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GREAT LAKES FISHERY COMMISSION
Research Completion Report[1]

Isolation of Bioactive Components of Commercial TFM Formulations

by

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Final Report to the Great Lakes Fishery Commission:

Isolation of bioactive components of commercial TFM formulations

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Executive Summary

Significant progress has been made to isolate and identify the chemical(s) in TFM formulations which are responsible for the MFO enzyme induction in fish. TFM formulations (Hoescht 1990-2) have been fractionated using solid phase cartridges (SPEs) to isolate and aid in the identification of the inducing components. The TFM was removed from the formulation by shifting the pH of the methanol/water mobile phase; this separation did not remove any of the MFO induction and therefore allows the concentration of the MFO inducing components to be increased without the toxicity associated with the TFM. This extract is not acutely toxic at 50 times the equivalent concentration (5.8 mg/L TFM) and could be separated into distinct sub-fractions using a prep scale C_{18}-HPLC system with a buffered methanol/water mobile phase. After numerous trials to optimize the extraction and chromatography, MFO induction was isolated into two distinct reproducible fractions (identified as 19-3, 19-5). This indicates that there was at least two different compounds in the formulation causing the MFO response; there may be additional compounds capable of induction but not present in sufficient concentrations in the formulation (there is no guarantee that chronic exposure may not result in bioaccumulation to a degree sufficient to cause effects). Both fractions 19-3 and 19-5 were found to contain one major component and several minor components. The major components in each inducing fraction of the Hoescht 1990-2 formulation (19-3, 19-5) were identified as diphenyl ethers (by High Resolution GC/MS) which most likely originated from TFM and/or p-chlorophenol during the synthesis of TFM. To confirm the identities, the compounds were synthesized, and the purified products were tested for MFO induction potential. All of the diphenyl ethers failed to cause MFO induction in fish exposed to several concentrations. The compounds associated with the induction must be minor components in the TFM formulation in fractions 19-3 (30-3) and 19-5 (34-2 and 31-3); these compounds would be present in receiving water during application of the formulation in very low levels. Work has been initiated to further separate the MFO inducing fractions to isolate the responsible components. The inducing fractions (19-3, 19-5) were collected, concentrated to three times the original concentration and reinjected on the HPLC column using an optimized mobile phase program. This resulted in the induction being further isolated into two major fractions in 19-5 and one in 19-3 which contained significantly fewer of the original contaminants (including the major diphenyl ethers). High Resolution GC/MS total ion chromatographs have been obtained on each bioactive fraction and controls. Mass spectra for each fraction have been examined and preliminary tentative identifications have been made for several chemicals including additional chlorinated diphenyl ethers and chlorotrifluromethylnitro-p-dioxin. Substitution of a "dioxin" with a trifluoromethyl or nitro groups would only slightly change its toxicity and could even increase it relative to a similarly substituted polychlorinated dibenzo-*p*-dioxins.

Testing of a variety of TFM formulation batches (1994) from two companies has confirmed that significant MFO induction exists in all current formulations. One chemical supplier consistently had induction several fold greater than the reference H1990-2 batch. This testing confirms that extensive efforts by the company to purify the formulation without knowing the identity of the inducing chemicals failed. Given the mass of TFM formulation discharged into the Great Lakes, the potential for components to be persistent and to cause metabolic disturbance (including circulating levels of sex steroids) in wild fish, and the potential for highly toxic chemicals such as dioxins to be present, it is critical that the components of the formulation which are responsible for biological effects be identified and removed from the formulations. The environmental fate and toxicology of the chemicals already discharged into the Great Lakes needs to be addressed.

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BACKGROUND

During the 1980s, fish collected from Whitefish and Batchawana Bays, Lake Superior showed unexplained elevations of hepatic mixed function oxygenase (MFO) activity (Luxon et al. 1987; Allan et al. 1991; Smith et al. 1991). MFO enzymes represent a class of membrane bound detoxification enzymes and induction of enzyme activity is usually associated with exposure to polycyclic aromatic compounds such as PCBs, dioxins, furans and PAHs (Hodson et al. 1991). The elevation of MFO activity at these relatively pristine, non-industrialized areas of eastern Lake Superior could not be accounted for by known contamination. The only anthropogenic source of chemical known for these pristine sites has been the periodic treatment of tributary streams with lampricides.

For more than 30 years, the main approach for controlling the abundance of lamprey (*Petromyzon marinus*) in the Great Lakes basin has been the application of pesticides to reduce numbers of larval lampreys in nursery streams (Scholefield and Seeley 1990). The main chemical used has been 3-trifluoromethyl-4-nitrophenol (TFM) (Meyer and Schnick 1983; GLFC 1985; Seelye et al. 1988), which has been applied to most nursery streams on a 3 year cycle; current usage approximates 50,000 kg per year of TFM. Some areas have also been treated with 2',5-dichloro-4-nitrosalicylanilide (Bayer 73) alone or in combination with TFM. The toxicity of the TFM field formulation is strongly influenced by pH, conductivity, alkalinity and hardness ((Gilderhus et al. 1992, Seelye and Scholefield 1990, NRCC 1985, Bills et al. 1988). TFM has a pK_a of 6.07 (NRCC 1985) and toxicity to rainbow trout (12 h LC50) ranged from 0.9 mg L^{-1} at pH 6.5 to >100 mg L^{-1} at pH 9.5 (Bills et al. 1988). Treatment concentrations around the Great Lakes would vary from <1.0 mg L^{-1} in soft, acid water to >14 mg L^{-1} of TFM in hard, alkaline water (T. Bills, pers. comm). Treatment concentrations of 6.9 mg L^{-1} were utilized in Lynde Creek (Carey et al. 1988) and 8.7 mg L^{-1} in Wilmot Creek (Beamish et al. 1987) in the Lake Ontario drainage.

White sucker exposed to a scheduled lampricide application showed a 5-fold induction of hepatic MFO activity 2 days after exposure (Munkittrick et al. 1994a). Laboratory experiments using the same batch of lampricide confirmed the potential of the formulation to induce EROD activity in fish. Further investigation of the individual components of the lampricide formulation identified the TFM formulation as the bioactive (inducing) fraction and no activity was evident in the Bayer 73 (niclosamide) component. Isolation of the TFM from the TFM formulation using HPLC has demonstrated that the TFM is not the bioactive ingredient in terms of MFO induction. This result is supported by the lack of induction in fish exposed to two independent sources of purified TFM.

Induction of P450IA in fish has been associated with multi-ringed planar compounds such as dioxins and furans (Muir et al. 1992; Parrott 1993), PCBs (Janz and Metcalfe 1991) and PAHs (Hodson et al. 1991). These compounds have been shown to induce MFO activity through activation of a cytosolic receptor, (the Ah-receptor; Lorenzen and Okey 1990), which is thought to require a specific molecular size and conformation for activation and subsequent induction (Poland and Knutson 1982). Single-ringed structures such as TFM would not be expected to

activate this pathway, so it is probable that the inducing component of the TFM formulation would be a multi-ring structure. Even though the chemical structure of Bayer 73 is similar to the structure of chemicals known to cause induction, no induction was seen with Bayer 73 at exposure levels close to the lethal concentration. Multi-ring MFO active compounds are known to be produced during synthesis of some phenolic derivatives (IARC 1982; Rappe 1984; Fiedler et al. 1990). It is critical to identify the chemical responsible for induction, and to establish if its origin is during manufacture of the formulation.

Although MFO induction is commonly used as an indicator of exposure to complex effluents, the significance of MFO induction is unknown. It is believed that MFO-mediated biotransformation of chemicals may act to increase mutagenicity of some compounds or that elevated MFO activity may lead to increased degradation of circulating levels of hormones required to control growth and reproduction. Neither of these impacts of MFO induction have been conclusively shown. There have been recent reports of elevated MFO activity and decreased circulating levels of steroid hormones near pulp mills (McMaster et al. 1991; Munkittrick et al. 1991, 1992a, 1994b), but the low hormonal level can not be directly linked to MFO activity (Van Der Kraak et al. 1992, Munkittrick et al. 1992a, 1992b, Van Der Kraak et al. 1992). However, disruption of circulating levels of steroid hormones by TFM formulations has been recently demonstrated (Munkittrick et al. 1994).

An essential step in identifying the inducing chemical(s) is to isolate it from other components of the pesticide formulation. HPLC separation was effective in separating the various components of the TFM formulation while retaining the inducing characteristics. The HPLC separations indicated that the inducing chemical(s) eluted from the preparative column after the TFM component (10 min), but prior to 32 min. Based on HPLC retention times and reported K_{ow}s of chlorophenols (Mackay et al. 1992), the log K_{ow} of the inducing chemical is predicted to be between 3.0 and 4.5. Because the induction was recovered from the column prior to 32 min, it is unlikely that the chemical is a highly hydrophobic compound (i.e., log $K_{ow} > 4.5$). Analysis of the TFM formulation (90-2) showed non-detectable levels of polychlorinated dioxins and furans (2,3,7,8-tetra- to octa- congeners <0.06 pg μL^{-1}) and only trace amounts of coplanar PCBs (<0.11 pg μL^{-1}) (Appendix 4).

PROJECT OVERVIEW

Introduction

This report documents systematically the approach taken to address the study objectives. The objectives were to:

To isolate and identify the chemical(s) in TFM formulations which are responsible for the MFO enzyme induction in fish by

a. developing and validating a method to isolate the MFO inducing chemicals from the TFM
b. optimizing an HPLC method to isolate the MFO inducing chemicals
c. obtaining total ion chromatograms of the fractions
d. identifying the dominant chemicals in the fractions by mass spectral analysis.

Although in several instances numerous trials were conducted, only the key experiments are presented here to avoid confusion (e.g. several trials were conducted to evaluate the performance of the solid phase extractions (SPE) and solvents but only the successful trials which were used in subsequent experiments are presented).

The methodology used to prepare TFM formulation fractions had to be refined and validated prior to conducting fractionation experiments. The major problem encountered was that the presence of TFM in the formulation resulted in toxicity at concentration only slightly above 5 mg/L. The high concentrations of TFM also hindered the quality of the HPLC chromatography. The TFM therefore had to be cleanly separated from the MFO inducing components in the formulation. This separation would allow an increased concentration of the bioactive fractions to be utilized in fish exposures to strengthen the consistency of the response and improve the chromatographic fractionation. The chromatographic conditions were then optimized to separate the major peaks and bioactive fractions. Major peaks were identified using High Resolution GC-MS. Each of the bioactive fractions were collected and reinjected on the HPLC under new chromatographic conditions to further optimize the separations. The bioactive fractions were concentrated and total ion chromatographs were run. Mass spectra of unique peaks in the bioactive fractions relative to the controls were obtained to facilitate chemical identification. Several chemicals which were tentatively identified were synthesized and used to confirm the chemical identifications and the MFO induction potential. Several chemicals were tentatively identified but standards were not available for confirmation.

To determine if changes to the production technologies had changed the MFO induction potency of individual batches supplied in 1994, several batches of TFM were obtained directly from the supplier or the Sault Ste. Marie Sea Lamprey Control Centre and assayed for MFO inducing potential.

GENERAL METHODS

Fish Exposures

TFM exposures were conducted in darkness and glass aquaria using juvenile rainbow trout (3-5 g) at a loading density of 2.5 g L^{-1}. Fish were acclimated to 13°C for at least 7 d and were not fed 6 d prior to exposures. Single exposures were 72h. Exposures to filtered TFM and any fractions generated were conducted in water used for holding (dechlorinated Burlington city tap water; pH 7.5-8.0, hardness 128-133mg L^{-1}). Unless otherwise stated, fish were exposed to 150µL of the TFM formulation in 12 L of laboratory water (5.8 mg/L TFM), or the equivalent in generated fractions.

MFO Analysis

MFO analyses were completed using 7-ethoxyresorufin (ER) as the substrate. For the measurement of ethoxyresorufin-o-deethylase (EROD) activity, liver samples were processed as fresh tissue, homogenized in a hand-held homogenizer in 1 mL of HEPES-KCl (pH 7.5, 0.15 M KCl, 0.02 M HEPES) homogenization buffer. The homogenates were spun at 10,000 x g for 20 min. at 4°C and the post-mitochondrial supernatant (PMS) drawn off with a pasteur pipette. EROD activity determinations were completed using the method of Pohl and Fouts (1980) as modified by McMaster et al. (1992). The reaction mixture contained 1250 µL of 0.1 M HEPES buffer (pH 7.8), 10 µL of magnesium sulphate (0.154 g mL^{-1}), 50 µL BSA (40 mg mL^{-1}), 30 µL reduced nicotinamide adenine dinucleotide phosphate (NADPH; 20 mg mL^{-1}), 100 µL of PMS and 20 µL of ER (0.022 mg mL^{-1}) in 13 x 100 mm borosilicate glass tubes. Reaction mixtures were incubated for 10 min at 25°C and stopped by addition of 3 mL of methanol. Blanks were prepared by adding 3 mL of methanol to the tubes prior to the addition of 20 µL ER. The samples were centrifuged at 6000 x g for 5 min to pellet the precipitated protein. The supernatants were transferred to square polycarbonate cuvettes and fluorescence measured on a Perkin Elmer LS50 spectrofluorometer, with an excitation wavelength of 530 nm and an emission wavelength of 585 nm. The excitation slit width was 2.5 nm and the emission slit width was 20 nm. The protein concentration of the PMS was determined using the Lowry method (Lowry et al. 1951). Results were converted to units of pmol mg $protein^{-1}$ min^{-1} using a resorufin standard curve.

During the fractionation experiments the emphasis was to track the induction in various HPLC fractions therefore the EROD assays were modified to increase sensitivity and reduce analysis time and cost. For these trials spectrofluorometric assays were performed on fresh liver homogenates using large (300µL) homogenate volumes. Duplicates samples were performed for each fish and one blank was determined for each aquaria. Protein analyses were not carried out for these assays and EROD activities are expressed in fluorescence units.

HPLC System

The HPLC system utilized was a Waters system equipped with a 717 autosampler, a 610 fluid and valve station, a 600E system controller and a model 481 spectrophotometer UV detector set at @254 nm. A semi-preparative reverse phase Partisil 10 ODS 2 column (500mm x 9.4mm ID) was utilized (Watman). HPLC conditions were optimized for peak separation using the following conditions:

reverse phase Partisil 10 ODS 2 column (500mm x 9.4mm ID)
flow rate 4 mL min^{-1}
1500 µL injection
UV detector @ 254nm
solvent program:
10:90 methanol: 0.2M pH 4 aceate buffer for 2.00 min
linear ramp to 100% methanol at 34.00 min
hold for 21.00 min

Characterization of the HPLC column

Several lipophilic compounds were run on the HPLC in order to determine the retention time relative to compounds present in the TFM formulation fractions which were being tested for EROD activity. These compounds, in order of increasing retention time and log K_{ow}, were; napthalene (38.3 min, 3.26), PCP (39.94 min, 5.12), fluorene (41.81 min, 4.38), pyrene (45.54 min, 5.18) and B[a]P (55.77 min, 6.5).

Gas Chromatography

TFM fraction extracts were prescreened on a Hewlett Packard 5890 gas chromatograph (GC) coupled directly to a 5970 mass selective detector, equipped with a 60m RT_x5 (Restek) column. High Resolution Mass Spectrometry (MS) was conducted with a VG Autospec-Q (Fisons, VG Analytical) connected to a Hewlett Packard 5890 GC.

Chemical analysis were conducted under the following conditions:

GC conditions,
60m x 0.25 I.D. DB5 (J&W Scientific) column with a 2m retention gap
on column 2uL injections
ultrapure helium carrier gas
initial 80°C for 0.1min
temperatures programmed at 4°C/min to 280
held at 280°C for 2 min
programmed at 4°C/min to 290°C and held for 10 min

MS Conditions,

full scan at 1000 resolution from 40 to 450 amu
1.00 scans/s
positive ion EI (electron impact) ionization
8000V accelerating voltage
source temp 270°C
electron energy 70eV
emission current 1.57mA
filament current 4.04A

EXPERIMENTAL RESULTS

TFM Batch Experiments

To evaluate the variability and MFO inducing potential of TFM formulation batches received by the Great Lakes Fishery Commission, Sea Lamprey Control Program in 1994, a series of trials were conducted with samples received from Sea Lamprey Control (SLC) in Sault Ste. Marie (ssm), Ontario: three Hoechst batches (B); ssmB161, ssmB831 & ssmB1778, and two Kinetics batches; ssmB317 and ssmB188, and from the Great Lakes Fishery Commission (GLFC or fc): five batches of Kinetics; fcB317, fcB188, fcB312, fcB310 and fcB338.

The first exposure (conducted July 22-25, 1994) compared the EROD activity of fish exposed to four of the Kinetics batches obtained from the GLFC; fcB317, etc., with those of a reference; Hoechst batch 1990-2 (Fig. 1). The reference, 1990-2, and analytical controls ran as expected. All of the Kinetics batches produced significantly higher EROD activity than in the 1990-2 treated fish.

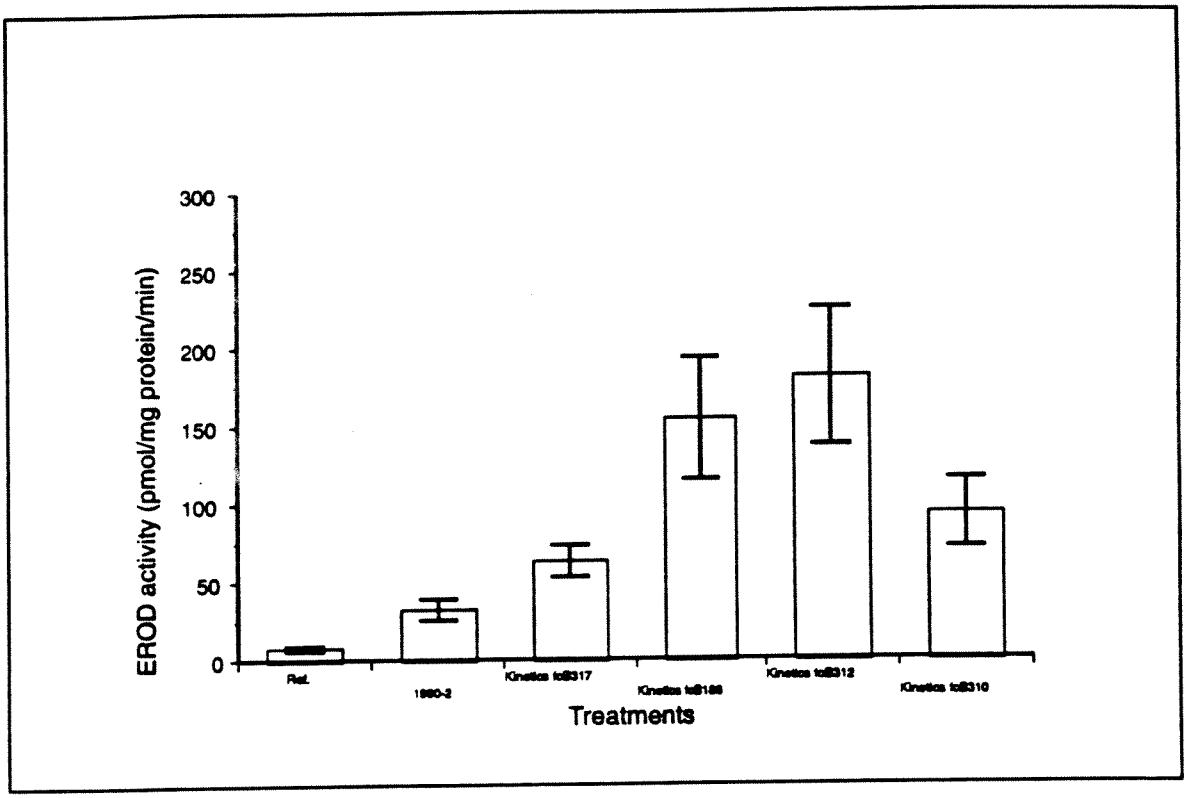


Figure 1. Exposure of rainbow trout to 1994 Kinetics TFM formulations (supplied by GLFC; Rep. 1).

A second exposure (August 03-06) tested all of the TFM batches sent by the SLC and GLFC (Fig. 2). The reference, 1990-2, and the analytical controls ran as expected. The Kinetics 1993 batch was comparable with the 1994 Kinetics batches from both SLC and GLFC. All of the Kinetics batches tested caused significantly higher induction than the Hoechst batches. There was comparable EROD activity observed for the GLFC batches fcB188 and fcB312 in both experiment 1 and 2 but the activity increased for fcB317 and fcB310 in the second exposure.

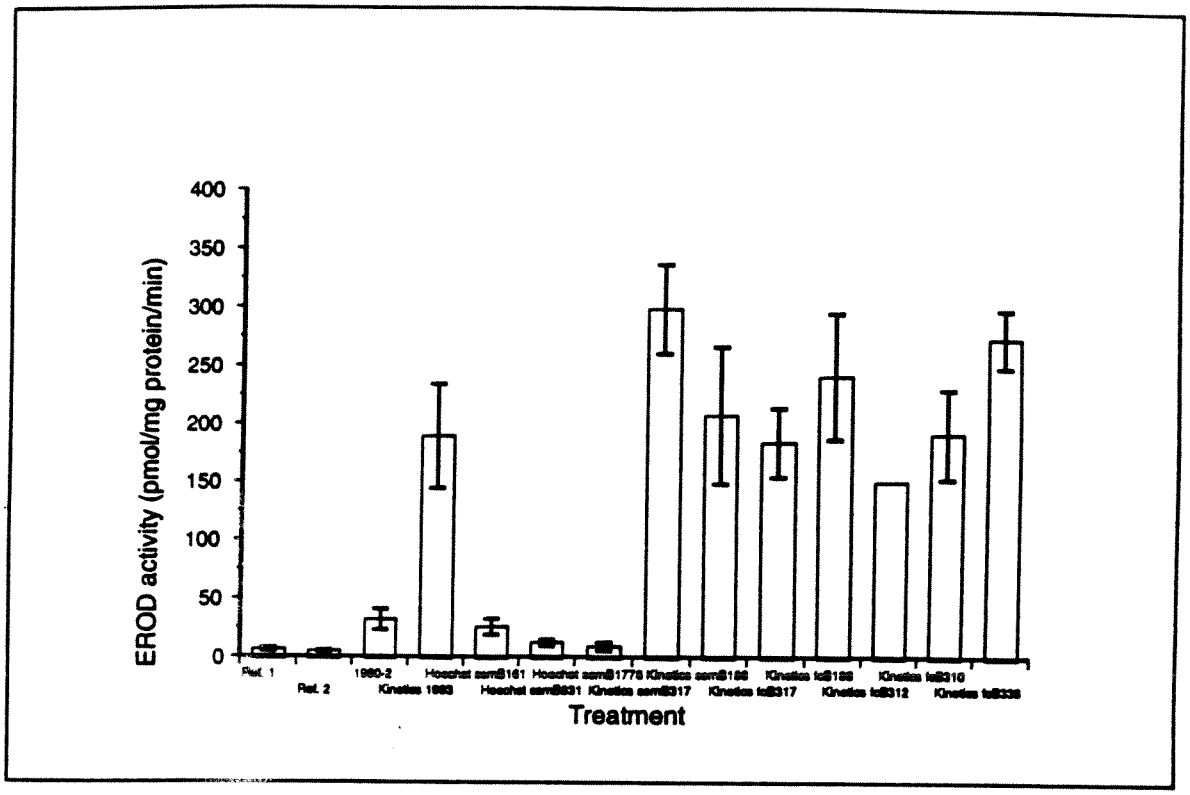


Figure 2. Exposure of rainbow trout to 1994 Hoechst and Kinetic TFM formulations (supplied by SSM and GLFC; Rep. 2).

The toxicity of both the Kinetics batches was found to have increased in the second experiment compared to the first (Appendix 1). This could not be explained by differences in either the physical or chemical water parameters that were measured during both experiments (see Appendix 2). Prior experience has indicated that changes in the water hardness can affect the toxicity of TFM (N.R.C.C. 1985), however this is not believed to be the cause in this case as the hardness (130 mg/L $CaCO_3$) values do not change sufficiently, nor do other water quality parameters measured (Appendix 2). A third batch exposure was conducted in order to verify the consistency of the MFO induction and toxicity (Fig. 3).

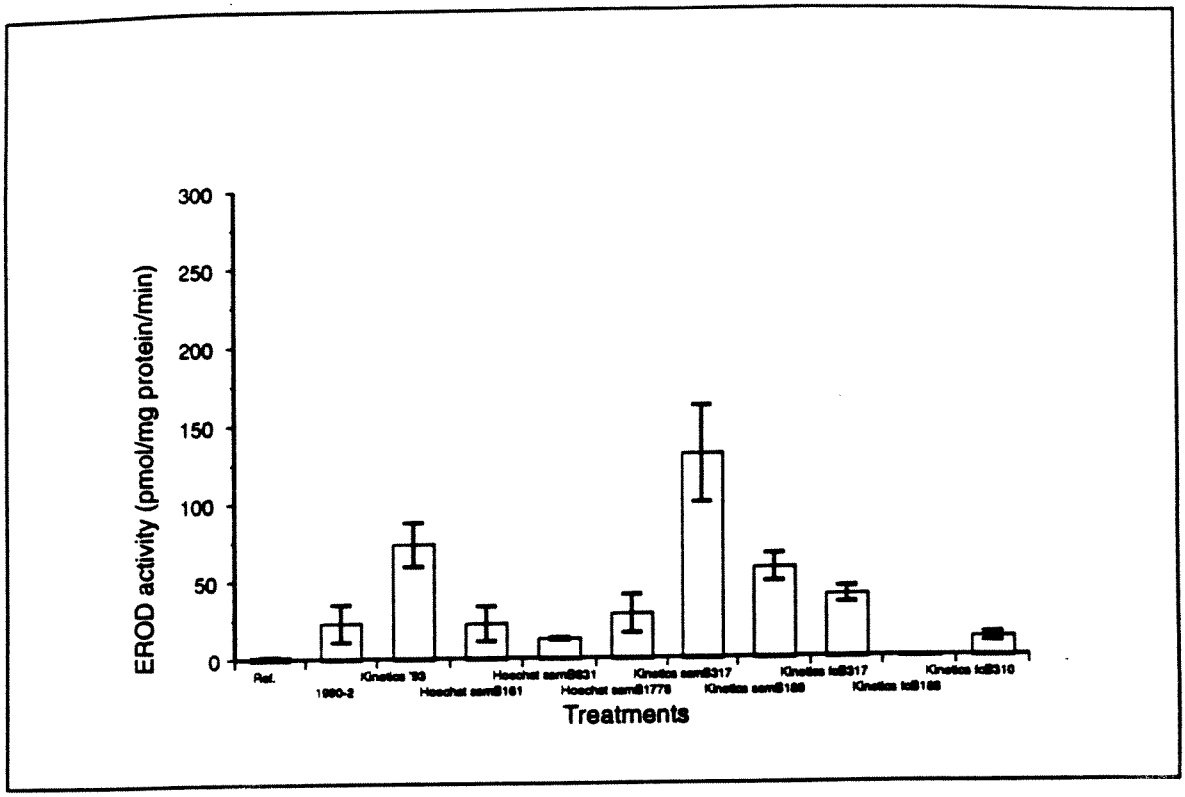


Figure 3. Exposure of rainbow trout to 1994 Hoechst and Kinetic TFM formulations (supplied by SSM and GLFC; Rep. 3).

The third exposure experiment (Aug. 23-26) confirmed that there was an increase in the toxicity of all TFM batches tested (Appendix 1) relative to previous trials. Overall however, the Kinetics batches produced higher toxicity at 5.8 mg L^{-1} (150 µL in 12 L) than the Hoechst batches (no control mortality). A dose-response experiment (Aug. 26-29) conducted with the reference batch H1990-2, and Kinetics batch ssmB317 determined that in both cases 100 µL of TFM in 12 L produced the expected EROD activity but did not cause the toxicity seen with exposure to 150 µL (Fig. 4).

The references, 1990-2 and Kinetics 1993, and analytical controls in the third exposure ran as expected and the Hoechst batches produced less MFO activity than the Kinetics batches (Fig. 3). However, the EROD activity for all of the Kinetics batch exposed fish was lower than in Experiment 2. This may in part be due to the higher toxicity of TFM observed in the third

experiment compared to the second (Appendix 1). These observations are corroborated by the fact that the dose-response experiment (Fig. 4) indicated that the EROD activity was higher at lower TFM doses (100 µL) than those which were more toxic (150 µL).

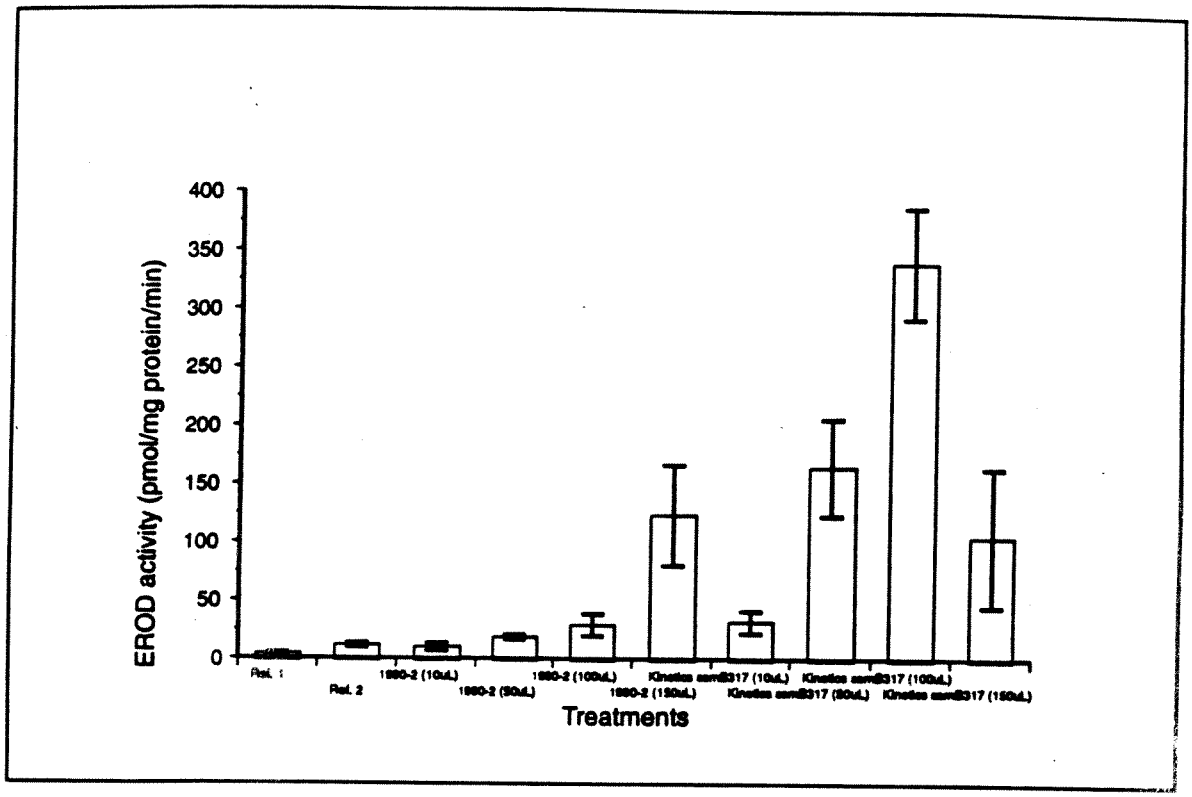


Figure 4. Dose response for Hoechst 1990-2 and Kinetics 317 (10, 50, 100, 150 µL).

The results of the TFM batch experiments indicates that there is a great deal of variability in the MFO induction potential and toxicity between batches and formulations (Hoechst and Kinetics). All TFM formulations, including the batch received in 1994 from both suppliers, result in the induction of MFO activity in fish. Even after considerable efforts by Kinetics to purify the TFM formulations, induction in fish exposed to these formulations remains as high or higher than previous batches or the Hoechst 1990-2 reference batch. Additional experiments in collaboration with Kinetics to isolate and remove the inducing chemicals are continuing. Preliminary results are reported in Appendix 3.

Solid Phase Extraction Experiments

The objective of these studies was to remove TFM from formulation contaminants so that a) the large TFM interference and acute toxicity will be removed, and b) the contaminants could be concentrated for exposures and HPLC separations.

Fractionations were performed using the 1990-2 batch from Hoescht. Separations utilized C_{18} solid phase extraction (SPE) cartridges since previous work has demonstrated that the inducing chemicals are less polar than TFM (Munkittrick et al., 1994). We hypothesized that after applying the formulation to a cartridge, it should be possible to elute TFM, leaving inducing contaminants adsorbed on the cartridge. The contaminants could then be eluted in a separate fraction and allow exposure of the fish to only the inducing chemicals without acute toxicity associated with the TFM. It was therefore necessary to first establish the solvent conditions which would achieve this separation.

All SPE separations were performed using 150 µL TFM formulation. Cartridges were preconditioned with 4 bed volumes of methanol followed by 4 bed volumes of the first solvent to be used in the fractionation. Cartridge elutions were performed under 5" Hg vacuum using a cartridge manifold and a vacuum pump. Reference fish were exposed to the maximum amount of solvents, etc. used in fraction generation.

The separations in the first trial used a 100 mg C18 cartridge and fractions were generated sequentially. Fraction 1 was collected with 1mL 25:75 methanol:pH4 0.2 M acetate buffer, fraction 2 with a subsequent 2 mL 85:15 methanol:buffer and fraction 2a with 2 mL methanol. The visible TFM was distributed between fraction 1 and 2 and separation from induction was unsuccessful. It was hypothesized that incomplete separation of TFM from inducing compounds may have been due to the insufficient chromatographic capacity of the 100 mg SPE cartridges.

The next series of exposures utilized i) solvent conditions analogous to those used in previous semi-preparative HPLC separations (which demonstrated a separation of TFM from inducing compounds with a similar column packing) and ii) cartridges with a larger packing. Fraction 1 was collected as the formulation volume drawn through the SPE cartridge, fractions 2 and 3 were each eluted with 0.5 mL 10:90 methanol:pH4 acetate buffer, and fraction 4 was eluted with 1.5 mL methanol. Most of the TFM was eluted in fractions 1 and 2 with a trace evident in fraction 3. Although there was induction associated with fraction 4 with no visible TFM, there was still induction associated with the first two TFM-containing fractions. Using the same methodology, a cartridge with more packing showed an enhanced separation of TFM from induction. Conditions were modified slightly with the larger packing. Fraction 1 was eluted with the formulation volume plus 0.5 mL of the 10:90 methanol:acetate buffer; fractions 2, 3 and 4 were eluted with 0.5 mL 10:90 methanol:buffer, and fraction 5 was eluted with 1.5 mL methanol. TFM was visibly absent from fractions 4 and 5. These results suggested that the capacity of the smaller cartridges to separate the components of the formulation may have been exceeded. All further fractionations used 500 mg SPE cartridges.

The methanol fraction causing induction was coloured and appeared to contain a mixture of several components. By modifying the solvent elution methodology, it was hoped that non-inducing formulation contaminants could be separated from this fraction. In the first experiment, further washes of the cartridge with 10:90 methanol:buffer prior to the collection of the methanol fraction showed that all induction was still associated with the methanol fraction. The following experiment utilized solvent combinations with polarities between those of 10:90 and methanol. Solvent mixtures over a narrow range of decreasing polarity were used. Fish exposures to fractions eluted with 1.5 mL of 15:85, followed by 1.5 mL 20:80, then 1.5 mL 25:75 after the initial 3 mL 10:90 showed no induction, while induction was still maintained in the less polar 100% methanol fraction.

Methanol fractions after SPE clean-ups were profiled by semi-preparative HPLC. HPLC profiles revealed that there were significantly more contaminants present in Hoescht formulations than was previously reported, and that there was residual TFM present in the methanol fraction using the present SPE clean-up. It was hypothesized that the pH 4 buffer was protonating TFM during SPE and the less polar protonated TFM (colourless) would elute with 100% methanol. Using SPE, two approaches were taken to further eliminate TFM from inducing fractions.

In the first approach, an elution of a solvent mixture less polar than 25:75 but more polar than methanol was applied after the visible TFM was removed with the initial 10:90 clean-up. A mixture of 75:25 methanol:acetate buffer chosen. During this elution, additional TFM was visibly removed. The second approach adopted a different buffer system. A buffer with a pH above the pK_a of TFM (ca. 6.1) should maintain TFM dissociation and thus facilitate its visible elution from the cartridge. A 0.2 M pH 8 Tris buffer was chosen for this. Trials using this buffer combined with low proportions of methanol demonstrated removal of TFM only after relatively large elution volumes (>10mL). Increasing the proportion of methanol to 40:60 methanol:tris buffer showed visible TFM removal after a 9 mL elution. HPLC profiles of methanol fractions after both clean-ups demonstrated >99.9% removal of TFM. EROD activity in fish exposed to methanol fractions from both revised clean-ups demonstrated that induction was maintained with the pH 8 clean-up method while some induction was lost in the pH 4 clean-ups (Fig. 5).

Standardized SPE Clean-up

A standard protocol was adopted. The TFM formulation was added directly to a prewashed (1.5 tube volume methanol followed by 1.5 tube volume of tris buffer, pH 8) 0.5 g C_{18}-SPE. The cartridge was washed with 9.0 mL of 40:60 methanol:Tris buffer (pH 8) and the eluent, which contained the TFM, was discarded. The inducing chemicals were recovered from the SPE cartridge by eluting with 0.5 mL x 3 (1.5 mL total) of methanol.

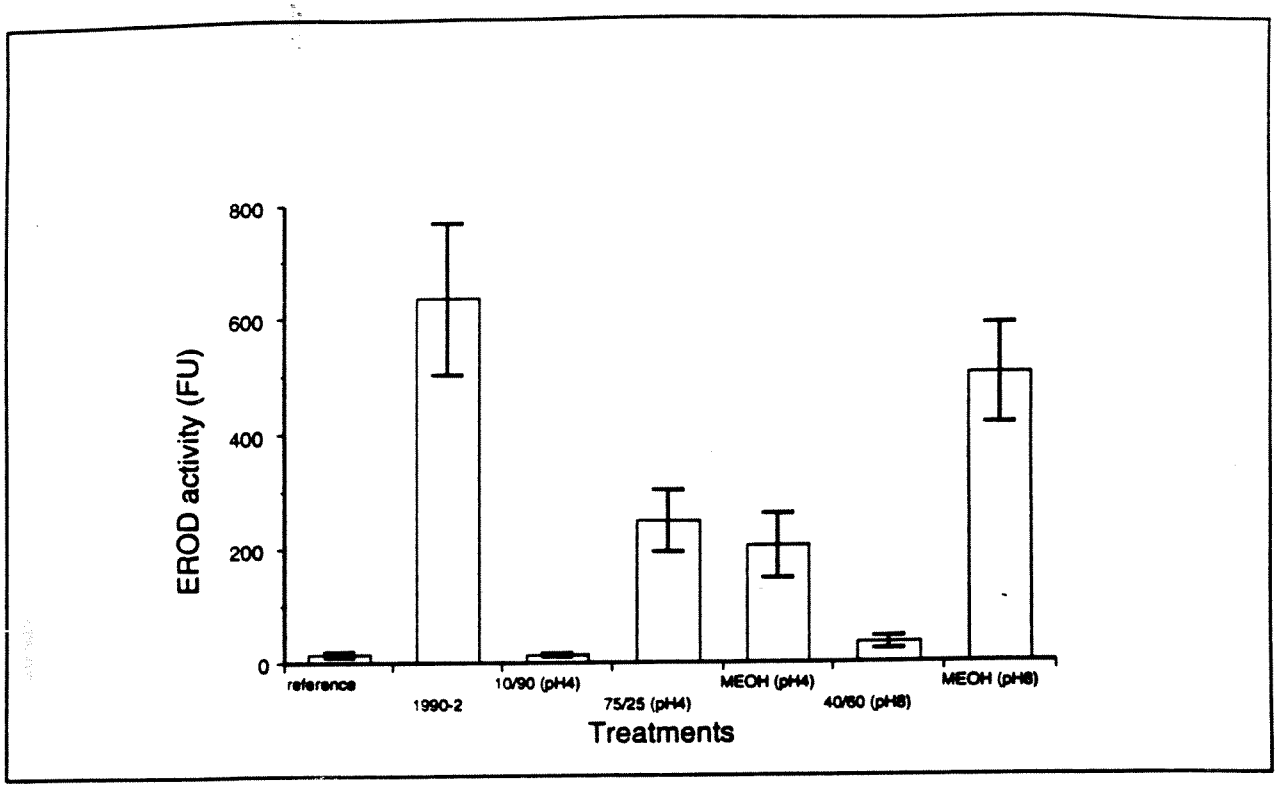


Figure 5. Rainbow trout hepatic EROD activity associated with SPE fractions: elution with 10:90 methanol:pH 4 buffer; 75:25 methanol:pH 4 buffer; 100% methanol after elution with methanol: pH 4 buffer; 60:40 methanol:pH 8 buffer; 100% methanol after elution with methanol:pH 8 buffer. The EROD activity is recovered in the 100% methanol after elution with methanol:pH 8 buffer fraction which also isolated the inducing chemicals from the TFM.

HPLC Fractionations

The next series of experiments utilized semi-preparative HPLC fractionation of the methanol soluble contaminants after the pH 8 SPE clean-up. The objective of these experiments was to isolate the inducing chemicals from the remaining formulation contaminants detected with this technique.

The approach taken was to collect the components of the mixture as they were separated by the HPLC and determine the MFO induction potential of each fraction. Inducing fractions could be broken down further until the limits of separation and detection of the system were reached. To maintain a consistent fraction identification system, HPLC fractions were numbered according

to the experiment in which they were derived. The first HPLC fractionation experiment (Experiment # 16) split the entire HPLC profile into three portions: the portion containing all peaks visible at 254 nm and the chromatographic segments before and after visible peaks. The methanol-SPE treatment involved exposure to the methanol fraction after pH 8 clean-up and prior to semi-preparative HPLC fractionation. The methanol-HPLC treatment was an exposure to the entire HPLC run collected from one equivalent of methanol soluble contaminants. Induction was recovered in the portion of the chromatograph containing the majority of the peaks visible at 254 nm, i.e., fraction 16-2 (Fig. 6).

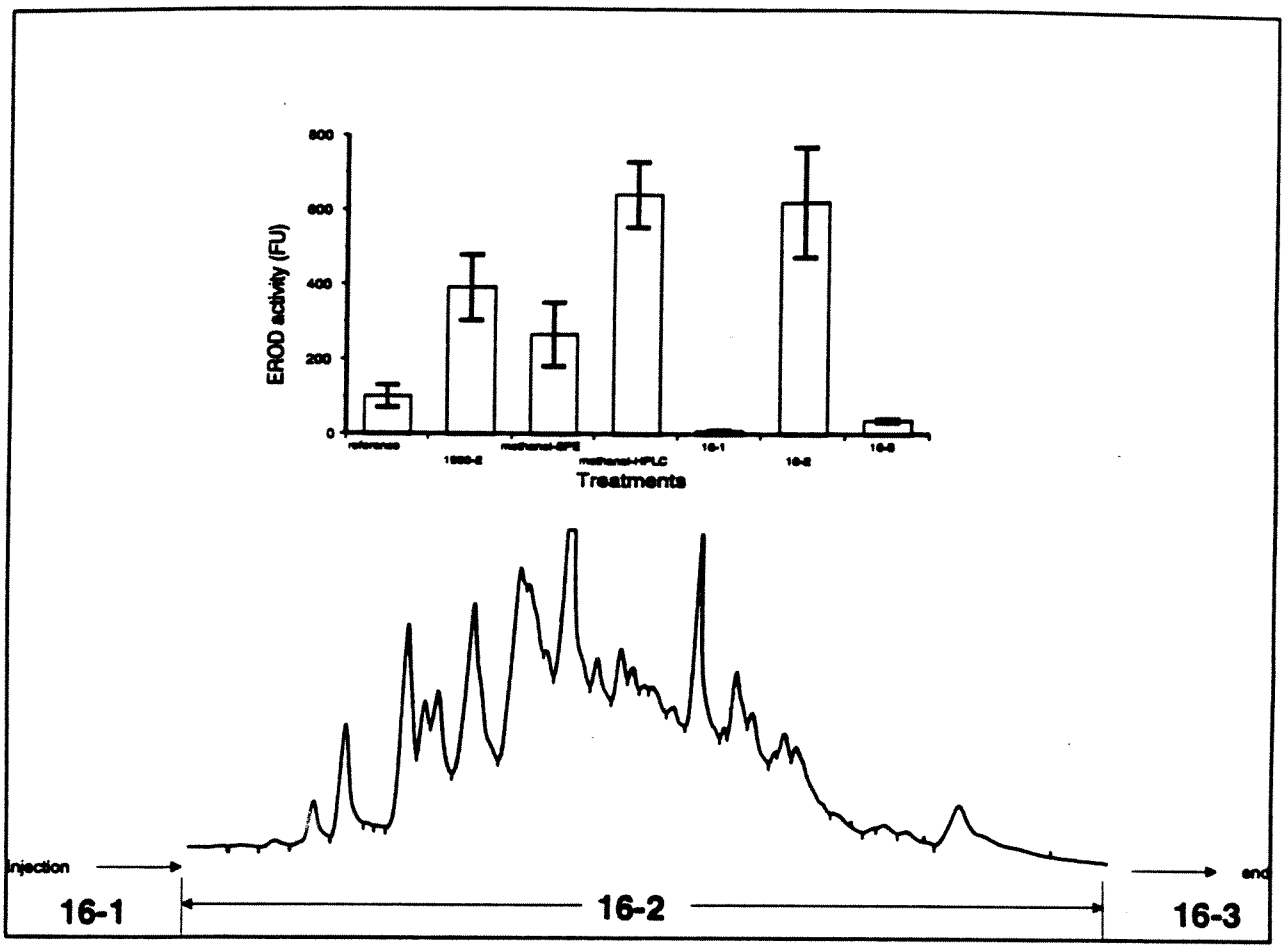


Figure 6. EROD induction from HPLC fractions (16-1,2,3) including recovery from the SPE and HPLC system and related HPLC chromatogram @254 nm.

The next fractionation split the visible peaks into two groups; the first group (17-1) were peaks unique to the pH 8 clean-up of the TFM formulation, the second group (17-2) contained peaks which were shared between the pH 8 and the pH 4 clean-ups (Fig. 7). The activities were compared to 16-2 and the results show that activity was recovered in 17-2. The next experiment fractionated 17-2 into three additional fractions, 18-1, 18-2 and 18-3 (Fig. 7). EROD activity in fish exposed to 18-1 and 18-2 were inconsistent in two trials of this experiment; in one series

there was induction associated with both 18-1 and 18-2 and in the other trial induction was confined to 18-2. In both series of exposures, no induction was associated with 18-3. It was hypothesized that an inducing compound could be eluting at the split between 18-1 and 18-2. In a subsequent trial (#19) 18-1 and 18-2 were fractionated into five fractions, 19-1 to 5 (Fig. 7). The exposures at higher concentrations (3x the 18-1&2) indicate that fractions 19-3 and 19-5 contained the inducing compounds.

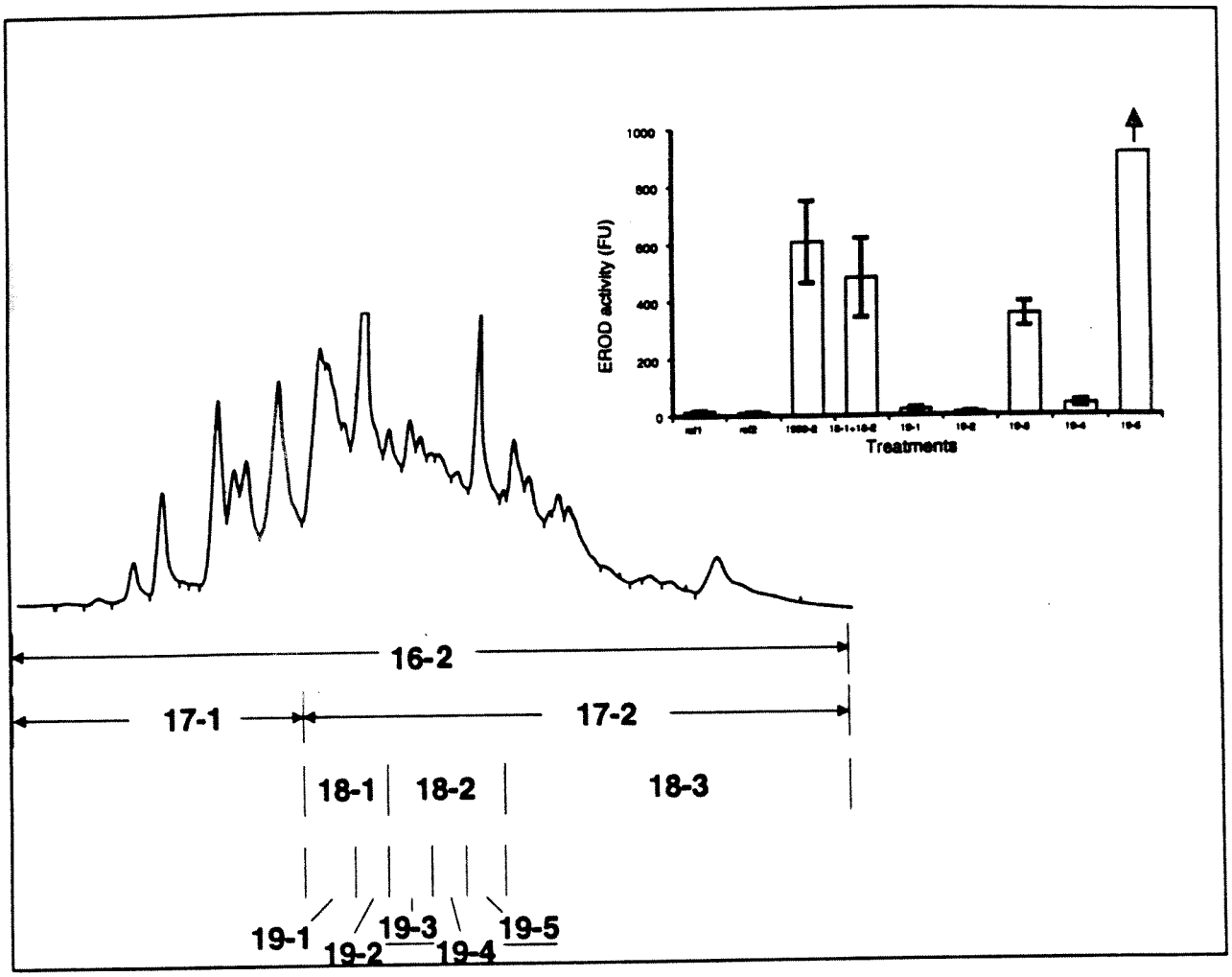


Figure 7. EROD activity associated with the HPLC fractions after SPE clean-up. Induction is isolated to fractions 19-3 and 19-5.

The same fractionation scheme was used to separate the inducing chemicals in the Kinetics 1993 formulation. The Kinetics formulation showed induction also occurred in the 19-3 and 19-5 fraction but induction was also associated with the 19-4 fraction (referred to in this trial as 36-1 to 5; Fig. 8). This indicates that there is an additional inducer in this fraction found in Kinetics formulations or it is present at concentrations above the threshold for induction in the Kinetics formulation but not the Hoechst formulations.

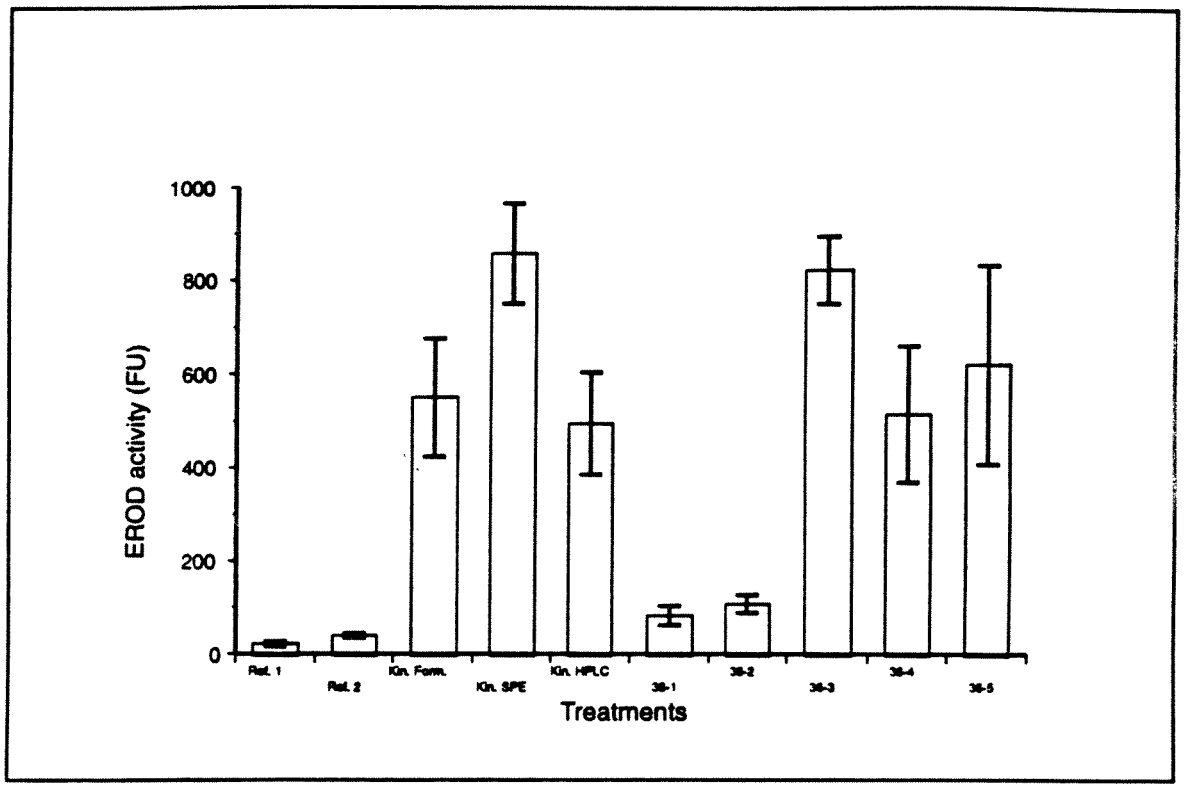


Figure 8. EROD induction in HPLC fractions generated from the Kinetics 1993 formulation. Induction occurs in fractions 36-3 and 5 as well as 36-4 (equivalent to 19-3, 4, 5).

Recovery in Toluene

To prepare SPE, HPLC fractions, etc. for analysis by gas chromatography (GC), toluene extractions were employed. To test whether toluene extractions would recover bioactive compounds, the methanol fraction after pH 8 SPE clean-up was directly reduced under nitrogen to dryness. The residue was then brought up in toluene, ultrasonicated, quantitatively removed and the insoluble residue was redissolved in methanol. The toluene soluble fraction was evaporated under nitrogen to dryness and redissolved in methanol. Both the toluene-soluble extract (now in methanol) and the toluene insoluble fractions were exposed to fish and the EROD activity assayed. The toluene extraction recovered the EROD activity and therefore validated that the bioactive compounds were in the final extract prepared for the gas chromatographic steps (Fig. 9).

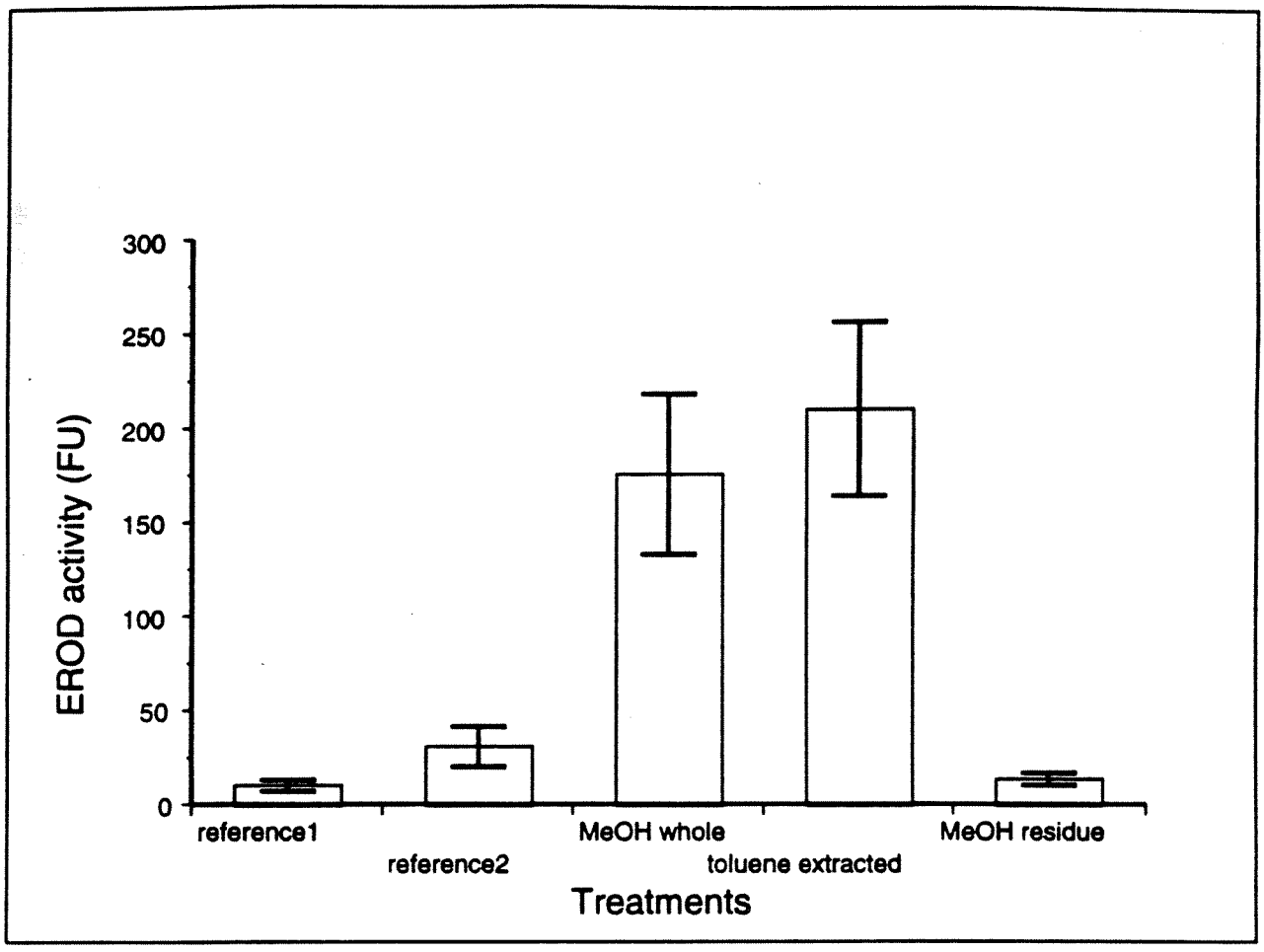


Figure 9. EROD activity in fish exposed to extracts after sample preparation for gas chromatography. Induction is recovered in the final toluene extracts thus insuring that the inducing chemicals are transferred to the gas chromatographs.

GC-MS of Fractions 19-3 and 19-5

Separate toluene extracts of 19-3 and 19-5 were prepared for analysis by gas chromatography-mass spectrometry (GC-MS). Full scan GC-MS analyses revealed that each of the 19-3 and 19-5 extracts contained one unidentified major component and several other minor unidentified components. Searches of spectral libraries produced matches of low probability. GC-MS analyses of the crude SPE toluene soluble extracts contained several minor components and 4 major components. Based on chromatographic retention times and mass spectra, two of the major components were the same unknowns found in ether extracts of fractions 19-3 and 19-5. The other two components were tentatively identified as 4-chlorophenol and 3-nitro-4-chlorophenol. Spectral interpretation of these unknowns was aided by the presence of the chlorophenol which could combine with TFM to produce a diphenyl ether; tentatively identified as the major component in fraction 19-5 (Fig. 10). The major 19-3 unknown was tentatively identified as the diphenyl ether of the condensation of TFM with itself (Fig. 10). The molecular formulas of 19-3 and 19-5 were confirmed by high resolution mass spectrometry.

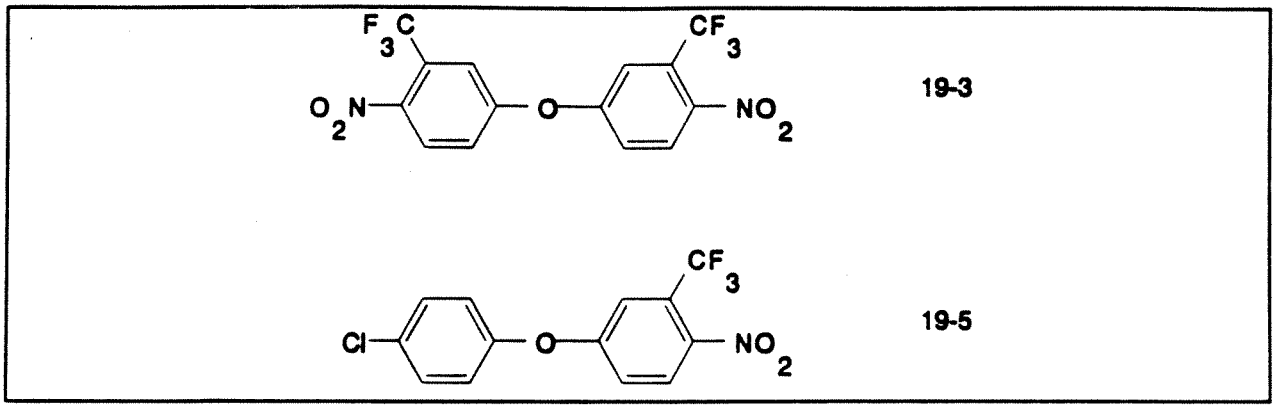


Figure 10. Structure of the two major components of fraction 19-3 and 19-5.

The recovery of induction in the toluene soluble portion of the Hoescht 1990-2 clean-up was further evidence that the major unknowns in 19-3 and 19-5 were potential inducers. Syntheses of the proposed compounds was undertaken for chemical and toxicological verification (Fig. 11).

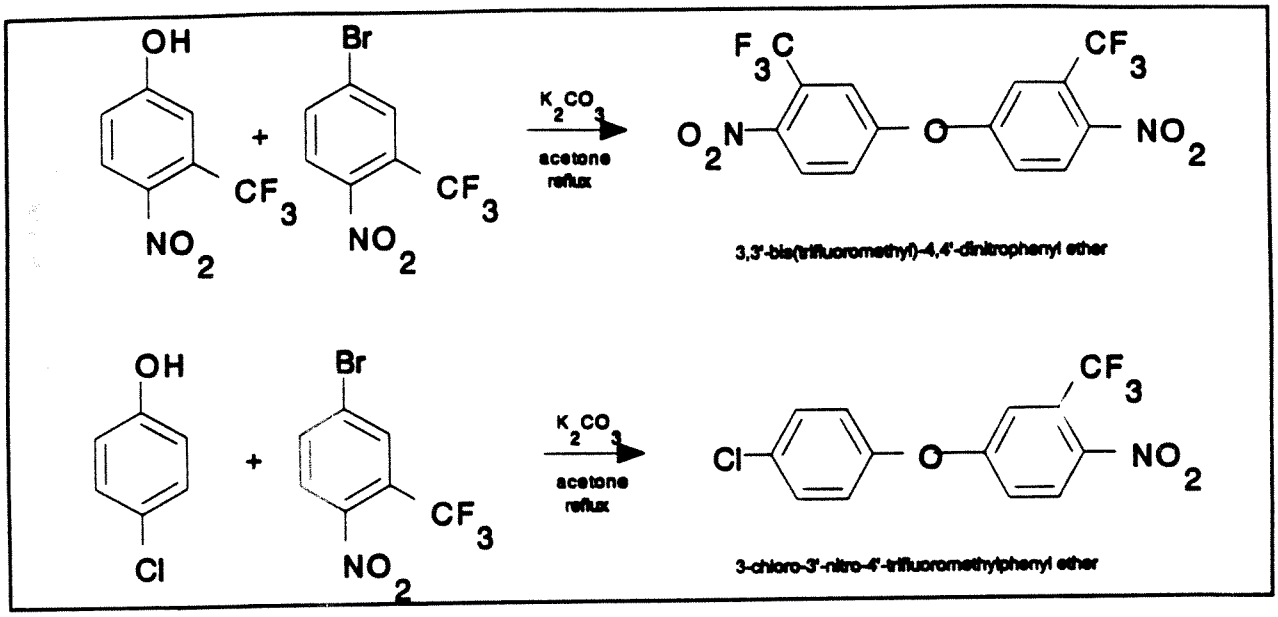


Figure 11. Synthesis pathway for the two diphenyl ethers identified in 19-3 and 19-5.

The products from both syntheses were purified by HPLC where the retention times of product peaks matched those found in the TFM contaminant fractionations. Aliquots of each product were collected, concentrated and analyzed by GC-MS. Confirmation was obtained when these materials exhibited both the same GC retention time and mass spectra as the unknowns in 19-3 and 19-5. Using response factors from the HPLC UV detector, enough of each product was collected to expose fish to several concentrations. After exposures to several concentrations of the synthesized materials it was apparent that although they were present in the formulation, these chemicals were not responsible for the observed induction (Fig. 12).

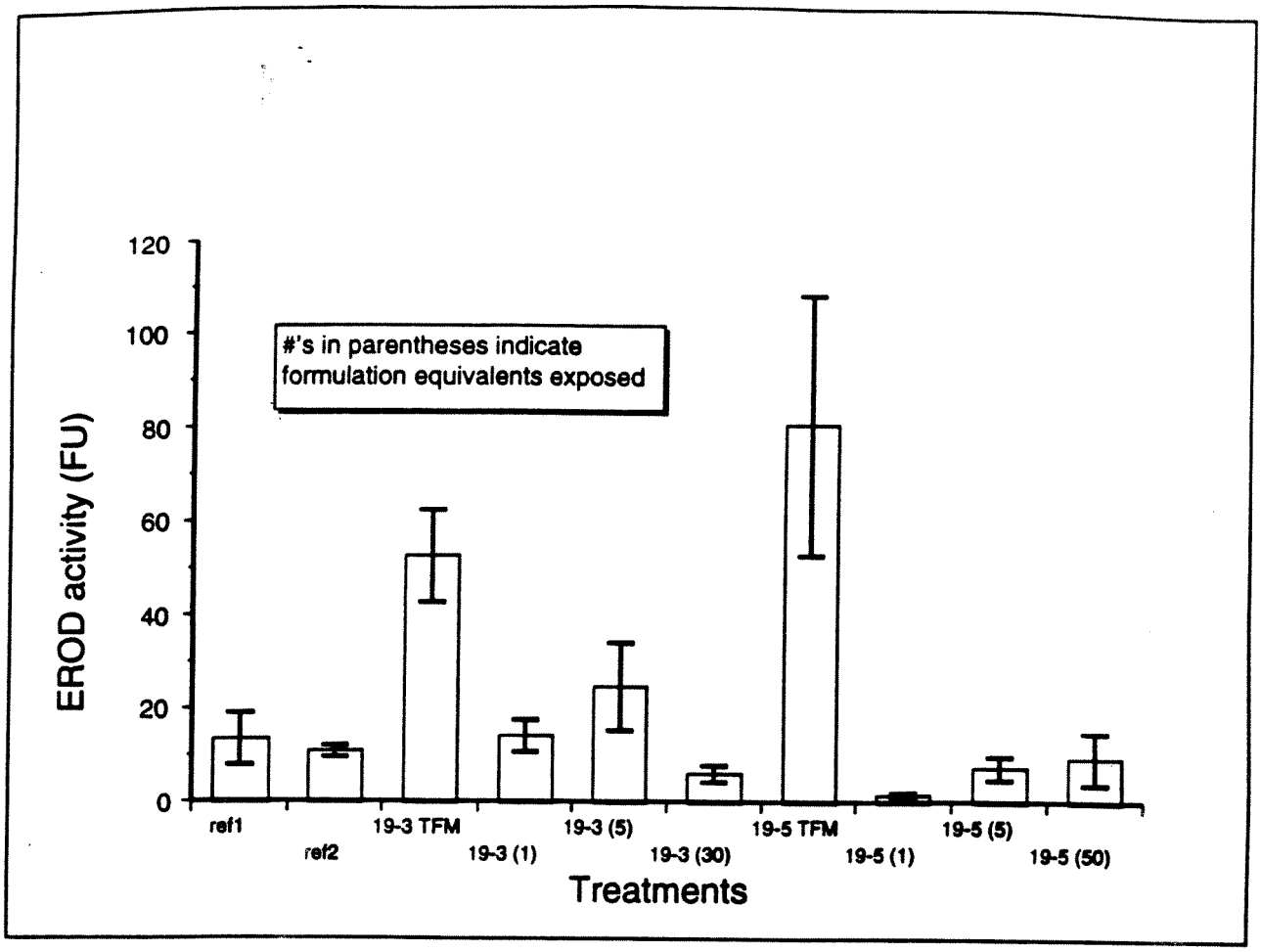


Figure 12. EROD activity of diphenyl ethers identified in 19-3 and 19-5 at 1, 5 or 50 times the equivalent concentration of the associated fractions. Neither chemical caused significant induction at even 50 times the equivalent concentration found in the TFM formulation.

Reinjection of bioactive fraction on HPLC

With the major components in both 19-3 and 19-5 eliminated as inducers, the inducing chemicals within fractions 19-3 and 19-5 remained unidentified. At this point, the approach taken was to collect and concentrate both bioactive fractions and reinject them on the HPLC to see if further separations of the components within these fractions were possible. Further separations were achieved by reinjection under modified HPLC conditions.

19-3 HPLC Fractionation Conditions

Column:
reverse phase Partisil 10 ODS 2 column (500mm x 9.4mm ID)

Solvent Conditions:

10:90 methanol: acetate buffer (pH 4, 0.2M) held for 2 min
linear gradient to 70:30 at 21 min
linear gradient to 100% methanol at 55 min
hold 100% methanol for 15 min
flow 4 mL/min
UV @ 254nm

Under these conditions the separations presented in Figure 13 are achieved. Fish were exposed to the fractions collected as with previous experiments. EROD activity was finally isolated into fraction 30-3.

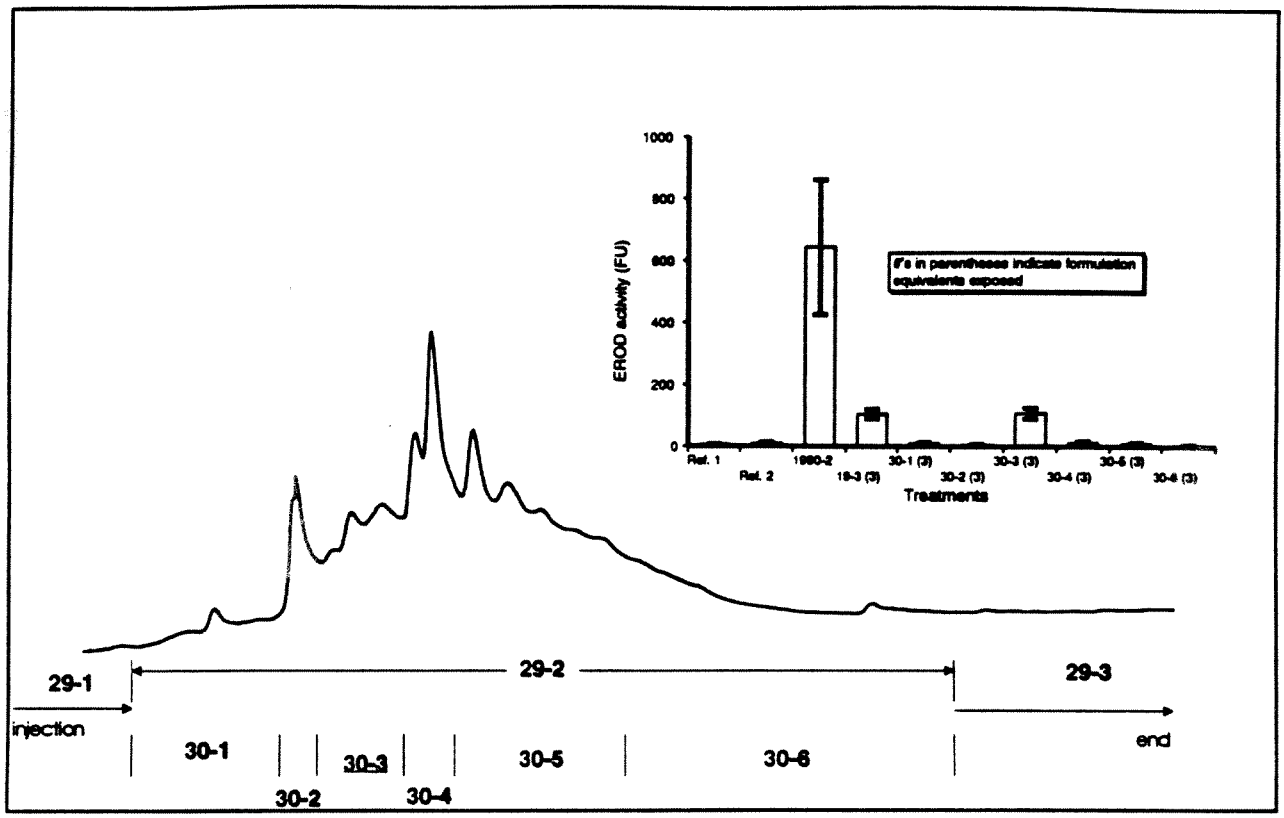


Figure 13. EROD induction of fractions from 19-3 after reinjection on the HPLC under different mobile phase conditions. Induction is isolated to fraction 30-3.

19-5 Fractionation Conditions

Column:
reverse phase Partisil 10 ODS 2 column (500mm x 9.4mm ID)
Solvent Conditions:
10:90 methanol: acetate buffer (pH 4, 0.2M) held for 2 min
linear gradient to 70:30 at 21 min
linear gradient to 100% methanol at 65 min
hold 100% methanol for 15 min
flow 4 mL/min
UV @ 254nm

Under these conditions the separations presented below are achieved and the fractions illustrated were exposed to fish (Fig. 14). The corresponding EROD activities associated with the final fractions are also presented. Since most EROD activity was associated with fraction 31-2, this fraction was further fractionated (Fig. 14). Subsequent fish exposures to these fractions revealed that most of the induction was associated with fraction 34-2. The majority of the induction associated with 19-5 and therefore the formulation as a whole, had now been isolated to this fraction.

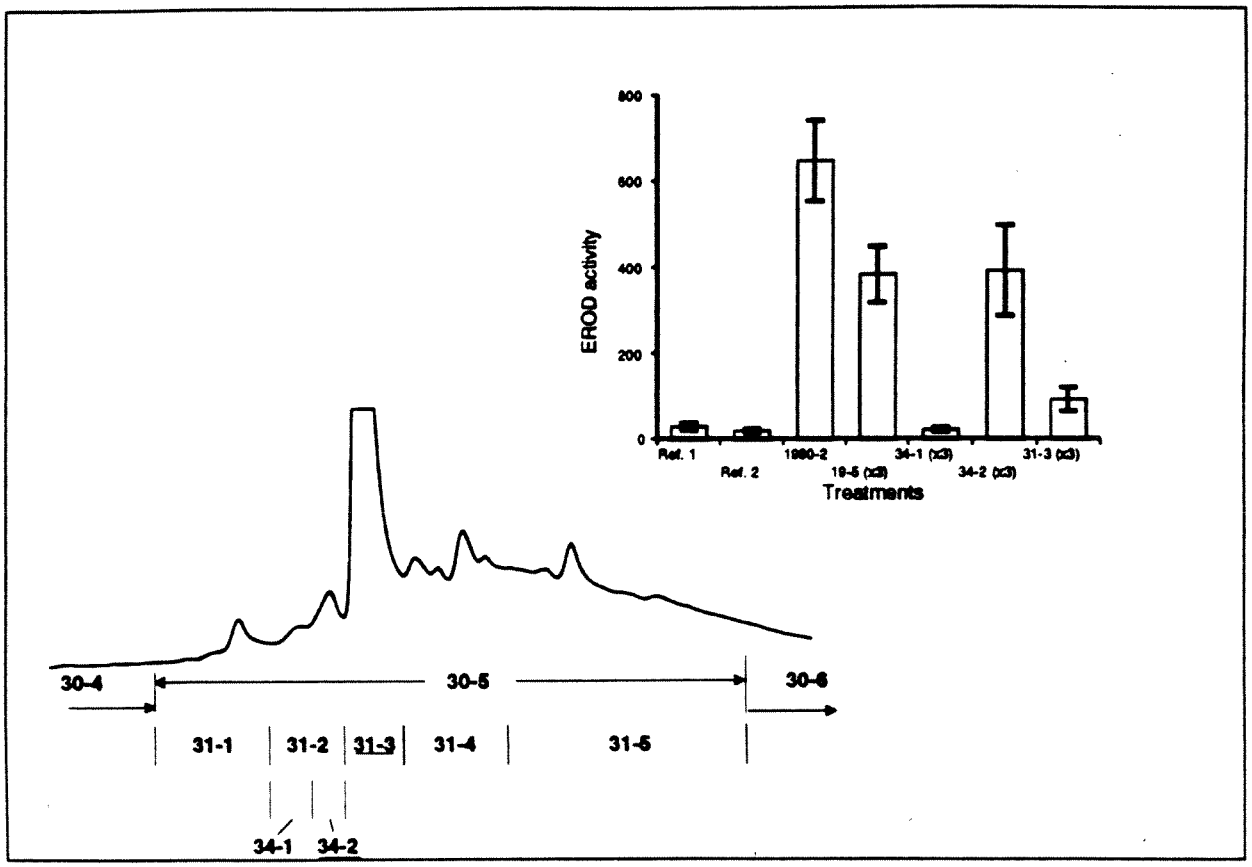
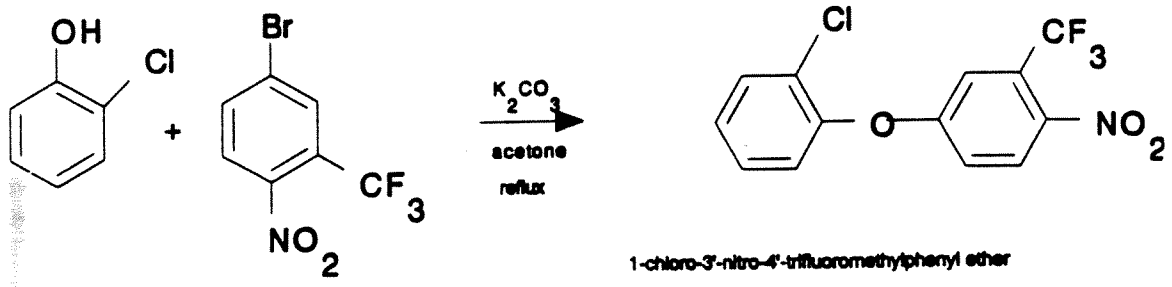


Figure 14. EROD induction of fractions from 19-5 after reinjection on the HPLC under different mobile phase conditions. Induction is isolated to fraction 34-2.

High Resolution GC-MS of reinjected bioactive fractions (30-3, 31-3 and 34-2)

High resolution mass spectrometry was initially run full scan on each of the fractions but little else was detectable with the exception of a moderately significant peak in fraction 31-3. This compound had the same mass spectra as the 3-chloro-3'nitro-4'-trifluoromethylphenyl ether previously synthesized and found within this fraction, but this compound eluted earlier by GC. A structure with the chlorine atom para to the ether linkage was proposed for this compound and synthesis using a similar route utilized previously, was performed. The product was purified by HPLC and using a GC-ECD response factor, fish exposures were performed at several relative concentrations. The synthetic route and structure are outlined below (Fig. 15).



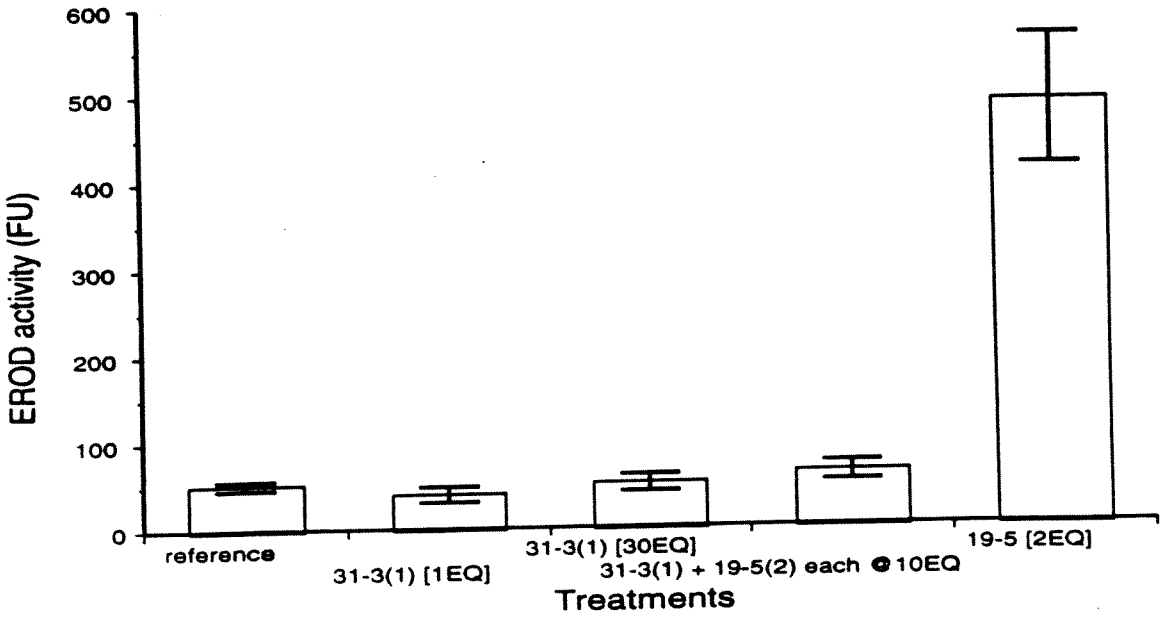


Figure 15. Synthesis pathway and EROD induction of 1-chloro-3'-nitro-4' -trifluoromethyldiphenyl ether.

Due to the reduced sensitivity resulting from acquiring mass spectral data by full scan and to the apparently low levels of inducing compounds present in these fractions, fraction extracts were concentrated in an attempt to detect the responsible chemicals. Extracts had to be concentrated to the point where approximately one formulation equivalent of each fraction was injected for each analysis. Fraction profiles were compared to a method blank and a highly concentrated toluene-soluble SPE extract which had been previously shown to recover all formulation induction. Total ion chromatograms (TICs) for these analyses follow (Fig. 16-20).

Each chromatogram was examined closely to identify constituents that were potential inducers; i.e. unknowns present in fractions and in the toluene extract but not in the blank. Mass spectra for several unknowns were acquired from these analyses (Fig. 21-27). Two more diphenyl ether derivatives were tentatively identified, each in fractions 30-3 and 31-3 (Fig. 28). The molecular formulae and several ion formulae of each were confirmed by scanning for the exact mass of these ions at 10,000 resolution. These compounds remain to be synthesized for complete chemical confirmation. However, based on the results with the phenyl ethers synthesized and exposed to date, these materials are unlikely to cause induction.

After highly concentrating fraction 34-2, the most potently inducing fraction, a single unknown was detected which was unique to this fraction. The mass spectra for this unknown is shown in Fig. 27) and was tentatively identified as 2-chloro-7-trifluoromethyl-8-nitrodibenzo-p-dioxin. Ion fragmentations and isotopic abundances are consistent with these substituents, which are expected, owing to the phenyl ethers previously confirmed. This structure is also not unreasonable as it is also the dioxin analogue of the major phenyl ether previously confirmed in fraction 19-5 and it is also analogous to the hydroxylated phenyl ether tentatively identified in 30-3. The molecular formula and several ion formulae of this unknown were confirmed by high resolution mass spectrometry (see below). This unknown also elutes just behind trichlorodioxin, which would be expected because the nitro group is more polar and would extend GC retention time. We are presently investigating means to have this material synthesized for us for chemical and toxicological confirmation.

Figure 16. Total ion chromatograph of the blank.

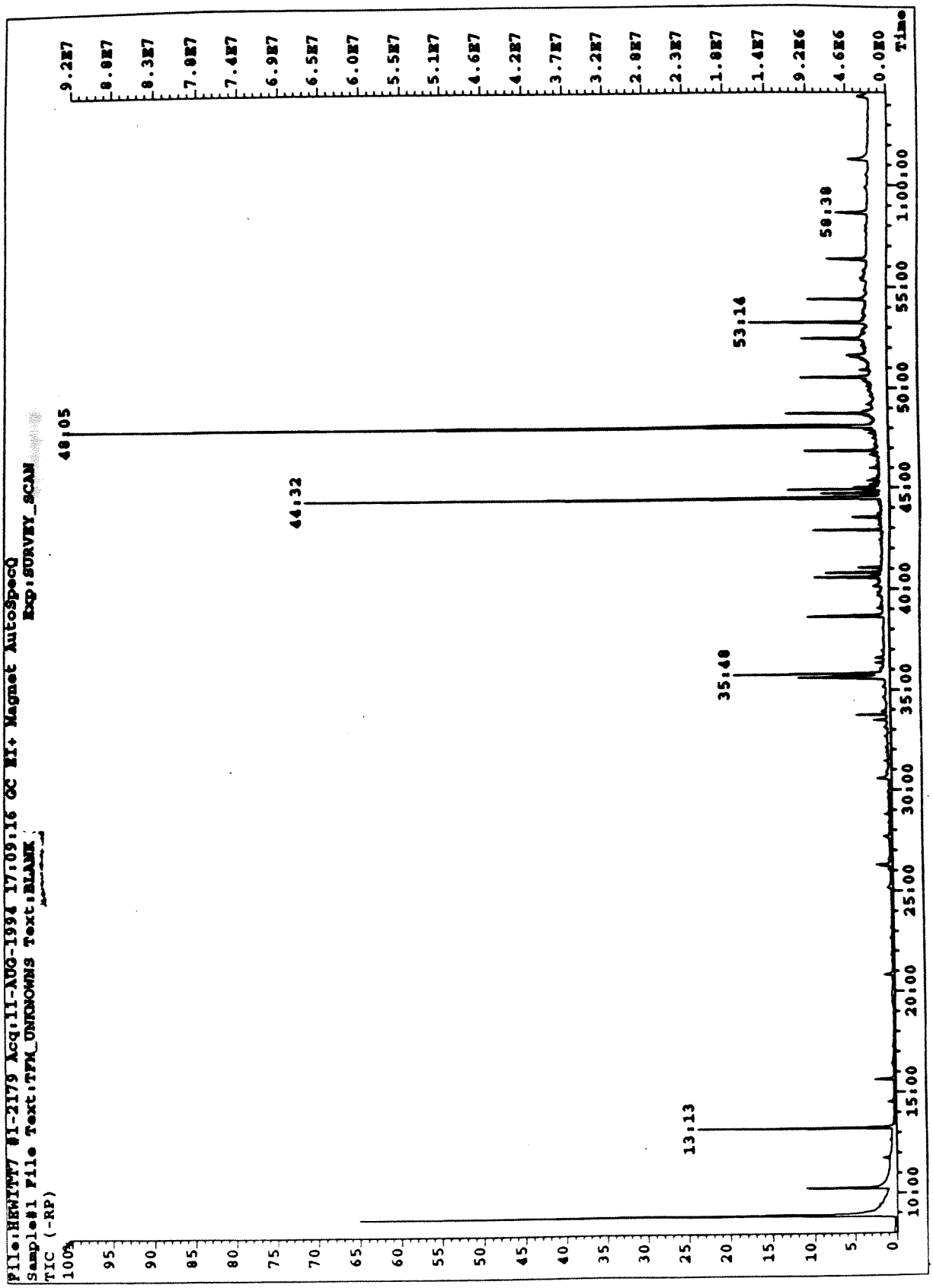


Figure 17. Total ion chromatograph of fraction 30-3.

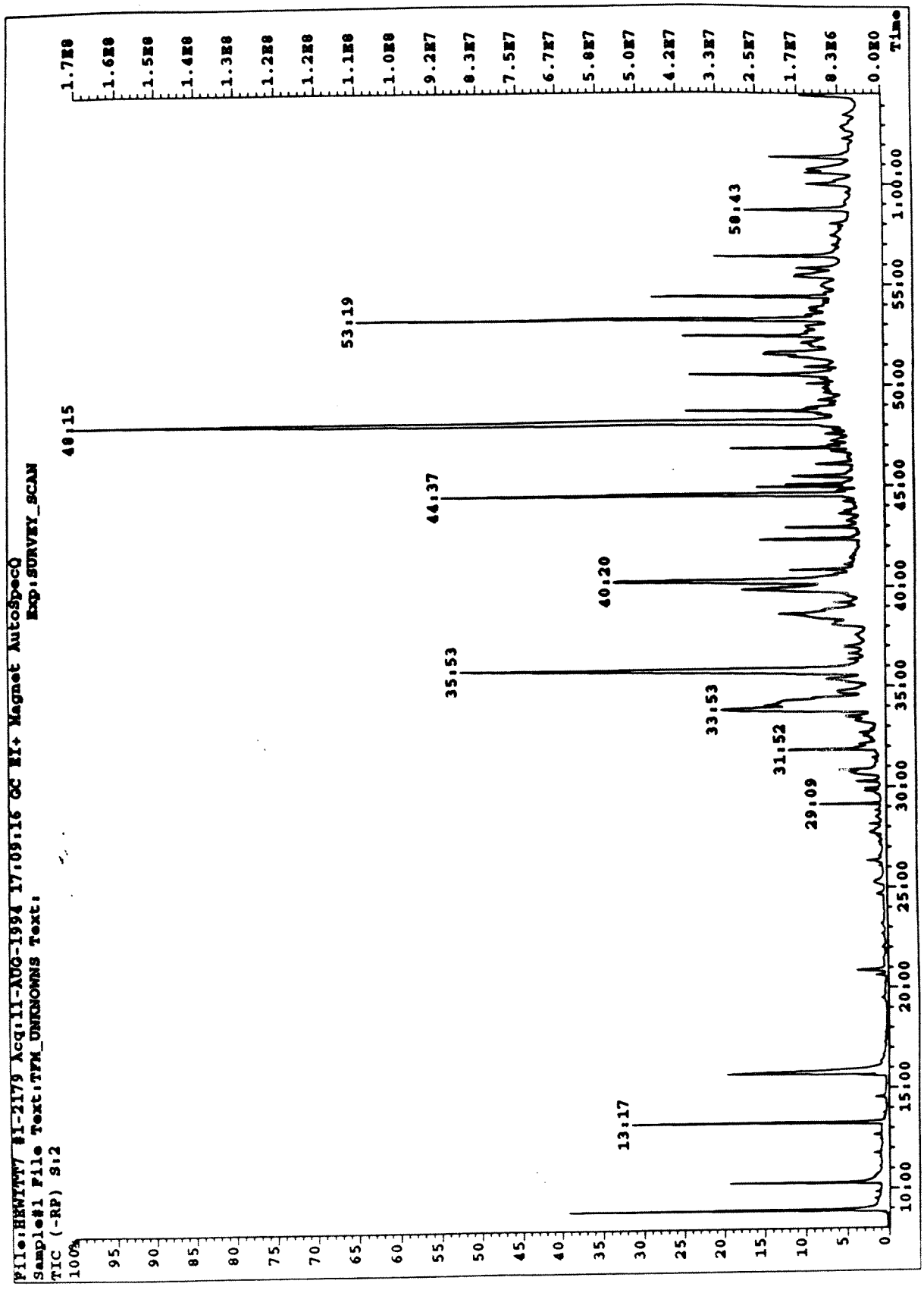


Figure 18. Total ion chromatograph of fraction 31-3.

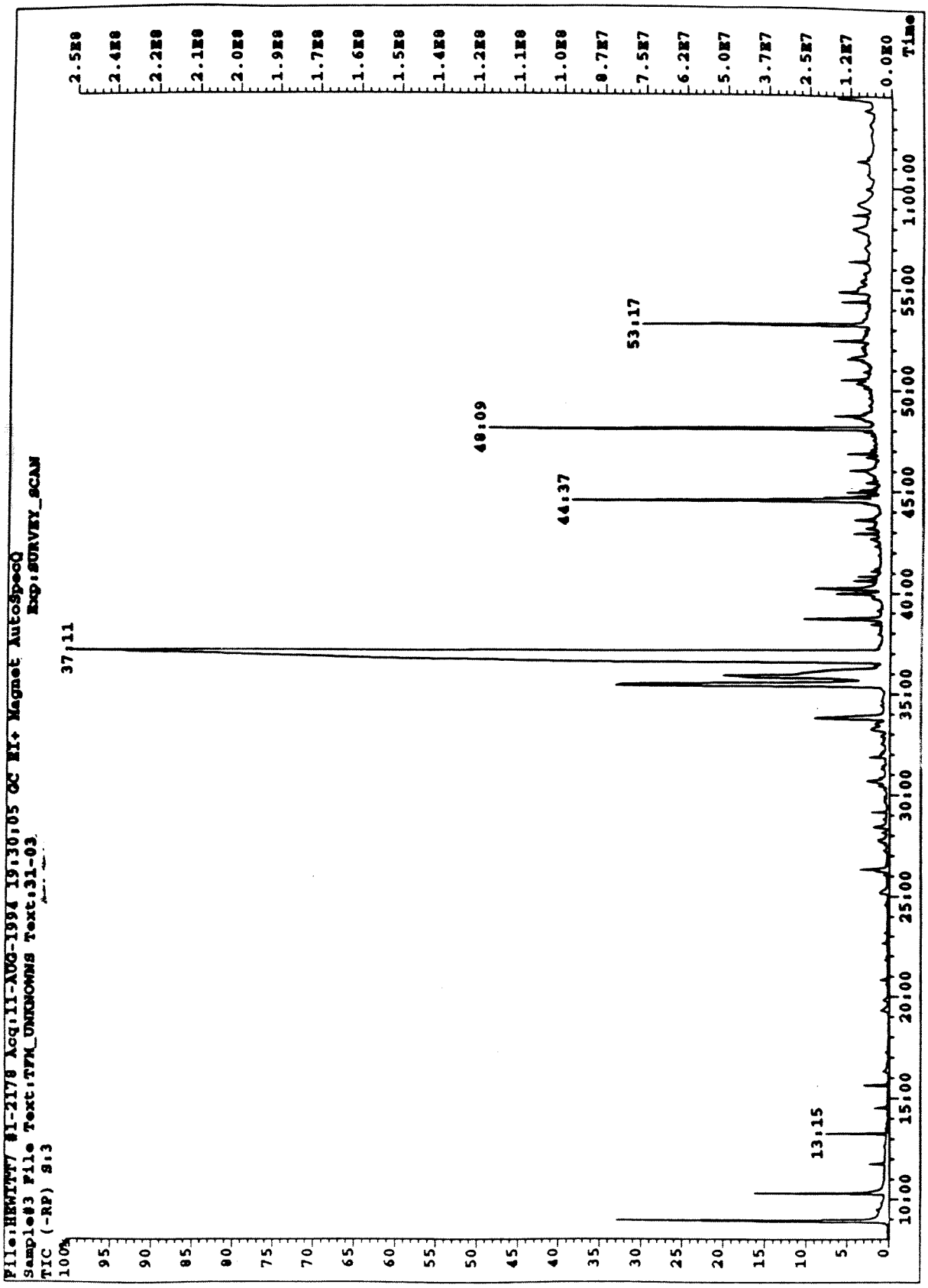


Figure 19. Total ion chromatograph of fraction 34-02

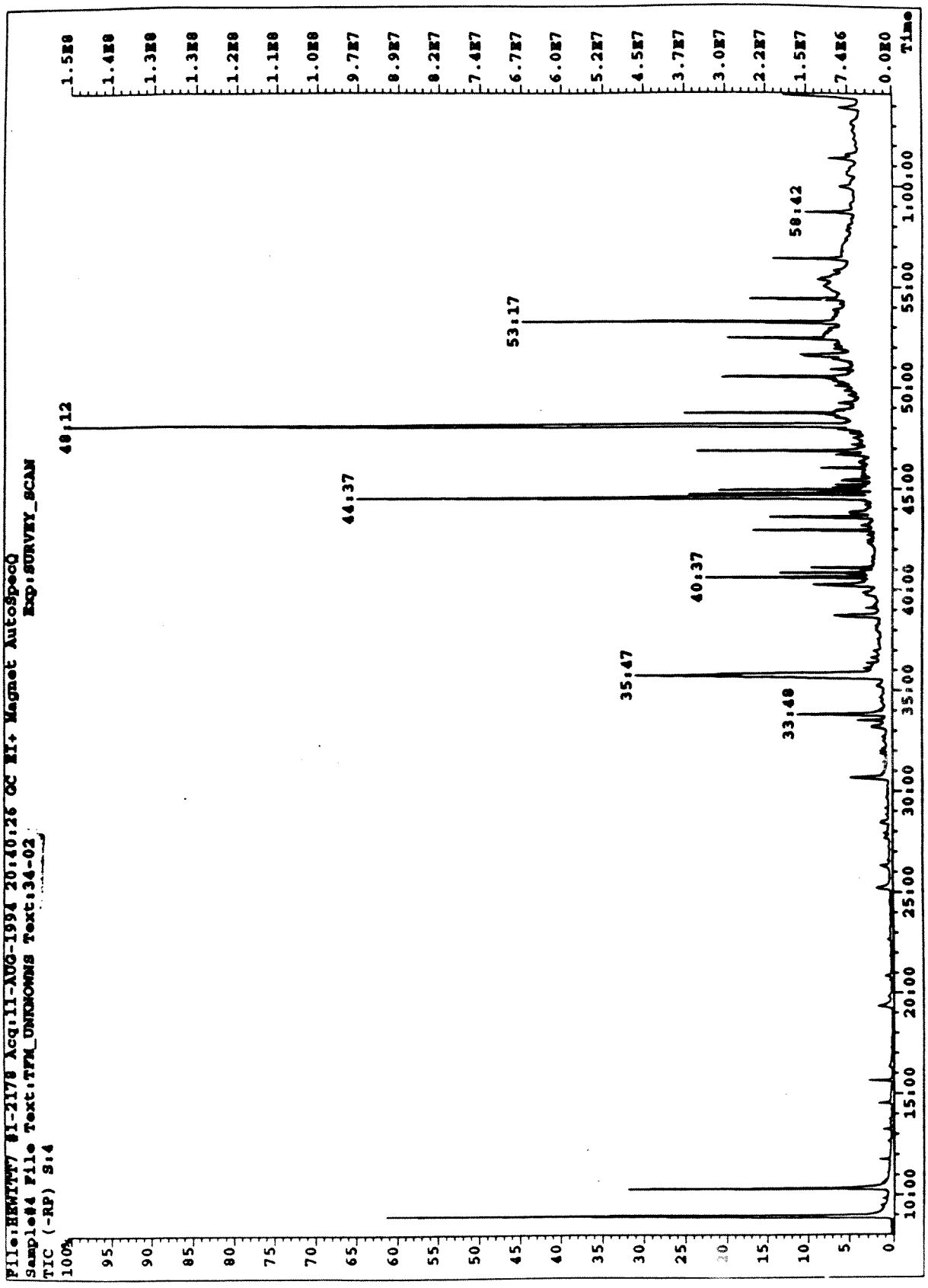


Figure 20. Total ion chromatograph of the toluene extract.

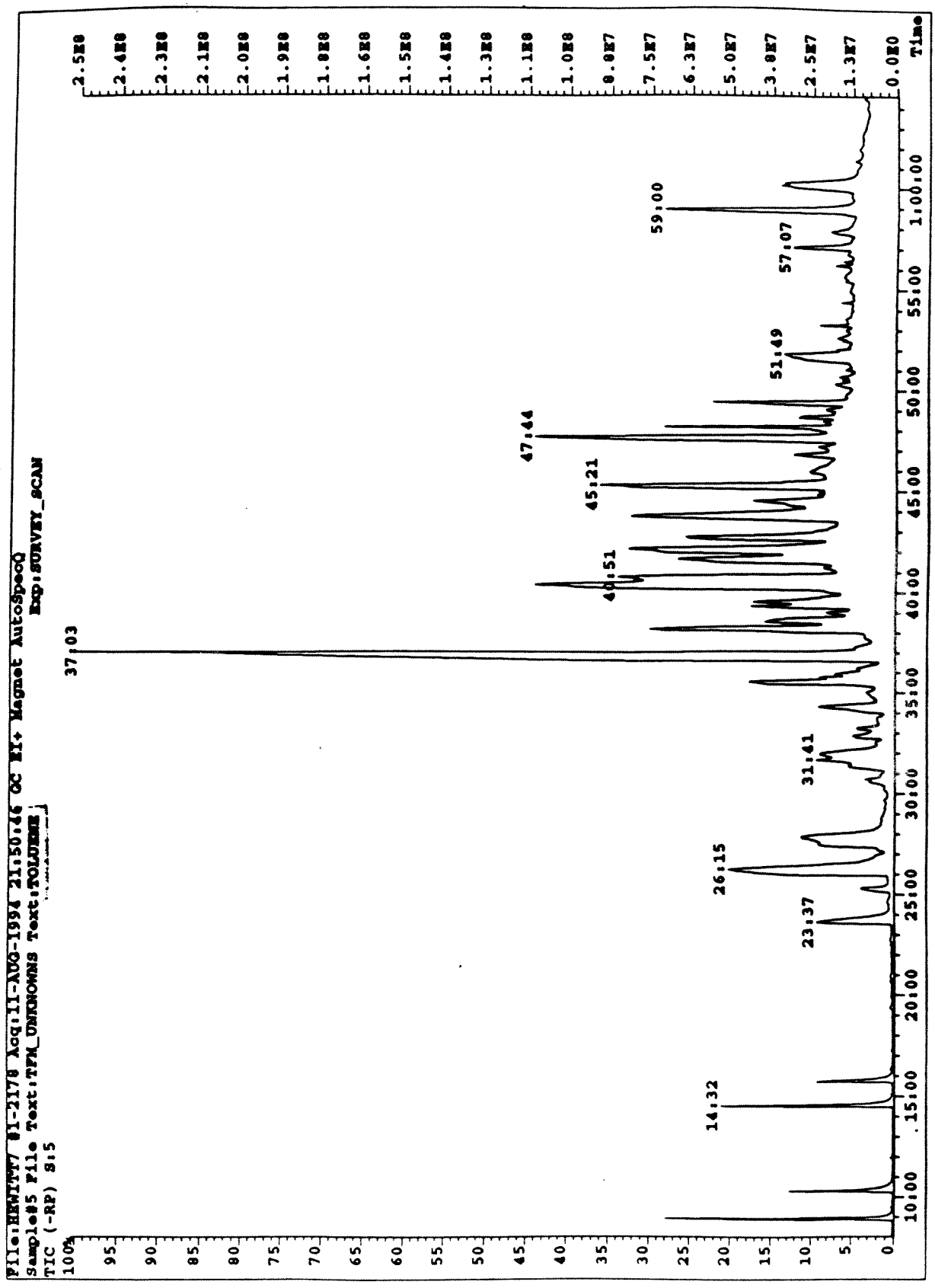


Figure 21. Mass spectra of unknown in 31.3.

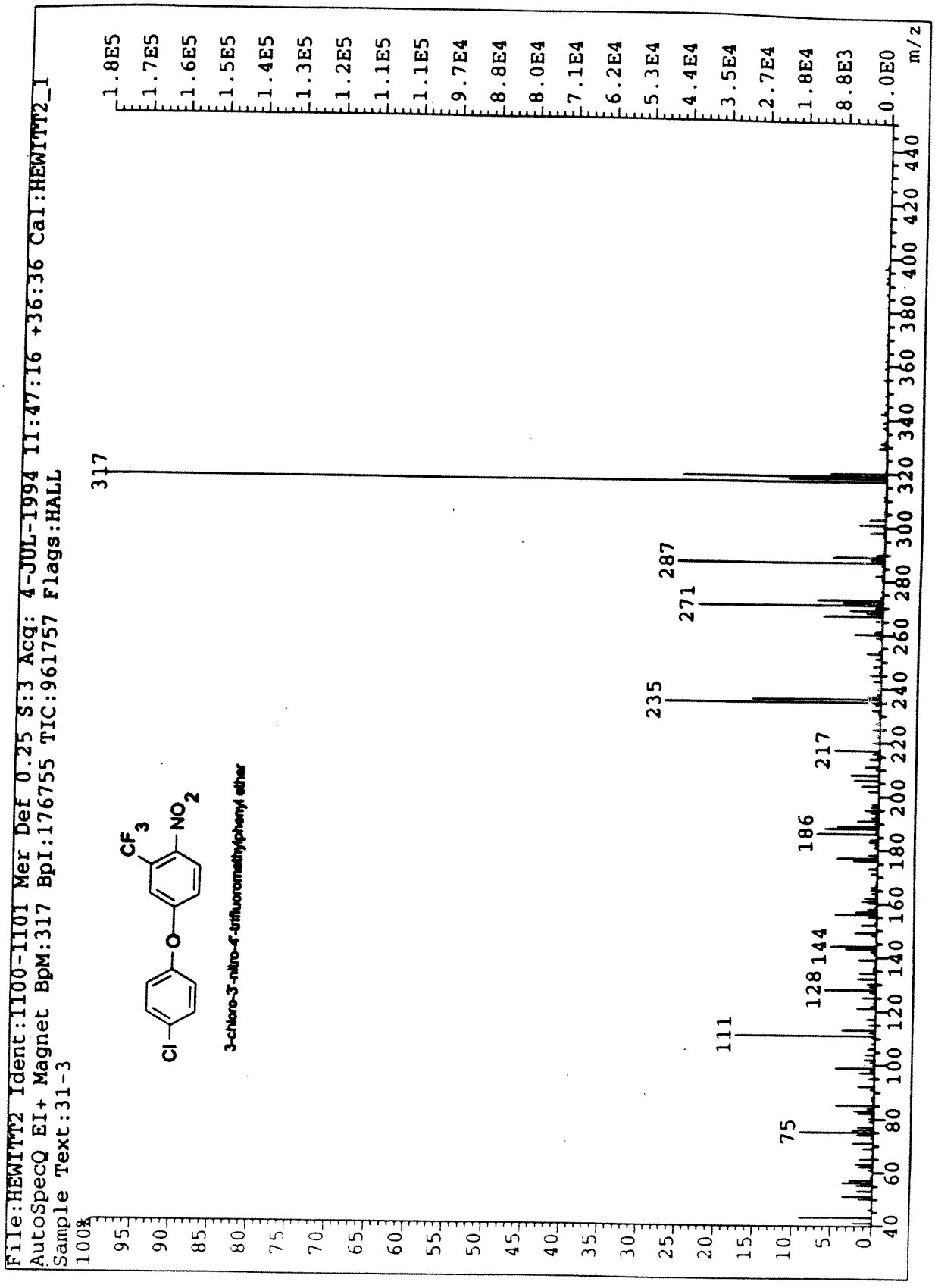


Figure 22. Mass spectra of unknown in 31.3.

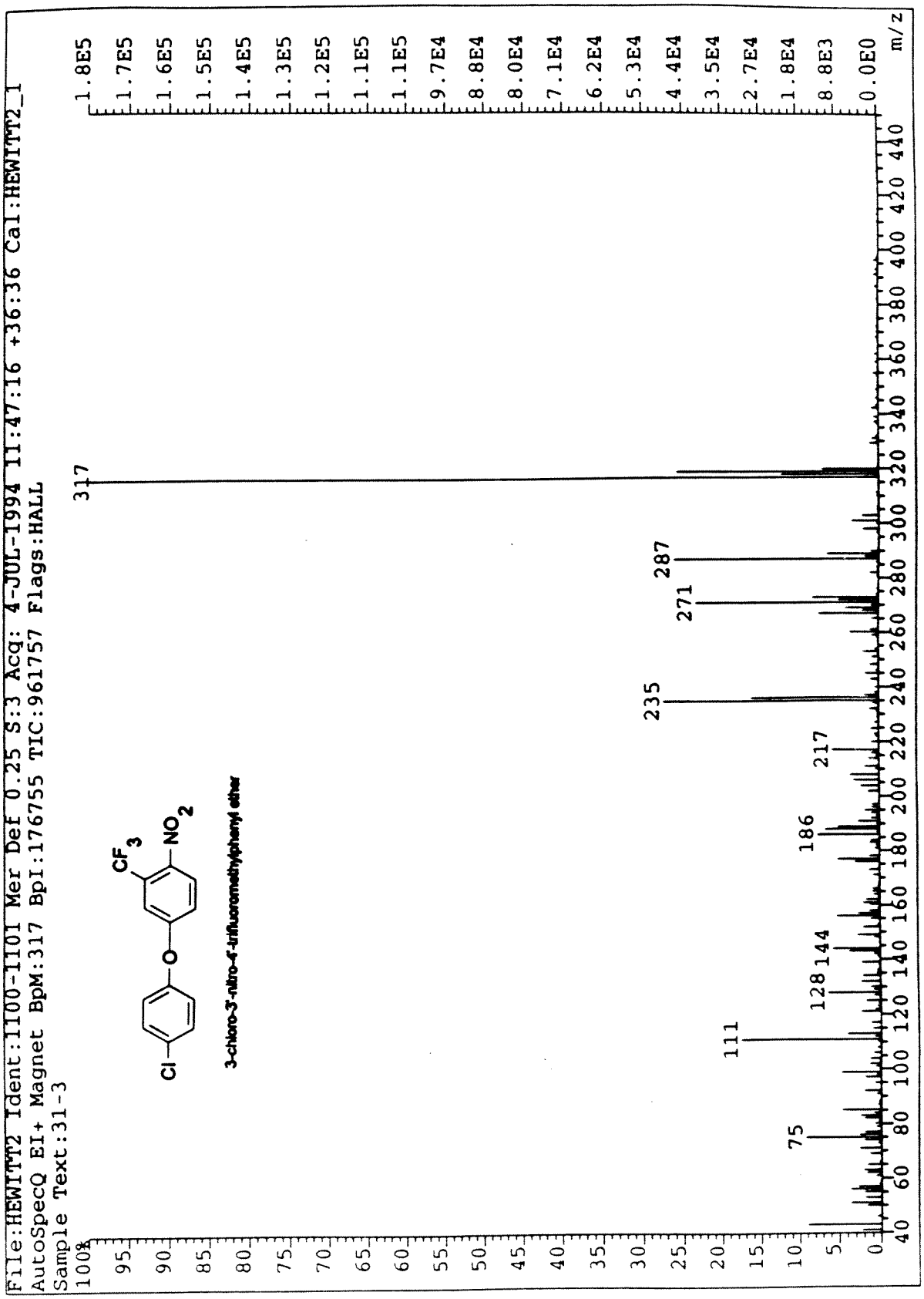


Figure 23. Mass spectra of unknown in 31.3.

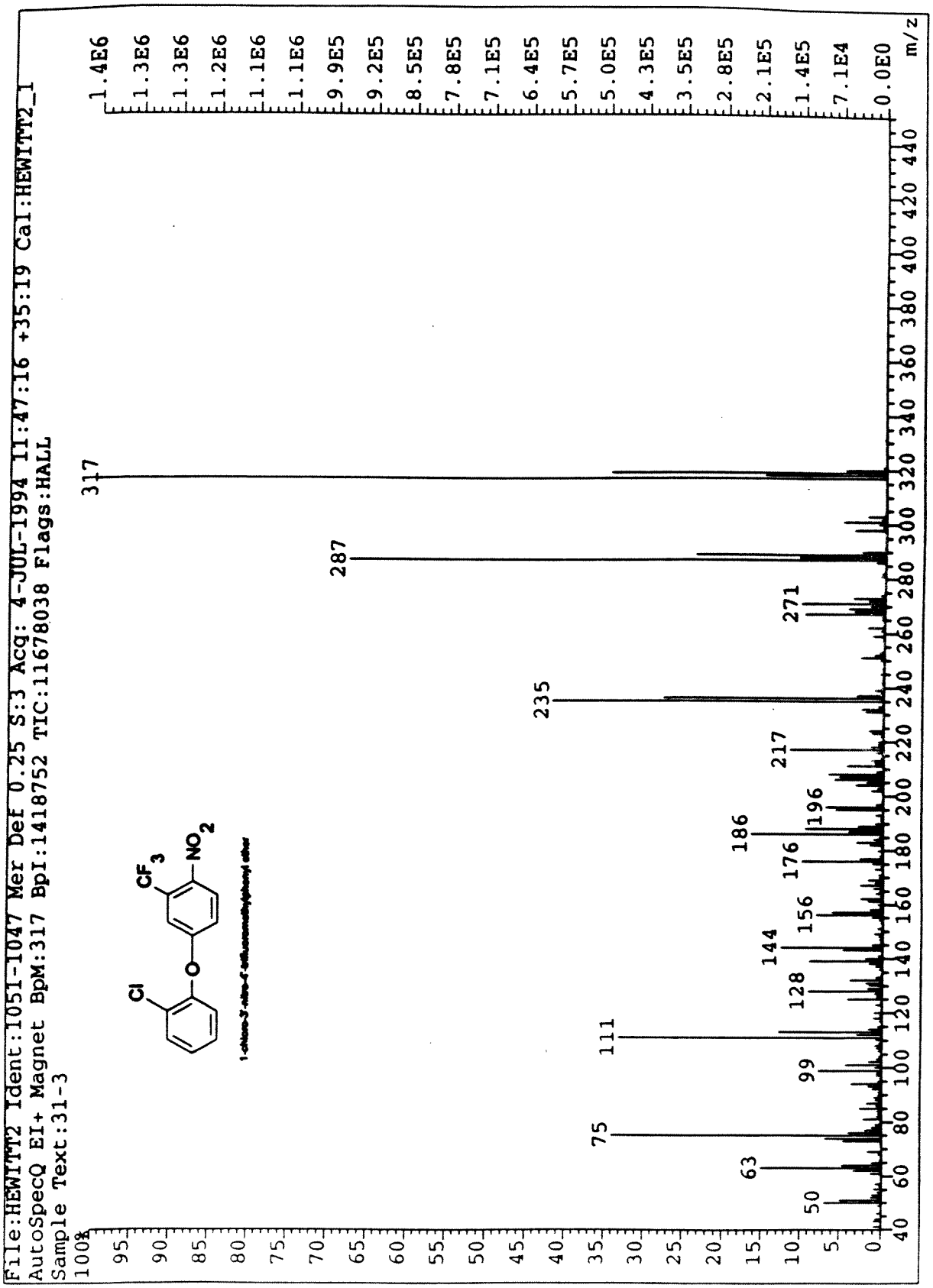


Figure 24. Mass spectra of unkown in toluene extract.

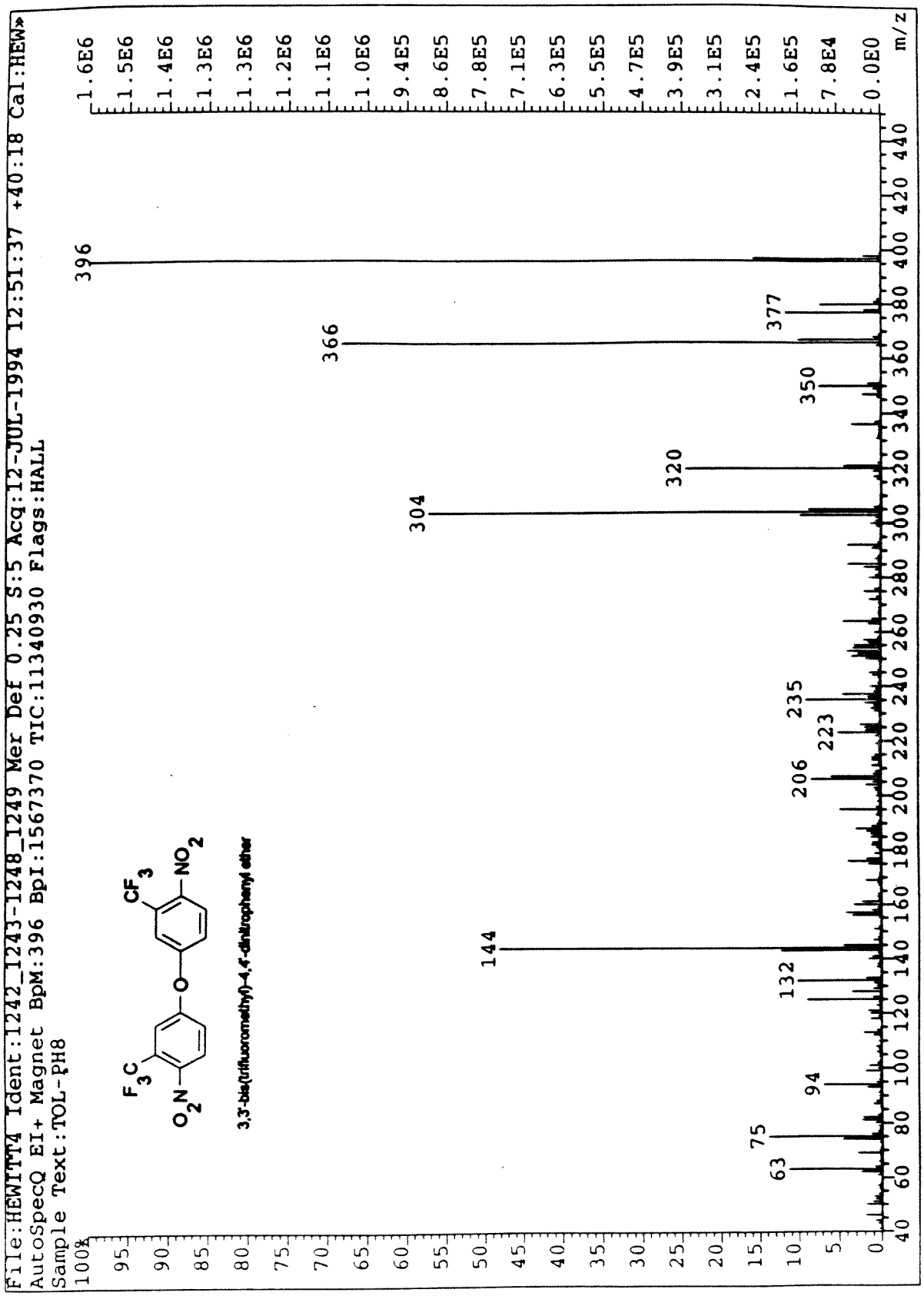


Figure 25. Mass spectra of unkown in fraction 30-3.

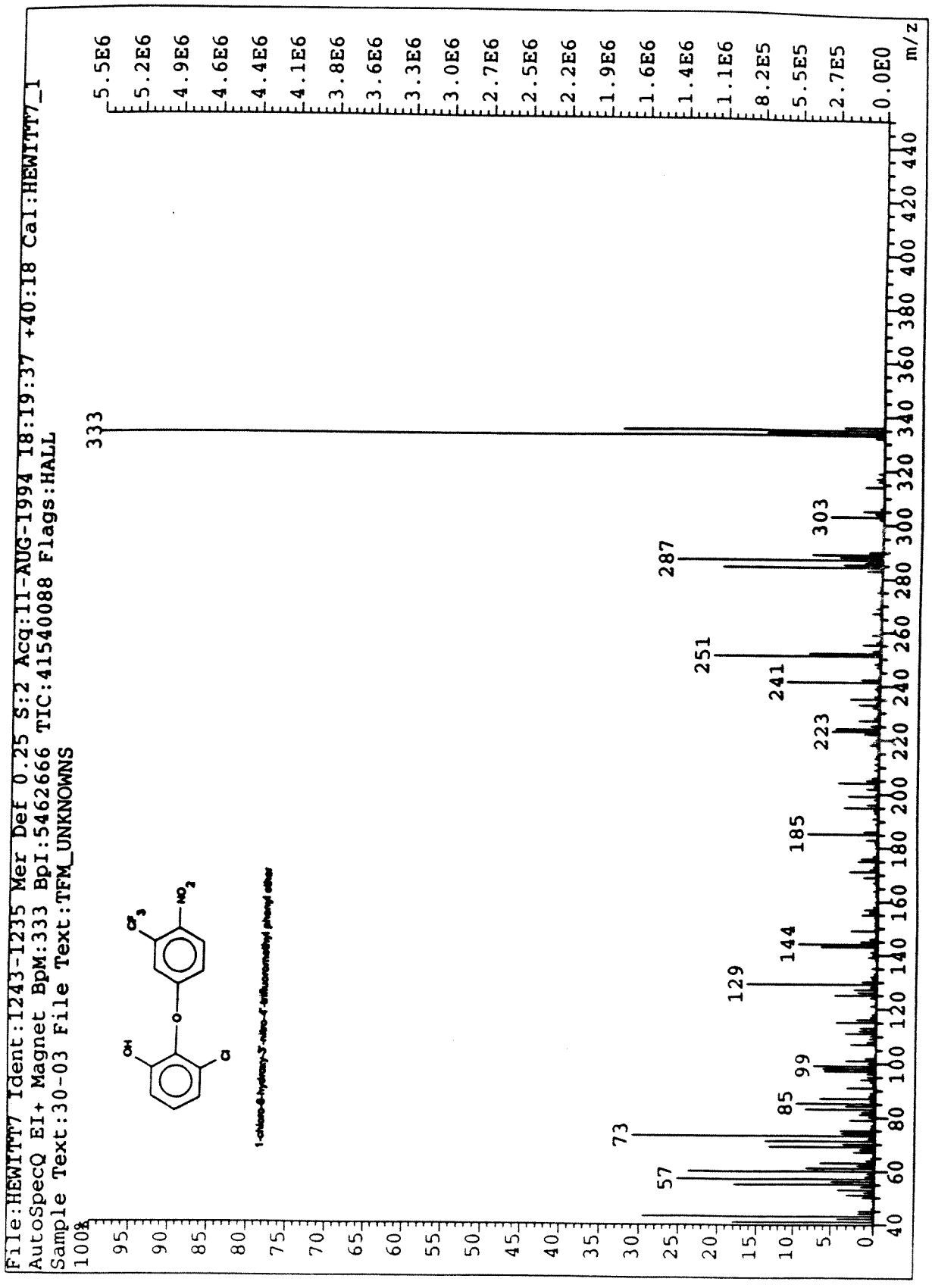


Figure 26. Mass spectra of unkown in fraction 30-3.

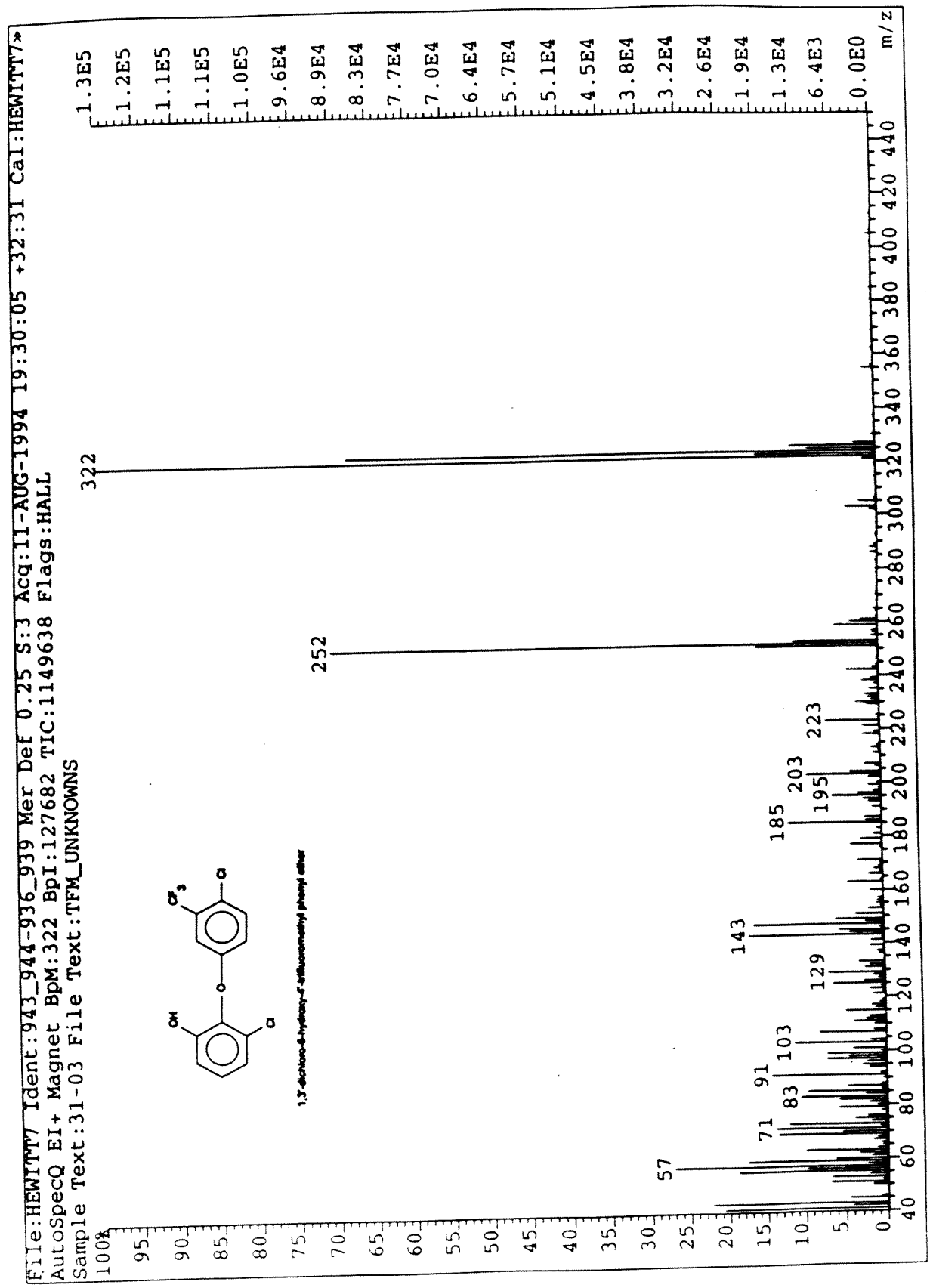
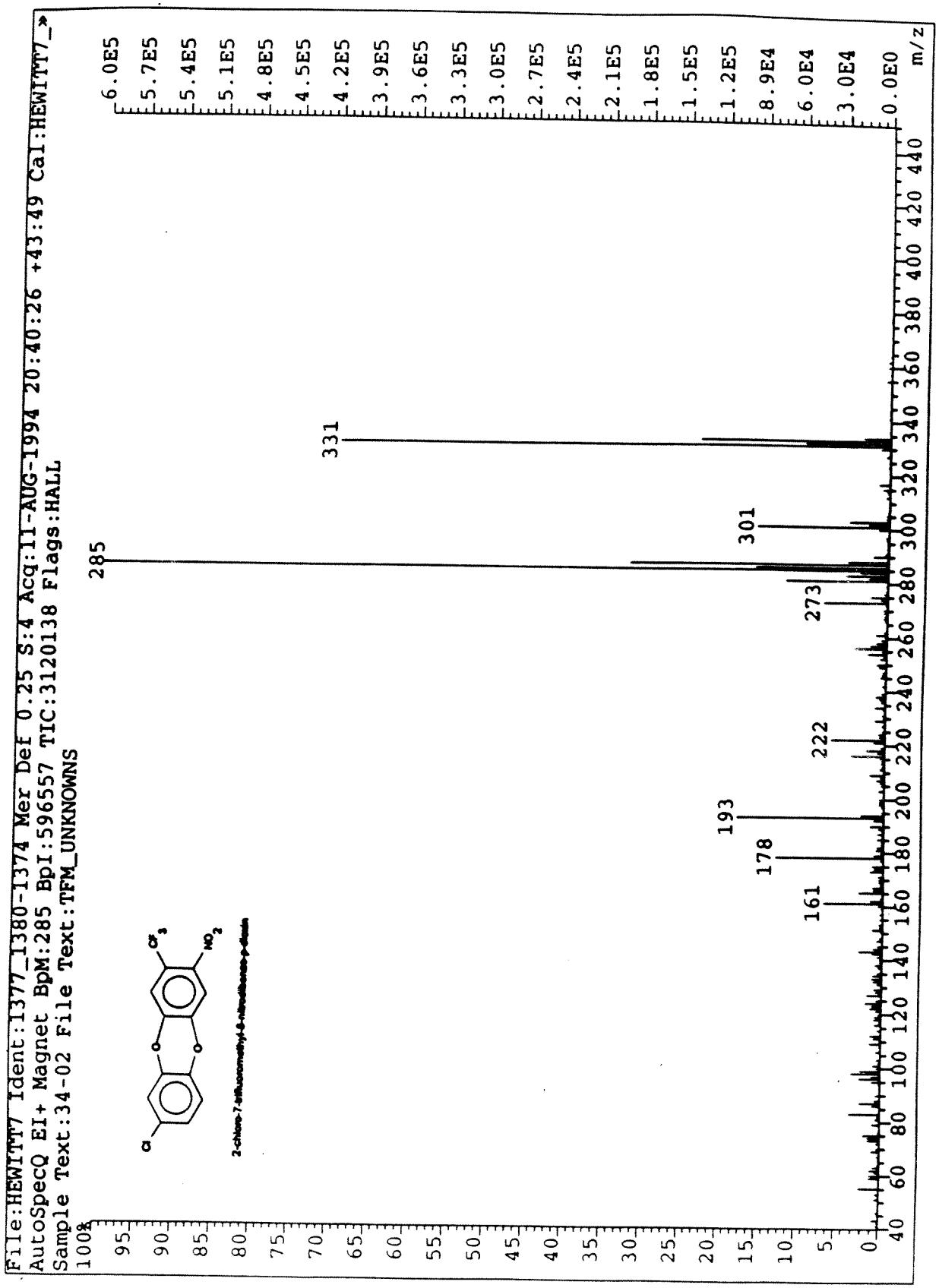
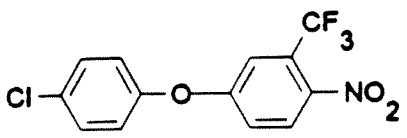


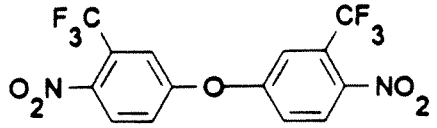
Figure 27. Mass spectra of unknown in fraction 34-02.



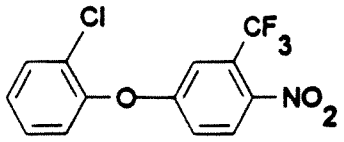
CONFIRMED BY SYNTHESIS



3-chloro-3'-nitro-4'-trifluoromethylphenyl ether

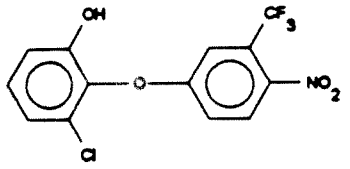


3,3'-bis(trifluoromethyl)-4,4'-dinitrophenyl ether

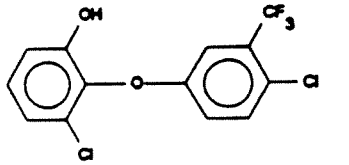


1-chloro-3'-nitro-4'-trifluoromethylphenyl ether

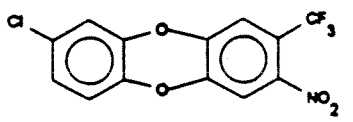
MOLECULAR FORMULAE CONFIRMATION



1-chloro-6-hydroxy-3'-nitro-4'-trifluoromethyl phenyl ether



1,3'-dichloro-6-hydroxy-4'-trifluoromethyl phenyl ether



2-chloro-7-trifluoromethyl-8-nitrodibenzo-p-dioxin

Figure 28. Summary of compounds found in bioactive fraction of TFM formulations.

CONCLUSIONS

1. Reliable methods were developed to isolate the bioactive fractions from the TFM component.

2. There are multiple inducers in each formulation and there may be an additional and different inducers in the Kinetics formulations.

3. The inducers behave as base-neutral, i.e. are not readily ionizable, and can therefore be easily separated from the TFM.

4. The log K_{ow} of the inducing chemicals is between 3.0 and 4.0 based on the retention times of standard compounds.

5. Several diphenyl ethers are found in the bioactive fraction but are not responsible for the observed MFO induction.

6. Several chemical were tentatively identified in the bioactive fractions including 2-chloro-7-trifuromethyl-8-nitro-dibenzo-*p*-dioxin. A number of nitro and trifluoromethyl substituted dioxins are expected to be present in the formulations.

7. Formulation supplied by two manufactures in 1994 resulted in significant MFO induction despite significant effort by the manufacturer to purify the product. All TFM formulations to date have the potential to cause MFO induction in fish.

The results imply that the responsible chemicals are present in extremely low concentrations and are more potent inducers than was previously thought. The task is complicated by their being multiple inducers, numerous contaminants and extremely low concentrations in the formulations. It is now known that there are at least three formulation contaminants that cause induction in the Hoescht formulation. Tests with the Kinetics formulations have indicated that there may be additional chemicals responsible for the induction in these formulations.

While the toxicity of the TFM field formulation is strongly influenced by pH, the inducing chemical(s) in the H1990-2 formulations are not strongly ionizable and therefore influenced by pH. This fact was used to separate the TFM from the inducing chemicals to allow an increase in the exposure concentration. This property could potentially be exploited to remove the inducing chemicals from the commercial formulation.

The inducing chemical(s) will have a very different environmental fate and persistence from TFM. Identification of the inducing chemical(s) in the TFM formulation is necessary before the temporal or spatial distribution of the chemical(s) and therefore the MFO and steroid response can be predicted. The rates of disappearance downstream reported during a lampricide treatment was chemical dependent and several of the contaminants detected in TFM formulations

previously are more persistent than TFM itself, specifically hydroxynitrobenzoic acid and nitrochlorobenzotrifluoride (Carey et al. 1988). Treated streams and their estuarine areas could therefore be exposed to the treatment-related chemical(s) for much longer periods of time than the duration of the lampricide treatment. This could have the potential to disrupt fish behaviour, especially if treatment took place in the fall or early spring when prespawning fish were staging around stream mouths.

Exposure to the TFM formulation resulted in alteration of testosterone profiles in goldfish, suggesting that the formulation also has the potential to disrupt hormonal control (Munkittrick et al. 1994a). These results clearly show the need for further reproductive evaluations. Since MFO induction lasts at least 4 d after exposure, steroidal disruptions may be present for at least this long after exposure subsides. The biological significance of these disruptions would relate to the duration and magnitude of exposure, as well as the life history characteristics of the fish exposed, and the timing of the exposure relative to the critical period in reproductive development. Techniques to directly test HPLC fractions for steroid disruptions have been develop using injections (*i.p.*) of the SPE fraction of TFM H-1990-2 formulation into gold fish (Servos, Munkittrick, Hewitt and Van Der Kraak, unpublished data). Significant reductions in the basal levels of circulating testosterone and 11-ketotestosterone result from TFM formulation exposures. Stimulation of steroid production with an injection of GnRH after day 4 resulted in similar levels indicating that the ability of the gonad to respond is not affected. This technique can now be applied to the TIE approach developed for MFO induction and will allow the separation the two responses.

Given the mass of TFM formulation discharged into the Great Lakes, the potential for some components to disappear at slower rates than TFM, and the potential of the unidentified compounds to cause metabolic disturbance in wild fish, it is critical that the components of the formulation which are responsible for biological effects be identified and their environmental fate investigated. Determination of the threshold, duration and magnitude of physiological effects is also required to evaluate the potential impact in the environment.

Based on the compounds already found in the formulation, particularly the presence of 2-chloro-7-trifluoromethyl-8-nitrodibenzo-p-dioxin it seems highly probable that a variety of (more than a dozen) other substituted dioxins will be found in the extracts. This question is currently being explored and detailed analyses of the fractions is continuing. The substitution of a Cl with a trifluoromethyl group in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin has been shown to make the molecule even more toxic while NO_2 substitution only slightly decreases the toxicity (Romkes et al. 1987, Prokipak et al. 1990, Safe et al. 1986). Dioxins and related compounds have also been documented to cause a variety of effects on the endocrine system (Whitlock 1994). One or more of the trifluoromethyl and/or nitro substituted dioxins could be responsible for the observed effects in the TFM formulations. This hypothesis needs to be followed up immediately.

RECOMENDATIONS

1. Work with the manufacturers of TFM formulations to assist in developing a process to eliminate or reduce the presence of bioactive contaminants.

2. Determine the extent to which the formulations are contaminated with chloro/trifluoromethyl/nitro-diphenyl ethers and dioxins.

3. Identify the chemicals in the bioactive fractions (30-3, 31-03 and 34-2). Concentrate the extract further and run TIC and mass spectra, confirming the identity of the unique and major peaks with authentic standards. Confirming the MFO induction potential of identified peaks. Authentic standards are not available for most of the proposed compounds and will have be synthesized.

4. Determine if the EROD induction is caused by the same chemicals in different formulations/batches of TFM.

5. Determine if the steroid disruption is caused by the same chemicals as the EROD induction.

6. Determine the temporal and spatial distribution of the EROD induction and steroid disruptions during a stream treatment of TFM.

7. Evaluate the environmental fate and persistence of the bioactive chemicals as well as the diphenyl ethers and dioxins in the TFM formulations.

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Appendices

Appendix 1: Percentage Mortality During TFM Batch Exposures

Treatments	Batch Exposure # 1	Batch Exposure # 2	Batch Exposure # 3	TFM Dose/ Response	Avg.
Ref.	0	0	0	0	0
1990-2	0	0	33	16	12
Kinetics 1993		16	50		33
Hoechst ssmB161		16	0		8
Hoechst ssmB831		16	33		25
Hoechst ssmB1778		16	33		25
Kinetics ssmB317		16	33	67	39
Kinetics ssmB188		33	50		41
Kinetics fcB317	0	67	67		45
Kinetics fcB188	0	0	100		33
Kinetics fcB312	0	84	67		50
Kinetics fcB310	16	0			8
Kinetics fcB338		41			41

Appendix 2: Temperature, pH, dissolved oxygen and conductivity for TFM batch experiments.

Batch	Expt. No.	Temp. (°C)	pH	D.O. (mg L^{-1})	Conductivity (mS cm^{-1})
Ref.	1	16-15	7.8-7.4	9.0-9.4	0.26-0.24
	2	14-15	7.4-7.4	10.1-10.3	0.24-0.27
	3	15-16	7.7-7.6	9.9-9.4	0.27-0.29
1990-2	1	15-14	7.8-7.7	8.9-9.9	0.16-0.25
	2	14-14	7.7-7.6	9.9-9.8	0.17-0.18
	3	15-16	7.8-7.5	10.0-9.5	0.17-0.18
Kinetics 1993	2	14-14	7.8-7.7	10.0-10.0	0.15-0.17
	3	15-16	7.7-7.7	10.1-9.7	0.17-0.19
Hoechst ssmB161	2	14-14	7.7-7.6	10.0-9.6	0.15-0.28
	3	15-16	7.8-7.8	10.0-9.7	0.16-0.17
Hoechst ssmB831	2	14-14	7.8-7.7	10.0-10.0	0.15-0.29
	3	15-16	7.9-7.8	10.1-9.5	0.16-0.18
Hoechst ssmB1778	2	14-14	7.7-7.7	10.0-9.9	0.16-0.16
	3	15-16	7.9-7.8	10.0-9.8	0.16-0.18
Kinetics ssmB317	2	14-14	7.7-7.7	10.0-10.0	0.15-0.16
	3	15-16	7.9-7.7	10.2-9.8	0.17-0.31
Kinetics ssmB188	2	14-14	7.7-7.7	9.8-10.0	0.15-0.16
	3	15-16	7.9-7.8	10.1-9.8	0.16-0.18
Kinetics fcB317	1	15-14	7.8-7.7	9.0-9.7	0.16-0.15

	2	14-14	7.7-7.7	10.0-9.9	0.15-0.18
	3	15-16	7.9-7.8	10.1-9.7	0.17-0.31
Batch	Expt. No.	Temp. (°C)	pH	D.O. (mg L^{-1})	Conductivity (mS cm^{-1})
Kinetics fcB188	1	15-14	7.9-7.7	9.1-9.7	0.16-0.15
	2	14-14	7.8-7.6	10.0-9.5	0.15-0.17
	3	15-16	7.9-7.2	10.3-7.1	0.17-0.33
Kinetics fcB312	1	15-14	7.9-7.8	9.1-9.6	0.16-0.15
	2	14-14	7.7-7.8	10.2-10.1	0.15-0.2
	3	15-16	7.9-7.8	10.1-9.7	0.18-0.31
Kinetics fsB310	1	15-14	7.9-7.6	9.2-9.7	0.16-0.15
	2	14-14	7.8-7.6	10.0-9.6	0.15-0.17
Kinetics fcB338	2	14-14	7.7-7.7	10.1-9.9	0.16-0.17

Appendix 3: Batch exposure with laboratory prepared TFM from Kinetics Inc.

This experiment was conducted in collaboration with Kinetics Inc. to evaluate methodologies to eliminate the MFO inducing chemical(s) from its TFM lampricide formulation. Kinetics provided 10 separate lots (No.'s CS-322-C1 to CS-322-C10) on August 30th from H & S Chemical Co., Ohio, 45217, which were received by GLLFAS on September 01, 1994. Both EROD activity and protein concentrations were determined for the rainbow trout exposed to the 10 lots. 100 µL volumes of each lot was used to reduce mortality observed in previous exposure (Appendix 1).

It is important to note that several fish had EROD activity that ran off scale in the highly induced tanks which means that the values from these treatments will be slightly higher than reported on the graph since they were given the maximum fluorescence reading of 1000 (see Appendix 3 for raw data) . The variability in fish response is normal and the reference, 1990-2, and analytical controls ran as expected.

Several of the treatments were toxic (Table 1) to the exposed fish and these lots generally produced lower EROD activity than those which were not toxic as was found in the previous batch experiments.

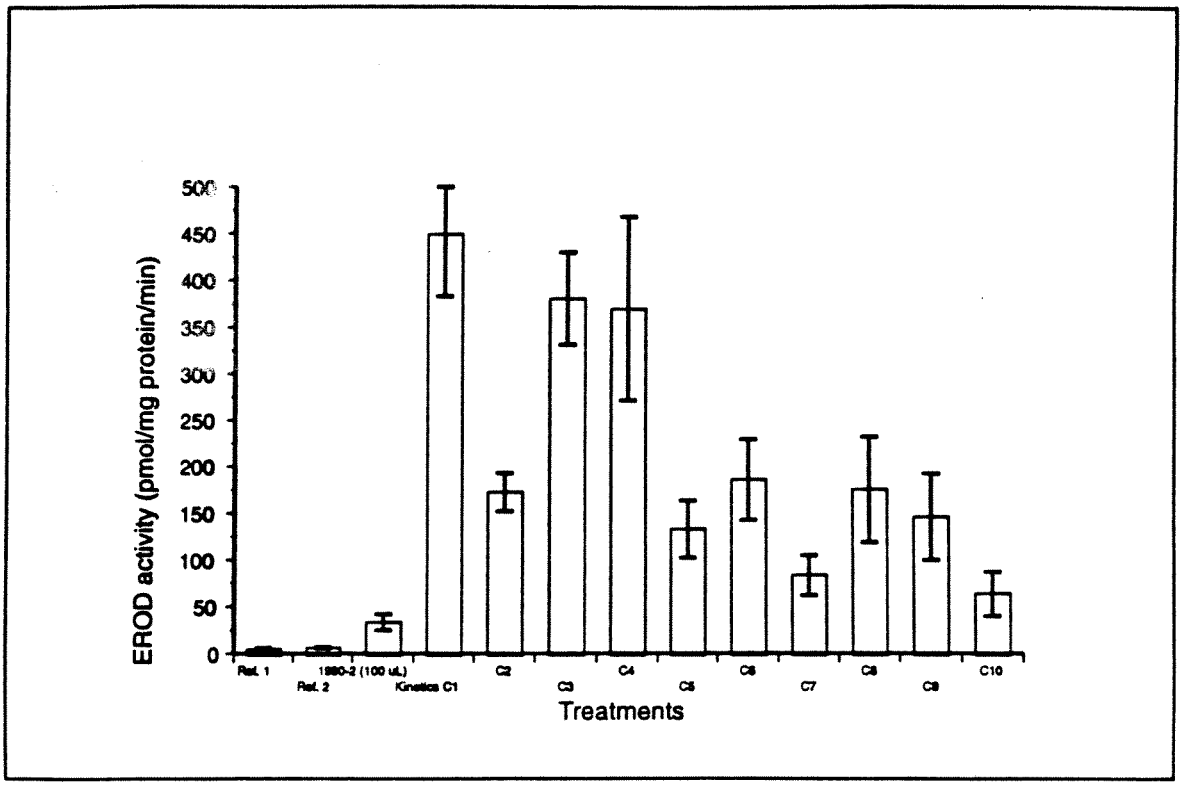

Figure 1. Exposure of rainbow trout to 1994 Kinetic batch lots CS-322-C1 to C10.

Appendix 3, Table 1: Percent mortality of fish exposed to TFM batches

	Ref.	1990-2	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
% Mort.	0	0	0	16	0	0	0	0	0	50	16	0

Appendix 3, Table 2: Temperature, pH, conductivity and dissolved oxygen measurements for Kinetics TFM exposures.

Treatment	Day	Temp. (C)	pH	Conduct. (mS/cm)	D.O. (mg/L)
Ref. 1	1	14.9	8.01	0.152	9.87
	3	15.1	7.9	0.143	9.49
	Diff.	0.2	-0.11	-0.009	-0.38
Ref. 2	1	14.9	8.07	0.15	9.57
	3	15.1	7.92	0.148	9.33
	Diff.	0.2	-0.15	-0.002	-0.24
1990-2	1	14.8	8.05	0.156	9.98
	3	15.1	8.12	0.153	9.89
	Diff.	0.3	0.07	-0.003	-0.09
C1	1	14.8	8.08	0.156	9.95
	3	15.1	8.1	0.155	9.83
	Diff.	0.3	0.02	-0.001	-0.12
C2	1	14.8	8.08	0.156	9.96
	3	15.1	8.1	0.16	9.85
	Diff.	0.3	0.02	0.004	-0.11
C3	1	14.8	8.01	0.158	10.01
	3	15.2	7.89	0.178	9.9
	Diff.	0.4	-0.12	0.02	-0.11
C4	1	15	7.95	0.16	9.93

	3	15.1	8.13	0.155	9.9
	Diff.	0.1	0.18	-0.005	-0.03
C5	1	14.8	8.08	0.166	10.03
	3	15.1	8.13	0.159	9.88
	Diff.	0.3	0.05	-0.007	-0.15
C6	1	15	7.97	0.17	10.06
	3	15.1	8.14	0.156	9.92
	Diff.	0.1	0.17	-0.014	-0.14
C7	1	14.8	8.09	0.16	9.89
	3	15.1	8	0.158	9.82
	Diff.	0.3	-0.09	-0.002	-0.07
C8	1	14.8	8	0.158	9.94
	3	15.1	8.15	0.174	9.84
	Diff.	0.3	0.15	0.016	-0.1
C9	1	14.8	8.08	0.263	9.98
	3	15.1	7.99	0.197	9.84
	Diff.	0.3	-0.09	-0.066	-0.14
C10	1	14.8	8.09	0.263	10.13
	3	15.1	8.01	0.16	9.76
	Diff.	0.3	-0.08	-0.103	-0.37

Appendix 4. 2,3,7,8-substituted dioxins and furans, coplanar PCBs and PAHs in TFM formulation H1990-2.

COMPOUND	92S208	DET. LIMIT	93S001	DET. LIMIT
	TFM (OLD) pg/g	pg/g	TFM (NEW) pg/g	pg/g
2378-TCDD	N.D.	0.06	N.D.	0.04
TCDD TOTAL	N.D.		N.D.	
12378-PCDD	N.D.	0.03	N.D.	0.02
PCDD TOTAL	N.D.		N.D.	
123478-H6CDD	N.D.	0.01	N.D.	0.02
123678-H6CDD	N.D.	0.01	N.D.	0.01
123789-H6CDD	N.D.	0.01	N.D.	0.01
H6CDD TOTAL	N.D.		N.D.	
1234678-H7CDD	N.D.	0.02	N.D.	0.01
H7CDD TOTAL	N.D.		N.D.	
OCDD	N.D.	0.02	N.D.	0.01
2378-TCDF	N.D.	0.01	N.D.	0.03
TCDF TOTAL	N.D.		N.D.	
12378-PCDF	N.D.	0.02	N.D.	0.01
23478-PCDF	N.D.	0.03	N.D.	0.02
PCDF TOTAL	N.D.		N.D.	
123478-H6CDF	N.D.	0.01	N.D.	0.01
234678-H6CDF	N.D.	0.01	N.D.	0.01
123678-H6CDF	N.D.	0.01	N.D.	0.01
123789-H6CDF	N.D.	0.01	N.D.	0.01
H6CDF TOTAL	N.D.		N.D.	
1234678-H7CDF	N.D.	0.02	N.D.	0.01
H7CDF TOTAL	N.D.		N.D.	
OCDF	0.08	0.01	0.06	0.01
Sample Volume(uL)	250.00		250.00	
Final Sample Vol.(ul)	20.00		20.00	
% RECOVERIES				
13C-TCDD	51.00		70.00	
13C-PCDD	61.00		76.00	
13C-HxCDD	70.00		80.00	
13C-HpCDD	69.00		77.00	
13C-O8CDD	68.00		74.00	

COMPOUND	1 92S208	2 DET. LIMIT	3 93S001	4 DET. LIMIT
SPECIES UNITS	TFM (OLD) pg/uL	pg/uL	TFM (NEW) pg/uL	pg/uL
33'44'-TPCB	0.108	0.001	0.086	0.001
233'44'-PePCB	0.019	0.002	0.021	0.001
33'44'5-PePCB	0.026	0.003	0.018	0.002
33'44'55'-HxPCB	0.006	0.002	0.002	0.004
% RECOVERIES				
13C-33'44'-TPCB	67.000		95.000	
13C-33'44'5-PePCB	65.000		98.000	
13C-33'44'55'-HxPCB	61.000		67.000	

0 COMPOUND	1 MASS	2 DET. LIMIT	3 '93E001	4 '93E002
1 Sample ID			1990	kinetics
2		ng/g	ng/g	ng/g
3				
4 Naphthalene	128	10	N.D.	N.D.
5 Acenaphthylene	152	10	N.D.	N.D.
6 Acenaphthene	153	10	N.D.	N.D.
7 Fluorene	166	10	N.D.	N.D.
8 Phenanthrene	178	10	N.D.	N.D.
9 Anthracene	178	10	N.D.	N.D.
10 Fluoranthen	202	10	N.D.	N.D.
11 Pyrene	202	10	N.D.	N.D.
12 Benz(a)anthracene	228	5	N.D.	N.D.
13 Chrysene	228	5	N.D.	N.D.
14 Benzo(b)fluoranthene	252	5	N.D.	N.D.
15 Benzo(k)fluoranthene	252	5	N.D.	N.D.
16 Benzo(a)pyrene	252	5	N.D.	N.D.
17 Dibenzo(a,h)anthracene	278	5	N.D.	N.D.
18 Indeno(1,2,3-cd)pyrene	276	5	N.D.	N.D.
19 Benzo(g,h,i)perylene	276	5	N.D.	N.D.

GREAT LAKES FISHERY COMMISSION
Research Completion Report[1]

Isolation of Bioactive Components of Commercial TFM Formulations

by

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September 30, 1994

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[1]Project completion reports of Commission-sponsored general research are made available to the Commission's cooperators in the interest of rapid dissemination of information that may be useful in Great Lakes fishery management, research, or administration. The reader should be aware that project completion reports have not been through a peer review process and that sponsorship of the project by the Commission does not necessarily imply that the findings or conclusions are endorsed by the Commission.

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