Metabolites of 2,2',4,4',5,5'-hexachlorobiphenyl: isolation from feces and characterization by mass spectrometry

METABOLITES OF 2,2',4,4',5,5'-HEXACHLOROBIPHENYL: ISOLATION FROM FECES AND CHARACTERIZATION BY MASS SPECTROMETRY

by

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ABSTRACT

Individual polychlorinated biphenyl (PCB) congeners and their isomers are metabolized to varying degrees depending on the position and extent of chlorination, and on the species studied. Highly ortho substituted PCBs have been found resistant to extensive biotransformation. The dog (beagle) has been found uniquely capable of extensive biotransformation of a completely ortho substituted chlorinated biphenyl, having no two adjacent, unsubstituted, positions (2,2',4,4', 5,5'-hexachlorobiphenyl). Two methods of metabolite isolation and cleanup, consisting of gel permeation chromatography (GPC) and concentrated sulfuric acid extraction from hexane, were investigated, along with GC-MS characterization of the metabolite methyl ethers. GPC results suggested the need for two solvent systems to take advantage of both adsoprtion and steric-exclusion in achieving cleanup and separation. Concentrated sulfuric acid cleanup was found to be a fast and efficient method for cleanup of diazomethane derivitized extracts from feces. Reverse-phase TLC of an acid-ether extract of fecal homogenate indicated the presence of four metabolites in the ratio of 53:32:10:5%. Mass fragmentation of the metabolite methoxy derivatives is highly characteristic of the position of the methoxy substituent. GC-MS suggested one of the metabolites in the derivitized acid-ether extract of fecal homogenate to be 3-methoxy-2,2',4,4',5,5'-hexachlorobiphenyl. Urinary excretion of parent compound or metabolites was negligible.

INTRODUCTION

Polychlorinated biphenyls are produced commercially by the direct chlorination of biphenyl, and marketed as homologue -- isomer mixtures classified on the basis of percent, by weight, of chlorine (Hubbard 1964, Webb and McCall 1972). There are 209 theoretical substitution possibilities resulting from the chlorination of biphenyl. These 209 homologues and their isomers are referred to collectively as polychlorinated biphenyls (PCBs) (Hutzinger, Safe, and Zitko 1974a).

PCBs have in the past found wide use in industry due to their thermal and chemical stability and insulating properties. Examples include such diverse uses as plasticizers, heat transfer agents, fire retardants, antioxidants in marine paints, and as pesticide additives to reduce volatility and prolong residue effectiveness (Hutzinger, Safe, and Zitko 1974a, Anonymous 1971).

The very properties which make PCBs commercially useful have contributed to their accumulation in the ecosystem (Bush, Tumasonis, and Baker 1974). Their presence as environmental pollutants was first documented by Jensen, in Sweden in 1966 (Jensen 1966). Since that time PCBs have been identified globally in marine and terrestrial life forms, including man (Jensen 1972, Peakall and Lincer 1970).

The PCB 2,2',4,4',5,5'-hexachlorobiphenyl (2,4,5 HCB) is of particular importance to those studying the behavior of PCBs in the environment, for two reasons: (1) it is a major component in PCB

commercial mixtures with over 50% chlorine, and (2) it has no two adjacent, unsubstituted carbon atoms (Jensen and Sundstrom 1974a, Brinkman et al. 1976). This structural requirement has generally been thought to be a prerequisite for microsomal drug metabolizing enzymes involved in biodegradation (Jensen and Sundstrom 1974a, Schulte and Acker 1974, Mathews and Anderson 1975).

Metabolism

Due to their chemical stability, PCBs were at first believed to be inert to biodegradation. Analysis of residues found in wild animals, however, showed that some isomers commonly found in significant quantities in PCB technical grades were at lowered levels or totally undetectable in animal tissues. Later studies of PCB technical mixtures fed to test animals confirmed these findings (Grant, Phillips, and Villanueva 1971, Jansson et al. 1975, Burse et al. 1976).

Subsequent investigations have shown that virtually all PCBs are capable of at least some biotransformation by some organism. Isolated metabolites are almost invariably phenolic, but instances of diol formation and dechlorination have been reported (Jensen and Sundstrom 1974b, Hutzinger et al. 1974b, Safe, Hutzinger, and Jones 1975).

Certain molecular configurations, however, do appear to be favored as a means to rapid hydroxylation. Similar to chlorinated benzenes, the presence of two adjacent, unsubstituted positions, on either phenyl ring, promotes rapid hydroxylation (Jondorf, Parke, and Williams 1955, Jensen and Sundstrom 1974a, Schulte and Acker 1974).

Extensive research with the biotransformation products of numerous life forms, from mammals to bacteria, have shown that this hydroxylation is accomplished through epoxidation of an aromatic double bond by monooxygenases (Daly, Jerina, and Witkop 1972, Jerina 1973, Jerina and Daly 1974). The lack of substitution at two vicinial positions on the aromatic ring facilitates this epoxidation.

In mammals, and particularly man, a second factor is important; the presence or absence of ortho (to the biphenyl bridge) chlorines, additionally, facilitates or limits metabolism (Jensen and Sundstrom 1974a). Jensen and Sundstrum showed that the number of ortho chlorines was probably the second single most significant structural feature in determining PCB isomer retention in human adipose tissue. Their research showed that virtually all 2,2',6,6'- or 2,2',6- substituted chlorobiphenyls, except maximally substituted 2,2',3,3',4,4',5,5',6,6'-decachlorobiphenyl and possibly 2,2',3,3',4,5,5',6,6'-nonachlorobiphenyl (for which they had no comparative standard), were undetectable in tissue, or significantly below their proportions in PCB technical mixtures. They contended that this disparity between human tissue levels, technical grade concentrations, and residue levels found in similar research with lower animals (unpublished), indicates extensive metabolism of chlorobiphenyls by mammals. In this same study (Jensen and Sundstrom 1974a) 2,2',4,4',5,5'-hexachlorobiphenyl, with only one ortho chlorine on each ring, was found in the highest abundance of all detectable PCB residues found in human tissue. It represented 21% of all measurable residues, which is far

in excess of its contribution to 50% and 60% technical grades, the mixtures in which it is normally found in its highest relative abundance.

The geometry of 2,4,5-HCB suggests that, spatially, the two phenyl groups would be virtually coplanar since electrostatic repulsion between the two chlorines would force them to opposite sides of the molecule. Based on the disproportionately low levels of 2,2',6,6'- and 2,2',6' substituted chlorobiphenyls, cited above, it would appear that the mutually perpendicular planes of the phenyl rings associated with these substitutions are the optimal configuration for microsomal drug metabolizing enzymes. The lack of this optimal configuration, together with a lack of adjacent unsubstituted positions could easily account for the observed low biotransformation and inordinately high concentration in human tissue.

A similar phenomenum was found in rhesus monkeys in a study on the distribution and excretion of 2,4,5-HCB administered intravenously. Excretion in the monkeys was approximately 10% after 90 days. This was contrasted with that of dogs (beagles), which, when similarly dosed, excreted 60% in only 15 days.

The rapid excretion of 2,4,5-HCB in dogs, compared with that of primate mammals has prompted a characterization of the metabolic end products as a clue to understanding this unique process of biotransformation in the dog.

Previous investigations into the metabolism of 2,4,5-HCB in the rat, mouse, and quail (Jensen and Sundstrom 1974b) revealed the

presence of a hydroxy metabolite in the feces of the rat and mouse. Mass spectral characterization of the methyl ethers of the respective metabolites strongly suggested substitution at the 3 position in both. No metabolites were detected in the excreta of quail.

A similar study in rabbits (Hutzinger, Jamieson, et al. 1974) demonstrated metabolic dechlorination, with the isolation of a hydroxy-pentachloro biphenyl from a rabbit fed 2,4,5-HCB. A hydroxyhexachloro biphenyl was also identified in the same animal.

Metabolite Separation and Characterization

Investigation of the biotransformation products of 2,4,5-HCB involved both a separation (extraction, cleanup, and chromatography) and a characterization (spectrometry) phase.

Gel Permeation Chromatography (GPC)

Gel Permeation Chromatography (GPC) has been used by various investigators as a cleanup technique prior to gas chromatographic analysis of PCB and other environmental pollutants (Jansson et al, 1975, Stahling, Tindle, and Johnson 1972, Johnson et al. 1976, Kuehl and Leonard 1978).

To characterize the separations produced by GPC it is necessary to understand some basic concepts about gels and gel chromatography.

Gels are three dimensional polymeric structures capable of absorbing discreet amounts of a particular solvent. Absorbtion of solvent by the gel matrix gives rise to swelling, the extent of which

is generally limited by the degree of crosslinkage. It is the phenomenon of swelling which determines the pore size of the individual gel particles, which in turn determines the separation capabilities (Freeman 1973).

In general, as the solubility coefficient (δ) of the solvent approaches that of the gel (becomes a better solvent for the gel itself), swelling increases (Mori 1978). Table 1 shows solvent solubility coefficients and swelling values for Bio-Beads SX-2 (200/400) (Bio-Rad Laboratories, Richmond, Ca.) in the solvents listed. Bio-Beads SX-2 is a copolymer of polystyrene, crossed-linked (2%) with divinyl benzene.

As the solvent passes through the column it will tend to diffuse into and out of pores which have a diameter equal to, or greater than its own effective diameter. The concept of effective diameter takes into account length to width ratio, and it is this property which permits separation of compounds of the same molecular weight, but different geometry (Johnson and Stevenson 1978).

All gel pores are not of the same diameter. Because of this, smaller molecules will tend to enter more pore spaces. The smallest molecules will permeate virtually all pores, and in so doing, elute at the total permeation volume of the column. If a sample is of such dimensions that it cannot enter any pore spaces, it will elute at the interstitial, or void, volume, which comprises that volume of solvent between gel particles (together with any additional volumes contributed by the column and tubing). In the ideal steric-exclusion system, all

Table 1. Solubility coefficient, and swelling values for solvents in use with Bio-Beads SX-2 (200/400).

Solvents	δ $(cal./cm^3)^{1/2}$*	Actual Swelling (ml/g)**
Cyclohexane	8.2	3.2
Ethyl acetate/Toluene (3:1)	9.05+	4.7
Methylene chloride	9.7	6.16
Benzene	9.2	6.2

* Brandrup and Immergut 1966

** Manufacturer's label values

\+ Sum of the relative contributions of each solvent (Mori 1978)

components of a mixture should elute somewhere between the void and total permeation volumes (Johnson and Stevenson 1978).

The distribution coefficient (K_D) for any compound can be calculated from the expression:

$$K_D = \frac{\text{Pore volume permeable to compound}}{\text{Total pore volume of the column}}$$

In terms of measurable volumes, this becomes:

$$\frac{\text{Elution volume } (V_E) \text{ -- Void volume } (V_O)}{\text{Total pore volume of the column } (V_{TP})}$$

V_{TP} is commonly measured as the elution volume of benzene. Because of its low molecular weight and non-polar character, it approaches the characteristics of an ideal solvent, capable of total column permeation. The presence of double bonds also makes it readily UV detectable.

Transposing the above equation to express the retention volume (V_R) of any solute, one gets:

$$V_R = V_O + K_D V_{TP}$$

From this equation it is evident that in pure GPC the degree of retention and separation is a function of pore size, which in turn, is a function of the eluant solvent solubility coefficient (δ).

Solvents such as cyclohexane are poor solvents for polystyrene gels, as indicated by the swelling and δ values in Table 1. Of the solvents listed, it will swell the gel matrix the least. This results in smaller pore sizes overall and a lower exclusion limit (effective molecular size which is totally excluded). This should produce a

chromatogram in which higher molecular weight compounds should elute early and together. Separation would then be confined to molecules of lower molecular weight, since their effective diameters would still be compatible with the remaining pore volumes.

If the components of interest elute after the total permeation volume of the column (V_{TP}), (taken as the elution volume of benzene), it would suggest that adsorption and/or partition effects are present (Johnson and Stevenson 1978). It is probable that metabolite fractions are of sufficient polarity as to adsorb onto the gel matrix due to electrostatic interactions of the van der Waals type. Cyclohexane, being a relatively inert solvent, would not be expected to be of sufficient polarity to neutralize these adsorptive effects, and elute the solute. Such non-ideal conditions as these can often be the major factor in producing the desired separation (Freeman and Angeles 1974).

Hexane/Concentrated Sulfuric Acid Cleanup

Various groups investigating PCBs and their phenolic metabolites have reported success with a cleanup technique involving concentrated sulfuric acid extraction of hexane solutions of PCBs, their metabolites, or their metabolite methyl ethers (Jensen et al. 1972, Murphy 1972, Jensen and Sundstrom 1974a, Jensen and Sundstrom 1974b, Jansson and Sundstrom 1974, Mowrer et al. 1974, Jansson et al. 1975, Tulp, Olie, and Hutzinger 1977).

In an early paper on the utility of concentrated sulfuric acid cleanup, Murphy (1972) describes the ease and efficiency of pesticide and PCB residue cleanup by simply shaking a hexane extract of tissue

with 10-20% of its volume of concentrated sulfuric acid. With the exception of Dieldrin and organophosphorous compounds, recoveries were 90-100%.

In another paper from the same year, Jensen et al. (1972) used much the same technique to effect sample cleanup of extracts from fish prior to analysis for PCB and DDT residues. Instead of 10-20% acid, these investigators used a 1 to 1 ratio of hexane extract to fuming sulfuric acid. The fuming sulfuric acid was composed of concentrated sulfuric acid with 7% sulfur trioxide.

In a novel method of phase transfer, the Jensen group (Jensen et al. 1972) froze the sulfuric acid layer in dry ice-acetone and simply poured off the organic layer, which they found acceptable for gas chromatography. Subsequent investigators have used this technique of hexane/concentrated sulfuric acid to clean up derivitized extracts of hydroxy metabolites of PCBs prior to gas chromatography-mass spectrometry (GC-MS) (Jansson and Sundstrom 1974, Jensen and Sundstrom 1974a, Tulp, Olie, and Hutzinger 1977).

Gas Chromatography-Mass Spectrometry of Methoxyhexachlorobiphenyls

For each group of PCB isomers with the same number of chlorines and hydroxyl groups, the mass spectra are similar (Hutzinger, Safe, and Zitko 1974b). Additional characterization is therefore necessary to assign substituent positions. Barnes et al. (1967), working with the mass fragmentation of methyl ethers of polycyclic compounds, and later, Jansson and Sundstrom (1974) working with the methyl ethers of

potential tetra, penta, and hexachlorobiphenyl metabolites, both demonstrated characteristic fragmentation patterns produced by methoxychlorobiphenyls.

STATEMENT OF THE PROBLEM

As mentioned above, the dog (beagle) has been found capable of rapidly excreting a compound (2,4,5-HCB) which is only slowly excreted, and little metabolized, by other animals, including man. As a clue to the mechanism of this unique metabolic phenomenon, an investigation of the metabolic end products was undertaken.

Since the compound and/or its metabolites are excreted primarily in the feces, certain analytical problems immediately present themselves. The presence of lipid in fecal homogenates and in extracts of these homogenates intereferes with chromatographic separations by both thin layer and gas chromatography. Solvent extraction, using solvents of varying polarity, has long been used to remove interfering substances from biological extracts. This method, however, has inherent problems of sample loss, through multiple manipulations of the sample, and sample alteration, due to chemical reaction with the solvent or other components in solution, not to mention the time involved in repetitive extractions.

To accomplish the task of metabolite identification in this particular study, and at the same time, attempt to develop a cleanup technique with broader applicability to biological extracts in general, two techniques, involving gel permeation chromatography and concentrated acid cleanup, were investigated.

An additional problem, concerning characterization, which is not unique to this investigation, is the problem of assigning substituent positions on the molecule from mass spectral data alone. Mass spectrometry has proved to be a valuable tool in assigning molecular weights and in detecting the addition or deletion on a molecule, but has, for the most part, given little indication of substituent position. For this information, investigators have had to rely on nuclear magnetic resonance spectrophotometry (NMR) with its associated problems of larger sample requirement, greater sample purity, and sample solubility requirements.

As mentioned above, previous investigators have demonstrated a relationship between fragment abundance values and substitution position in the mass fragmentation of methoxychlorobiphenyls. The success of these earlier investigations suggested a mass spectral investigation of the methyl ethers of potential 2,4,5-HCB metabolites as a means to unambiguous position assignment, without resorting to NMR.

METHODS AND MATERIALS

Extraction

Initial separation consisted of an acid-ether extract of a fecal homogenate. The feces were obtained from a beagle dog (Hazelton Research Animals, Cumberland, Va.) dosed orally, daily for four days, with 50 mg 2,4,5-HCB (Analabs, Inc., New Haven, Conn.) in its food. On day five a radioactive (^{14}C) tracer dose (sp. act. = 9.14 µCi/µm) of approximately 15 µCi was given intravenously.

Approximately 300 ml of fecal homogenate was extracted with 1000 ml of diethyl ether, together with 330 ml of 95% ethanol in a clear glass gallon jug with teflon cap. The ethanol was added to reduce emulsions, which could not be eliminated by centrifugation due to the container size (Perry 1978).

The ethanol remained with the aqueous layer on phase separation and was therefore not added to repetitive extracts of the same sample. Addition of the ethanol did increase the amount of water dissolved in the ether phase, compared with extraction with ether alone. Most of this could be removed by passage through anhydrous sodium sulfate (Matheson, Coleman, Bell, Inc., Norwood, Ohio).

Extraction efficiency by this technique was of the order of 78%, with three consecutive extractions removing 98% of the radioactivity present.

During the remainder of the separation phase, several techniques were tried to achieve further sample cleanup. These techniques included gel permeation chromatography and derivatization with liquid-liquid extraction in hexane/concentrated sulfuric acid.

Gel Permeation Chromatography

Gel permeation chromatography (GPC) separations were performed on several different glass columns, the dimensions of which will be described later. In all cases, however, the column packing was Bio-Beads SX-2 (200/400 mesh) (Bio-Rad Laboratories, Richmond, Ca.).

The solvent delivery system consisted of a Chromatronix CPM-2 metering pump with variable flow rates of 2.4, 6, 12, 24, 60, and 120 ml/hr. Sample injection was accomplished by both on-column syringe injection, and via an in-line Valco (Alltech Assoc., Arlington Heights, Illinois) six port stainless steel injector. Tubing and fittings were Teflon® (Dupont Inc., Wilmington, Delaware).

UV detector traces were recorded from a 254 nm Varian differential photometer UV detector. Bar graphs of sample radioactivity were constructed from samples of column eluant counted in a Beckman 100C Liquid Scintillation Counter (Beckman Instr. Co., Fullerton, Calif.), using external standard ratio quench correction. The cocktail used was Instagel® (Packard Inst. Co., Downers Grove, Ill.), a general purpose cocktail for both organic and aqueous samples.

Stahling et al. (1972) utilized Bio-Beads SX-2 (200/400) and cyclohexane to effectively separate PCB and pesticide residues from fish lipid. This same system was tried as a method to separate

hydroxy metabolites of 2,4,5-HCB, from not only lipid, but from each other.

A second solvent system, utilizing ethyl acetate and toluene (3:1) was also used to achieve this separation. This system is similar to that of Johnson et al. (1976) who used Bio-Beads SX-3 (3% crosslinkage) and this mixture to effect GPC cleanup of plant and animal extracts for pesticide residue determination. Since both components of this system have great UV absorbtivity at 254 nm, only radioactivity was measured. In order to characterize separations by this system, the total permeation volume of the column had to be arrived at indirectly, by calculation, rather than being measured directly as the R_V of benzene.

Investigations involving the ethyl acetate/toluene system were performed exclusively on a 1.5 x 50 cm jacketed glass column (Altex Scientific Inc., Berkeley, CA.) with in-line sample injection.

Measured from the outside of the column, the gel bed measured 42 cm in height, which would make the column bed volume equal to approximately 74 ml. Subtracting the volume of the gel matrix (as calculated from the density of the dry gel and the swelling value in ethyl acetate/toluene), one gets a total permeation volume of approximately 55 ml (neglecting instrumentation dead volume effects). This value was used in the analysis of chromatograms obtained from this solvent system.

The third GPC system investigated, utilized 100% methylene dichloride. This system was one of several tested by Kuehl and

Leonard (1978) as a means to efficiently separate lipids from low molecular weight hydrocarbons in tissue samples. Their investigations utilized two, 2.5 x 25 cm glass columns connected in series filled with Bio-Beads SX-2 (100/200). Of the combinations tested, which included: 100% CH_2Cl_2, 10% CH_2Cl_2/90% C_6H_{12}, 33% CH_2Cl_2/67% C_6H_{12}, 50% CH_2Cl_2/ 50% C_6H_{12}, and 75% CH_2Cl_2/25% C_6H_{12}, the 50/50 mixture of methylene dichloride and cyclohexane gave the best balance of high recovery and compound separation. For simple separation of lipid from other organics, however, 100% methylene chloride gave excellent recovery with low retention volumes.

Derivatization and Hexane/Concentrated Sulfuric Acid Cleanup

The first investigation undertaken, involving derivatization and hexane/H_2SO_4 cleanup, was to examine the effect of acid cleanup on the actual metabolites themselves before they were rendered more inert by derivatization.

Two 250 ml samples from the same liter of acid-ether extract of fecal homogenate were evaporated to dryness in a rotary evaporator. One sample was set aside to be derivatized with diazomethane, and the other was taken up in 10 ml of hexane and reacted, by gentle inversion (x30), with concentrated sulfuric acid. The sample was centrifuged and the acid layer frozen in dry ice-acetone. Following this procedure, the previously dark green, opaque, hexane layer, became clear and virtually colorless. The hexane layer was decanted and concentrated under a stream of nitrogen for thin layer chromatography.

The second of the two matched samples was methylated by reaction with ethereal ethanolic-diazomethane. The diazomethane was produced by ethanolic-potassium hydroxide treatment of N-methyl-N-nitroso-p-toluenesulfonamide (Diazald® - Aldrich Chem. Co., Milwaukee, Wisconsin) in ether, at 60°C, as per manufacturer's label instructions for ethereal alcoholic solutions of diazomethane.

The ether solution of the derivatized extract was evaporated to dryness and redissolved in 10 ml of hexane. The hexane solution was then carried through the concentrated sulfuric acid cleanup described above, and analyzed by thin layer chromatography.

Actual sample preparation for GC-MS involved acid-ether extraction of approximately 3 kg of fecal homogenate, by the method described earlier. The sample consisted of daily collections from the three days immediately following the administration of the radioactive tracer dose. Each 300 ml aliquot was extracted twice, the individual extracts pooled, and then derivatized as above.

Thin Layer Chromatography

Thin layer chromatographic separations were accomplished through the use of both reverse and normal-phase modes.

Reverse-phase thin layer chromatography (RPTLC) was performed on Whatman KC-18 (Whatman, Inc., Clifton, New Jersey) 5x20 cm, precoated TLC plates, using methanol, acetonitrile, and water (56:32:12%). This stationary and mobile phase constitute TLC System #1.

Normal-phase thin layer chromatography (TLC) was carried out on silica gel 60 F-254, 5x20 cm, precoated TLC plates (E. Merck,

Darmstadt, Germany), using xylene and glacial acetic acid (96:4%). This stationary and mobile phase constitute TLC System #2.

In all use of RPTLC and TLC, sample application (spotting) was made at 1.5 cm from the bottom edge of the plate and the sample was allowed to migrate to a height of 15 cm above this point.

Characterization

Synthesized methoxy derivitives of four possible metabolites of 2,4,5-HCB; 3-methoxy-2,2',4,4',5,5'-hexachlorobiphenyl, 4-methoxy-2,2',3,4',5,5'-hexachlorobiphenyl, 3-methoxy-2,2',4',5,5'-pentachlorobiphenyl, and 4-methoxy-2,2',4',5,5'-pentachlorobiphenyl, were provided through the courtesy of Dr. H. B. Mathews, Chief, National Institute of Environmental Health Sciences, Research Triangle Park, N.C.

Mass spectra of the methoxychlorobiphenyl standards and the derivitized metabolites were recorded from a Hewlett-Packard Model 5930 A Mass Spectrometer (Hewlett-Packard, Avondale, Penn.) by electron ionization at 70 electron volts. The mass spectrometer was joined to a Hewlett-Packard 5700 Gas Chromatograph which was fitted with a 6 ft. stainless steel column of 9% OV-101. The helium carrier gas flow was 30 ml/min. and the oven was programmed at 2 minutes at 200°C and then the temperature was increased by 32°/min. to 240°C.

RESULTS

Gel Permeation Chromatography

Figure 1 shows a composite chromatogram composed of the UV traces of GPC chromatograms of biphenyl and four substituted biphenyls. The compounds were chromatographed on a 1.5x 30cm glass column (Pharmacia Fine Chemicals, Uppsala, Sweden) with direct on-column sample injection. The elution solvent, cyclohexane, was delivered at 2 ml/min.

The retention volumes of 2,3,6-HCB and 2,4,5-HCB are graphic examples of the ability of GPC to resolve even compounds of the same molecular weight, based on differences in their effective diameters. Equally graphic is the separation achieved between biphenyl and its 2-hydroxy analogue. Though different by only 17 A.M.U. in molecular weight, better separation is achieved between them than between 2,3,6-HCB and the long, rod-like molecule 4,4'-DCB, a difference of 119 A.M.U.

Figures 2A and 2B show the UV and radioactivity chromatogram obtained from columns of 1.5x30 cm and 1.0x63cm (constructed) connected in series, utilizing this same cyclohexane solvent system. The sample chromatographed was an actual acid-ether extract of fecal homogenate containing metabolites of 245-HCB. The sample was injected on-column and solvent was delivered at 2 ml/min. Figure 2A represents the chromatogram produced by the first 240 ml of column eluant which includes fraction #1, the first of two eluant fractions with high

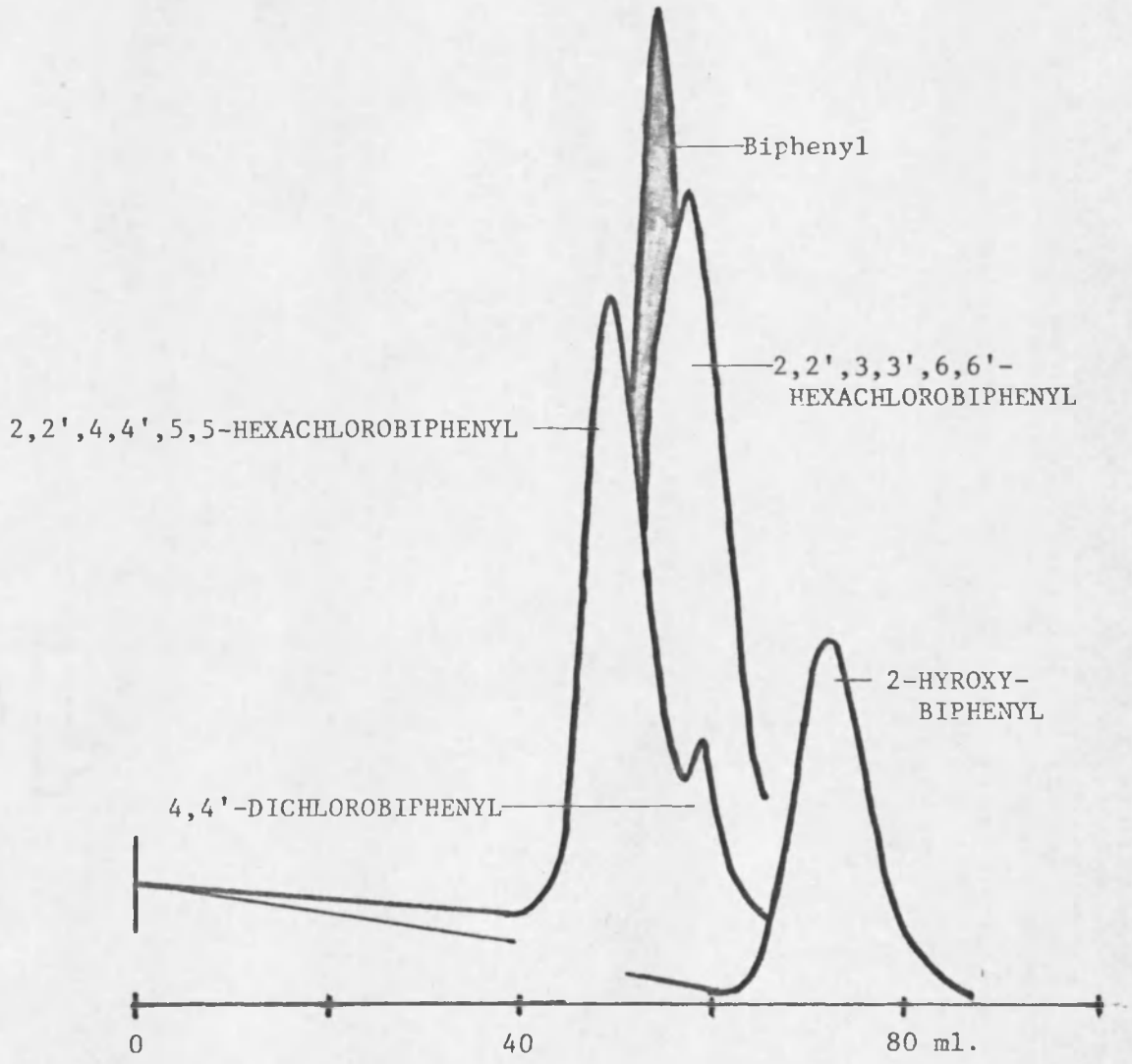

Figure 1. Composite chromatogram of biphenyl and four substituted biphenyls chromatographed in a solvent system of 100% cyclohexane on 1.5 x 30cm and 1.0 x 63cm columns of Bio-Beads SX-2 (200-400) connected in series.

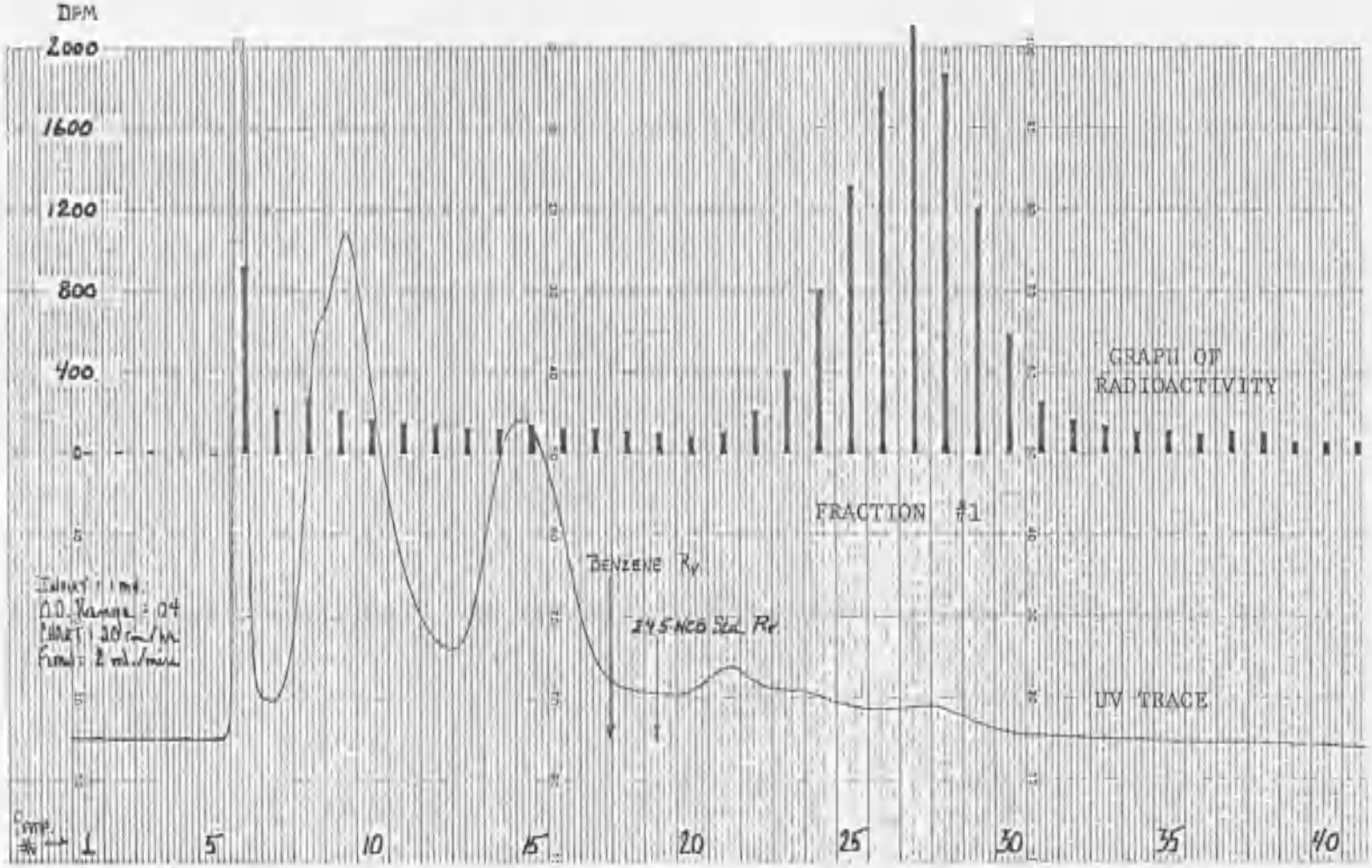

Figure 2. UV and radioactivity chromatogram of 498 ml of column eluant from a sample of an acid-ether extract of fecal homogenate containing metabolites of 2,4,5-HCB. The sample was chromatographed on 1.5 x 30cm and 1.0 x 63cm columns connected in series, in a system of 100% cyclohexane.

A. First 240 ml, Fraction #1.

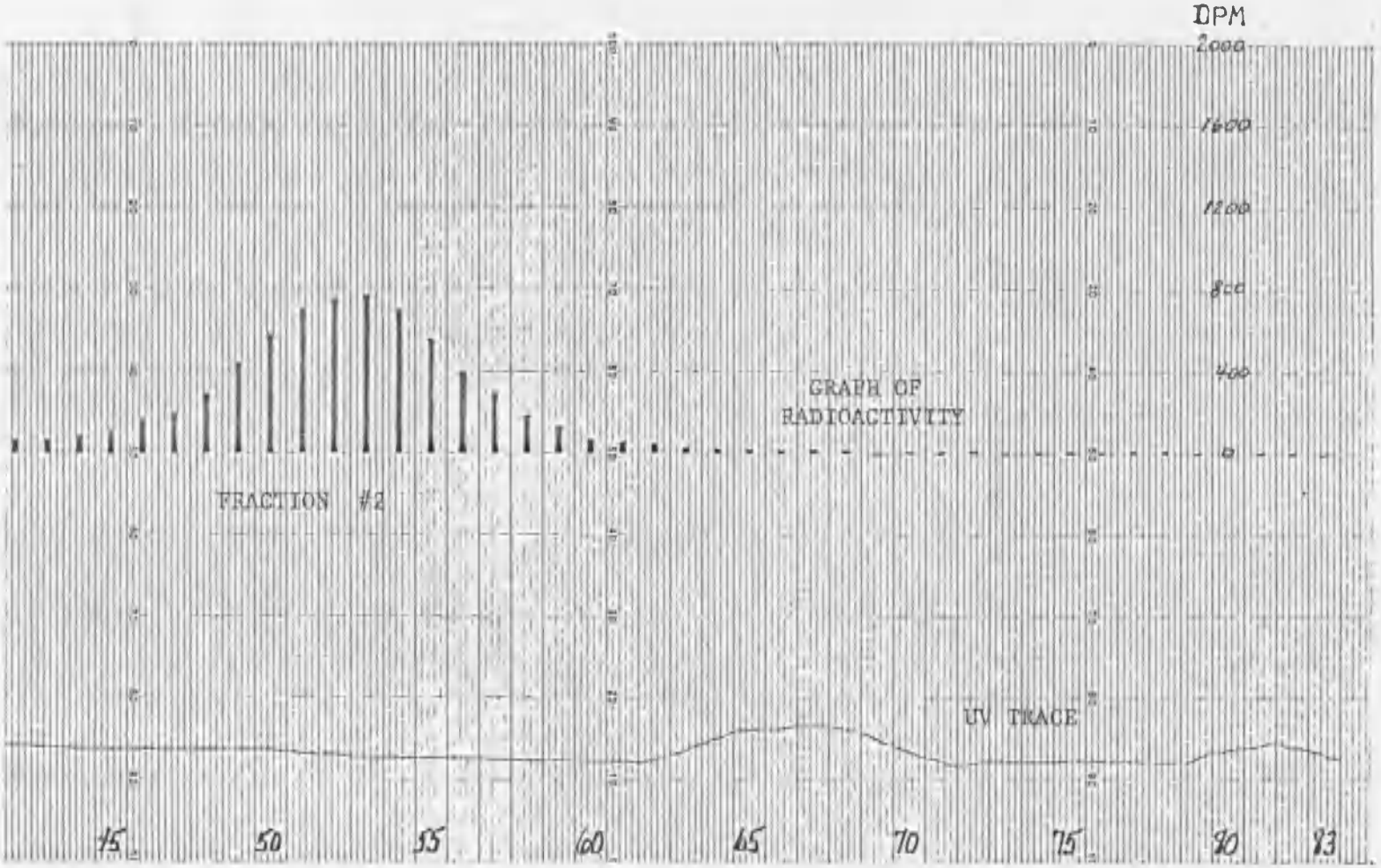

Figure 2. Continued

B. Second 240 ml, Fraction #2.

relative activity. Similarly, Figure 2B shows the second 240 ml of column eluant, including the second eluant fraction with high relative radioactivity, fraction #2.

In Figure 3, reverse-phase thin layer chromatography (TLC System #1) of the two main activity peaks (fractions #1 and #2, Figures 2A and 2B) indicates the presence of four metabolites, in the ratio of approximately 53:32:10:5%.

While the cyclohexane system achieved most of the desired separations (metabolites from lipid and partially from each other) the analysis time of three hours, made it totally unacceptable without automated sample collection.

Figure 4 shows the GPC radiochromatogram obtained from a sample of the crude acid-ether extract chromatographed on a 1.5x50cm column utilizing the ethyl acetate/toluene (3:1) solvent system (see Methods and Materials-GPC).

Solvent delivery was maximal, at 2.5 ml/min. At this flow rate, and a total permeation volume of approximately 55 ml (see Methods and Materials-GPC), all sample components should elute within 55 ml, or approximately 19 chart centimeters, to be classified as having undergone predominantly steric exclusion (GPC). Figure 4 shows that, to a large extent, this is the case with this separation, with only the tailing edge of the major activity peak lying outside this limit. Well resolved individual peaks were not produced by this system. Figure 5 shows TLC separations (System #1) of fractions 19, 20, and 22 from this GPC separation. Column fractions 9, and 14

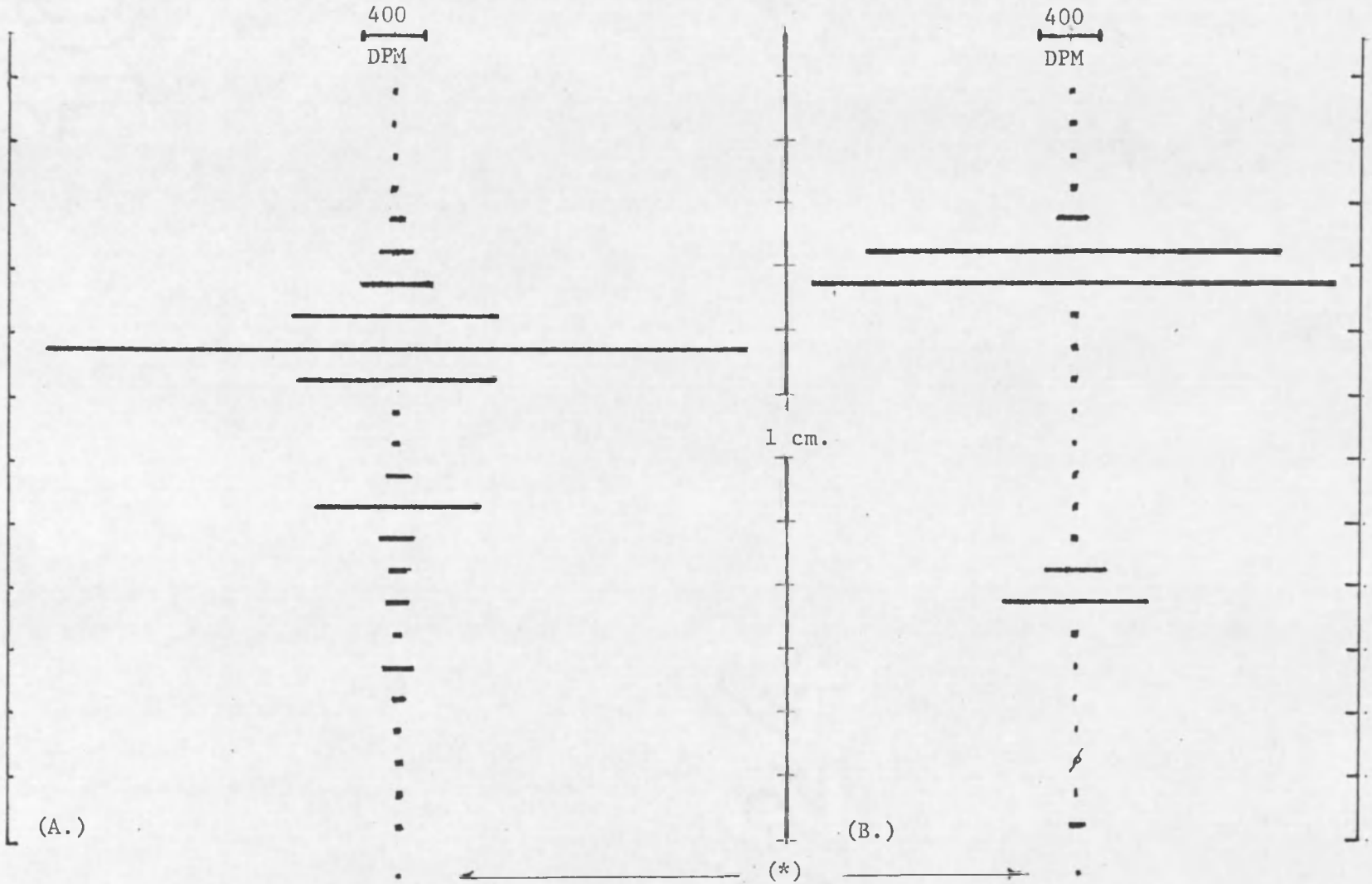

Figure 3. Thin layer radiochromatogram (TLC System #1) of cyclohexane fraction #1 (A.). Thin layer radiochromatogram (TLC System #1) of cyclohexane fraction #2 (B.). (*) Points of sample application.

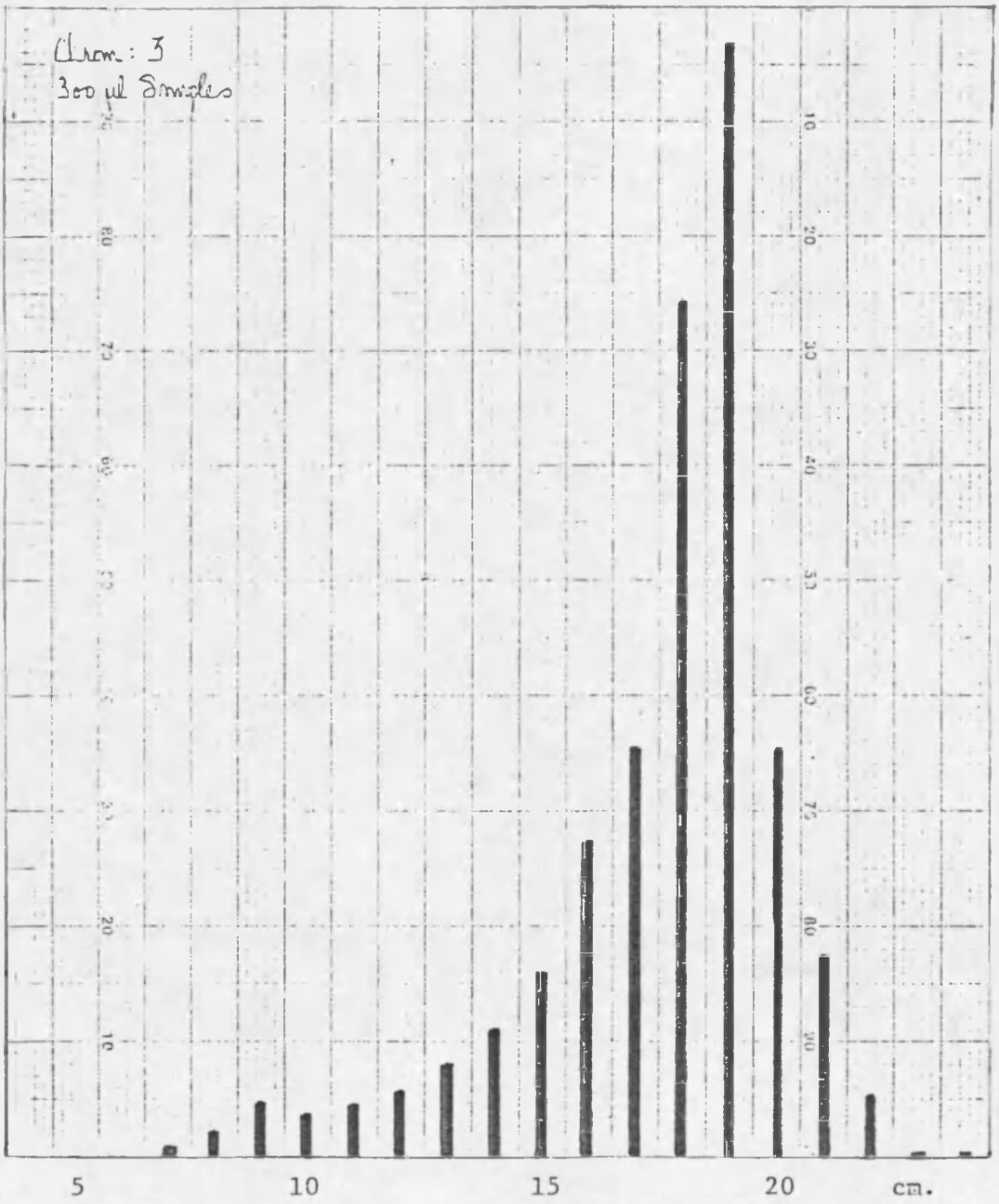

Figure 4. GPC radioactivity chromatogram of the crude acid-ether extract on a 1.5 x 50 cm column of Bio-Beads SX-2 in a solvent system of ethyl acetate/toluene (3:1). (Chart: 40cm/hr.; solvent flow: 2 ml/min).

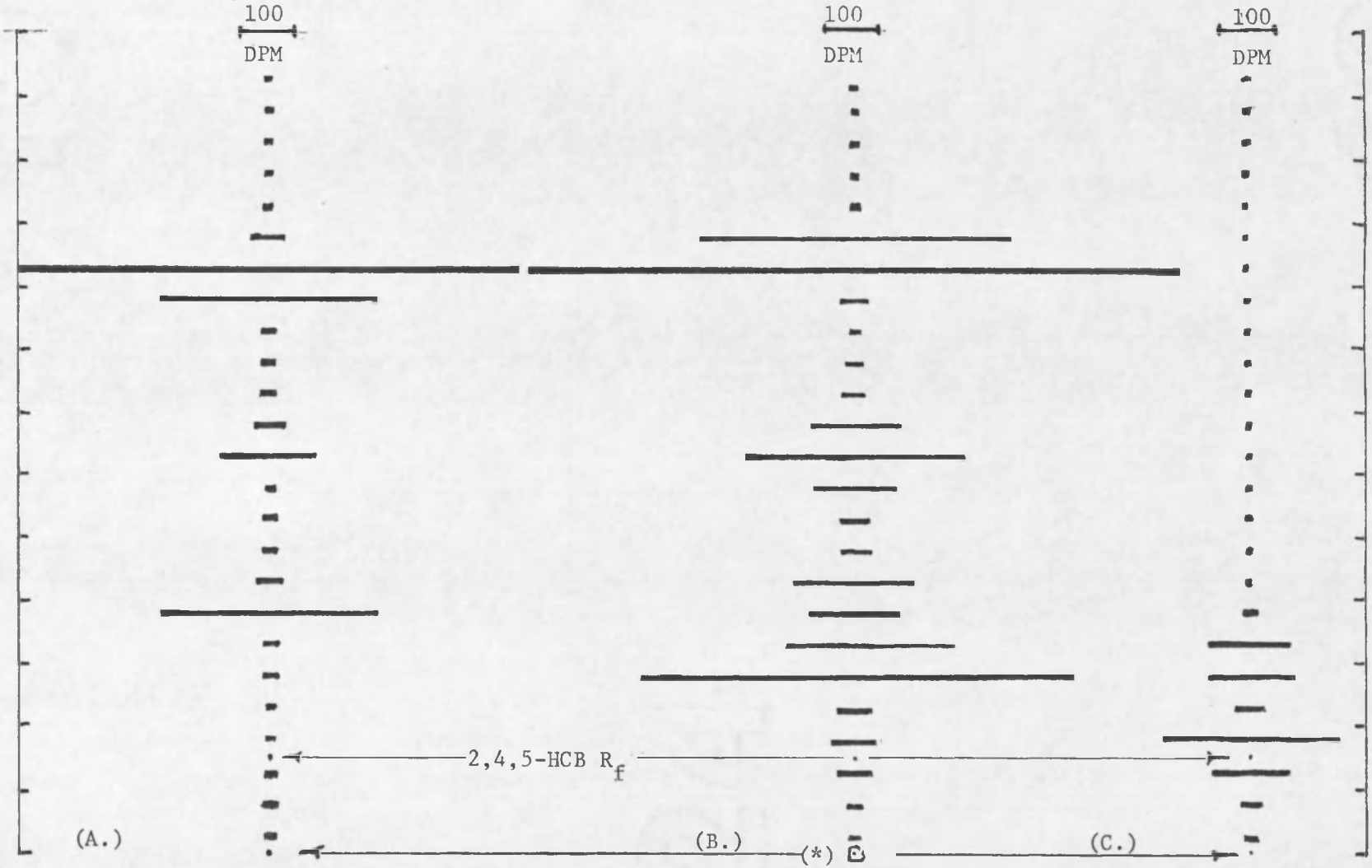

Figure 5. Thin layer radiochromatogrsms (TLC System #1) of fractions 19 (A.), 20 (B.), and 22 (C.) from the column chromatogram in Figure 4 (ethyl acetate/toluene 3:1). (*) Points of sample application.

(Figure 6) from this same GPC separation had insufficient total counts to produce satisfactory spot localization, other than for the most polar compound (highest R_f) which appeared to be in the highest abundance in both fraction 9 and 14.

The solvent system ethyl acetate/toluene (3:1) is both more polar than that of cyclohexane, and much closer to the solubility coefficient of the gel (δ = 9.4 $[cal/cm^3]^{1/2}$). This can easily account for the observed lack of separation. The increased polarity decreases the solute-gel interactions and the higher δ value increases the pore size permitting entrance of a wider range of molecules thereby broadening the separation spectrum (Freeman and Angeles 1974).

In an attempt to improve the separation by this system, elevated temperatures were employed. The effect can be seen in Figures 7,8, 9, and 10. The first increase, to 35°C (Figure 7) from approximately 25°C, caused an increase in the peak height of fraction 18, relative to fraction 19 (i.e., the chromatogram peak maximum shifted to earlier elution). Increasing the temperature another 5°C (Figure 8), caused a dramatic shift in the peak maximum, with fraction 18 now containing the highest activity. Further increases, up to 55°C, produced no additional dramatic changes. These results are consistent with Giddings' (1968) description of the effects of temperature on the GPC separation. He describes the effect as two-fold: (1) a decrease in solvent viscosity, and (2) a relaxation of intermolecular bonds (increased configurational entropy). Both of these phenomena decrease elution volume. Decreased viscosity facilitates mass transfer and increased

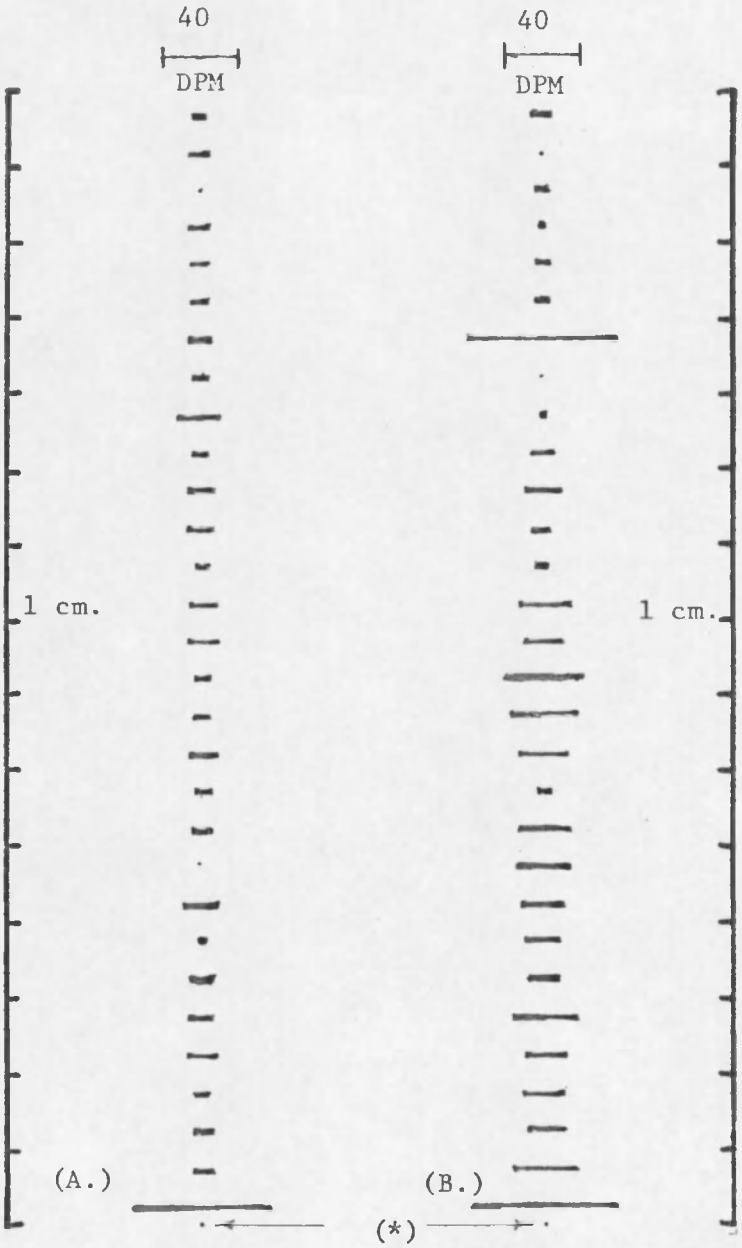

Figure 6. Thin layer radiochromatograms (TLC System #1) of fractions 9 (A.), and 14 (B.), from the column chromatogram in Figure 4. (*) Points of sample application.

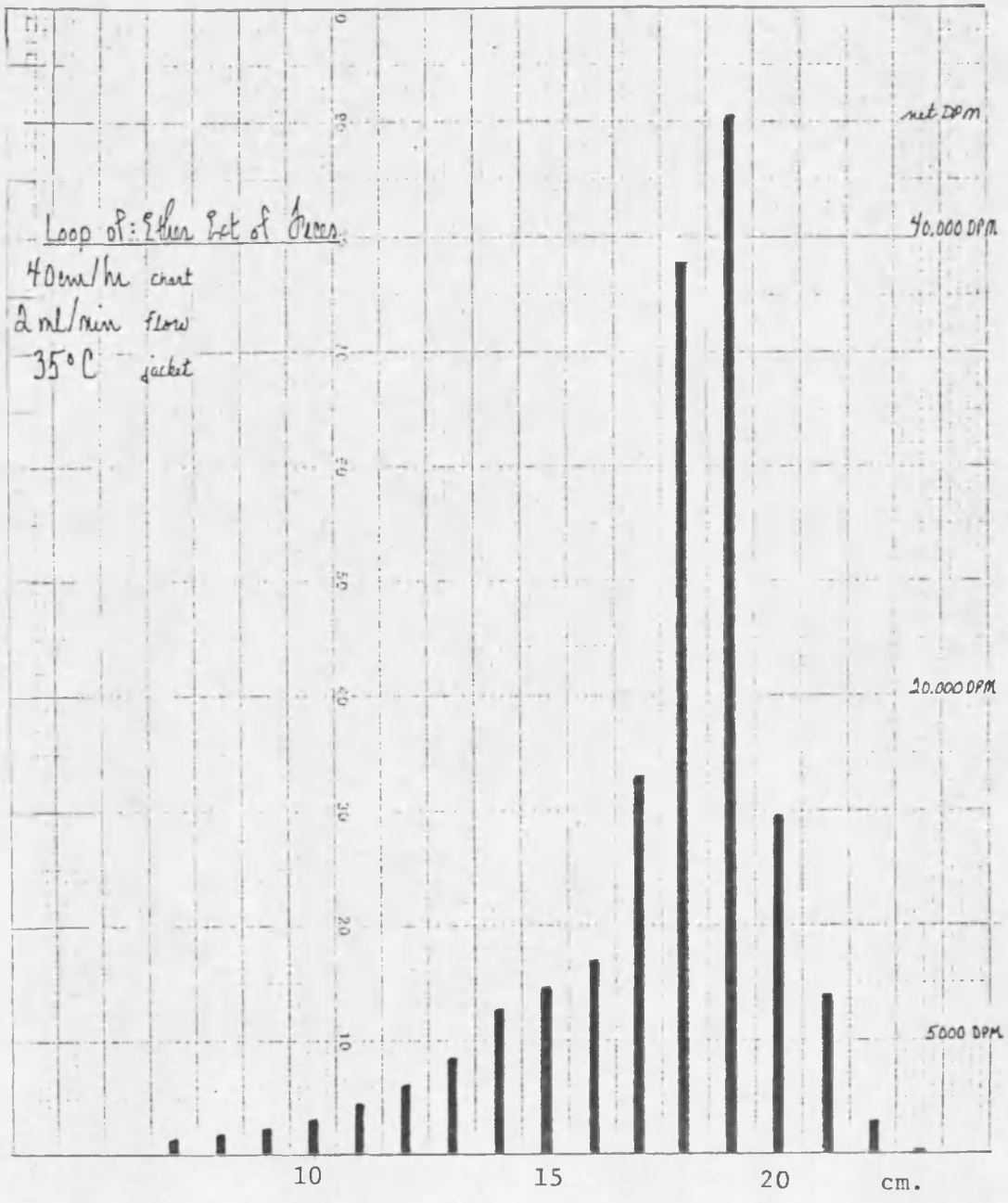

Figure 7. Temperature effects on GPC separations: 35°C. Crude acid-ether extract of fecal homogenate chromatographed on a 1.5 x 50cm column using ethyl acetate/toluene 3:1.

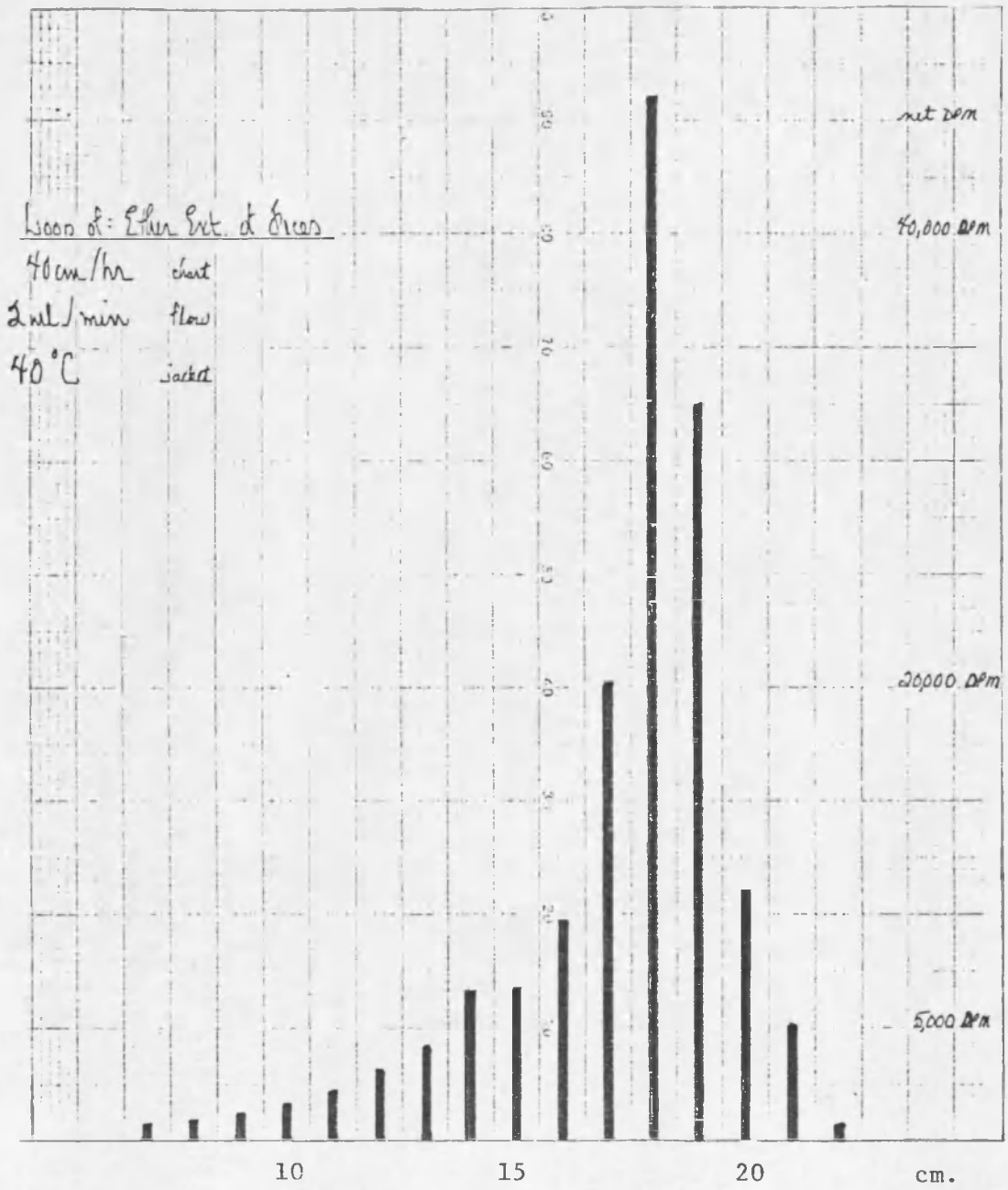

Figure 8. Temperature effects on GPC separations: 40°C. Crude acid-ether extract of fecal homogenate chromatographed on a 1.5 x 50cm column using ethyl acetate/toluene 3:1.

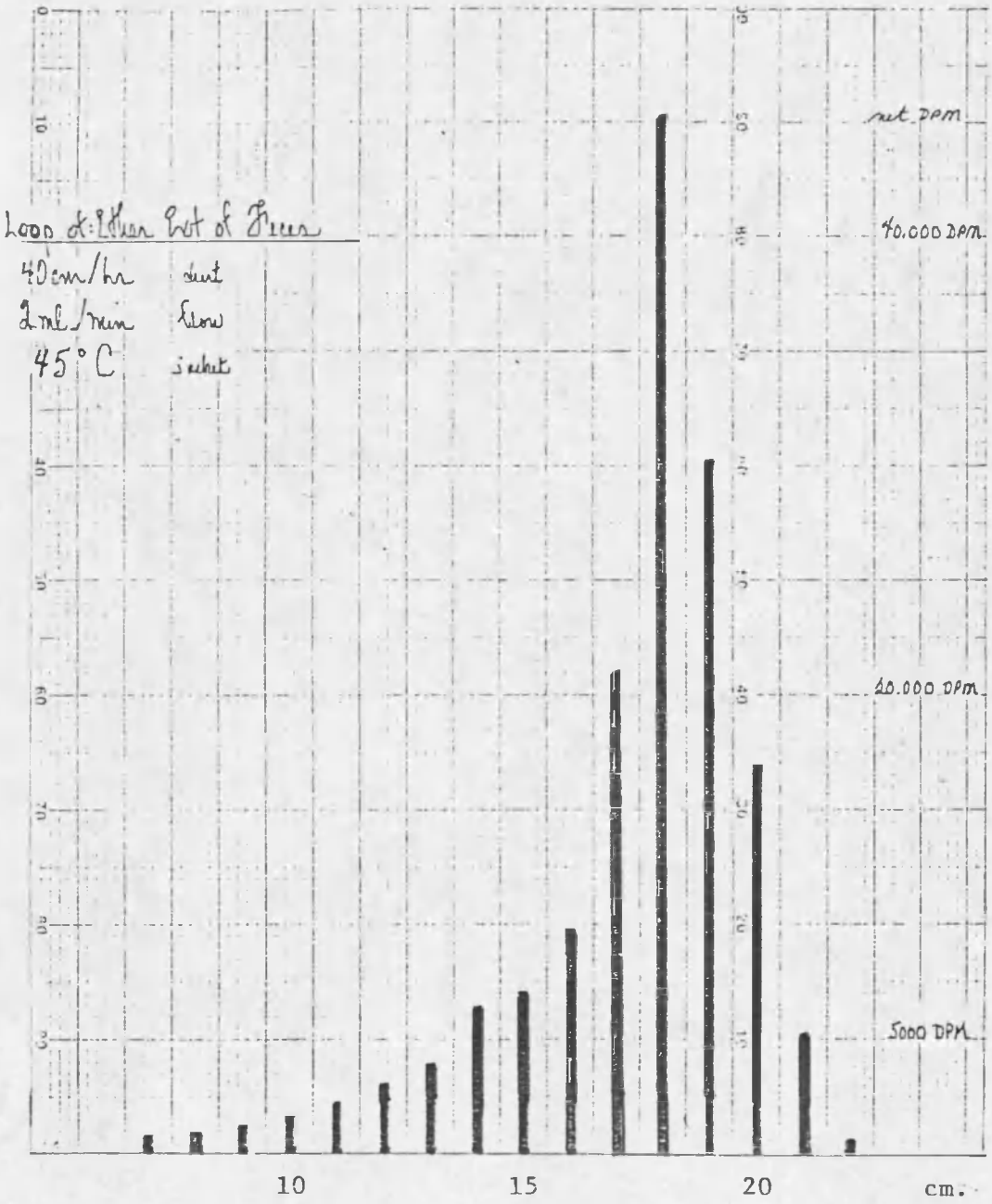

Figure 9. Temperature effects on GPC separations: 45°C. Crude acid-ether extract of fecal homogenate chromatographed on a 1.5 x 50 cm column using ethyl acetate/toluene 3:1.

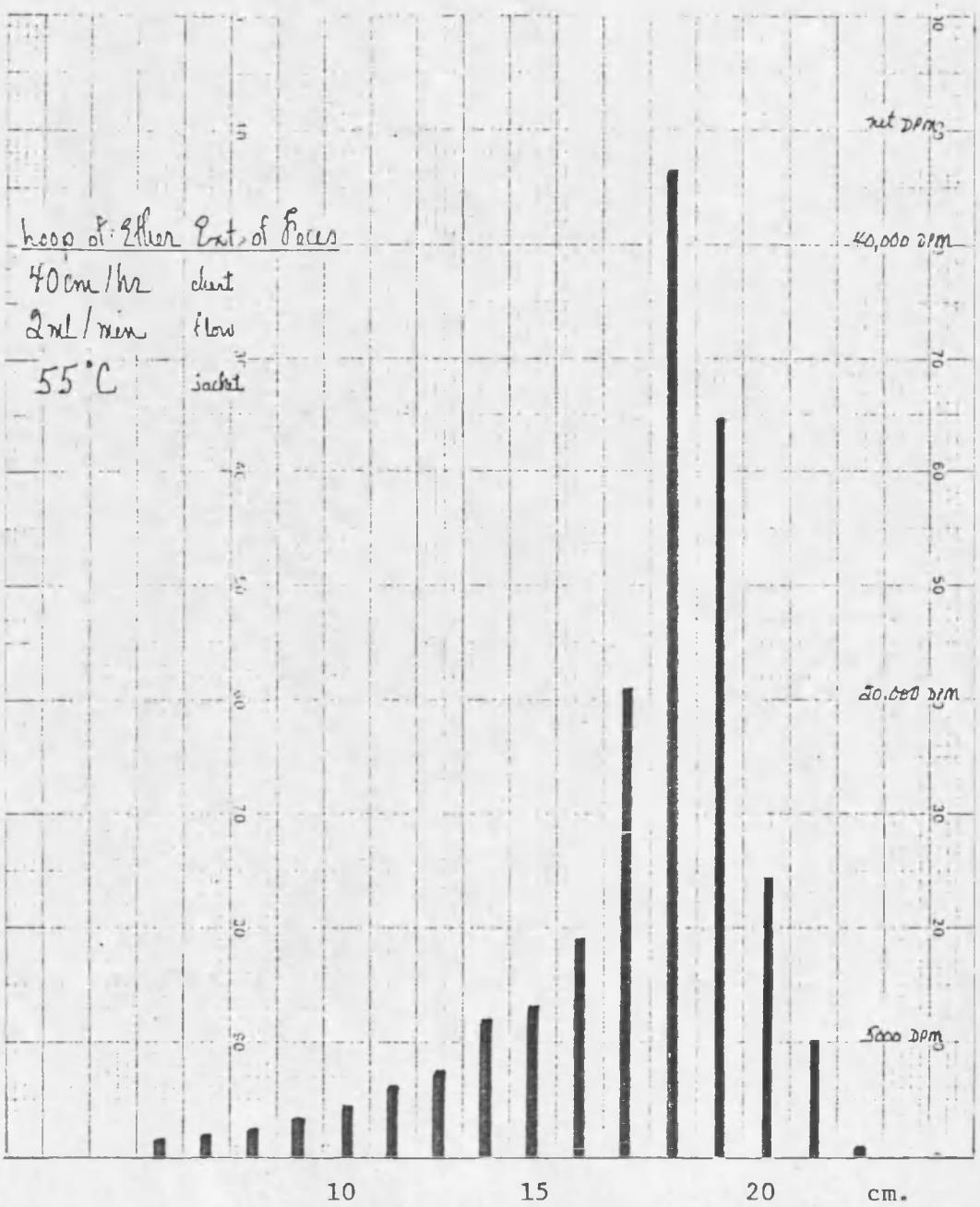

Figure 10. Temperature effects on GPC separations: 55°C. Crude acid-ether extract of fecal homogenate chromatographed on a 1.5 x 50 cm column using ethyl acetate/toluene 3:1.

configurational entropy produces larger effective diameters in large molecules, resulting in greater exclusion and earlier elution (Little et al. 1970).

The third GPC system tested, 100% methylene dichloride, produced the UV and radioactivity chromatogram shown in Figure 11. The sample chromatographed was the most polar fraction isolated from the cyclohexane system (fraction #2). The chromatogram was produced on a 1.0x63 cm column with on-column sample injection. Figures 12 and 13 are UV and activity chromatograms of a pooled sample of cyclohexane fractions #1 and #2 (Figure 12) and a sample of the crude acid-ether extract of fecal homogenate (Figure 13). Both chromatograms were produced on a 4mm x 104cm column with on-column injection, and solvent delivery of 24 ml/hr.

A generalized characterization of these separations would suggest that the primary mode of separation is steric exclusion. The fact that significant activity elutes after benzene, does, however, indicate some column-solute adsorptive interaction.

Figure 14 shows a UV and radioactivity chromatogram produced by a sample of the acid-ether extract in the same solvent system (100% methylene dichloride) but on a larger (1.5x50cm) column, with in-line injection. Solvent delivery was 2.4 ml/min. Figure 15 shows a normal-phase TLC radiochromatogram (system #2) of peak maxima fractions from the GPC chromatogram in Figure 14.

TLC shows that there was no significant improvement in separation of individual metabolites over that produced with ethyl acetate/toluene (3:1) (Figure 5, page 27). Methylene dichloride does have the

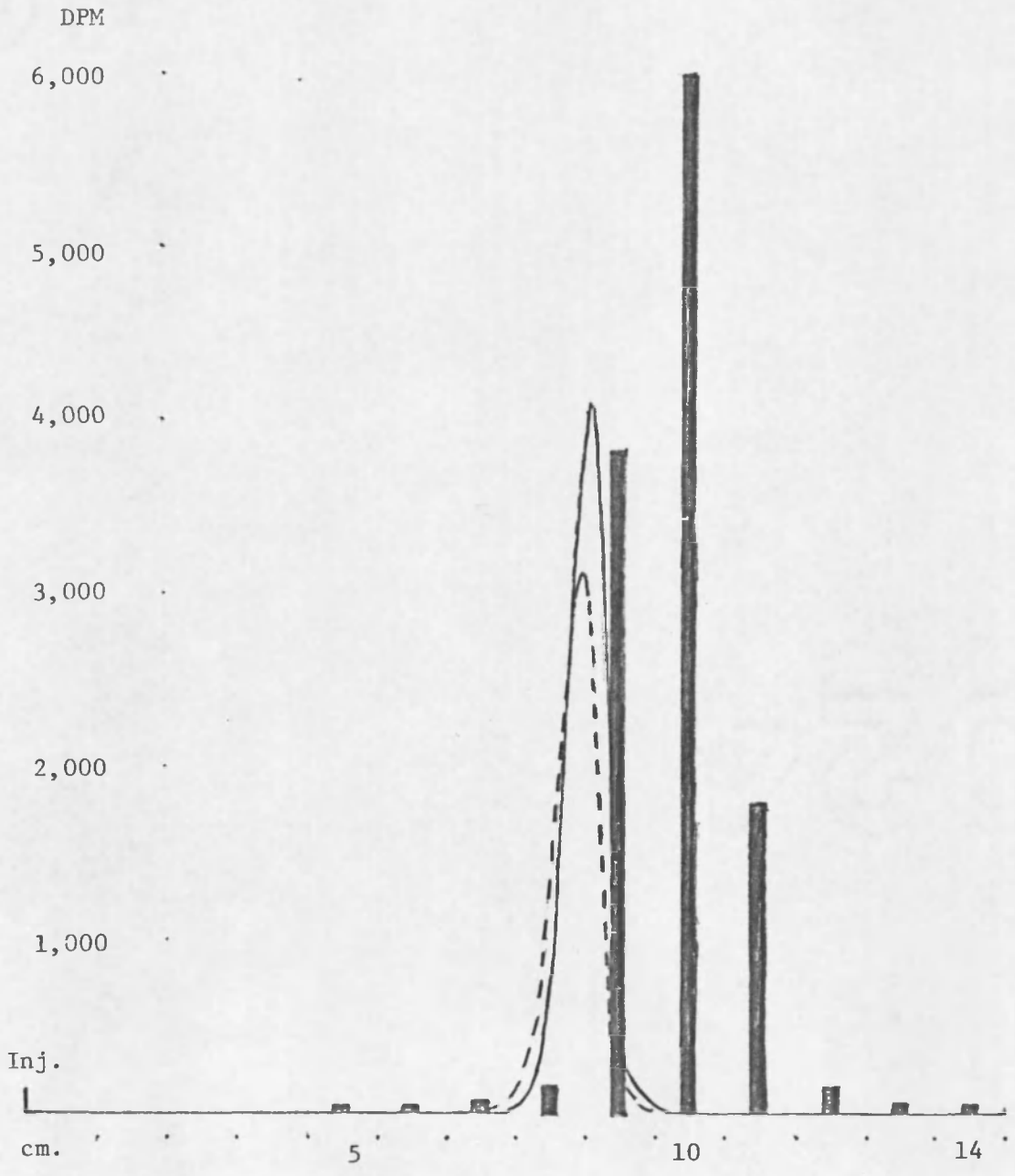

Figure 11. Column radioactivity chromatogram of a sample of a crude acid-ether extract of fecal homogenate chromatographed on a 2mm x 104cm column using 100% methylene dichloride. Peak retention volumes of benzene and 2,4,5-HCB are superimposed.

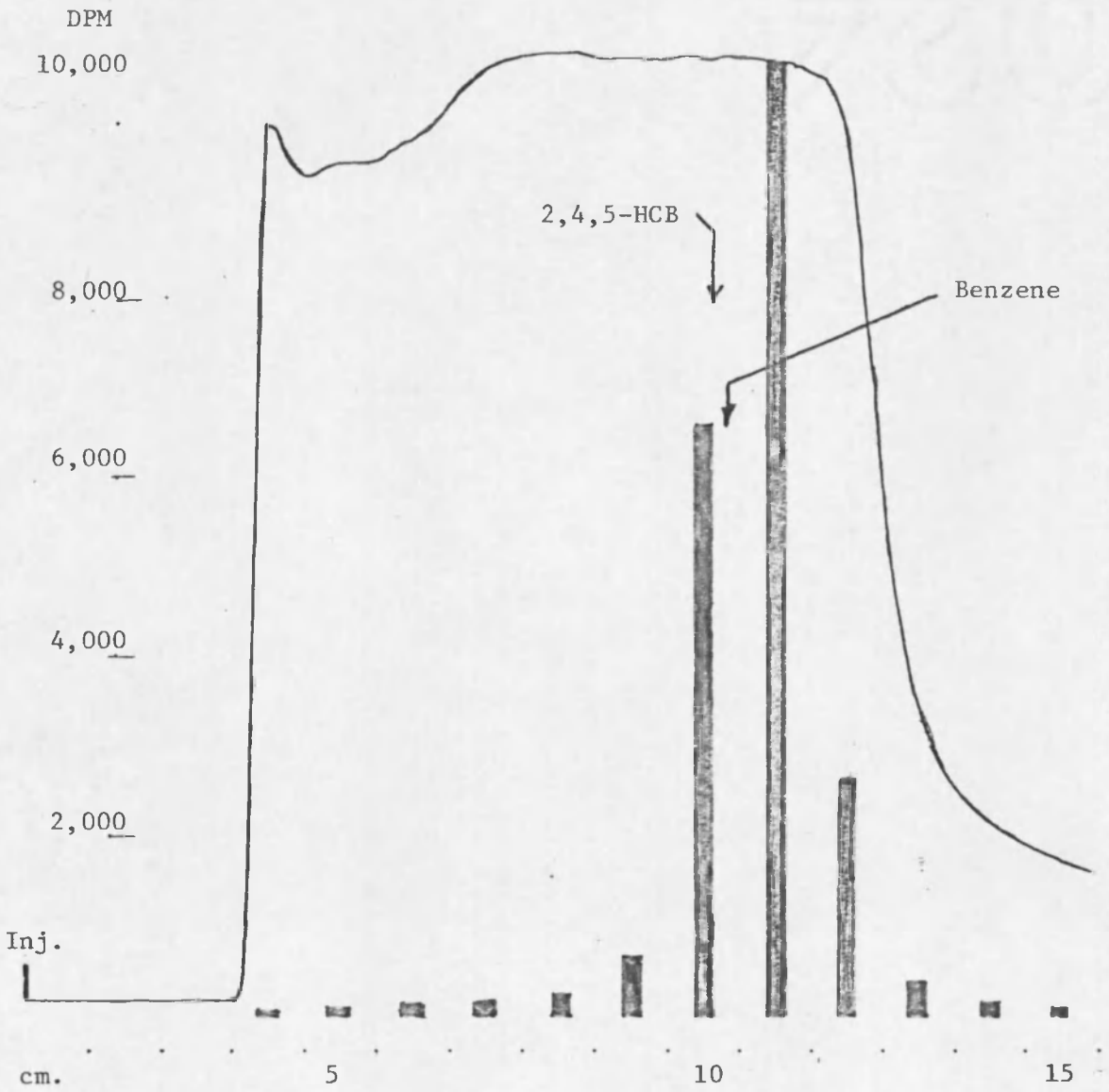

Figure 12. UV and radioactivity chromatogram of a pooled sample of fractions #1 and #2 from the cyclohexane solvent system (Figure 2A and 2B), chromatographed on a 2mm x 104 cm column using 100% methylene dichloride.

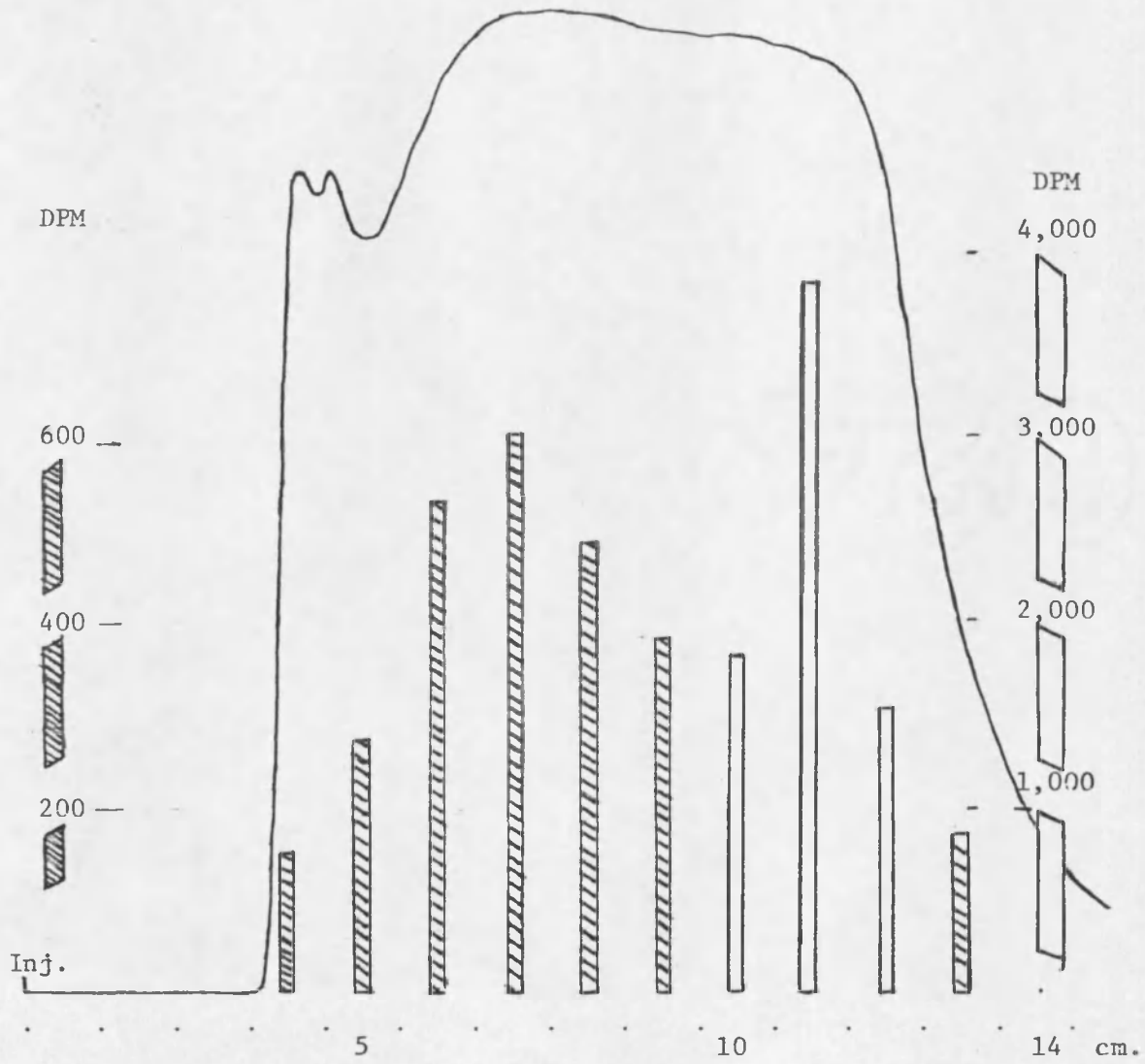

Figure 13. UV and radioactivity chromatogram of a sample of a crude acid-ether extract chromatographed on a 4mm x 104cm column using 100% methylene dichloride.

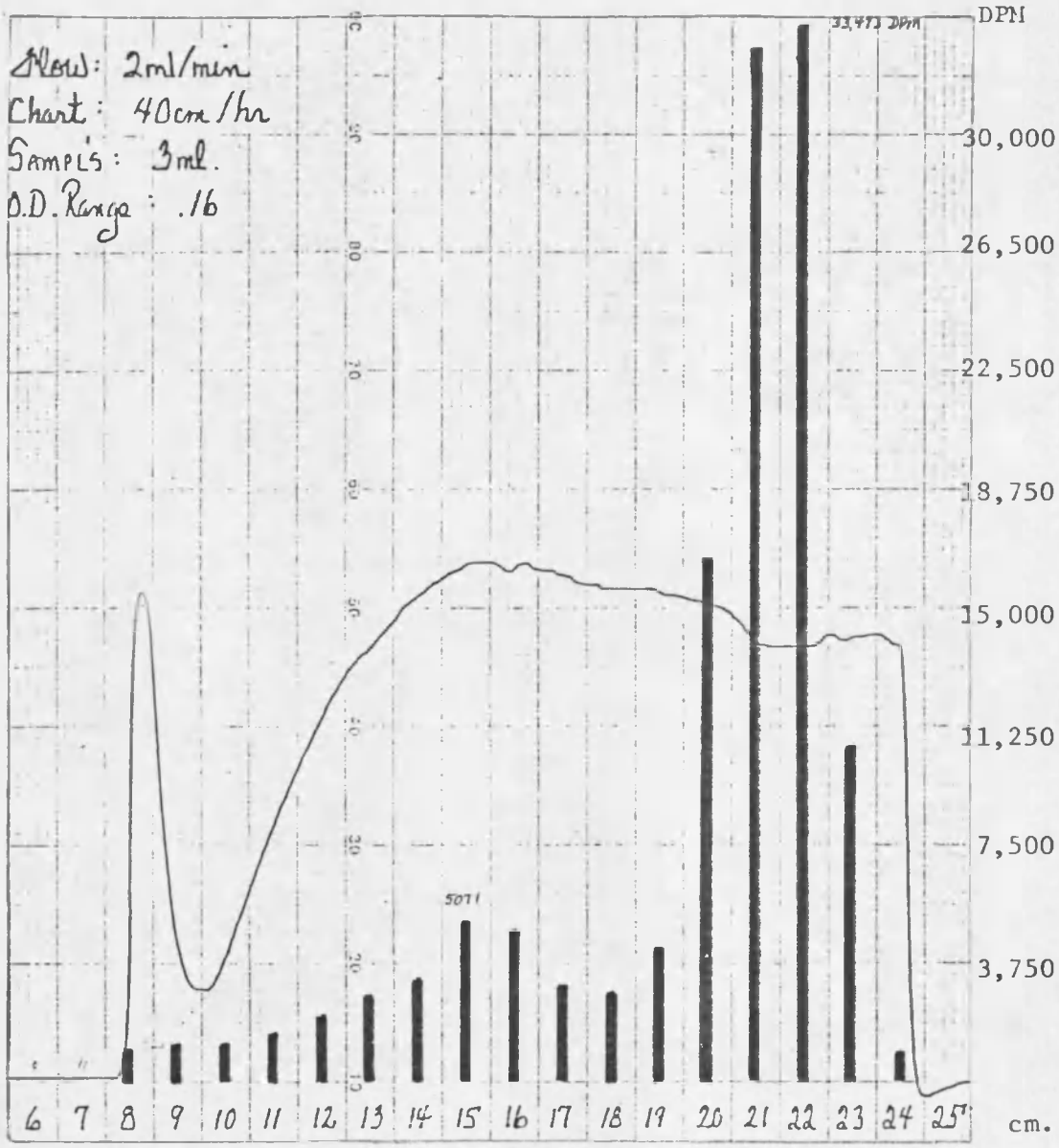

Figure 14. UV and radioactivity chromatogram of a sample of a crude acid-ether extract chromatographed on a 1.5 x 5.0 cm column using 100% methylene dichloride.

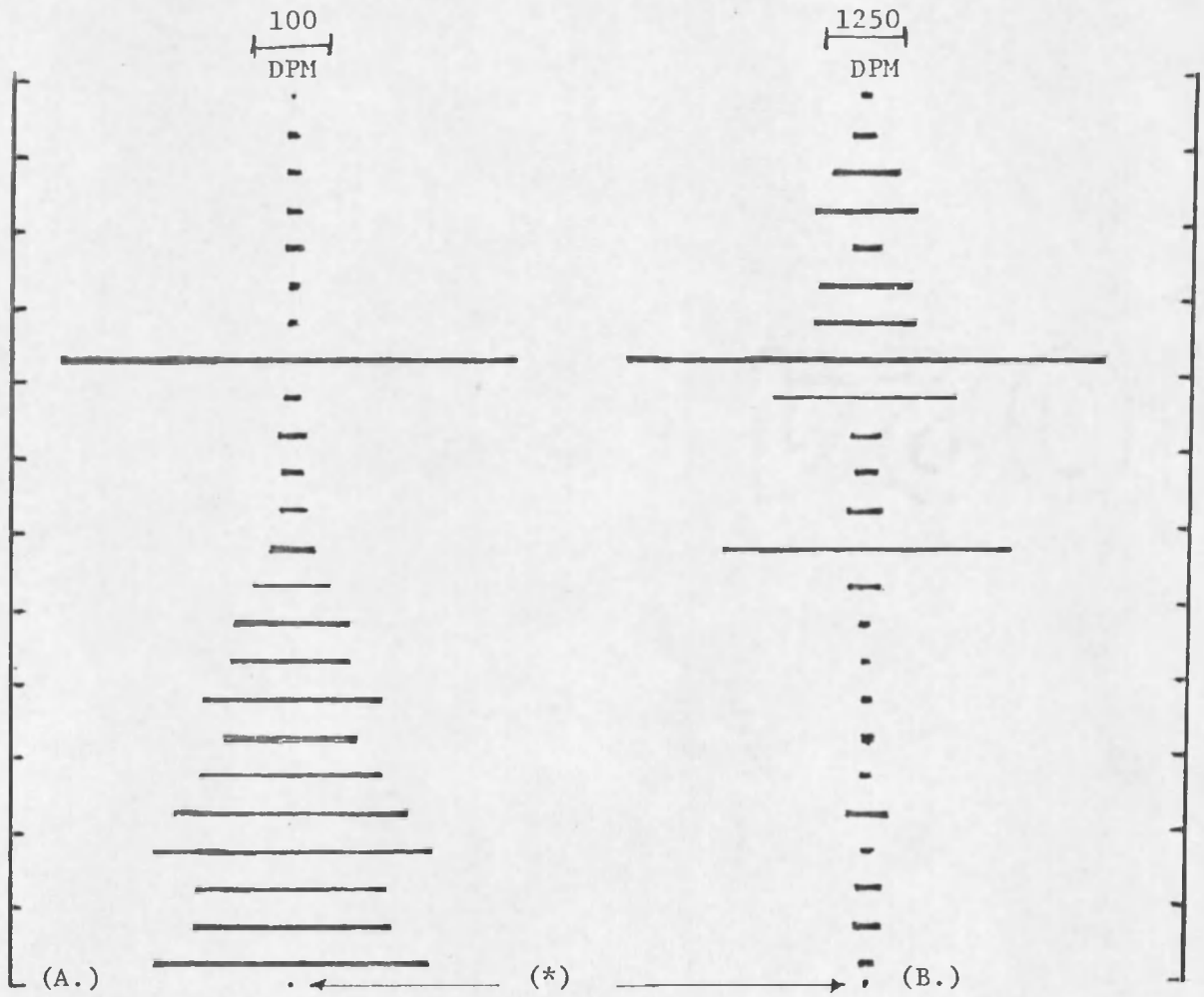

Figure 15. Normal-phase thin layer chromatogram (TLC System #2) of pooled fractions 14 and 15 (A.), and pooled fractions 21 and 22 (B.), from the chromatogram in Figure 14.

advantage of low UV absorbance and high volatility, which permits UV detection and rapid fraction evaporation.

Derivitization, Purification, and Spectrometry

Figure 16 shows an RPTLC (A), and a normal-phase TLC (B) of the underivatized extract after concentrated sulfuric acid purification.

A comparison of the RPTLC (16A) with RPTLCs of fractions 20 and 22 of the ethyl acetate/toluene system (Figure 5, A., B., and C., page 27) shows good correlations with regard to the R_fs of the various compounds. These results were taken to indicate that, though metabolite-acid reaction products may have been produced, no detectable reaction products were retained in the hexane fraction and no metabolite disappeared from the mixture.

The problem of indistinguishable mass spectra from chlorobiphenyls, equally chlorine and hydroxy substituted, is largely solved by the methylation of the crude extract. This procedure serves multiple purposes. It protects the metabolite hydroxyl groups from the possible effects of acid cleanup, renders the hydroxychlorobiphenyl molecule less polar (more hexane soluble), and last, but most importantly, produces a molecule whose mass fragmentation is characteristic of specific substitution positions (Barnes et al. 1967, Jansson and Sundstrom 1974).

Figure 17 shows a normal-phase TLC of the purified, derivitized ether extract. Localization of virtually all activity at an R_F at, or above, the maximum attained by the underivitized sample (Figure 16A), suggests that the sample was quantitatively derivitized,

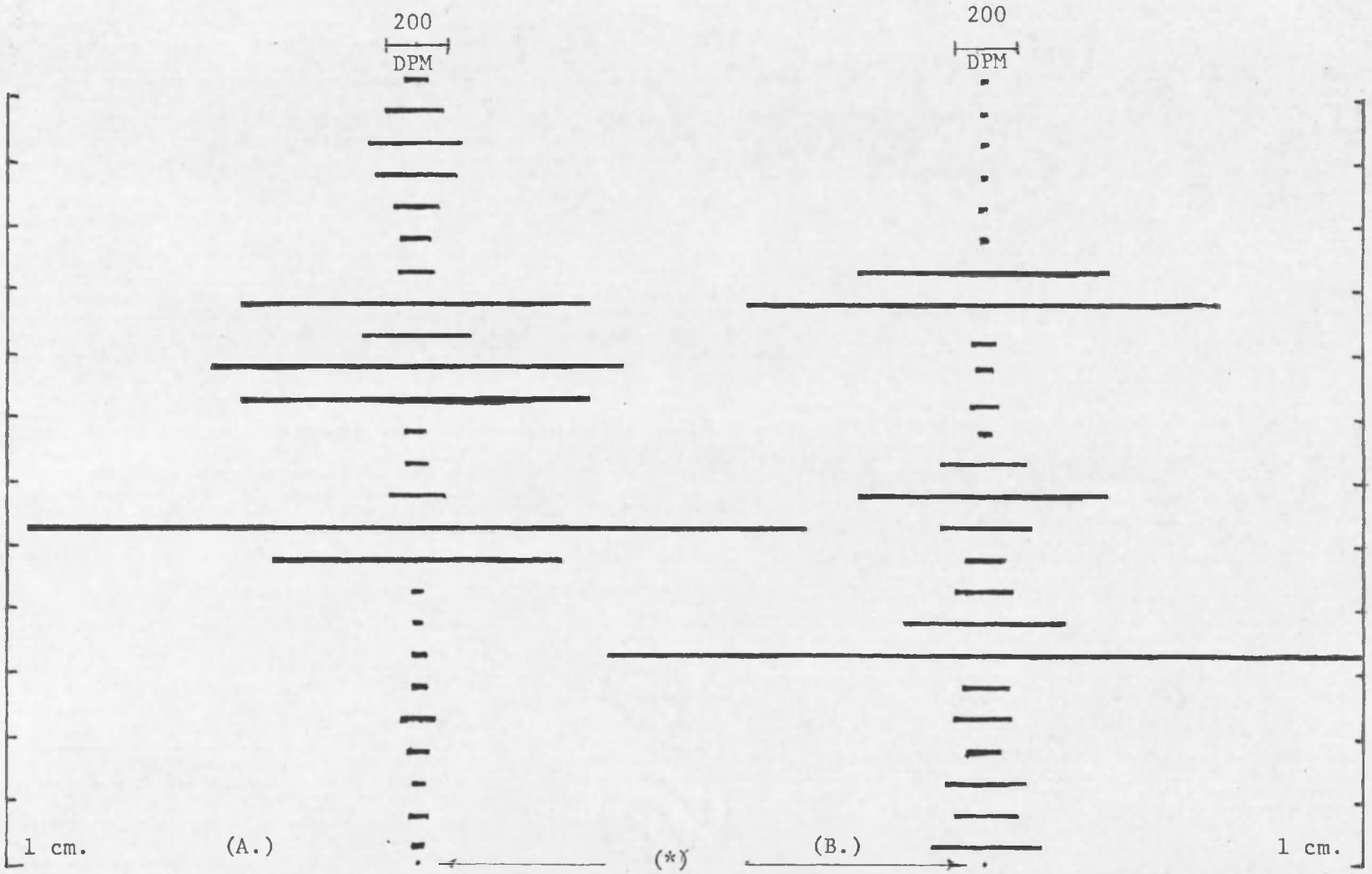

Figure 16. Normal-phase (A.), and reverse-phase (B.) radiochromatograms of an underivatized acid-ether extract of fecal homogenate after concentrated sulfuric acid cleanup. (*) Points of sample application.

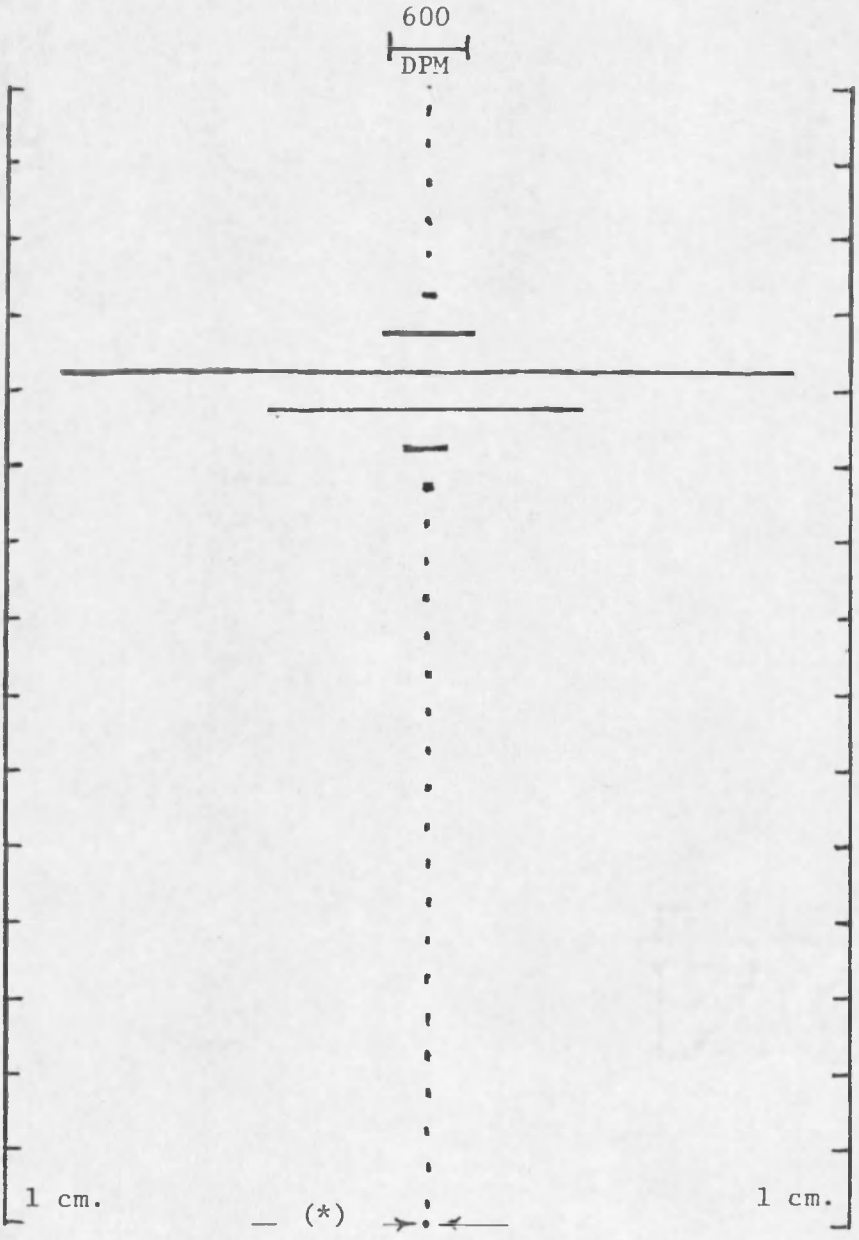

Figure 17. Normal-phase thin layer radiochromatogram (TLC System #2) of a hexane/concentrated sulfuric acid cleanup and diazomethane derivatized sample of the crude acid-ether extract of fecal homogenate (*) Point of sample application.

and that this process has rendered the hydroxyl metabolites significantly less polar.

A comparison of the extraction efficiencies of the two samples indicated recovery (hexane retention) of the derivitized sample to be approximately 20% better than that of the underivitized sample.

Figures 18, 19, 20, and 21 show mass spectra of synthesized penta- and hexachloro- biphenyls. The fragmentation patterns, while similar for each isomeric group, do exhibit certain characteristic relative abundances which are consistent with the relevent literature.

Penta- and hexachloro- compounds can readily be differentiated by the molecular ion (M^+), or more commonly, by the base peak at M^{+2} in the molecular cluster. An exception to this may occur in cases of ortho substitution, in which the base peak may appear to M-50.

The M-50 fragment varies from 84 to 234% of the molecular ion (M^+) abundance (Jensen and Sundstrom 1974b). This fragment is virtually definitive of ortho (2- or 6-) substitution and is believed to result from the formation of a cyclic structure with the loss of CH_3Cl, as depicted in Figure 22.

Jansson and Sundstrom (1974) showed that the loss of CH_3Cl is not dependent on the presence of an ortho chlorine on the adjacent phenyl ring. They demonstrated that even in a 2-methoxy compound with a total absence of chlorines on the opposite ring, the M-50 fragment still forms the base peak, or has very high relative abundance. They associate this with a randomization phenomenon.

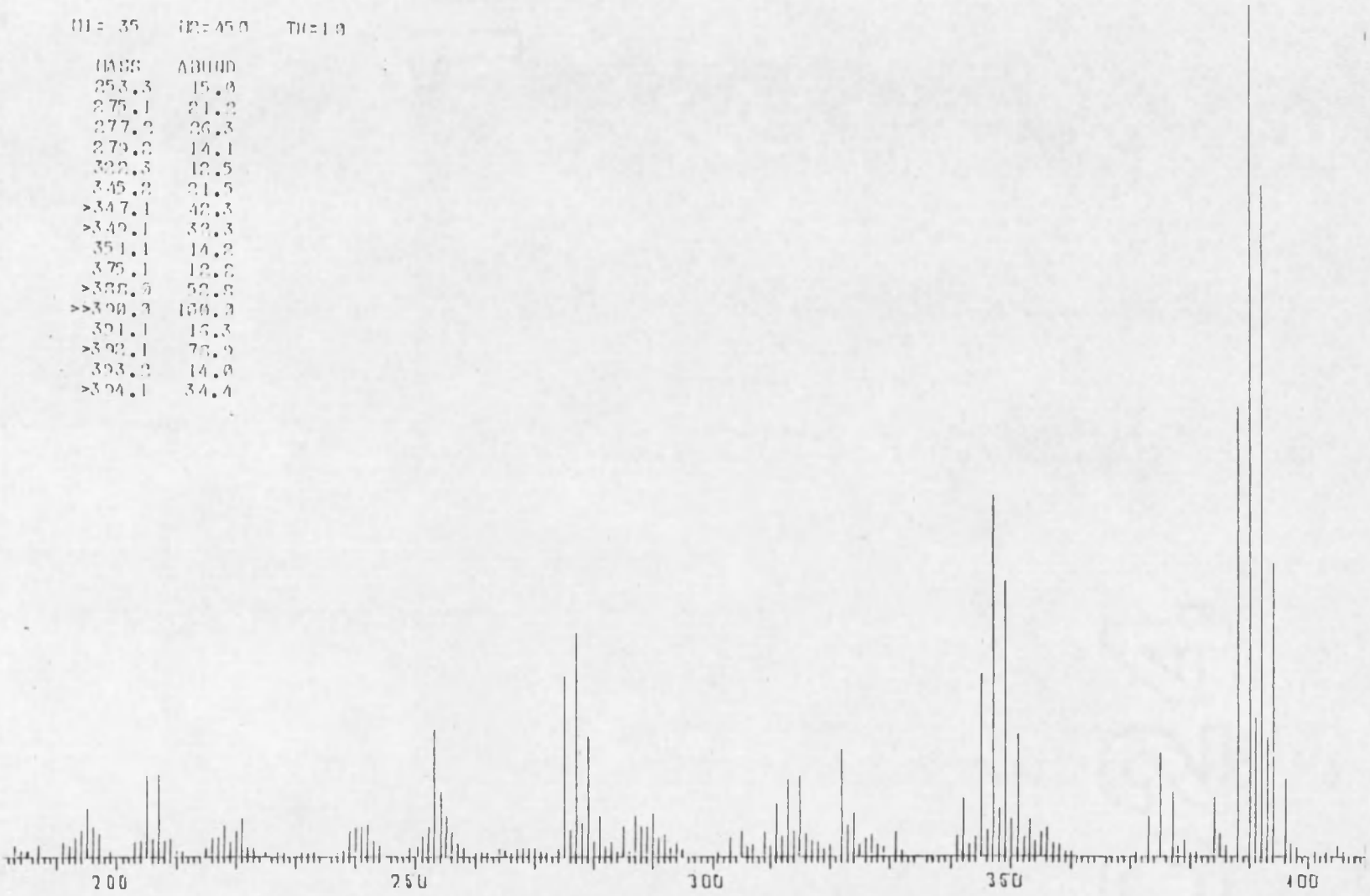

Figure 18. Mass spectrum of 3-methoxy-2,2',4,4',5,5'-hexachlorobiphenyl.

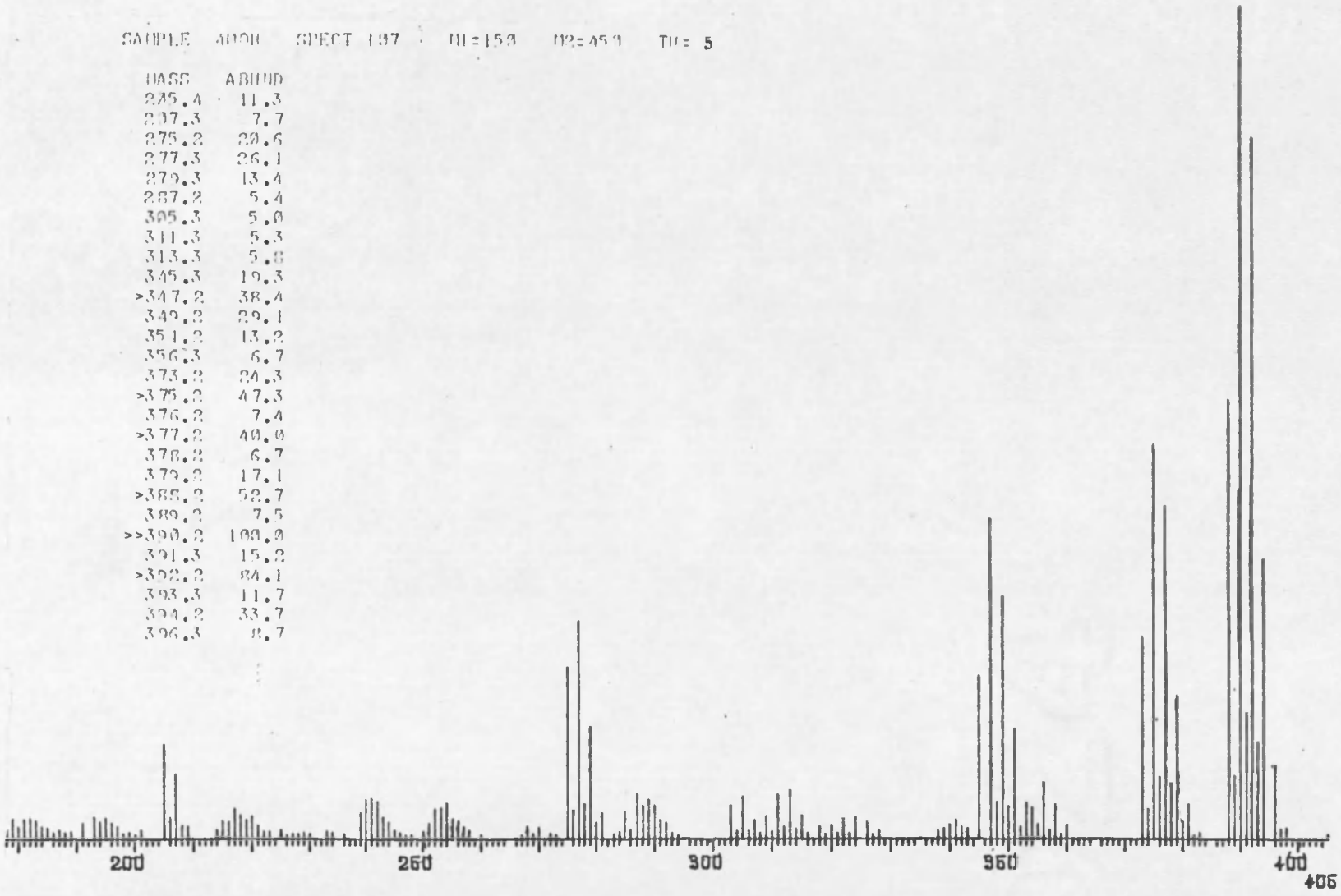

Figure 19. Mass spectrum of 4-methoxy-2,2',3,4',5,5'-hexachlorobiphenyl.

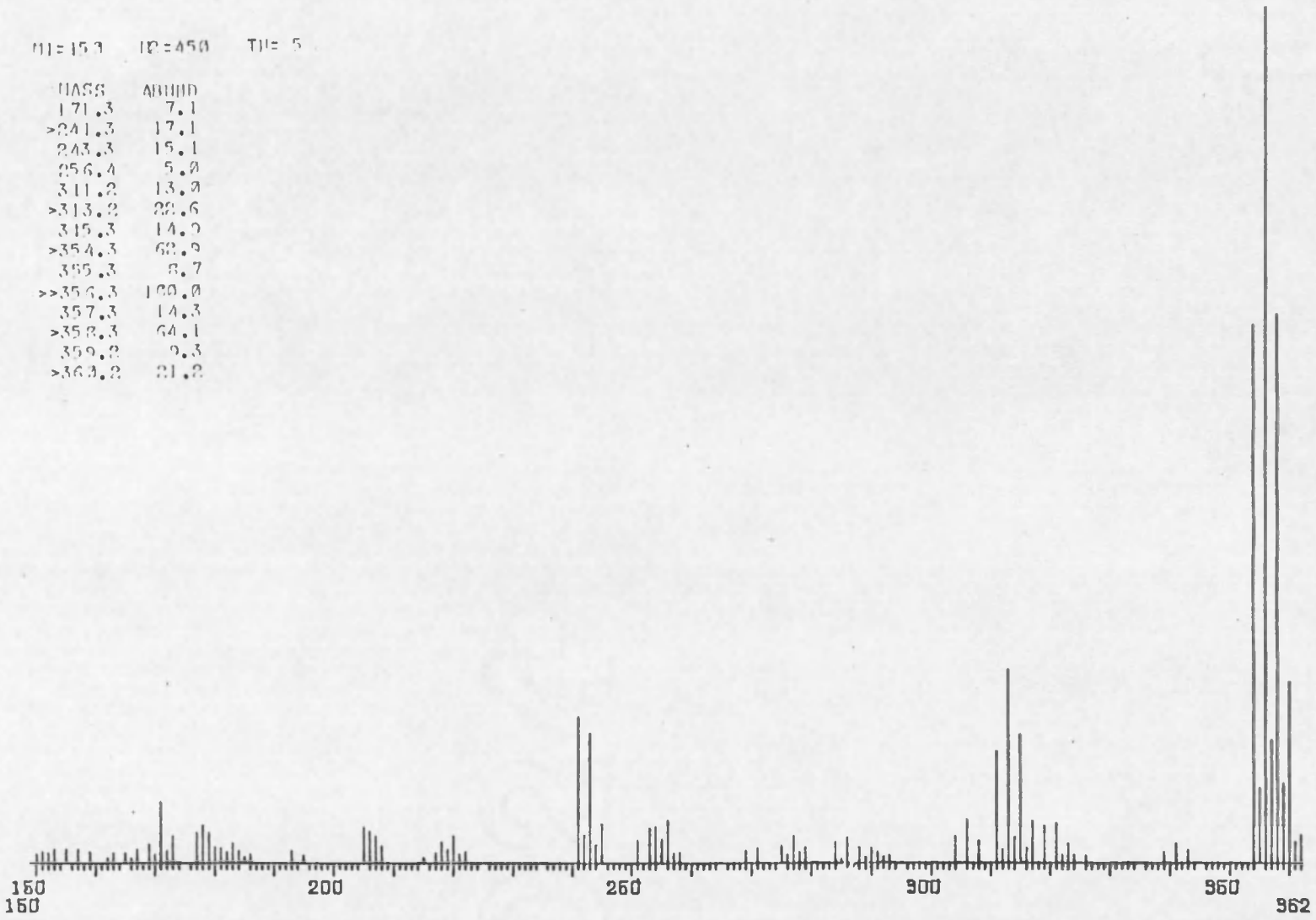

Figure 20. Mass spectrum of 3-methoxy-2,2',4',5,5'-pentachlorobiphenyl.

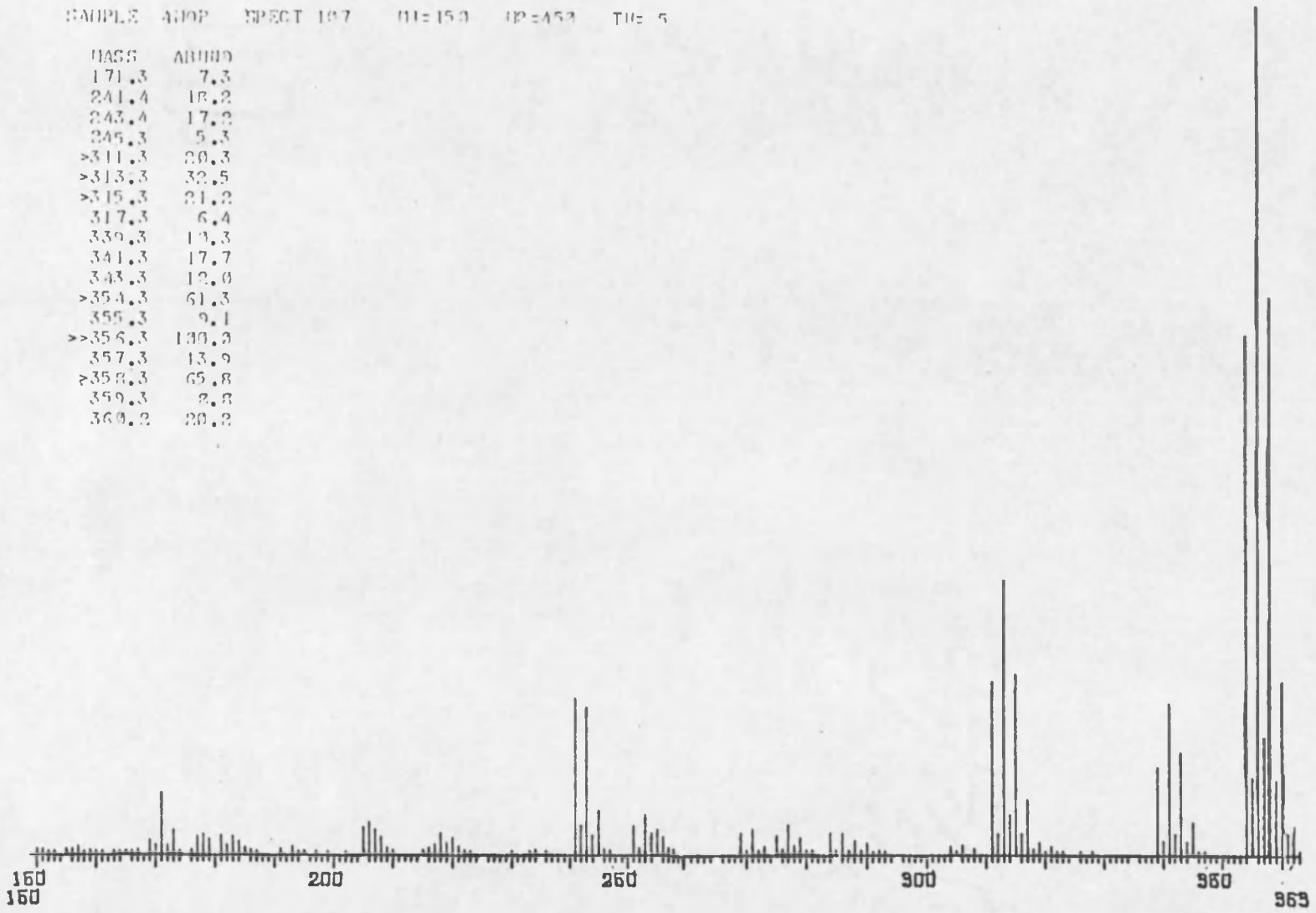

Figure 21. Mass spectrum of 4-methoxy-2,2',4',5,5'-pentachlorobiphenyl.

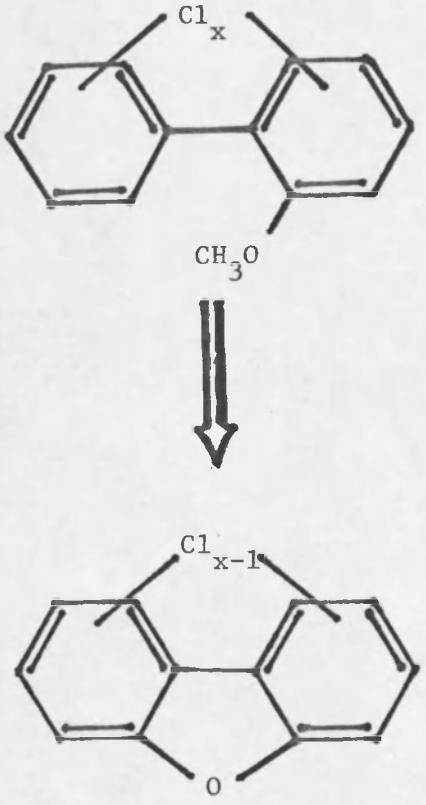

Figure 22. Proposed mechanism for the formation of the M-50 fragment on electron fragmentation of ortho substituted methoxy-chlorobiphenyls. (From Jansson and Sundstrom 1974).

McLafferty (1973) describes random rearrangements as being associated mainly with compounds possessing bonds of equivalent energy which are difficult to cleave. The resulting higher energy and longer reaction time requirements permit isomerization and atom exchange to occur, prior to decomposition. He mentions that the most common examples of this are aliphatic hydrocarbons and perhalocarbons.

An additional characteristic breakdown associated with ortho substitution is the loss of one or two chlorines (M-35 and M-70), respectively, from the molecular ion.

Differentiation of 3- and 4- methoxy derivatives can be made with relative assurance by the relative abundance of the M-15 fragment. This fragment is considerably more abundant in the 4-methoxy hexa- and pentachloro (24.3 and 10.3%), than in the 3-methoxy hexa- and pentachloro compounds (4.5 and 1.1%). This is considered attributable to a redistribution of charge around the phenyl ring to produce a quinoid ion, as shown in Figure 23.

Initial GC-MS of the derivatized extract produced the computer reconstructed chromatogram in Figure 24. A computer search was made for peaks producing mass fragments with m/e ratios of 390 (M+2 from methoxy-hexachloro) and 356 (M+2 from methoxypentachloro biphenyls).

Spectrum 122, Figure 25, contained a 356 m/e fragment, but the fragment did not show the characteristic high absorbance at M+, M+2, M+4, and M+6, seen in penta- and hexachloro biphenyls (Rote and Morris 1973; Figure 26, p. 53).

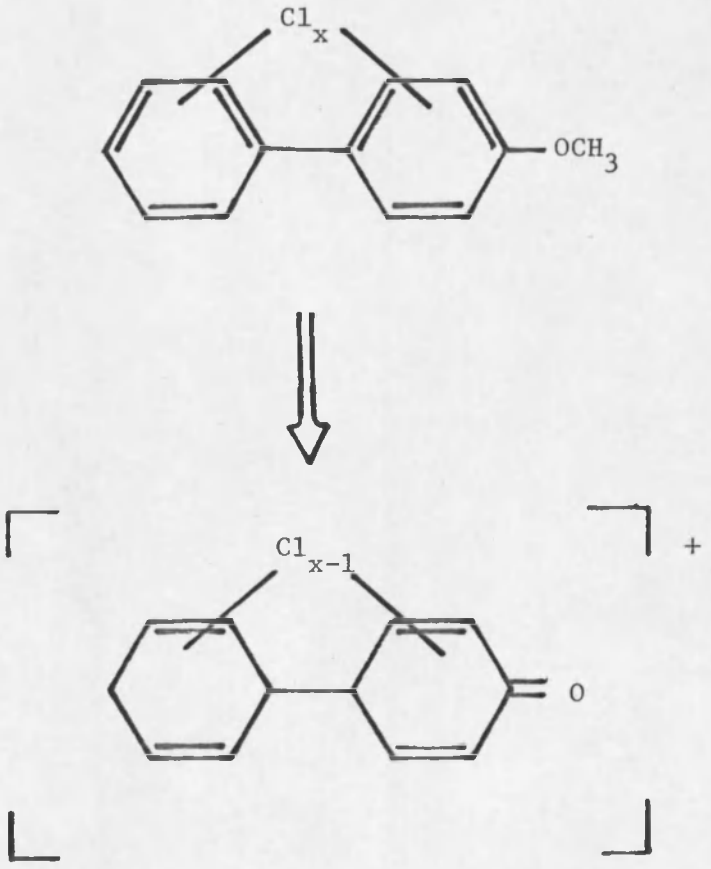

Figure 23. Proposed mechanism for the formation of the M-15 fragment on electron fragmentation of <u>para</u> substituted methoxy-chlorobiphenyls. (From Jansson and Sundstrom 1974).

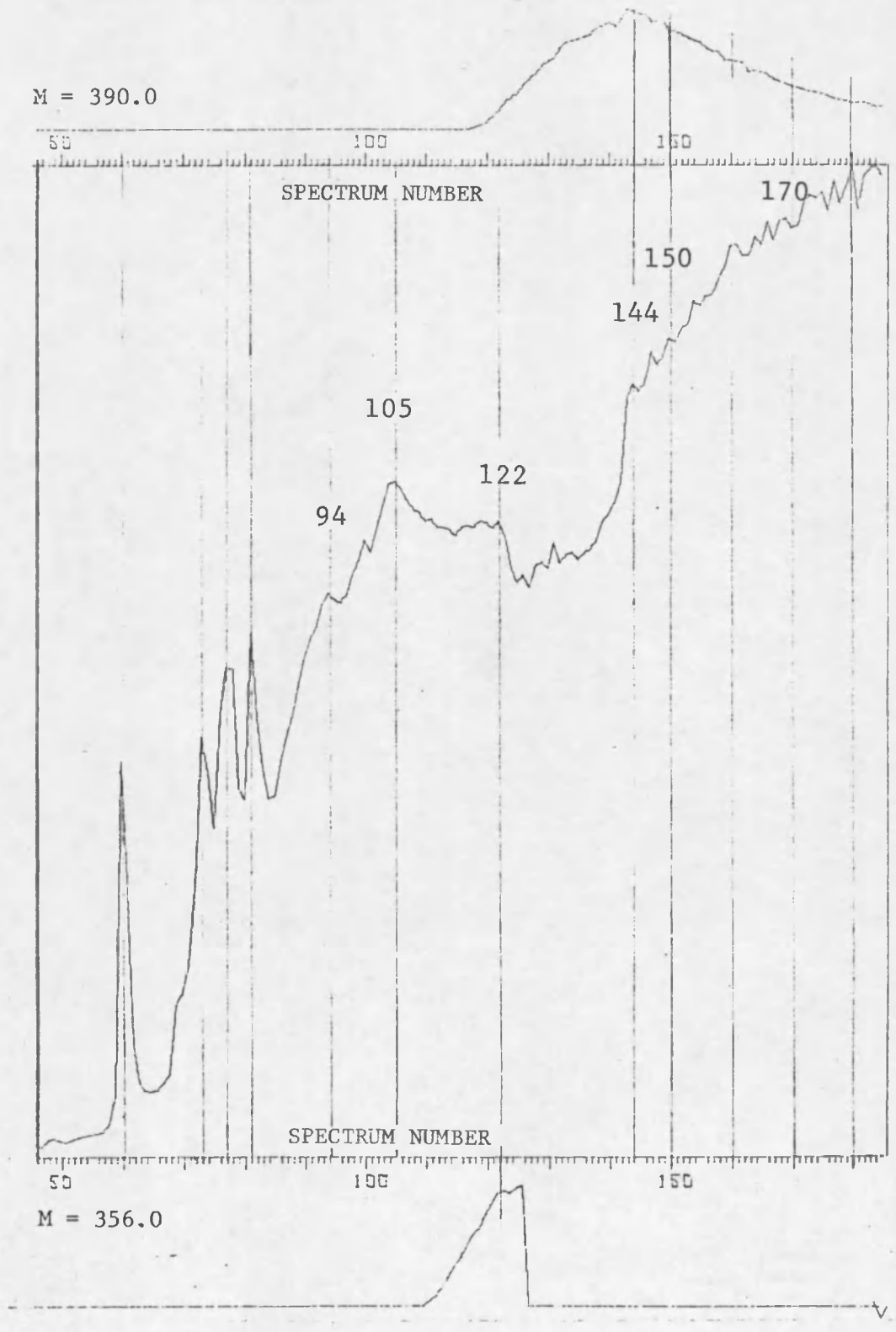

Figure 24. Computer reconstructed GLC chromatogram.

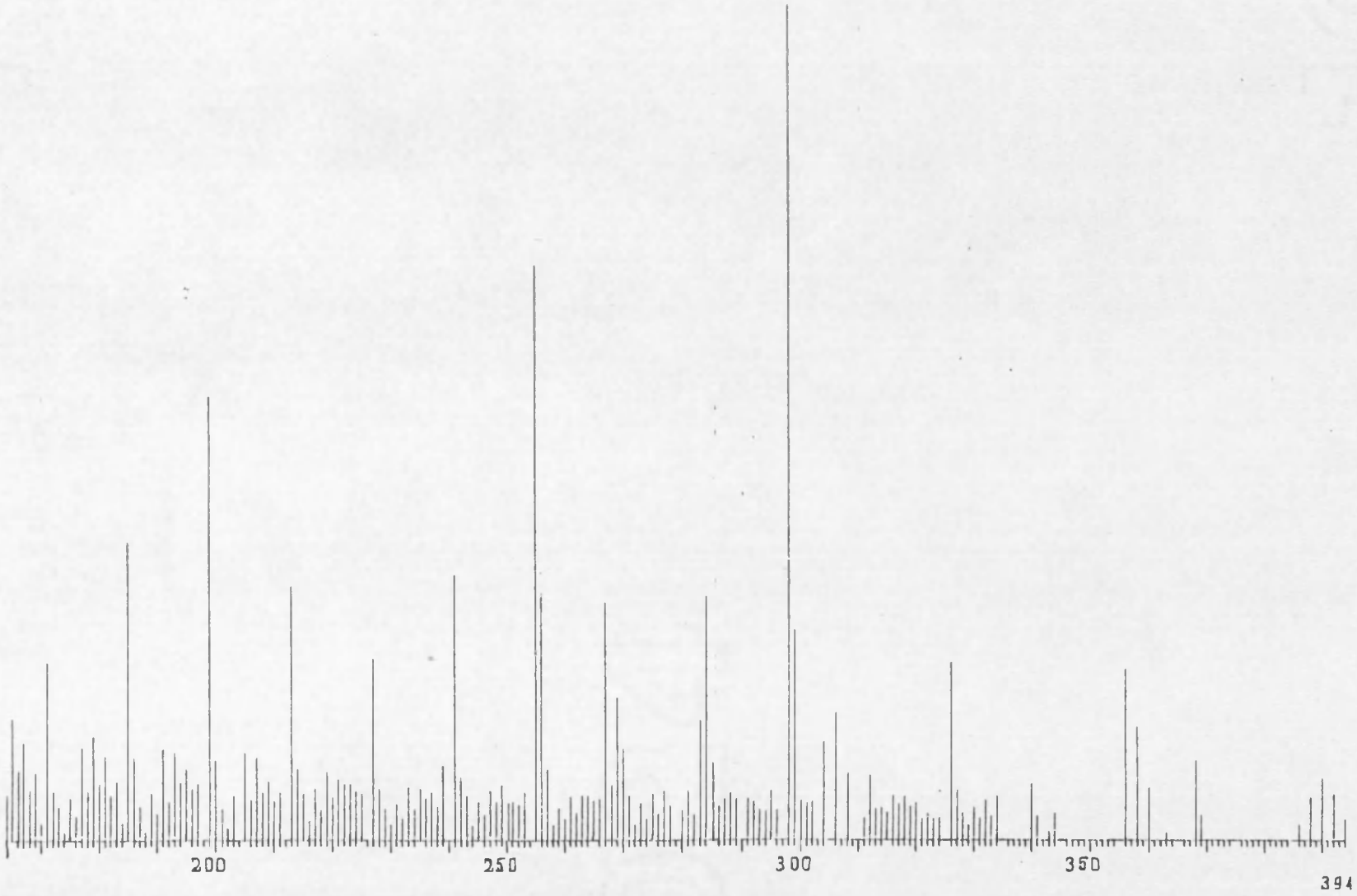

Figure 25. Mass spectrum number 122 from Figure 24.

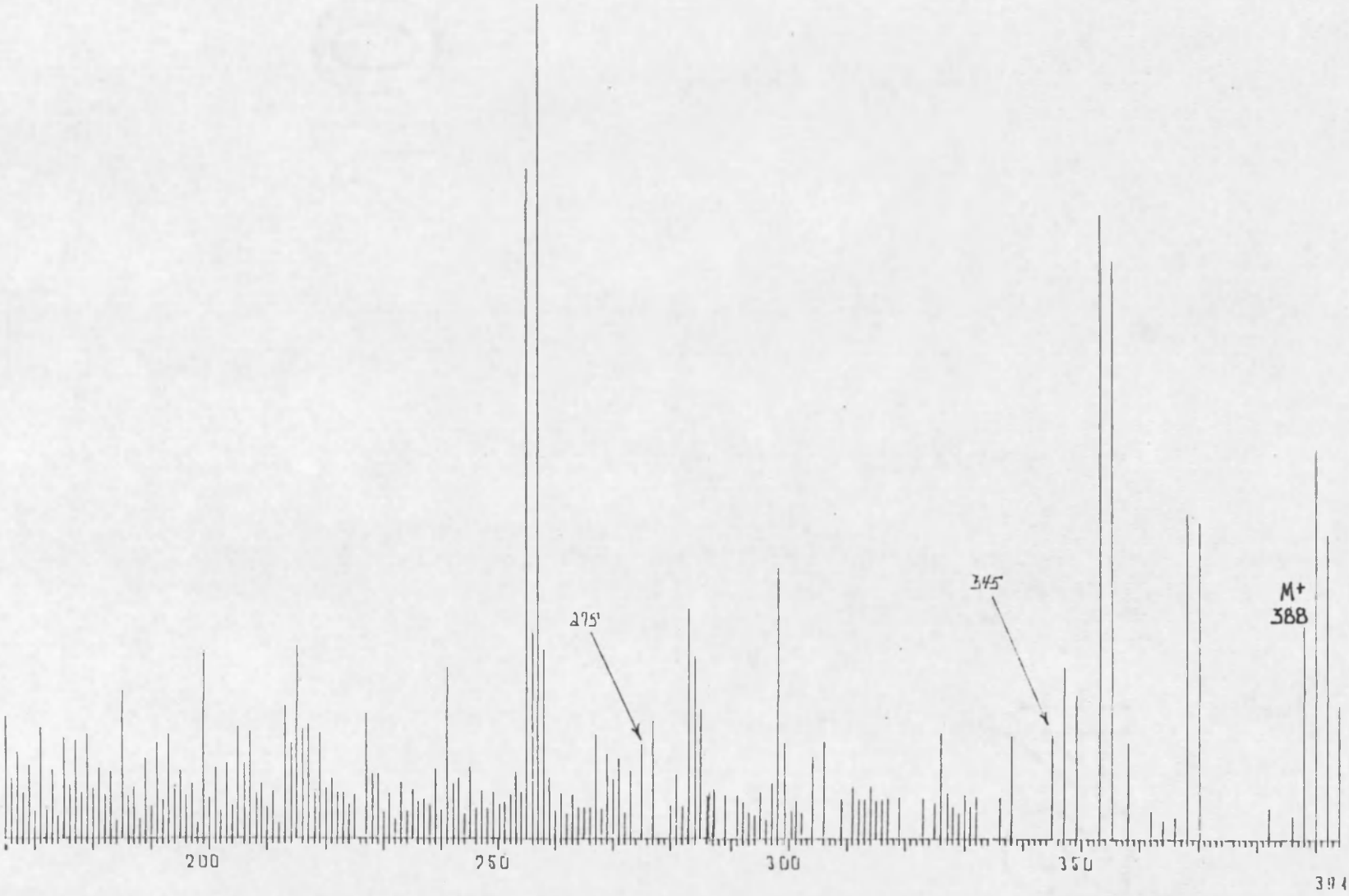

Figure 26. Mass spectrum number 144 from Figure 24.

Figures 27 and 28 show GLC chromatograms of the four compounds whose mass spectra appear in Figures 18, 19, 20, and 21. These chromatograms serve to illustrate the main obstacle encountered in the present investigation; separation.

Separation, while significant between 4-methoxy-2,2',3,4',5,5'-hexachloro- and -methoxy-2,2',4',5,5'-pentachloro (Figure 27), was not sufficient between each of these, and the other two compounds (Figure 28) to overcome the effects of sample impurities and GC-MS transfer line peak broadening. Better GLC separation, or fractionation by some other technique, will be required to produce separation sufficient for definitive characterization.

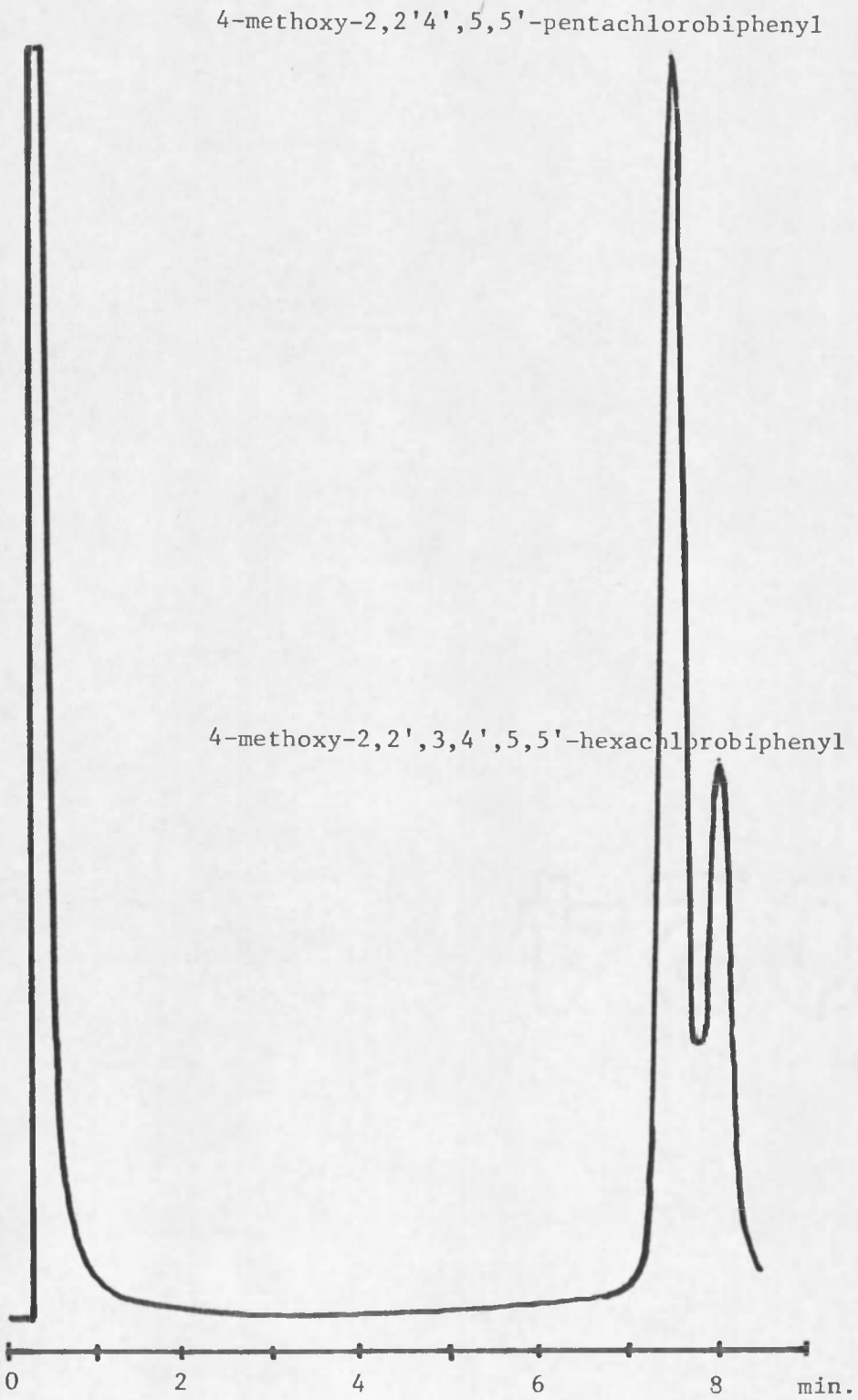

Figure 27. GLC chromatogram of 4-methoxy-2,2',4',5,5'-pentachlorobiphenyl and 4-methoxy-2,2',3,4',5,5'-hexachlorobiphenyl. (6 ft., 3.5% OV-225, glass column.)

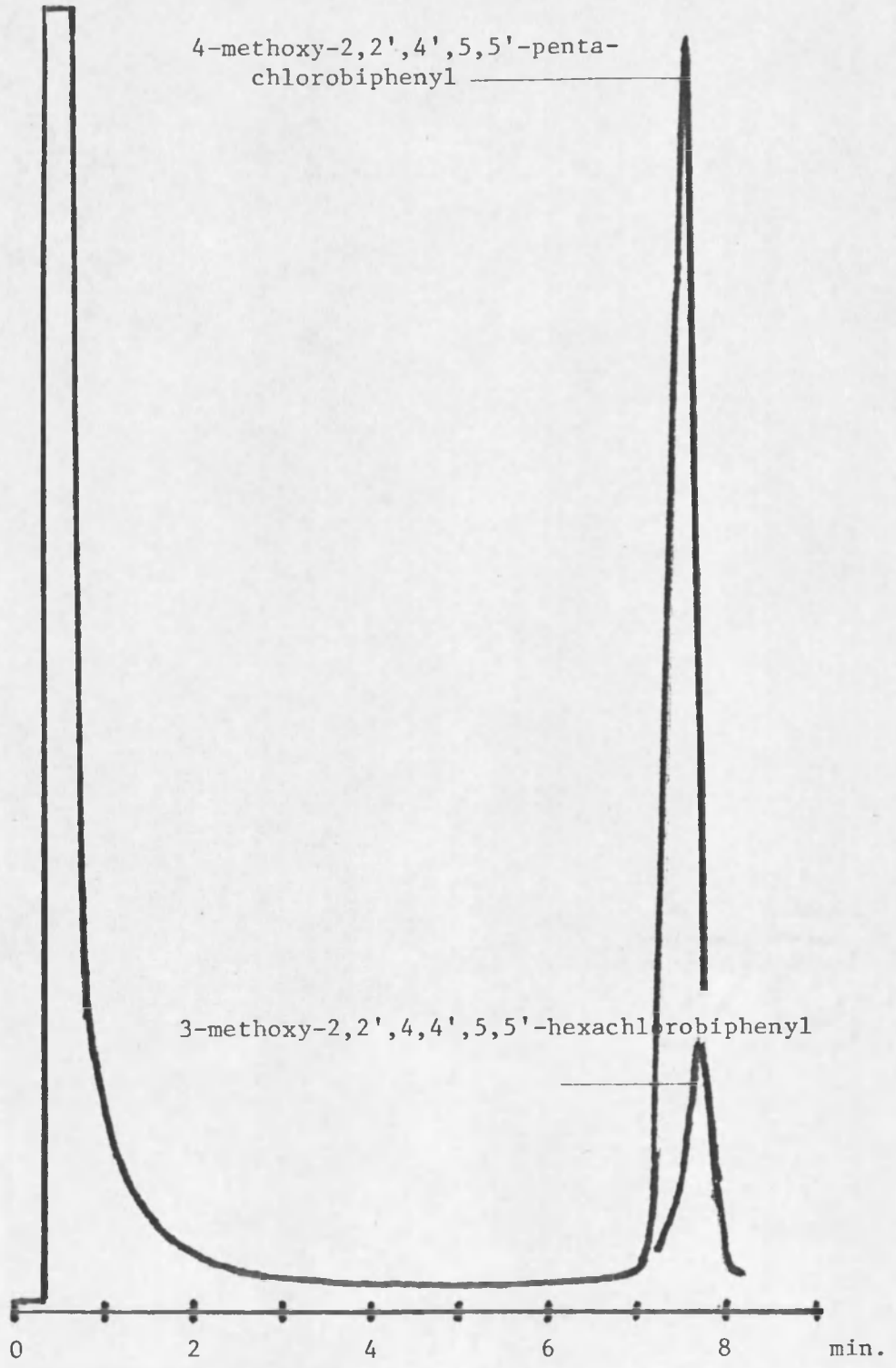

Figure 28. GLC chromatogram of 4-methoxy-2,2',4',5,5'-pentachlorobiphenyl and 3-methoxy-2,2',4,4',5,5'-hexachlorobiphenyl (3-methoxy-2,4,5-HCB). (6 ft., 3.5% OV-225, glass column).

DISCUSSION

In utilizing GPC to affect sample cleanup it was hoped that a system might be developed which would not only separate metabolites from lipid, which has been extensively documented, but metabolites from each other. In addition, it was hoped that this separation could be accomplished with the available equipment. The lack of an automated fraction collecter placed additional limits on just what would constitute an acceptable retention volume (retention time).

Analysis of the data from the three systems gives rise to several conclusions. Some metabolite separations, in addition to separation from lipid, do appear possible with the use of inert solvents. Use of this type of solvent permits maximum use to be made of solute-column adsorptive effects. Use of these solvents does, however, produce very high retention volumes (times) which can only be overcome by increasing the flow rate. This was not possible in the preceding investigations since, in the case of all but the 4mm x 104cm column, solvent flow was already at the maximum obtainable from the pump (2ml/min).

Interestingly, increasing the flow rate need not necessarily decrease peak resolution. In his paper on the mathematical treatment of resolution and optimization in GPC, Giddings (1968) also addresses the problem of resolution vs. elution volume. He demonstrates that with an increase in flow rate and a corresponding increase in temperature, resolution remains the same but the compound will elute faster. This

same phenomenon was demonstrated experimentally by Little et al. (1970). Using a mixture of a low and a high molecular weight polystyrene chromatographed on Styragel® (a polystyrene-divinylbenzene copolymer), they showed that increasing the flow rate by approximately three-fold, decreased resolution $\frac{2[V_{R2}-V_{R1}]}{W_1-W_2}$ by 30%. Increasing the temperature from 28 to 8o° C, however, resulted in almost the original resolution, in one-third the analysis time. Though Johnson and Stevenson (1978) associate temperature effects primarily with high molecular weight compounds, the peak shift seen with increased temperature in the ethyl acetate/toluene system suggests that advantage can be made of this, even in the molecular weight range encountered here.

In an earlier paper on GPC resolution, Giddings (1967) showed that in pure steric exclusion there is a definite limit to baseline resolvability. This limit is a function of the number of theoretical plates (N); expressed as $n \cong 1 + 0.2N^{1/2}$, where n is the number of peaks capable of baseline resolution.

Since polar solvents tend to suppress column-solute adsorptive interactions, they tend to produce more ideal steric exclusion conditions. Because of this, it would appear that multiple columns in series, would be necessary to increase resolution, particularly in more polar solvent systems.

One of the factors not previously mentioned, which contributes to separation, is the packing density. Yau, Malone, and Suchan (1970) demonstrated experimentally that the number of theoretical plates (N), decreases as the packing density decreases.

Using the slurry packing technique, it is generally suggested that the column be packed so as to give approximately 3-5 psi pressure at analytical flow rate (Johnson et al.1976). For the most part this was impossible with the equipment available, for the maximum flow rate of 2ml/min was insufficient to produce any detectable internal pressure.

Based on the data presented here and in the literature, to effect maximal cleanup and separation of biological extracts containing multiple compounds of interest, two discreet solvent systems will almost certainly be necessary.

Initial separation should be performed on a moderately polar solvent system, whose δ value approaches that of the gel. This permits significant swelling of the gel, with a resultant greater number, and distribution of pore volumes. Such a system produces separations based primarily on true steric-exclusion. This is accomplished by suppressing inherent dipole-dipole interactions between the polar hydroxyl metabolites and the gel.

Methylene dichloride would be preferable to ethyl acetate/toluene for this purpose because methylene dichloride more closely approaches the δ value of the gel without being so polar as to significantly effect the recovery of non-polar compounds (e.g., lipid).

Secondary separation of a crude extract should be performed on a solvent system of the inert, aliphatic type. This would maximize the adsorptive effects described above. Aliphatic solvents are generally poor swelling agents for Bio-Beads S gels, which would result in early elution (exclusion) of higher molecular weight compounds and retention

of polar compounds capable of hydrogen bonding. By performing a secondary separation on selected fractions of interest from the initial polar separation, it should be possible to remove residual contamination of higher molecular weight compounds and produce a separation based on polarity and effective diameter (substitution position).

CONCLUSIONS

Data from three GPC systems have been presented. Results suggest the feasibility of metabolite separation and cleanup from biological material by GPC, sufficient for GC-MS analysis.

It was shown that an effective GPC system should be, in fact, two systems; one of moderate polarity, and one with inert, non-polar character.

To increase resolution, particularly in the more polar solvent system, several columns in series, may be required. This may be necessary to offset the resolution limiting effect of insufficient plate number (N), which is partly a function of column length.

The increased analysis time associated with this added column length can largely be offset by an adequate solvent pumping system and an increased column temperature. An adequate solvent pumping system, (with maximum flow of at least 5-10 ml/min.) is also necessary to effect efficient column packing density, a major contributor to resolution.

For achieving rapid sample cleanup, concentrated sulfuric acid cleanup of derivatized metabolite extracts was found to be very efficient and effective.

Mass spectral abundance values for synthesized metabolite methyl ethers were found to be characteristic of specific substitution patterns, as previously reported elsewhere with less highly chlorinated methyoxychlorobiphenyls.

In this study the limiting factor in the actual GC-MS characterization of biological samples involved the gas chromatographic separation of individual isomers from other congeners, which was not adequate for definitive characterization.

REFERENCES

Anonymous. The PCB Story Unfolds, Tox. Appl. Pharmacol. 9 (4): 568-571, 1971.

Barnes, C.S., Collins, D.J., Hobbs, J.J., Mortimer, P.I., and Sasse, W. H.F. The Mass Spectra of Phenyl Methyl Ethers II. Polycyclic Compounds, Aust. J. Chem. 20: 699-712, 1967.

Brandup, J., and Immergut, E.H. Polymer Handbook, Interscience Publishers, 1966.

Burse, V.W., Moseman, R.F., Sovocool, G.W., and Villanueva, E.C. PCB Metabolism in Rats Following Prolonged Exposure to Aroclor 1242 and Aroclor 1016, Bull. Envir. Contam. Tox. 15(1): 122-128, 1976.

Bush, B., Tumasonis, C.F., and Baker, F.D. Toxicity and Persistence of PCB Isomers in the Avian System, Arch. Envir. Contam. Tox. 2 (3): 195-212, 1974.

Brinkman, U.A., DeKok, A., DeVries, G., and Reymer, H.G.M. High-Speed Liquid and Thin Layer Chromatography of Polychlorinated Biphenyls, J. Chrom. 128: 101-110, 1976.

Daly, J.W., Jerina, D.M., and Witkop, B. Arene Oxides and the NIH Shift: The Metabolism, Toxicity and Carcinogenicity of Aromatic Compounds, Experimentia 28: 1129-1149, 1972.

Freeman, D.H. The Gels for Liquid Chromatography, J. Chrom. Sci. 11: 175-180, 1973.

Freeman, D.H., and Angeles, R.M. The Influence of Hydrogen Bonding Upon Gel Permeation Chromatography, J. Chrom. Sci. 12: 730-735, 1974.

Giddings, J.C. Maximum Number of Compounds Resolvable by Gel Filtration and Other Elution Chromatographic Methods, Anal. Chem. 39 (8): 1027-1028, 1967.

Giddings, J.C. Resolution and Optimization in Gel Chromatography, Anal. Chem. 40: 2143-2149, 1968.

Grant, D.L., Phillips, W.E.J., and Villeneuve, D.C. Metabolism of a Polychlorinated Biphenyl (Aroclor 1254) Mixture in the Rat, Bull. Envir. Contam. Tox. 6: 102-112, 1971.

Hubbard, H.L. "Chlorinated Biphenyl and Related Compounds", in Kirk-Othmer Encyclopedia of Chemical Technology, 2nd Edition, pp. 289-297, John Wiley & Sons, Inc., 1964.

Hutzinger, O., Jamieson, W.D., and Safe, S., Paulmann, L., and Ammon, R. Identification of Metabolic Dechlorination of Highly Chlorinated Biphenyl in Rabbit, Nature 252: 698-699, 1974.

Hutzinger, O., Safe, S., and Zitko, V. The Chemistry of PCB, pp. 7, and 22, Chemical Rubber Company Press, 1974a.

Hutzinger, O., Safe, S., and Zitko, V. Preparation, Gas Chromatographic Behavior, and Spectroscopic Properties of Hydroxylated Chlorobiphenyls, J.A.O.A.C. 57(5): 1061-1067, 1974b.

Jansson, B., Jensen, S., Olsson, M., Renberg, L., Sundstrom, G., and Vaz, R. Identification by GC-MS of Phenolic Metabolites of PCB and p,p'-DDE Isolated from Baltic Guillemot and Seal, Ambio 4 (2): 93-97, 1975.

Jansson, B., and Sundstrom, G. Mass Spectrometry of the Methyl Ethers of Isomeric Hydroxychlorobiphenyls - Potential Metabolites of Chlorobiphenyls, Biomed. Mass Spectrom. 1: 386-392, 1974.

Jensen, S. Report on a New Chemical Hazard, New Scientist 32 (254): 612, 1966.

Jensen, S. The PCB Story, Ambio 1(4): 123-134, 1972.

Jensen, S., Johnels, A.G., Olsson, M., and Otterlind, G. DDT and PCB in Herring and Cod from the Baltic, Kattegat, and the Skagerrak 1st. Soviet-Swedish Symposium on the Pollution of the Baltic, Ambio Spec. Report 1: 71-85, 1972.

Jensen, S., and Sundstrom, G. Structures and Levels of Most Chlorobiphenyls in Two Technical PCB Products and in Human Adipose Tissue, Ambio 3(2): 70-76, 1974a.

Jensen, S., and Sundstrom, G. Metabolic hydroxylation of a Chlorobiphenyl Containing Only Unsubstituted Positions - 2,2',4,4',5,5'-Hexachlorobiphenyl, Nature 251: 219-220, 1974b.

Jerina, D.M. Hydroxylation of Aromatics - Chemical Models for the Biological Processes, Chemtech 3: 120-127, 1973.

Jerina, D.M., and Daly, J.W. Arene Oxides: A New Aspect of Drug Metabolism, Science 185 (4151): 573-582, 1974.

Johnson, E.L., and Stevenson, R. Basic Liquid Chromotagraphy, pp. 149-164, Varian Associates, Inc., 1978.

Johnson, L.D., Waltz, R., Ussary, J.P., and Kaiser, F.E. Automated Gel Permeation Chromatographic Cleanup of Animal and Plant Extracts for Pesticide Residue Determinations, J.A.O.A.C. 59(1): 174-187, 1976.

Jondorf, W.R., Parke, D.V., and Williams, R.T. Studies in Detoxification 66. The Metabolism of Halobenzenes.1,2,3-, 1,2,4-, and 1,3,5- Trichlorobenzene, Biochem. J. 61: 512-521, 1955.

Kuehl, D.W., and Leonard, E.W. Isolation of Xenobiotic Chemicals from Tissue Samples by Gel Permeation Chromatography, ANal. Chem. 50(1): 182-185, 1978.

Little, J.N., Waters, J.L., Bombaugh, K.J., and Pauplis, W.J. Fast Gel Permeation Chromatography, Sep. Sci. 5(6): 765-775, 1970.

Mathews, H.B., and Anderson, M.W. Effects of Chlorination on the Distribution and Excretion of Polychlorinated Biphenyls, Drug Metab. Dist. 3(5): 371-380, 1975.

McLafferty, F.W. Interpretation of Mass Spectra, 2nd Edition, W.A. Benjamin, Inc., 1973.

Mori, S. Elution Behavior of Some Solutes on a Porous Polystyrene Gel in High Performance Liquid Chromatography, Anal. Chem. 50 (6): 745-748, 1978.

Mowrer, J., Calambokidis, J., Musgrove, N., Drager, B., Beug, M.W., and Herman, S.G. Polychlorinated Biphenyls in Cottlids, Mussels, and Sediment in Puget Sound, Washington, Bull. Envir. Contam. Tox. 18(5): 588-594, 1974.

Murphy, P.G. Sulfuric Acid for the Cleanup of Animal Tissues for Analysis of Acid-Stable Chlorinated Hydrocarbon Residues, J.A. O.A.C. 55(6): 1360-1362, 1972.

Peakall, D.B., and Lincer, J.L. Polychlorinated Biphenyl- Another Long-Lived, Widespread, Chemical in the Environment, Bioscience 20(17): 958-964, 1970.

Perry, D.F. Research Assistant, Toxicology Program, Univ. of Arizona, Personal Communication, 1978.

Rote, J.W., and Morris, W.J. Use of Isotopic Abundance Ratios in Identification of Polychlorinated Biphenyls by Mass Spectrometry, J.A.O.A.C. 56(1): 188-198, 1973.

Safe, S., Hutzinger, O., and Jones, D. The Mechanism of Chlorobiphenyl Metabolism, J. Agric. Food Chem. 23(5): 851-853, 1975.

Schulte, E., and Acker, L. Identifizierung und Metabolisierbarkeit von Polychlorierten Biphenylen, Naturwissenschaften 61: 79-80, 1974.

Stahling, D.L., Tindle, R.C., and Johnson, J.L. Cleanup of Pesticide and Polychlorinated Biphenyl Residues in Fish Extracts by Gel Permeation Chromatography, J.A.O.A.C. 55(1): 32-38, 1972.

Tulp, M.Th.M., Olie, K., and Hutzinger, O. Identification of Hydroxyhalobiphenyls as their Methyl Ethers by Gas Chromatography-Mass Spectroscopy, Biomed. Mass Spectrom. 4(5): 310-316, 1977.

Webb, R.G., and McCall, A.C. Identities of Polychlorinated Biphenyl Isomers in Aroclors, J.A.O.A.C. 55(3): 746-752, 1972.

Yau, W.W., Malone, C.P., and Suchan, H.L. Separation Mechanisms in Gel Permeation Chromatography, Sep. Sci. 5(3): 259-271, 1970.