12	7

RA = relative abundance

Careful study of Table 8 reveals that ions characteristic of both compounds are present in spectrum 1025. For example, m/z 120, which is specific only to acetophenone, is present in this spectrum; furthermore, m/z's 79, 106 and 107, specific to benzoin, are also present in this spectrum. The observation that ions characteristic of both compounds appear in spectrum 1025, suggests that benzoin and acetophenone are co-eluting, i.e. these compounds are not sufficiently separated from one another on the analytical column to yield independent mass spectra. Careful examination of the total ion chromatogram depicted in Figure 47 supports the notion of co-eluting compounds since the chromatographic peak appearing at scan time 1025 contains a visible shoulder. However, a search of the Merck Index (1976) revealed that the boiling points of benzoin and acetophenone were 344^{o}C and 202^{o}C respectively, thereby dispelling any notion that these compounds are co-eluting since the boiling point differences are too great. This means that some compound other than benzoin is co-eluting with acetophenone. The presence of acetophenone in Fraction 2-Site VIII was confirmed by comparing a GC/MS analysis of acetophenone to the analysis of Fraction 2- Site VIII. The analytical conditions are described in Section 2.2.9, Method C, however, a 25 m column was used instead of a 50m column in order to speed up the analysis time. Figure 51 depicts a total ion chromatogram for acetophenone (upper portion) and Fraction 2 - Site VIII (bottom portion). From this figure we can see that acetophenone (scan time 561 - upper portion) and our unknown

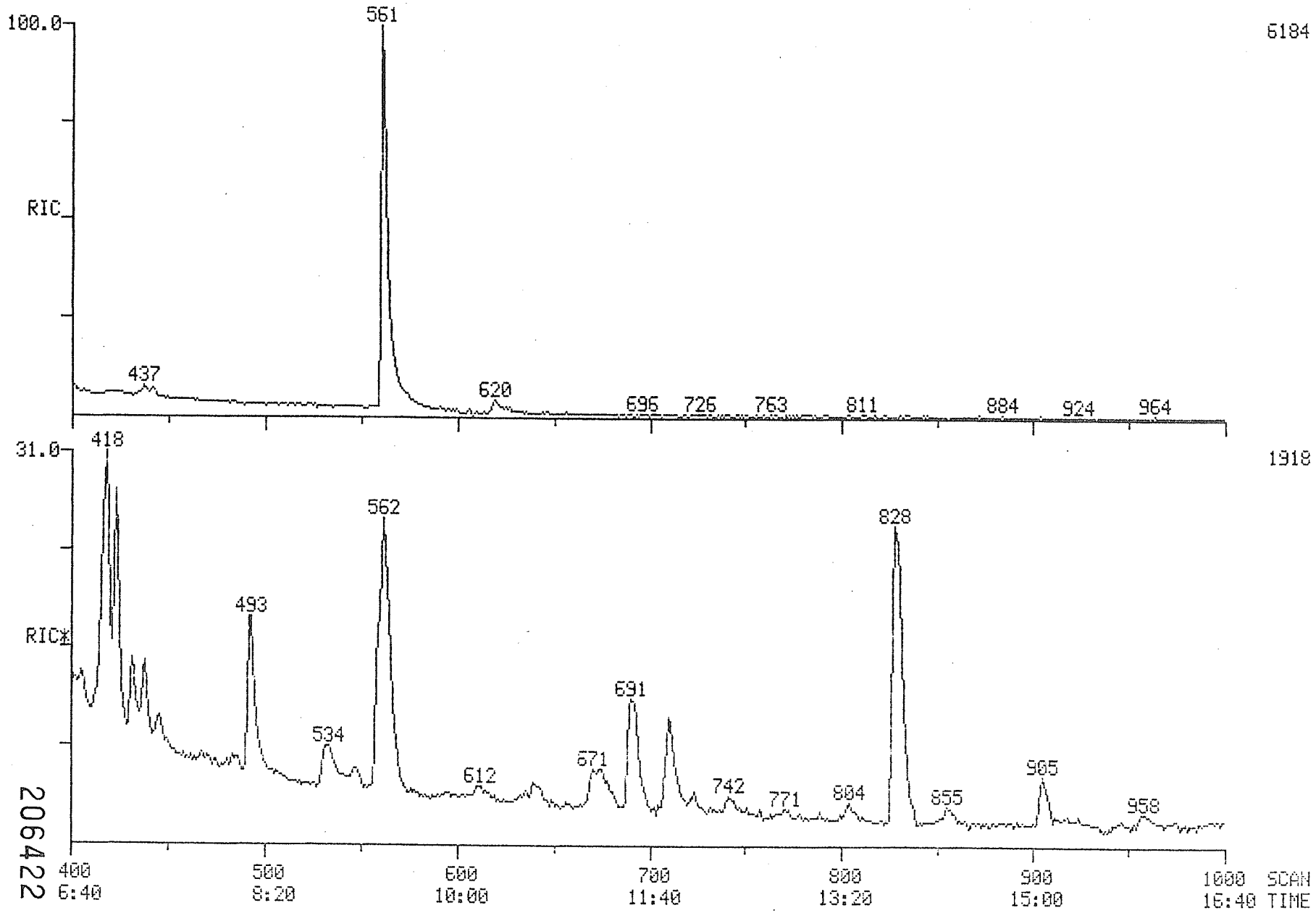


Figure 51 Total ion chromatogram for acetophenone (upper portion) and Fraction 2-Site VIII (bottom portion)

(scan time 562 - bottom portion) have, within experimental error, identical retention times. Furthermore, a comparison of the mass spectrum of scan number 562 (Figure 52 upper portion) to the mass spectrum of acetophenone (Figure 52 bottom portion) shows that the characteristic ions of acetophenone are present in the unknown (scan 562), however, the unknown contains additional ions which are not part of the mass spectrum for acetophenone. These ions include m/z 79, m/z 107 and m/z 122. In order to ascertain what co-eluting compound was contributing these ions, the mass spectrum for acetophenone was subtracted from spectrum 562, using the computer software available with the Finnigan GC/MS. The remaining ions were subjected to a computerized deconvolution/library search and compound identification using the NBS mass spectral library as a reference. For the remaining ions a good fit was observed for 1-phenylethanol. Figure 53 compares the mass spectrum for scan 562 (upper portion) to 1-phenylethanol (bottom portion).

Comparison of the mass spectrum for scan number 562 (Figure 53, upper portion) to the NBS mass spectrum for 1-phenylethanol (Figure 53, bottom portion indicates that the unknown (scan 562, Figure 53, upper portion) contains many of the ions characteristic of 1-phenylethanol. For example, ions with m/z's of 122, 107, 105, 79, 78, 77, 51 and 43 which are present in the mass spectrum for 1-phenylethanol are present in the mass specturm for scan 562. The absence of ions with m/z's of 121, 108, 104, 103, 80, 52 and 50 in mass spectrum

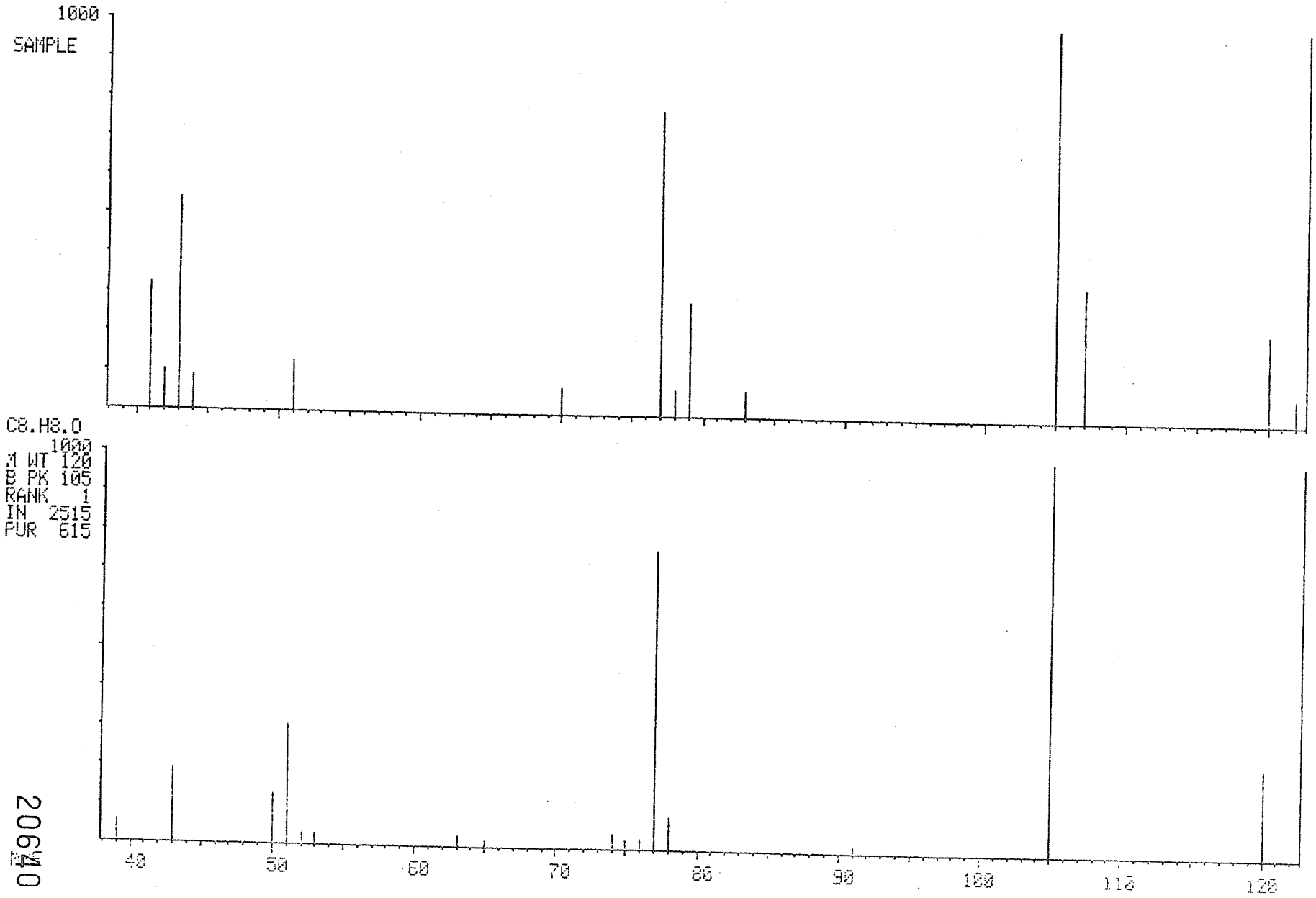


Figure 52 EI mass spectrum for scan number 562 (upper portion) and acetophenone (bottom portion)

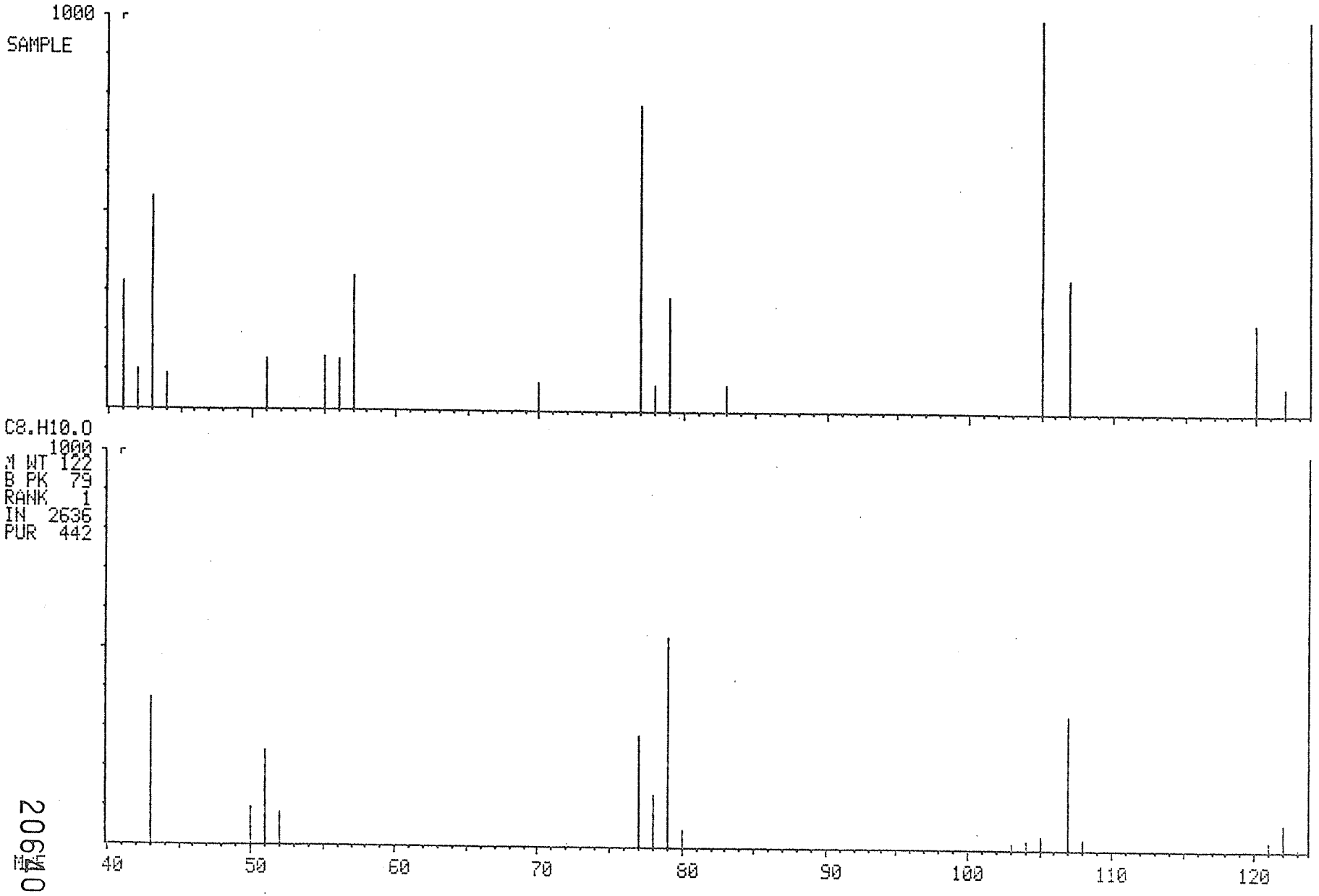
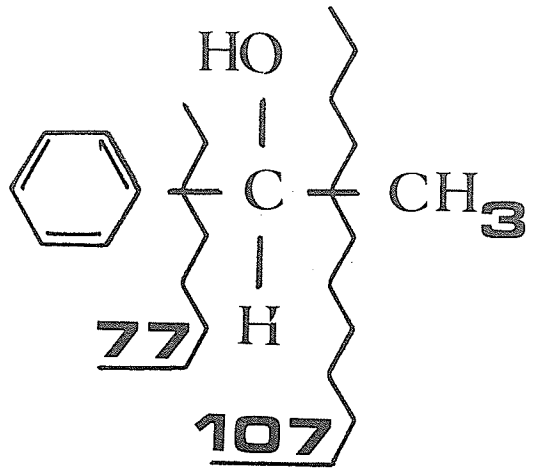
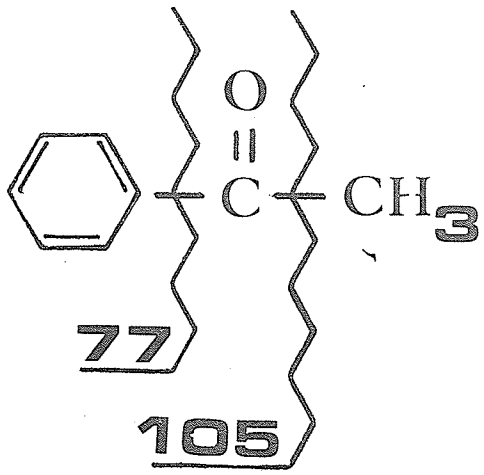


Figure 53 EI mass spectrum for scan number 562 (upper portion) and 1-phenylethanol (bottom portion)

562, but which are present in the mass spectrum for 1-phenylethanol, can be explained in part by the fact that the NBS mass spectrum for 1-phenylethanol (Figure 53, bottom portion) was obtained on a different instrument from that used to generate the mass spectrum for scan 562 (Figure 53, bottom portion) and therefore sensitivity differences of these instruments may explain the absence of these weakly abundant ions. Careful study of spectrum 562 (Figure 53, upper portion) also reveals the presence of ions which are not present in the mass spectrum of 1-phenylethanol. These ions include those with m/z values of 120, 83, 70, 55, 56, 57, 41, 42 and 44. The ion with m/z 120 is probably due to acetophenone (Figure 52-bottom). The other ions with m/z valves of 83, 70, 55, 56, 57, 41 and 42 can be attributed to background noise or a third eluting compound.

Figure 54 depicts the structural formula for 1-phenylethanol and acetophenone as well as the EI/MS fragmentation of these compounds. From this figure it is apparent that these compounds have similar structures, furthermore, their molecular weights differ by only 2 amu. A search of the CRC Handbook of Chemistry and Physics indicated that these compounds have boiling points which differ by only $1^{o}C$, thus lending support to the hypothesis of co-elution. From Figure 54 we can see that if one were to monitor for m/z 120 and m/z 107 over the retention span of the peak whose apex is scan 562 (Figure 51-bottom portion), then one would be able to ascertain from





MW = 122

MW = 120

BP = 203

BP = 202

1-PHENYLETHANOL

ACETOPHENONE

Figure 54 Structural formulae and EI/MS fragmentation for 1-phenyl-ethanol and acetophenone

the resulting ion chromatogram which compound is contributing most to scan 562.

Figure 55 depicts an ion chromatogram for m/z 107 and m/z 120 over the retention span of the peak whose apex is scan 562 (Figure 51-bottom). From this figure we can see that 1-phenylethanol (m/z 107) elutes first and reaches its maximum concentration at scan 561. Acetophenone (m/z 120), on the other hand, elutes second and reaches its maximum concentration at scan 565. Since the area under these ion chromatograms is proportional to concentration, it is obvious from Figure 55 that acetophenone (m/z 120) contributes the most to the peak appearing at scan 562 (Figure 51-bottom) since the area under the ion chromatogram for m/z 120 is greater than that for m/z 107.

A search of literature revealed that both acetophenone and 1-phenylethanol had been tested for carcinogenicity using E. coli cultures and found to be non-carcinogenic (Fluck et al., 1976).

The incorrect computer identification of benzoin for scan 1025 (Figure 47) and the subsequent correct identification of acetophenone and 1-phenylethanol for this same chromatographic peak, illustrates that a computerized/deconvolution library search and compound identification represents the lowest level of confidence for the identification of unknown compounds. These techniques should only be used for qualitative purposes and all compounds identified using this technique must be confirmed by comparing retention times and the mass spectrum of the unknown to a chemically pure standard.

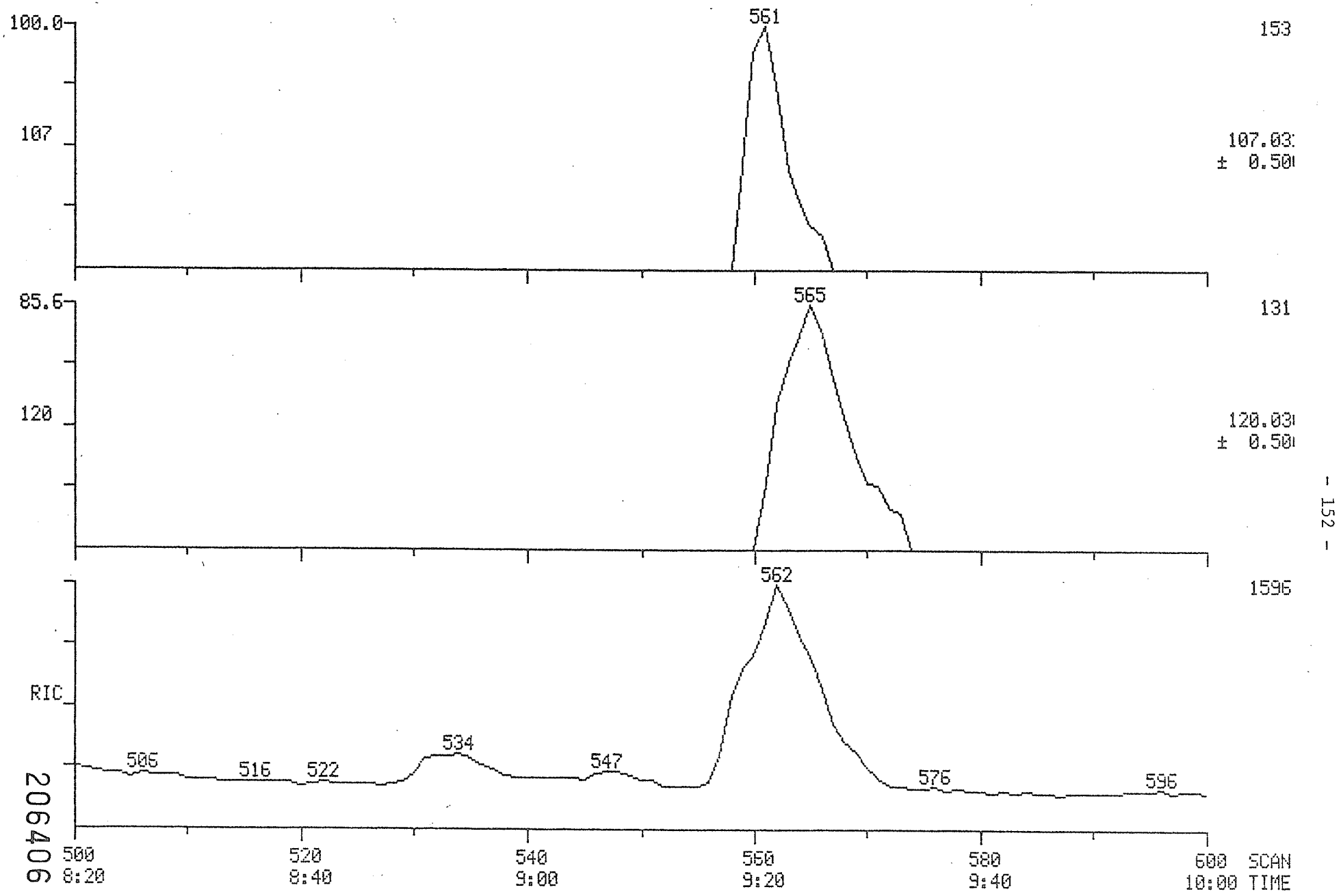


Figure 55 Mass chromatogram for m/z 120 and m/z 105.

Another peak in the total ion chromatogram for Fraction 2-Site VIII (Figure 47), namely the peak eluting at scan 1029, was identified by the computer as 2-methylbenzaldehyde. This compound was identified in the NIOSH-Registry of Toxic Effects of Chemical Substances as being oncogenic, therefore its spectrum was retrieved and visually compared with the NBS mass spectrum for this compound. Spectrum 1029 is presented in Figure 56. Visual comparison of this spectrum to the NBS mass spectrum for 2-methylbenzaldehyde (Figure 57) shows a good fit for the major ions.

Another tumor promoting agent was identified by the computer in spectrum 1071 which is presented in Figure 58. Comparison of this spectrum to the NBS spectrum for 4-methylbenzaldehyde (Figure 59) shows similarity with the extra ions in Spectrum 1071 indicative of background material.

Figure 60 compares the total EI ion chromatograms of the Fraction 2 extracts from Site VII (upper portion) to Site VIII (bottom portion). In both sites intense peaks were observed in the scan region 2500 to 3500. A computerized deconvolution/library search and compound identification of the ensuing spectra in this region indicated that these compounds were sterols. Good computer matches to the NBS mass spectral library were observed for cholesterol, campesterol and γ-sitosterol with the largest peak being cholesterol. Since a search of the NIOSH Registry of the Toxic Effects of Chemical Substances indicated that these compounds were non-toxic, no further effort was expended to confirm the presence of these compounds.

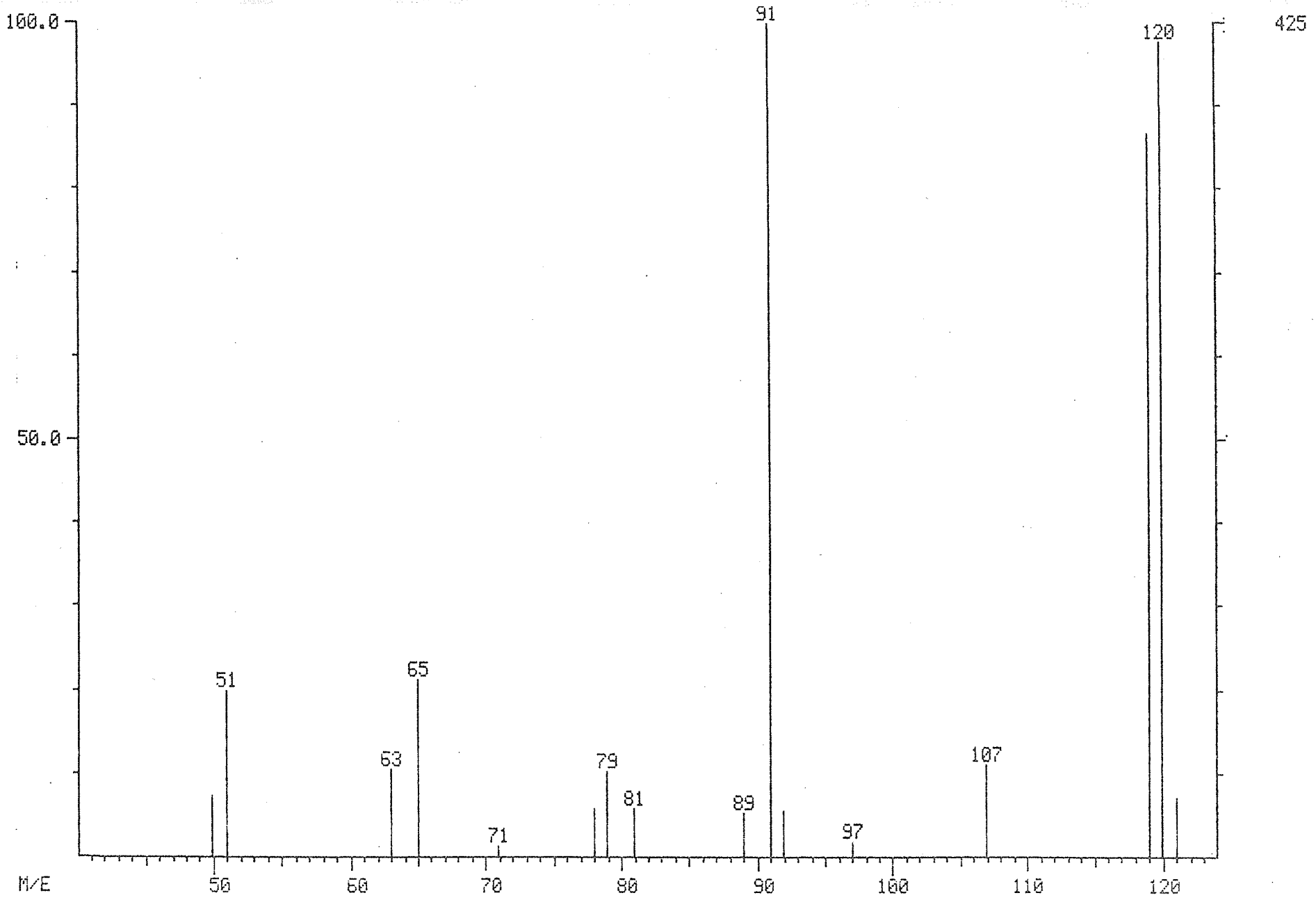


Figure 56 Spectrum 1029, Fraction 2-Site VIII

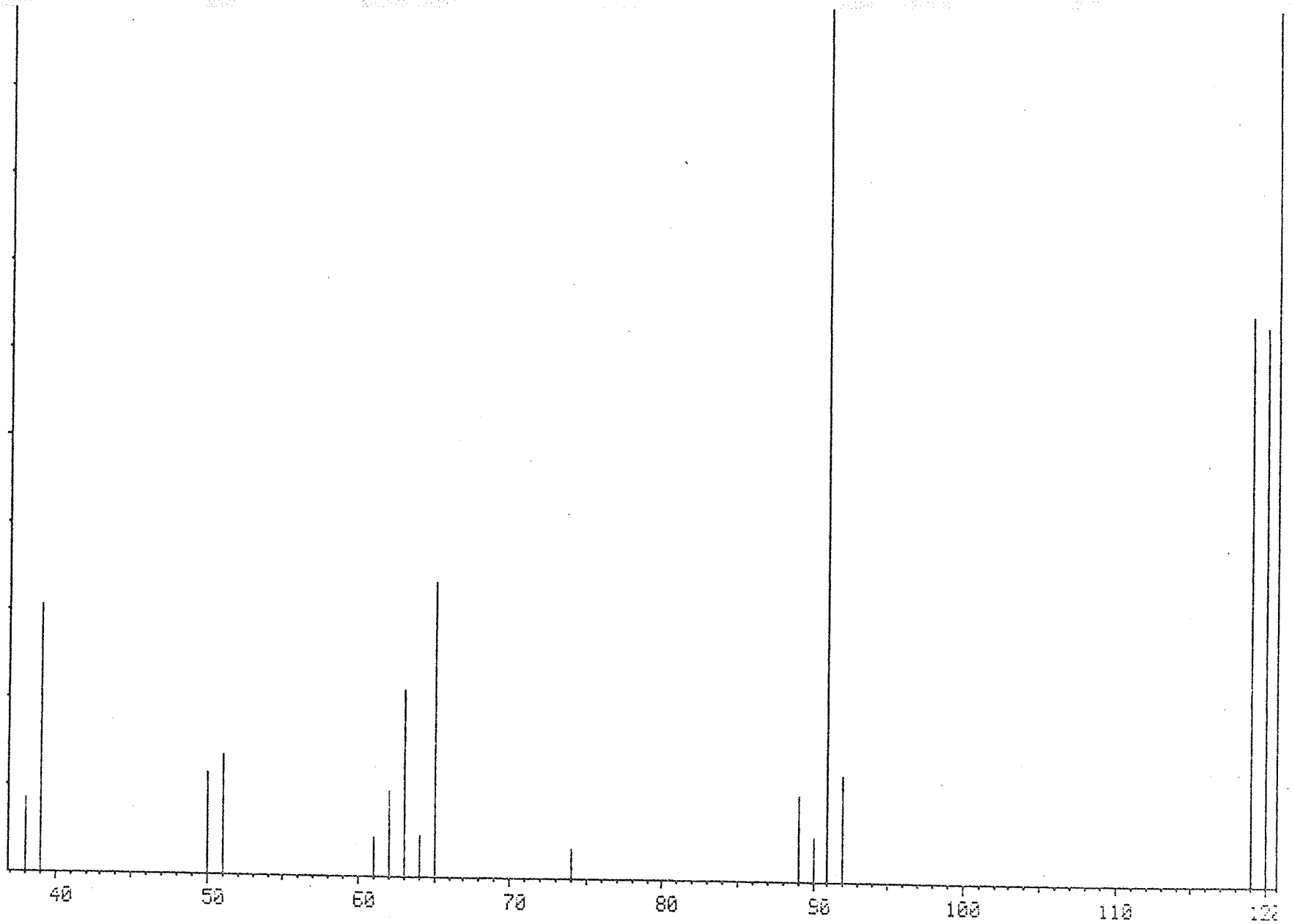


Figure 57

NBS mass spectrum for 2-methylbenzaldehyde

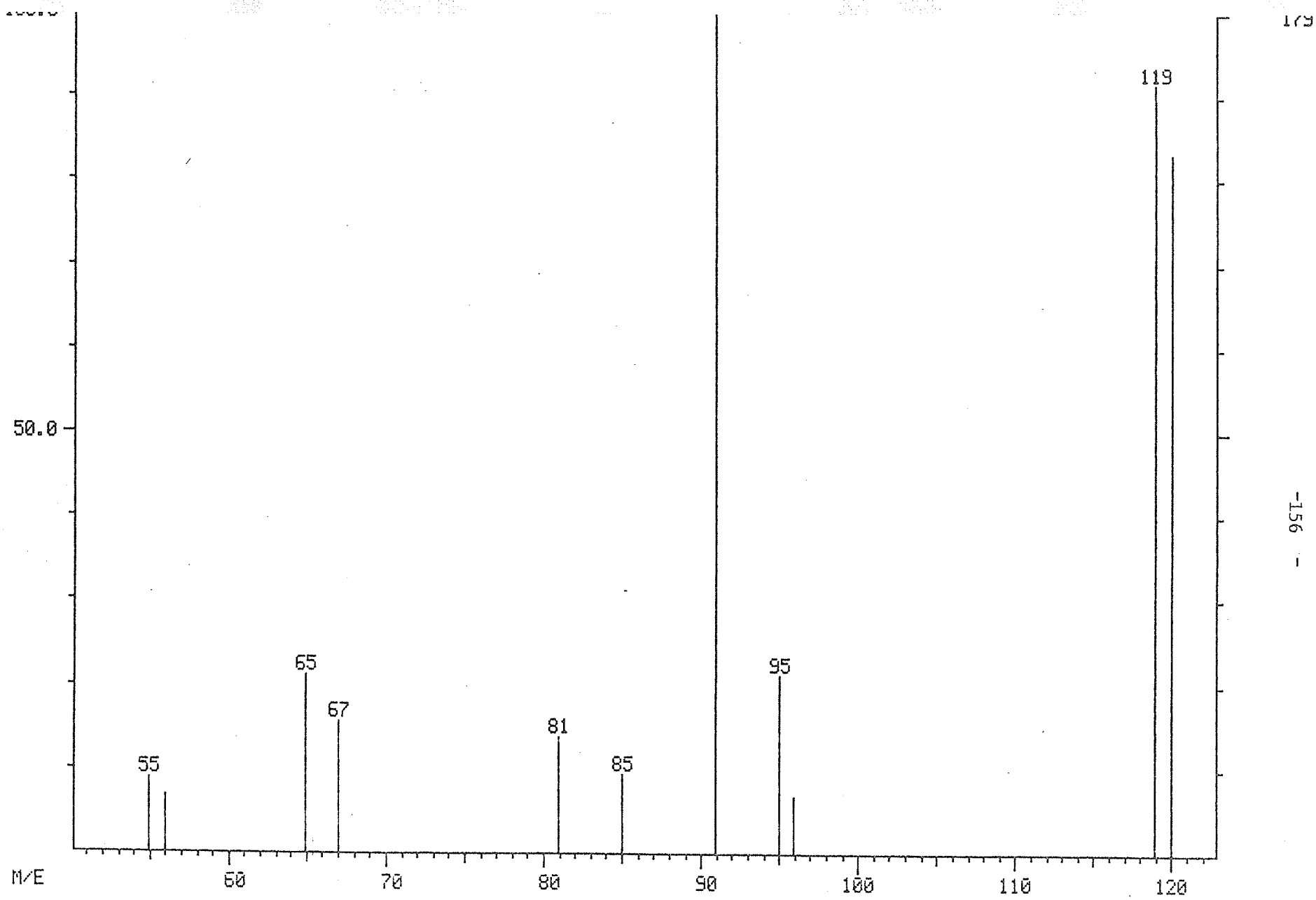


Figure 58 Spectrum 1071, Fraction 2-Site VIII

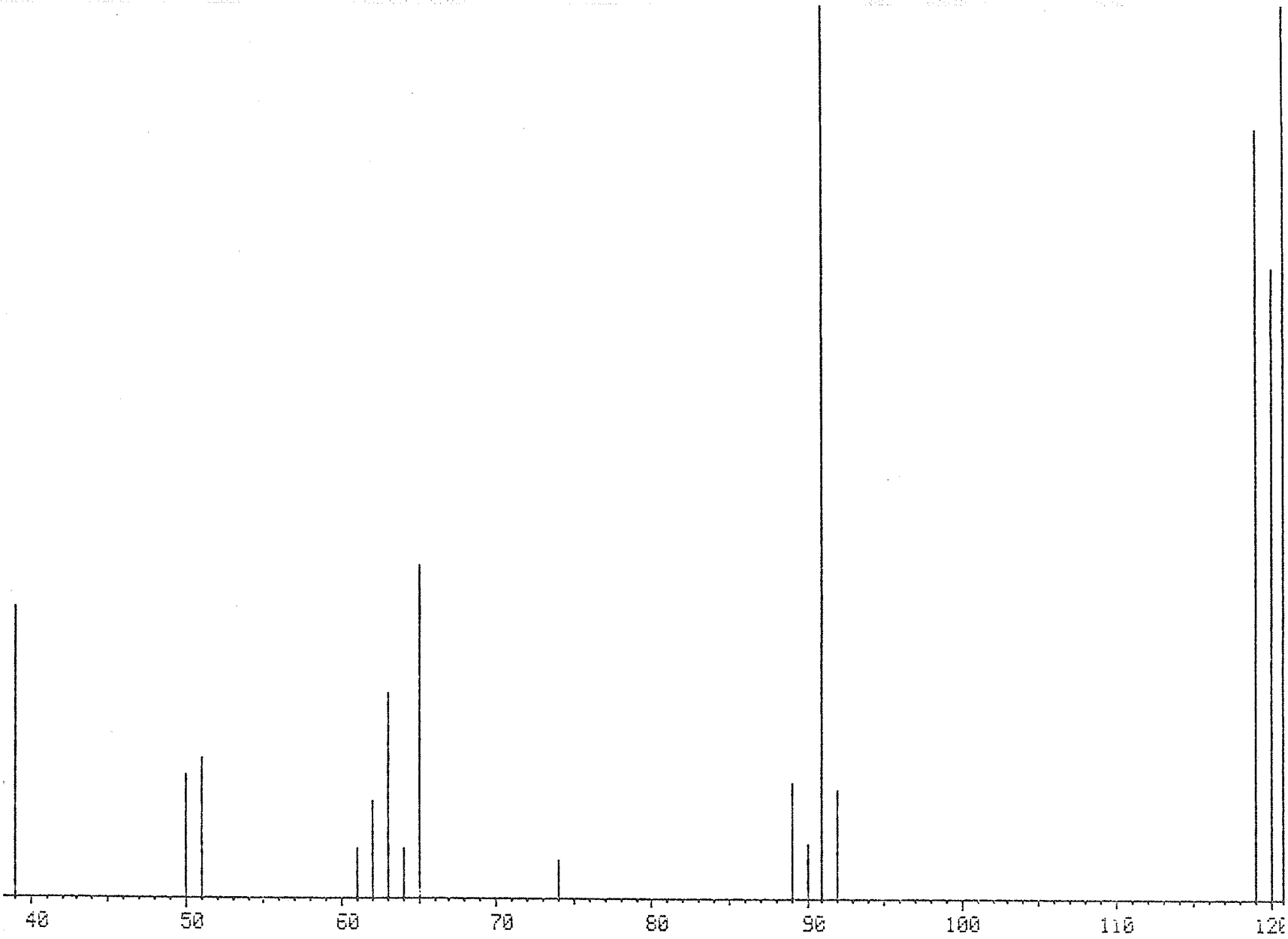


Figure 59 NBS mass spectrum for 4-methylbenzaldehyde

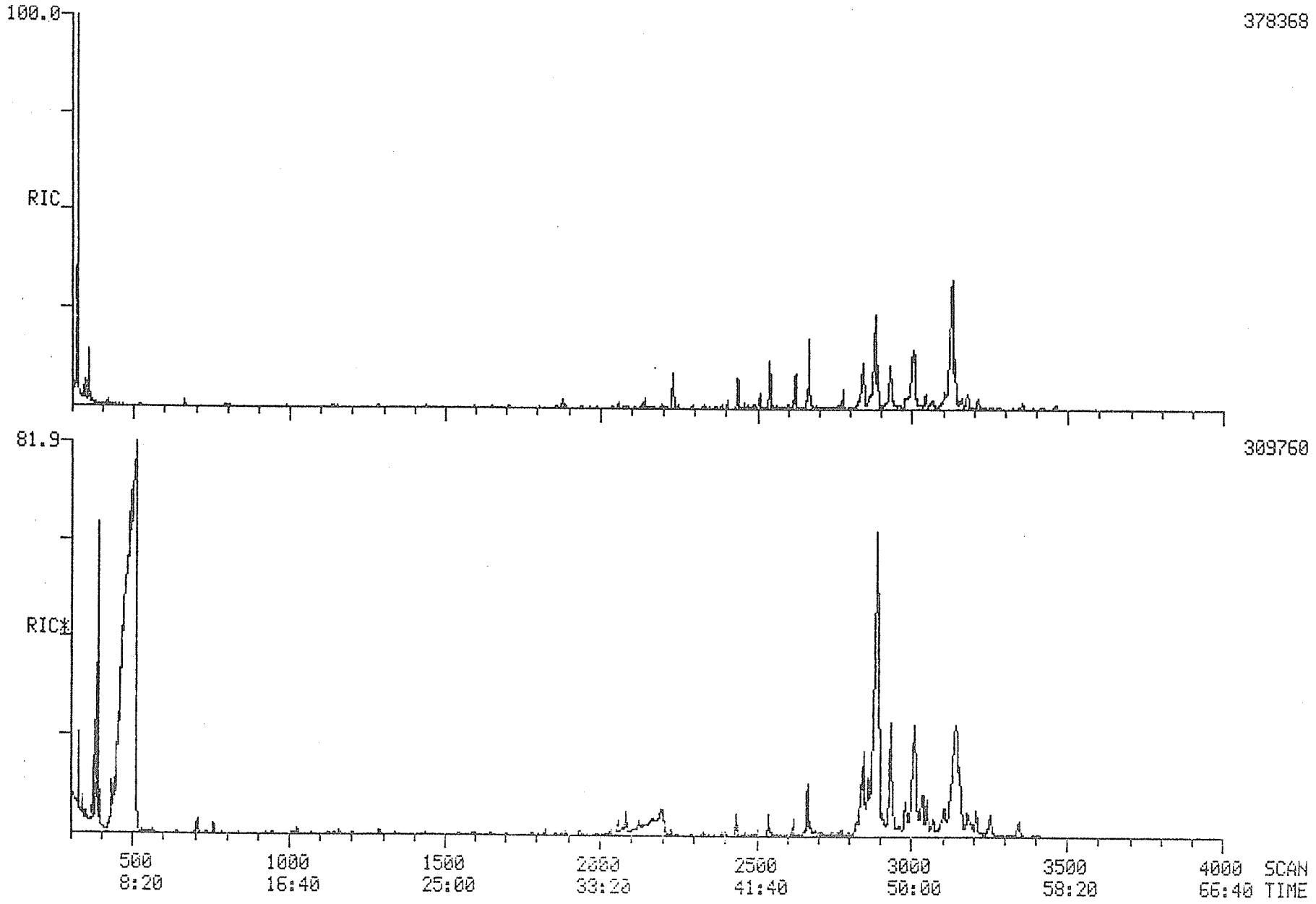


Figure 60 Comparison of total ion chromatograms from Fraction 2-Site VII and Fraction 2-Site VIII

A very large peak was observed around scan number 500 for Fraction 2-Site VIII (Figure 60, bottom portion). Comparison of the mass spectrum of this peak to the NBS library demonstrated a good fit for diacetone alcohol. This compound was searched in the NIOSH-Registry of Toxic Effects of Chemical Substances and found to be a skin and eye irrantant but non-toxic. Therefore, confirmation of the presence of this compound was not pursued.

Figure 60 shows that the most concentrated components present in Fraction 2 elute from the chromatographic column at retention times of 5 to 9 min. and 33 to 58 min. A computerized deconvolution/library search and compound identification of spectra taken in these retention windows indicated that the compounds present in greatest concentration were likely sterols and diacetone alcohol. These compounds are not generally considered to be toxic. On the other hand, toxic compounds such as styrene, benzaldehyde and o-xylene elute at retention times of 9 to 25 min. These compounds are barely detectable in the normalized ion chromatograms depicted in Figure 60. This observation demonstrates that residues of most concern are not necessarily the most abundant compounds present.

(3.3) CONCLUSION

Bioassay of fractionated sediment extracts from Site VII and VIII using the P. redivivus assay and the Salmonella/microsomal assay demonstrated that neutral compounds present in the sediment, and which elute from a 5% water deactivated Florisil column with hexane:dichloromethane (1:1), were responsible for the bulk of the observed toxicity. These compounds were designated as Fraction 2 and are characterized by being slightly polar. The degree of polarity can be compared to that of methoxychlor which was experimentally determined to elute 98 percent in this fraction when subjected to column chromatography.

Analysis of this fraction from Sites VII and VIII using capillary gas chromatography-mass spectroscopy indicated a complex mixture of compounds present at both sites. Mass spectra of eluting compounds were obtained and subjected to a computerized deconvolution/library search and compound identification. The most abundant compounds observed using this technique were branched chain ketones (C_5-C_9) aromatic ketones (C_8 and C_{14}), unsaturated cyclic ketones (C_7 and C_8), aromatic aldehydes (C_7 and C_8), straight chain aldehydes (C_6 and C_{14}), branched chain aldehydes (C_7 - C_8), cyclic alcohols (C_6 and C_9) branched chain alcohols (C_8 - C_{13}), diols (C_9), straight chain alcohols (C_6 and C_{32}), branched chain alkanes (C_8 - C_9), cyclic alkanes (C_8 - C_{12}), branched chain alkenes (C_7 - C_8), straight chain alkenes (C_{13}), benzenoids (C_7 - C_8), plant and animal sterols and alkane thiols.

The chemical abstract numbers (CAS numbers) for these compounds were obtained using the Chemical Abstracts Collective Indexes. The CAS numbers were then used to obtain toxicity data for these compounds by searching the NIH/EPA Chemical Information System, file NIOSH Registry of Toxic Effects of Chemical Substances. This data file indicated that toluene, alkane thiols, styrene, benzaldehyde, methylbenzaldehyde, o-xylene and benzoin are toxic. According to this data base, styrene and benzaldehyde are mutagenic, o-xylene has a high aquatic toxicity rating, benzoin and methylbenzaldehyde are tumorigenic and alkane thiols and toluene are regulated under occupational health and safety regulations.

As a result of this information the mass spectra of these compounds were retrieved and compared manually to the NBS mass spectra for these compounds. Chemical standards for styrene, benzaldehyde, toluene and o-xylene were obtained and subjected to high resolution gas chromatography and the retention times of these standards were found to match those of the suspected compounds thus confirming their presence.

Close study of the mass spectrum for benzoin indicated that this spectrum was the result of the co-elution of 1-phenylethanone and 1-phenylethanol, two compounds which have a similar structure to benzoin. The presence of 1-phenylethanone was confirmed by comparing the retention time and mass spectrum of a chemical standard of 1-phenylethanone to the suspected compound. A search of the NIOSH-Registry of Toxic Effects of Chemical Substances revealed that neither of these chemicals were toxic.

The mass spectra for the compounds suspected of being methylbenzaldehyde and 2-hexanethiol were retrieved and compared to the NBS mass spectra for these compounds. A close resemblance was observed between the spectra for the suspected compounds and the NBS reference spectra, however, due to the lack of chemical standards, the retention times of the suspected compounds could not be matched to pure standards, and therefore absolute confirmation could not be effected.

A reagent blank was analyzed for the presence of styrene, benzaldehyde, o-xylene and toluene. Toluene was found to be present and traced as a contaminant of the hexane that was used. As a result the presence of toluene in the sample extracts was discounted.

The presence of styrene and benzaldehyde should have been detected using the Salmonella/microsomal assay and the P. redivivus assay since these compounds are mutagenic. The concentrations of styrene and benzaldehyde were determined to be 0.90 and 0.54 µg per gram (ppm) of freeze dried sediment.

In Fraction 2 -Sites VII and VIII, both mutagenic and sublethal effects were observed with the Salmonella/microsomal assay and the P. redivivus assay. Part of the observed mutagenesis can be explained by the presence of styrene and benzaldehyde. The sublethal effects cannot be explained. This is not surprising since toxicological information was not available for many of the compounds tentatively identified by computer search. Furthermore, we know very little

of the effects of methylbenzaldehyde, 2-hexanethiol, and o-xylene upon our test organisms. Synergistic and antagonistic interactions of chemicals have to be considered as possible contributors towards the observed toxicity in Fraction 2.

The technique employed to detect and identify the toxic chemicals present in Fraction 2 from Site VII and VIII was GC/MS. In order to be detected by this technique, compounds have to be sufficiently volatile to allow them to be vaporized in the injection port of a gas-chromatograph. There may be toxic compounds present which may not be sufficiently volatile to lend themselves to identification by this method.

In order to determine which compounds are responsible for the observed toxicity, in Fraction 2-Site VII and VIII, these fractions should be subjected to further fractionation using preparative normal phase high pressure liquid chromatography. Much more sediment would have to be extracted to allow this type of additional fractionation. At least 400 g of freeze dried sediment would have to be extracted and chromatographed prior to HPLC fractionation in order to have sufficient material for a second bioassay. Refractionation followed by rebioassay would reduce the complexity of this fraction and allow one to focus on the most toxic compounds; however, techniques other than GC/MS might have to be used to effect identification. These techniques might include HPLC/mass spectrometry (EI, CI, and FAB), HPLC/Fourier

transform infrared spectroscopy, HPLC/mass spectrometry-mass spectrometry and HPLC/derivitization prior to mass spectrometry.

From this thesis it is evident that analytical fractionation of extracted sediment followed by bioassay is an effective means of isolating those components present in an ecosystem which generate the greatest risk to that system. For this reason, the method outlined in this thesis is being used in a multidisciplinary, comprehensive study, the Tobin Lake Project. Using this method, it is anticipated that anthropogenic compounds present in Tobin Lake which are causing the greatest risk to the ecosystem will be isolated and identified. This would allow Environment Canada to trace the origins of these compounds and eventually curb their release into the environment.

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