GREAT LAKES FISHERY COMMISSION

Project Completion Report[1]

Identification of MFO and Estrogenic Compounds in TFM Formulations

by:

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March 1997

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Identification of MFO and estrogenic compounds in TFM Formulations

March 3, 1997

Mark R. Servos

Priority Substances: Exposure/Effects Project
Aquatic Ecosystems Research Branch
National Water Research Institute
Environment Canada

Final Report to the Great Lakes Fishery Commission:
Identification of MFO and estrogenic compounds in TFM formulations.
March 3, 1997

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Summary

This report outlines results of studies to examine the MFO induction and contaminant concentrations in batches of TFM formulations. Almost every available batch of TFM has now been tested and shown to have the potential the potential to induce MFO activity in fish. Experiments have been conducted in the laboratory and in co-operation with a manufacturer to reduce and eliminate the MFO induction in TFM formulations. Enormous progress has been made in identifying contaminants in the formulations. The critical contaminants suspected to be in the formulations, including several diphenyl ethers and dioxins (2-chloro-7-nitro-8-trifluoromethyldibenzodioxin and 2,7-bis(trifluoromethyl)-3,8-dichloro-dibenzodioxin) have been synthesized, mass spectra determined and compared to those in the bioactive fractions of the formulations. While the presence of congeners of 2-chloro-7-nitro-8-trifluoromethyl-dibenzodioxin has been confirmed, 2,7-bis(trifluoro-methyl)-3,8-dichlorodibenzodioxin has not been confirmed in any batch tested. Although the specific congener of "tri substituted" chloro-nitro-trifluoro-methyl-dibenzodioxin in the formulations has not been synthesised the mass spectra in the formulations are consistent with this structure. 2-chloro-7-nitro-8-trifluoro-methyldibenzodioxin was confirmed to be an inducer of MFO activity in fish. Although it has not been absolutely confirmed it is highly probable that a "tri substituted" chloro-nitro-trifluoromethyl-dibenzodioxin congener is responsible for the MFO induction observed.

Previous laboratory studies have documented depressed steroids and induction of vitelogenin in fish exposed to TFM. Results of the current study using a estrogen receptor binding as an endpoint for TIE analysis suggests that unlike MFO induction which is a result of contaminants in the formulation TFM itself is estrogenic. However, whether or not these reproductive responses will be seen during normal field treatments is unknown.

Emphasis of the work was changed during this study to address several priorities of the Great Lakes Fishery Commission including: addressing concerns of the Expert Panel, additional batch testing (MFO and chemistry), work with the manufacturers to reduce MFO activity of batches. A field study and additional testing of samples from the Bad River were funded separately and will be report elsewhere.

This report highlights the major studies designed to achieve the objectives in the original proposal. Rather than have a long description of each study the major conclusions are highlighted and the more detailed manuscripts or reports are included in the Appendices.

Original Objectives

Deliverables in the original proposal:

1. obtain authentic standards of the possible dioxins and test for biological endpoints (dependent on standards (dependent on standards being provided by GLFC),
2. test new batches of Kinetics and Hoescht for EROD induction,
3. conduct an evaluation of the induction potential of HPLC fractions from the two different manufacturers formulations to determine if the inducers are the same,
4. evaluate the potential of different formulations to cause steroid disruptions in fish,
5. develop a method to evaluate the steroid disruption effects using HPLC fractions (including estrogen receptor binding),
6. determine if the EROD response and steroid response are caused by the same chemicals (fractions),

Objectives not funded in the original proposal:

7. conduct a field study to evaluate the temporal and spatial extent of EROD induction and steroid disruptions during a TFM treatment (not funded),
8. evaluate the persistence and environmental fate of phenyl ether and dioxin derivatives in the receiving environment (not funded).

Isolation and Identification of MFO inducing Chemicals in TFM Formulations

Efforts have continued to isolate and identify the chemicals responsible for MFO induction in TFM formulations. The main results of these studies have been published (see Hewitt et al. 1996a, b, c) and are included in Appendix 1. The reader is referred to these paper and the results are only highlighted here. These studies identified a series of contaminants in the TFM formulations including a number of chloro-nitro-trifuoromethyl-substituted diphenyl ethers and dioxins (Fig. 1). The development of a TIE approach has allowed the isolation of the bioactive compounds into specific HPLC fractions (Fig. 2,3). The acquisition or synthesis of authentic standards allowed for the confirmation of the MFO inducing capability of the suspected structures. The compound identified as the most likely cause was 2-chloro-7-nitro-8-trifluoromethyl-dibenzodioxin However, the exact retention time of the 2-chloro-7-nitro-8-trifluoromethyl-dibenzodioxin is not matched in the formulation, even though the spectra match. Numerous attempts have been made to synthesize other "tri-substituted" dioxins but none have had an exact chromatographic match on retention time. However, based on the weight of evidence it is highly probable that a congener of 2-chloro-7-nitro-8-trifluoromethyl-dibenzodioxin is the responsible chemical resulting in MFO induction in fish.

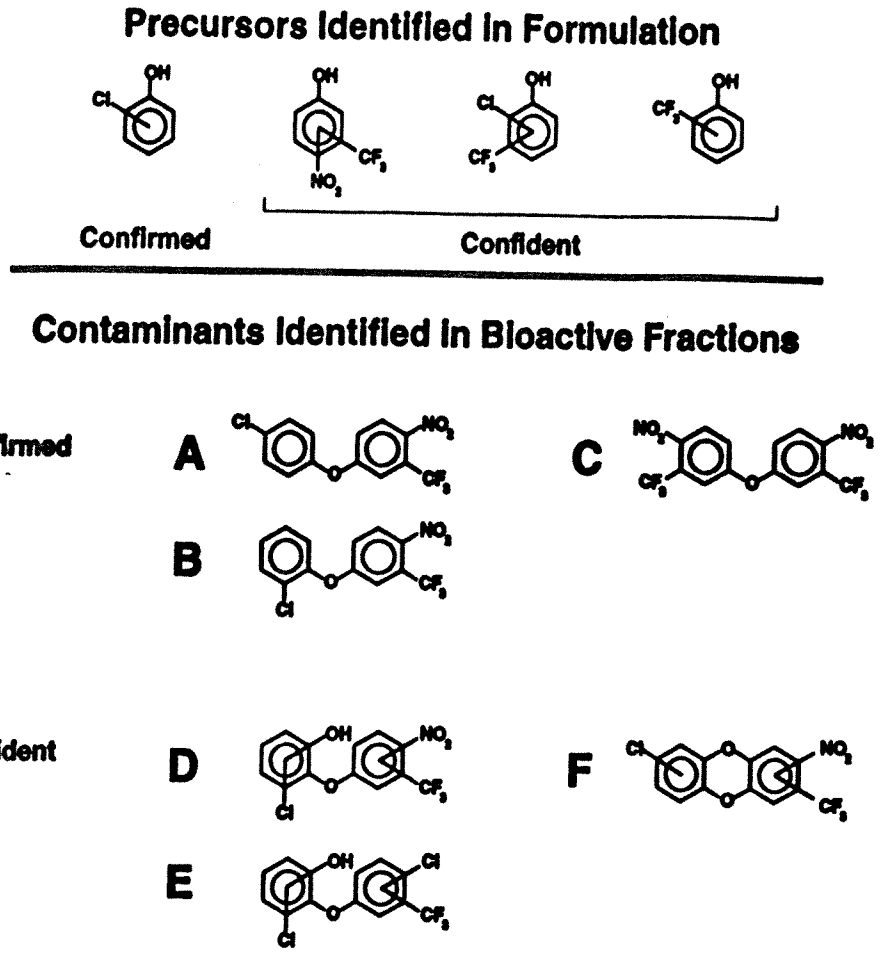

Figure 1. Chemical structures observed in the TFM formulations.

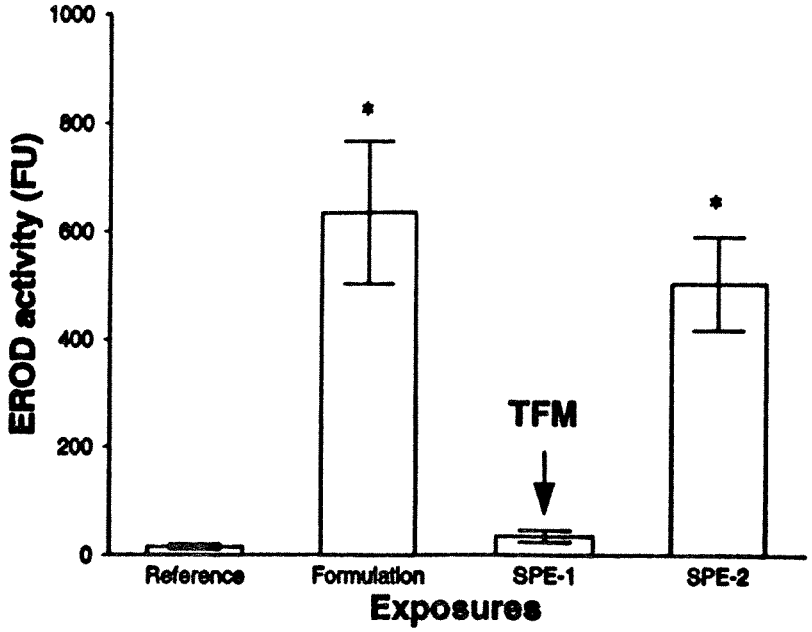

Figure 2. Rainbow trout hepatic EROD activity for fish exposed to fractions generated from SPE of TFM formulation H1990-2 (see Hewitt et al. 1996b).

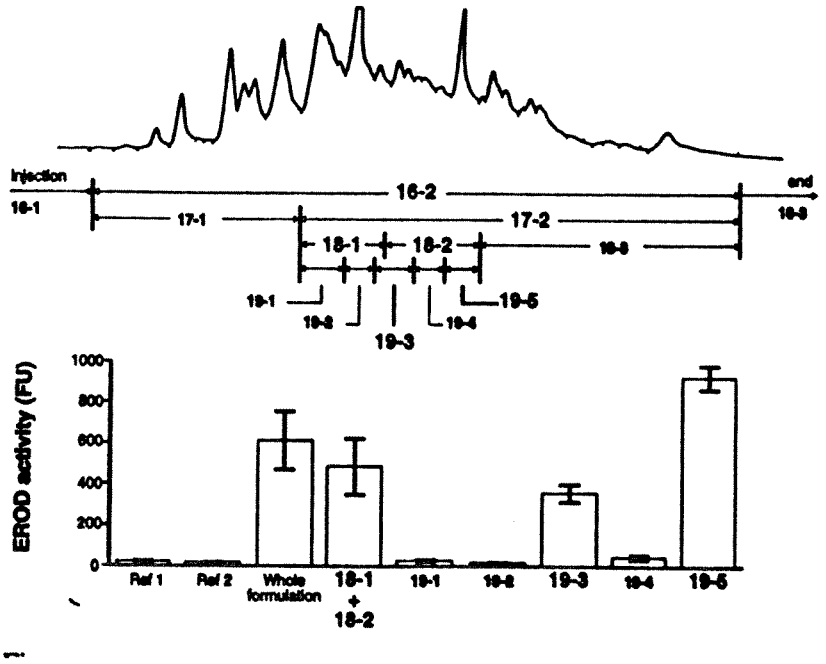

Figure 3. HPLC fractionation of SPE-2 showing the associated EROD activity (see Hewitt et al. 1996b).

Objective 1. Obtain authentic standards of the possible dioxins and test for biological endpoints.

1.1. Synthesis of "Dioxin" Standards

A commercial laboratory (ChemSyn, Kansas City, Kansas) conducted a synthesis of both 2-chloro-7-nitro-8-trifluoromethyl-dibenzodioxin and 2,7-bis(trifluoromethyl)-3,8-dichlorodibenzodioxin (see Appendix 2).The mass spectra have been determined for each of the major components of the standards (Figs. 4,5).

2-chloro-7-nitro-8-trifluoromethyldibenzodioxin
Mass spectra of the unknowns in the formulation were compared to the standard. The mass spectra matched identically (Figs. 4a, 4b), however the retention time differed (Figs 6). This indicates that the "tri-substituted" dioxin in the formulations is most likely a congener of the dioxins in the standard mixture. Several attempts have been made to synthesis a number of these congeners but to date we have not been successful at making a congener with the same retention time. It has therefore not been possible to determine the specific substitution pattern of the dioxins in the formulations.

2,7-bis(trifluoromethyl)-3,8-dichlorodibenzodioxin
Mass spectra of the "tetra-substituted" dioxin were determined from the standard. SIM GC-MS analysis of the batch samples indicated that although M and M^{+2} ions were detectable at the appropriate ratio in some samples the retention time did not match. Sixteen batches have been extracted and examined for the "tetra-substituted" dioxin but detectable levels of this compound were not found (Figs. 7,8). All evidence suggests that the 2,7-bis(trifluoromethyl)-3,8-dichlorodibenzodioxin does not exist at detectable levels in the formulations tested. See attached memo Nov. 15, 1996 (Appendix 3).

1.2. MFO activity of "Dioxins"

Exposure of 2-chloro-7-nitro-8-trifluoromethyldibenzodioxin provided by ChemSyn resulted in significant induction of EROD activity in fish (Fig 9.). Preliminary results (not presented here) from testing the various congeners, which were synthesized by ChemSyn or in our laboratory in Burlington, as well as extracts of TFM, using H4IIE cell line bioassays, suggest that the "tri-substituted" dioxins have the potential to cause the MFO induction observed.

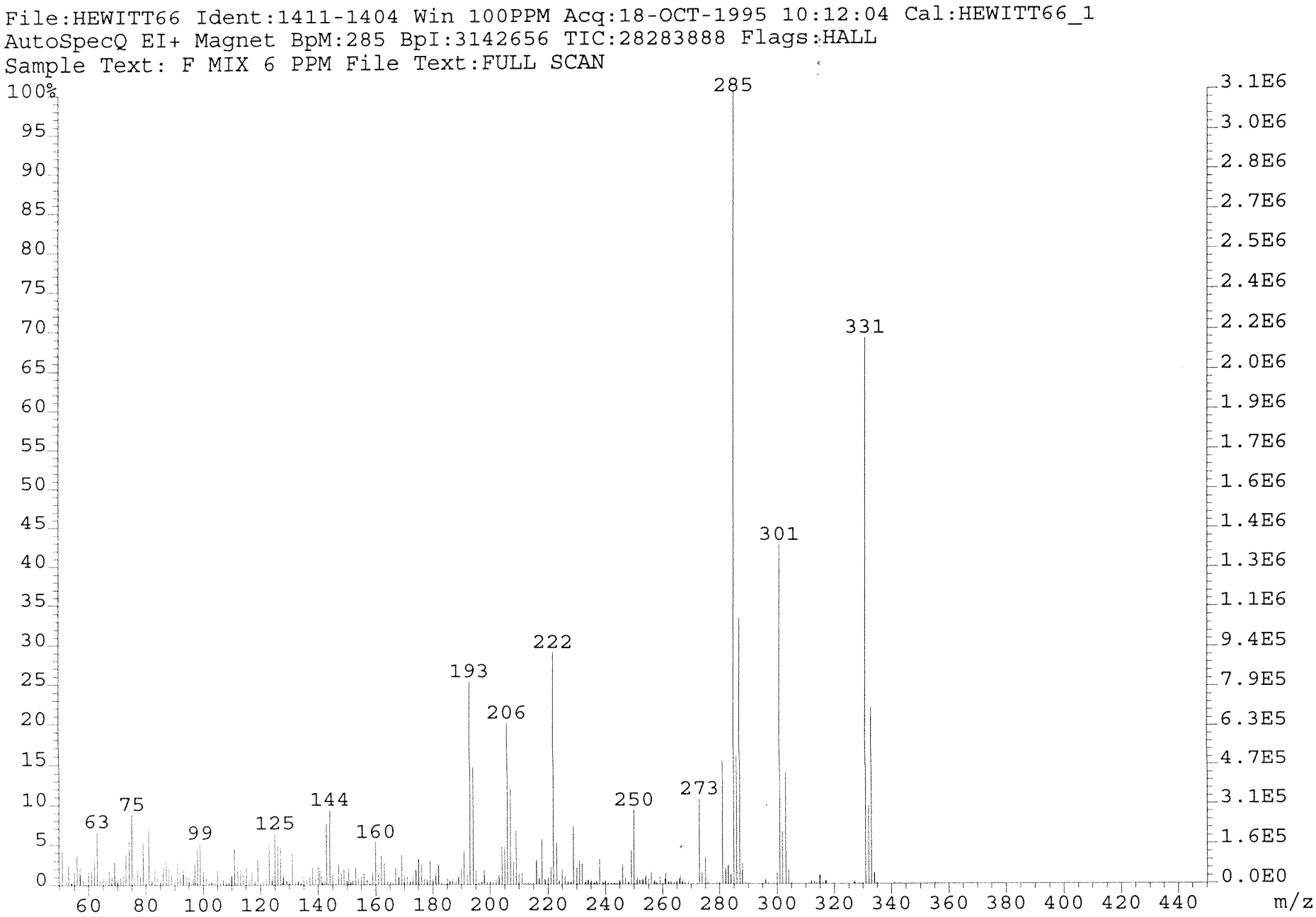

Figure 4a. Mass spectra of 2-chloro-7-nitro-8-trifluoromethyldibenzodioxin (Chemsyn standard).

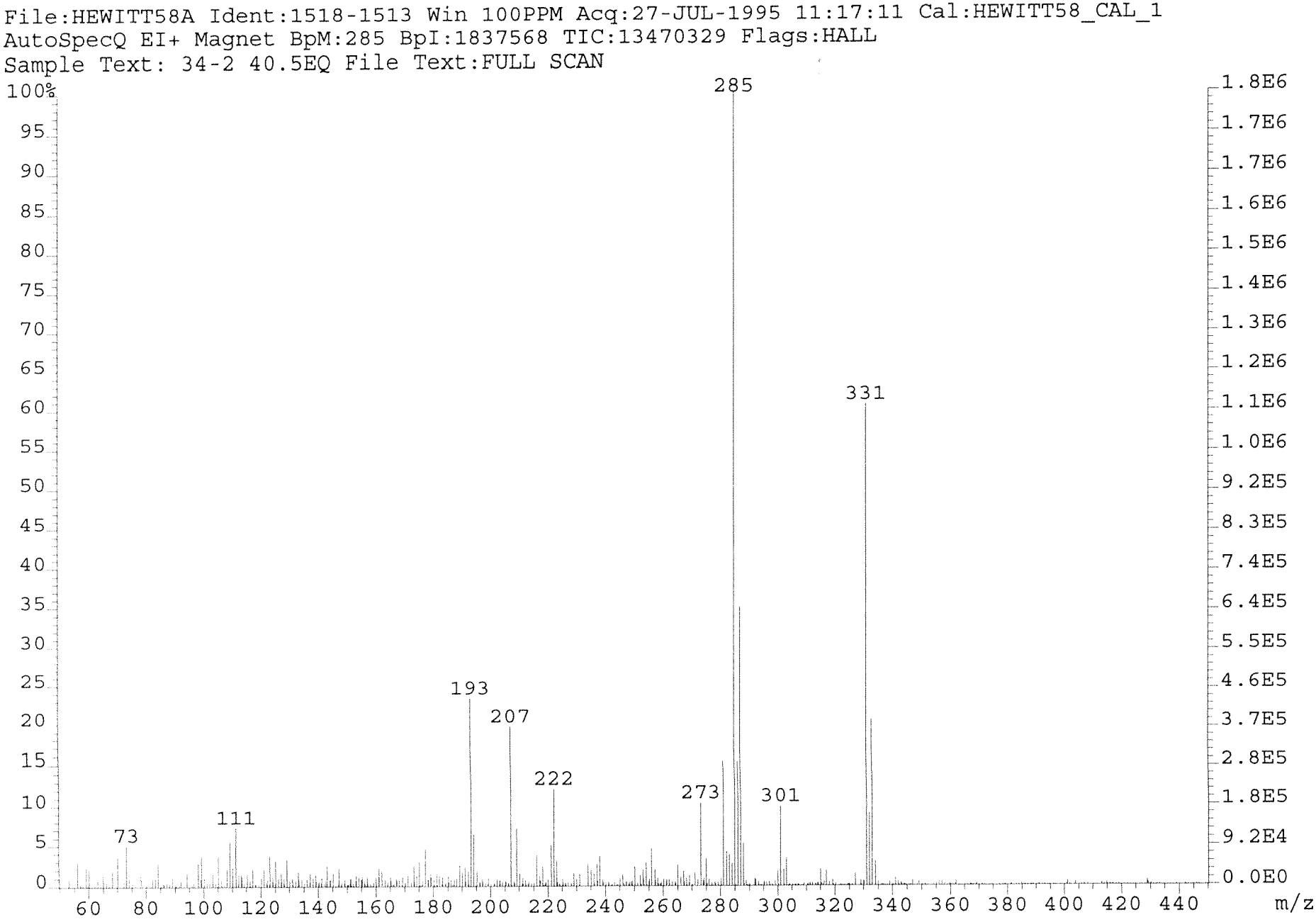

Figure 4b. Mass spectra of unknown in H1990-2, fraction 34-2.

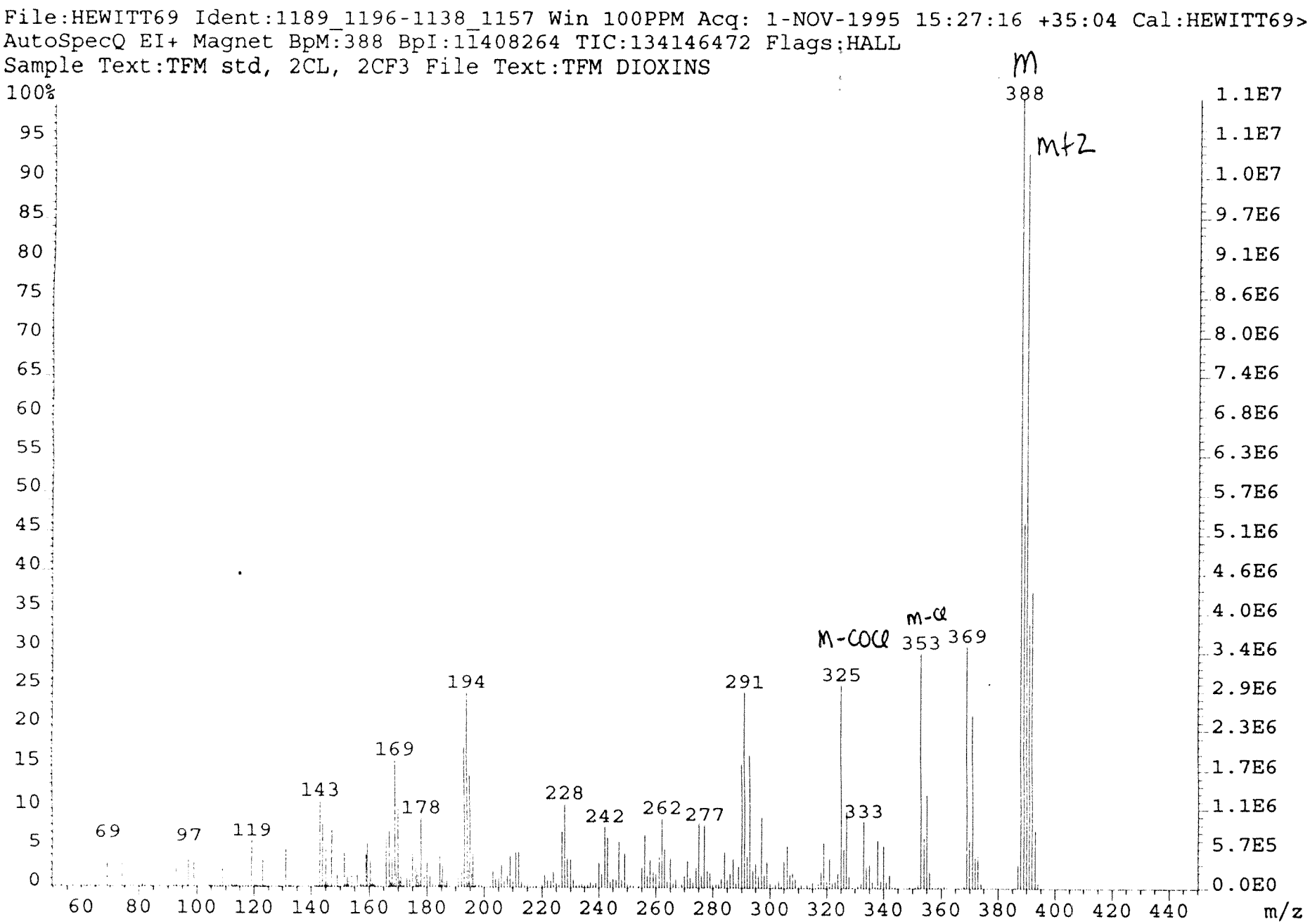

Figure 5. Mass spectra of 2,7-Bis(trifluoromethyl)-3,8-dichlorodibenzodioxin (Chemsyn standard).

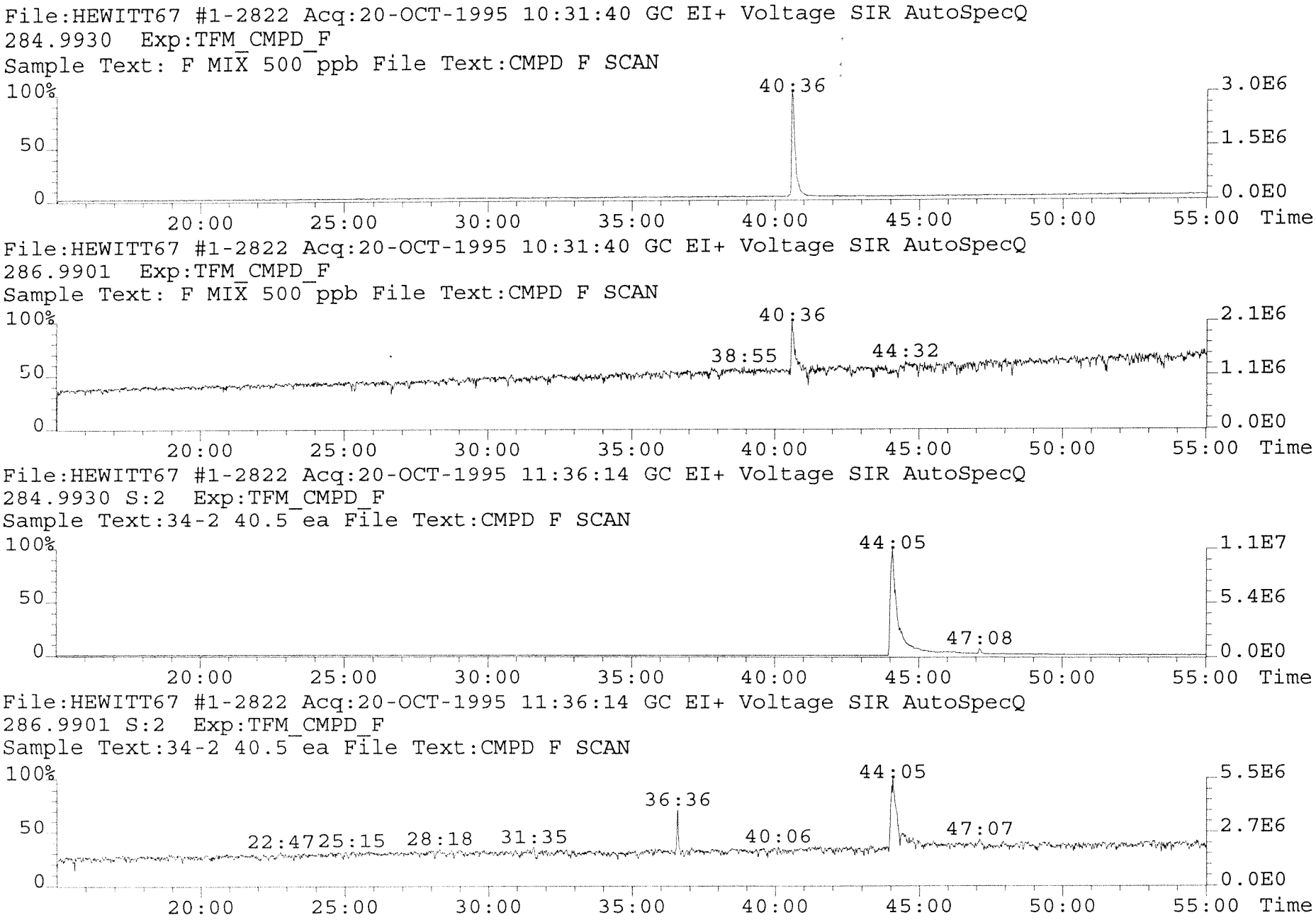

Figure 6. GC/MS (SIM) analysis of the 2-chloro-7-nitro-8-trifluoromethyldibenzodioxin, Chemsyn standard and H1990-2, fraction 34-2.

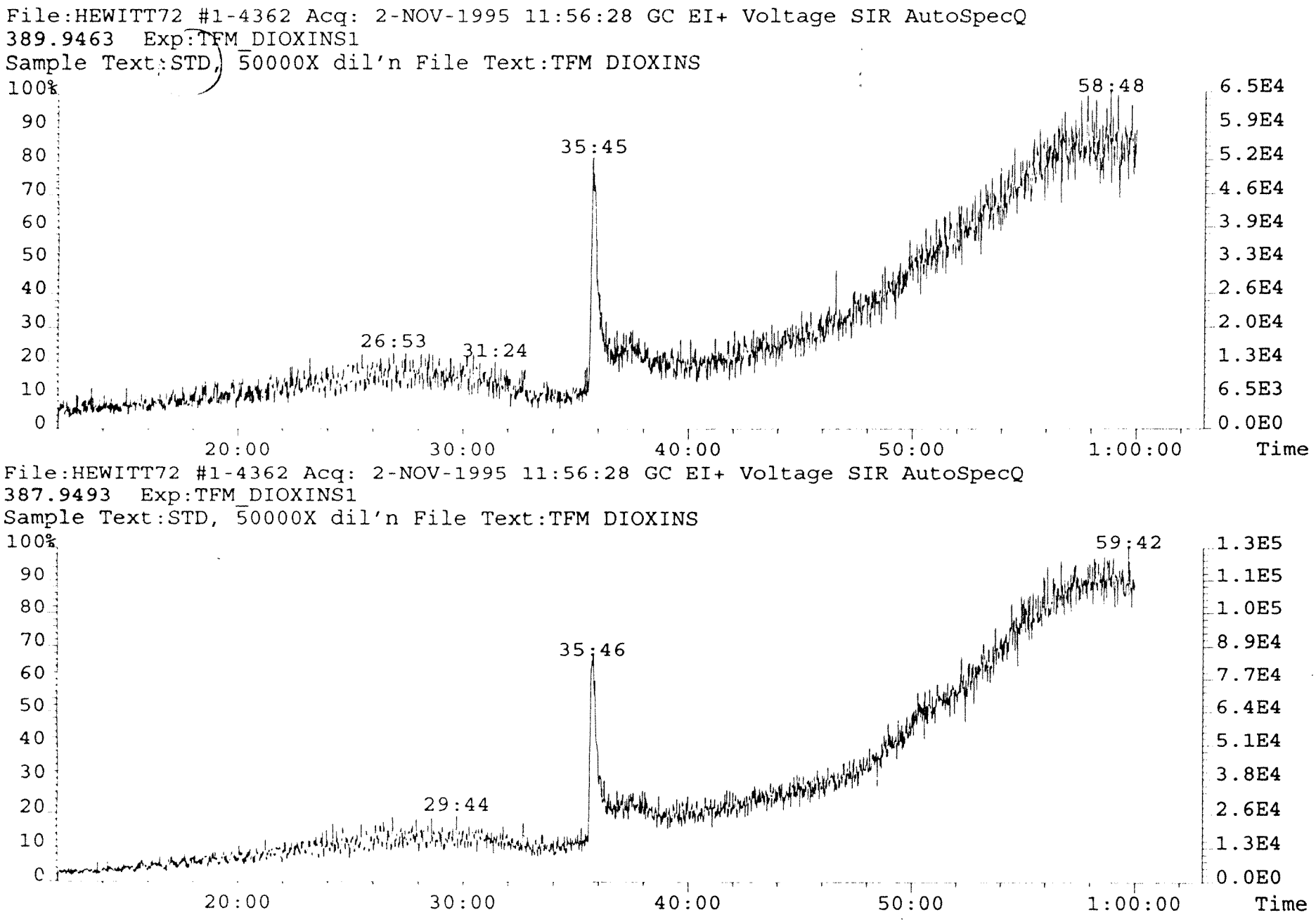

Figure 7. GC/MS (SIM) anaylsis of 2,7-Bis(trifluoromethyl)-3,8-dichlorodibenzodioxin, Chemsyn standard.

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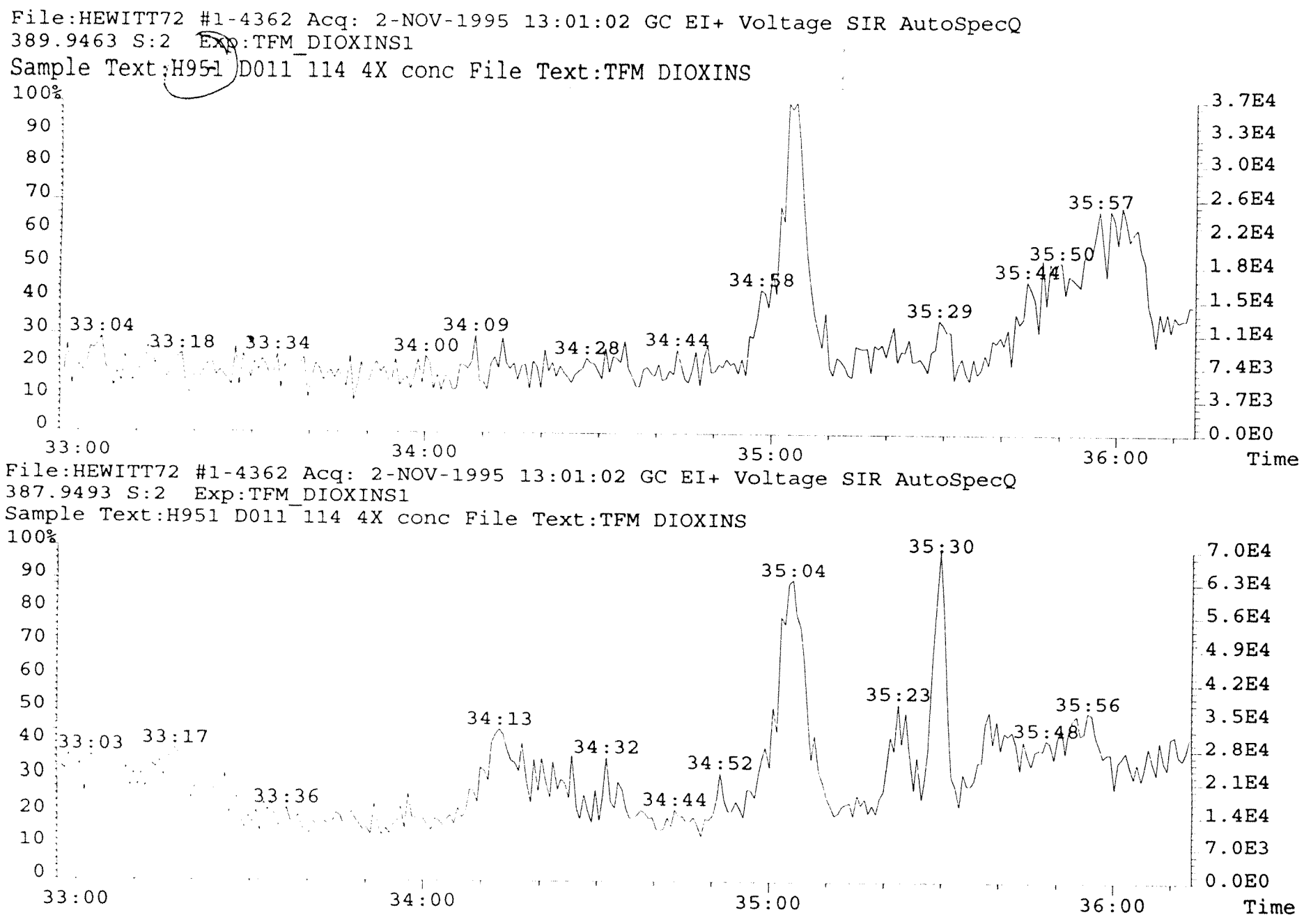

Figure 8. GC/MS (SIM) anaylsis of H95-1 for 2,7-Bis(trifluoromethyl)-3,8-dichlorodibenzodioxin, Chemsyn standard.

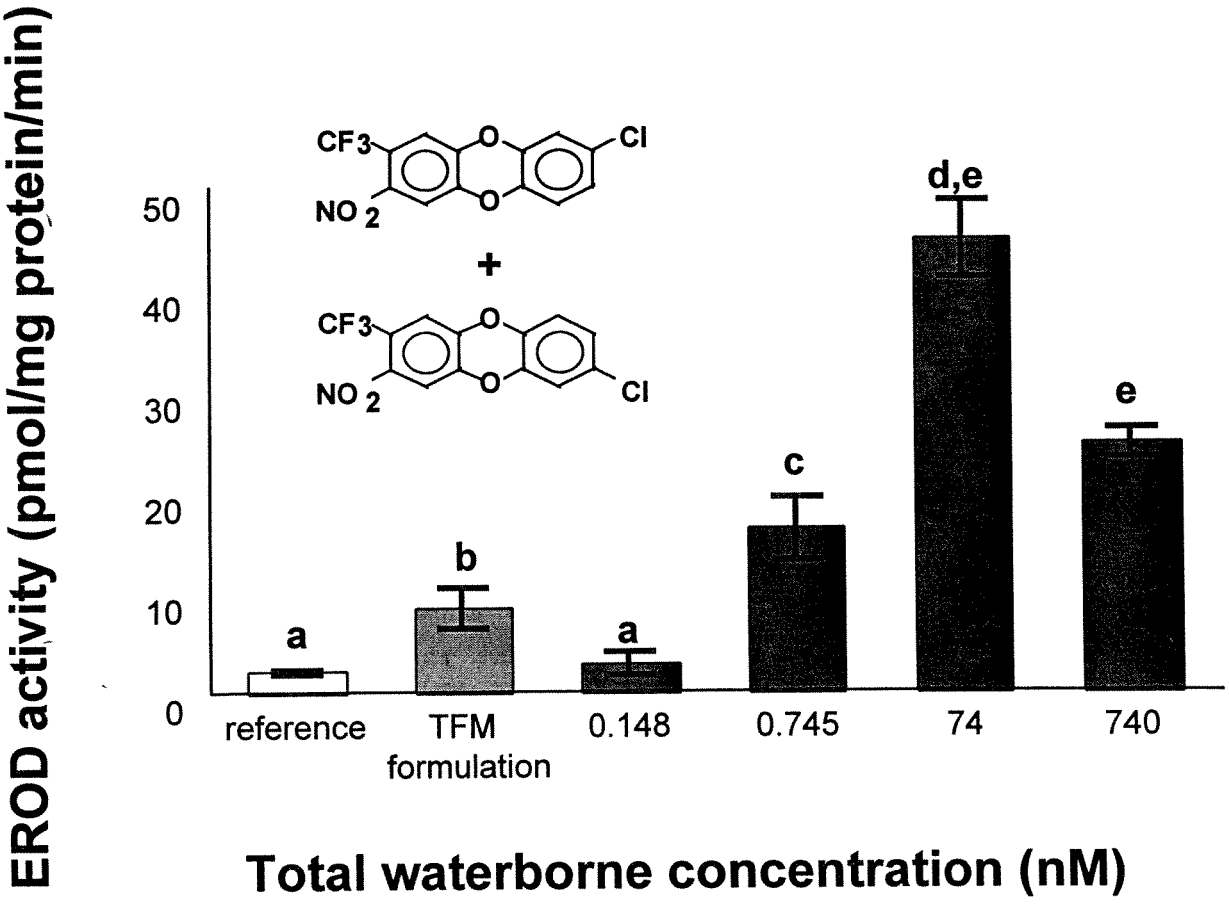

Figure 9. EROD induction in rainbow trout exposed to 2-chloro-7-nitro-8-trifluoromethyldibenzodioxin.

Objective 2. Testing of new batches of Kinetics and Hoescht for EROD induction.

2.1 MFO Induction in TFM Batches

Numerous batches of TFM formulations from two manufacturers were tested (see Appendix 4). These included batches provided directly by the manufacturer or random samples from the "cans" delivered to DFO or US-FWS. Although there was considerable variability, all batches tested produced some induction of EROD activity in rainbow trout. Batches of Kinetics in 1994 showed much higher induction than the H1990 reference sample. However, 1995 Kinetics batches showed induction similar to the H1990 reference batch (see Appendix 4). Raw data and summaries of these experiments were previously provided to the GLFC and US-FWS. The Kinetics batches tested in 1996 were also significantly induced (Fig. 10).

2.3 Reduction of MFO Induction in TFM Batches

Numerous experiments were conducted to determine if EROD induction could be reduced or eliminated from selected batches of TFM. Trials included C_{18}, activated carbon, gravitized carbon and toluene extractions. Using gravitized carbon the EROD activity in Kinetics batches could be reduced to nearly background. However, this required very high carbon/TFM ratios (Fig. 11). After discussions with Kinetics Inc. they incorporated a carbon column clean-up into their production process. This resulted in a very significant reduction in the EROD induction potential of these formulations such that they are now similar to the H1990 level. Appendix 5 describes these experiments in more detail.

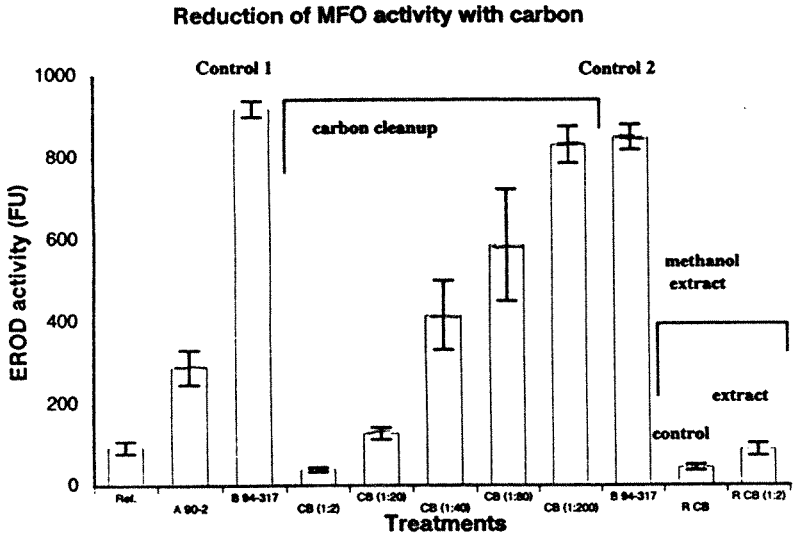

Figure 11. The effects of carbon treatment on the MFO activity of TFM formulations (see Appendix 5).

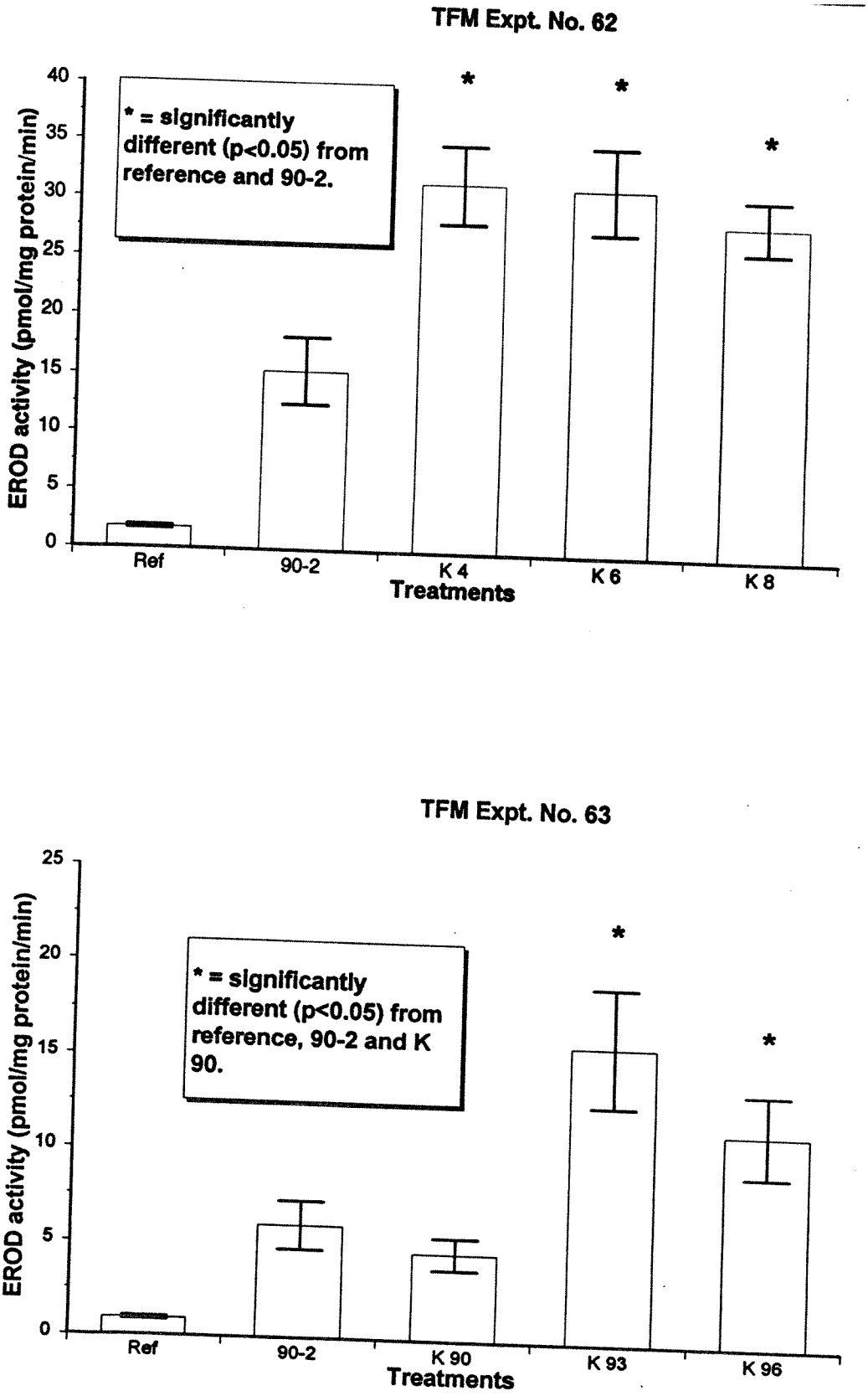

Figure 10. EROD induction in fish exposed to 1996 batches of TFM (see Appendix 4).

Objective 3. Conduct an evaluation of the induction potential of HPLC fractions from the two different manufacturers formulations to determine if the inducers are the same.

Experiments were conducted to compare the MFO induction profile of two manufacturers. The Hoescht 1990-2 results were presented in Hewitt et al. 1996. In comparison the Kinetic Batch tested showed a different response profile suggesting that the responsible chemicals were also different. There would appear to be additional compounds in the Kinetic formulations which are MFO responsive.

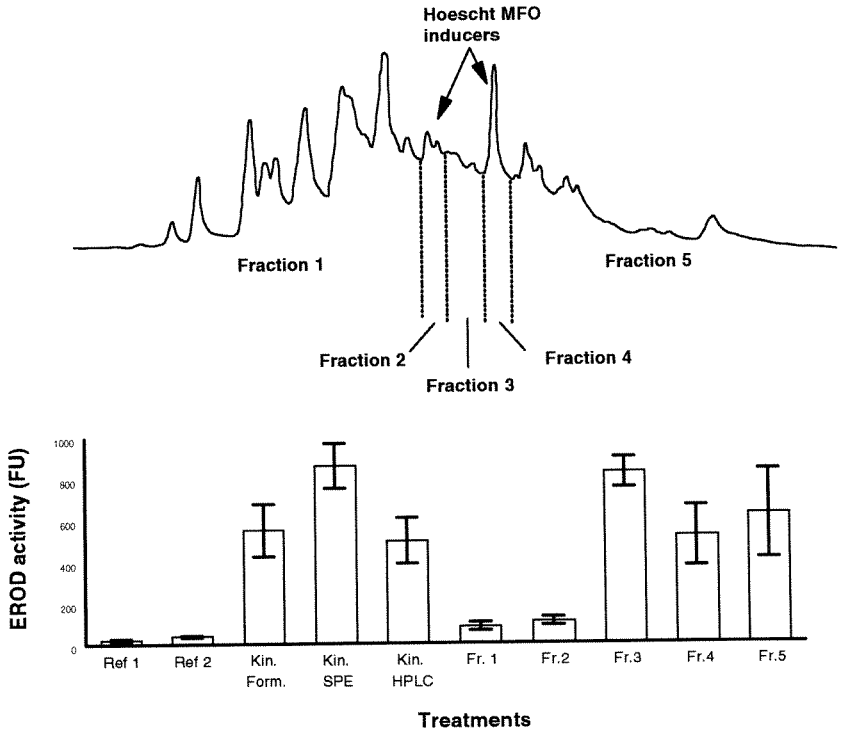

Figure 12. Rainbow trout hepatic EROD activity associated with HPLC fractions derived from one batch of Kinetics product.

Objectives 4-6. Potential reproductive responses

The last three objectives all relate to the potential reproductive responses in fish:

- evaluate the potential of different formulations to cause steroid disruptions in fish,
- develop a method to evaluate the steroid disruption effects using HPLC fractions (including estrogen receptor binding),
- determine if the EROD response and steroid response are caused by the same chemicals (fractions).

The first experiments conducted were aimed at determining if the same chemicals causing MFO induction were responsible for reproductive responses such as estrogen receptor binding and steroid depression. The same SPE fractionation used for MFO induction studies were used in a TIE using estrogen receptor binding as an endpoint. Unlike the MFO induction almost all of the activity was associated with the polar SPE-1 fraction (Fig. 13). HPLC fractionation indicated that the activity was associated with fractions which did not cause MFO induction (Fig. 14). The estrogen receptor binding of purified TFM demonstrated that it had a binding affinity for the estrogen receptor that was similar to other environmental contaminants, such as nonylphenol (Fig. 15). The receptor binding was shown to relate to a biological response, vitellogenin induction in cell culture (Fig. 16). Previous study has shown that exposure of fish to TFM resulted in vitellogenin induction in whole fish.

The results of these studies leads to the conclusion that TFM itself is the chemical responsible for estrogen receptor binding. And vitellogenin induction. TFM is therefore estrogenic an likely responsible for other reproductive effects in fish such as alter steroid profiles in fish exposed to the formulations. The results of these studies has been summarized in a manuscript which has been submitted to the Journal, Environmental Toxicology and Chemistry and is attached as part of this document. Whether or not these responses are translated into reproductive responses in the field needs to be evaluated and is currently unknown.

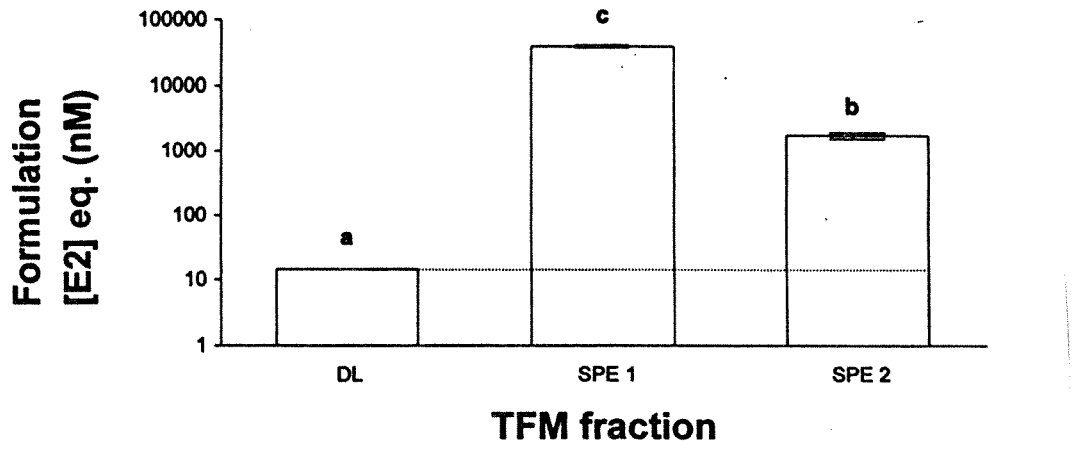

Figure 13. The relative E2 binding of SPE fractions of TFM formulation H1990-2 (from Hewitt et al. 1997; see Appendix 1)

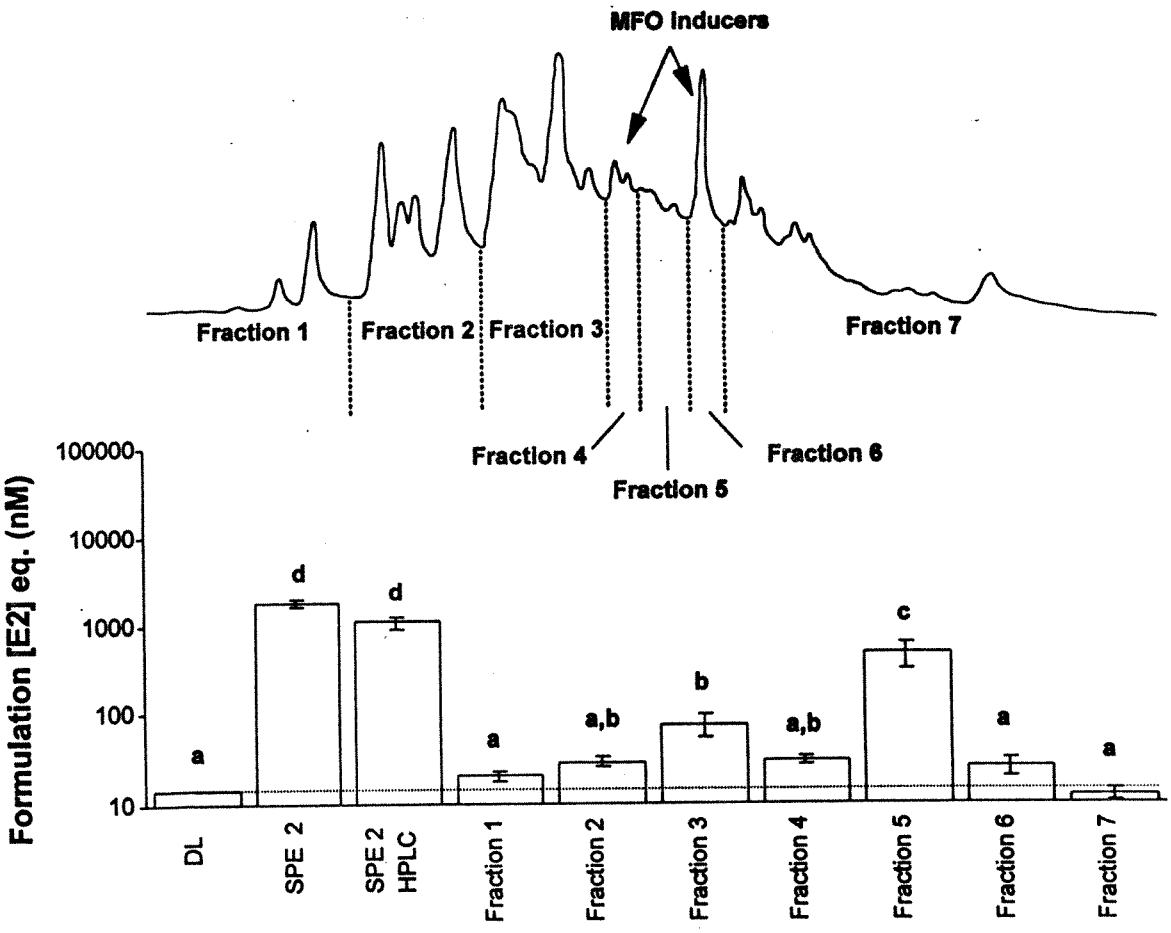

Figure 14. The HPLC fractionation of a TFM formulation and resulting E2 binding (from Hewitt et al. 1997; see Appendix 1).

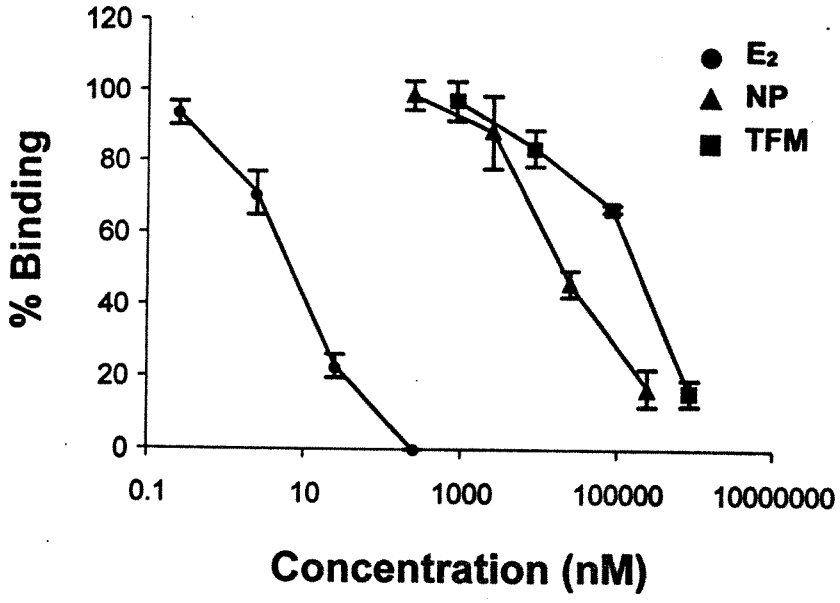

Figure 15. The relative binding of TFM, nonylphenol and estradiol (from Hewitt et al. 1997; see Appendix 1).

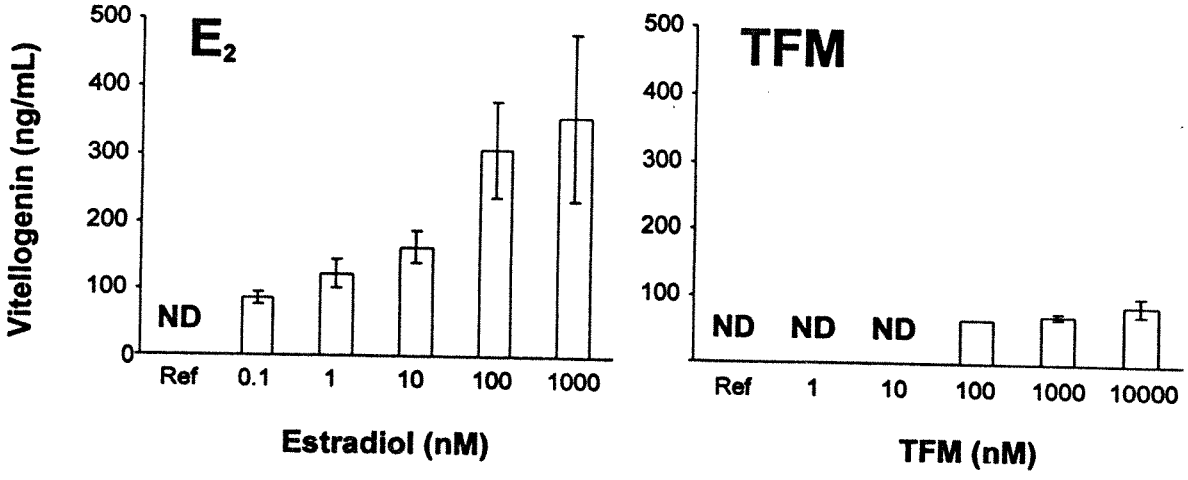

Figure 16. The relative potential of TFM compared to estradiol for vitellogenin induction (from Hewitt et al. 1997; see Appendix 1).

Recommendations

- The isolation and identification of the chemicals responsible for MFO induction in TFM formulations has proven difficult. Although there would be considerable advantages to identifying the specific congeners any further steps would be expensive and labour intensive. There would be no guarantee that further research would be successful. The GLFC should determine the consequences and/or advantages of proceeding further before pursuing this research.

- The confirmation of the 2,7-bis(trifluoromethyl)-3,8-dichlorodibenzodioxin congener in the formulations has been difficult because of the potentially low levels and nature of the chemical and analysis. It would appear that the levels are non-detectable or at best extremely low. If the definitive answer is required then the GLFC should follow this question up with a specific study to address this question.

- The environmental fate and bioavailability of the identified contaminants, diphenyl ethers, dioxins etc., should be determined under field conditions.

- TFM itself appear to be estrogenic. Although past use of TFM has not indicated any reproductive problems in fish populations a more detailed examination of this needs to be conducted to address this question. All indications from lab studies suggest that if exposure is long enough these types of responses may be seen in sensitive species in the field. The GLFC should 1) consider evaluating the risk of using an estrogenic compound in the environment, 2) conduct further lab and field studies to determine the significance of these responses.

Appendix 1.

Manuscripts resulting directly from this GLFC project to date.

1. Hewitt, L.M., I.M. Scott, K.R. Munkittrick, G.J. Van Der Kraak, K.R. Solomon and M.R. Servos. 1996. Development of TIEs for complex mixtures using physiological responses in fish. pp 37-52. *In* Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment (5th Volume), ASTM STP 1306, [*Eds.*] D.A. Bengtson and D.S. Henshel. American Society for Testing and Materials, Phiadelphia.

2. Hewitt, L.M., M.R. Servos, I.M. Scott, K. Solomon, J.C. Carey and K.R. Munkittrick. 1996. Use of a MFO-directed toxicity identification evaluation to isolate and characterize bioactive impurities from a lampricide formulation. Environ. Toxicol. Chem.15:894-905.

3. Hewitt, M, M. Servos, G. Van Der Kraak, K. Solomon, K. Munkittrick. 1996. Characterization of bioactive 3-trifluoromethyl-4-nitrophenol (TFM) lampricide formulation impurities. Proceedings of Dioxin '96, Amsterdam, The Netherlands, Aug. 11-15, 1996.

4. Hewitt, M.L., L. Tremblay, G.J. Van Der Kraak, K.R. Solomon and M.R. Servos. 1997. Identification of lampricide 3-trifluoromethyl-4-nitrophenol (TFM) as an agonist for the rainbow trout estrogen receptor. Environmental Toxicology and Chemistry *submitted.*

Several additional manuscripts are in preparation, including a Ph.D. which was directly supported by this project.

Presentations made as a direct result of this project (since 1995):

Hewitt, M., L. Tremblay, G. Van Der Kraak, K. Solomon and M. Servos. Identification of the lampricide 3,trifluromethyl-4-nitrophenol (TFM) as a ligand for the estrogen receptor in rainbow trout. Society of Environmental Toxicology and Chemistry Annual Meeting, Washington, DC. Nov. 17-21, 1996.

Hewitt, M, M. Servos, G. Van Der Kraak, K. Solomon, K. Munkittrick. Characterization of bioactive 3-trifluoromethyl-4-nitrophenol (TFM) lampricide formulation impurities. Dioxin '96, Amsterdam, The Netherlands, Aug. 11-15, 1996.

M.R. Servos. TFM Contaminants. Interim Meeting, Great Lakes Fishery Commission, Ann Arbor, MI, Nov. 30, 1995.

M.R. Servos. Unexpected biological impacts of lampricide formulations. Institute of Ichthylogy, University of Guelph, Guelph, Ont., Nov. 28, 1995

Scott, I. M. Hewitt, M. Servos, K. Munkittrick and G. Van Der Kraak. Methods for eliminating bioactive impurities from lampricide formulations. Society of Environmental Toxicology and Chemistry - Second World Congress, Vancouver, BC, Nov. 5-9, 1995.

Hewitt, M., L. Tremblay, M. Servos, I. Scott, G. Van Der Kraak, K. Solomon and K. Munkittrick. Development of a TIE directed by biochemical endpoints using the lampricide TFM. Society of Environmental Toxicology and Chemistry - Second World Congress, Vancouver, BC, Nov. 5-9, 1995.

Hewitt, L.M., M.R. Servos, J.H. Carey, G.J. Van Der Kraak and K.R. Munkittrick. Isolation and characterization of contaminants in a lampricide formulation associated with MFO induction in rainbow trout. Board of Technical Experts, Great Lakes Fishery Commission, Guelph, Ont. Oct. 18, 1995.

Servos, M.R. Toxicological implications of nitro-trifluoromethyl-chloro substituted dioxins in batches of TFM selected for use in the Bad River. Bad River Band Council, Ashland Wisconsin, August 2, 1995.

Servos, M.R. Trace contaminants in TFM formulations: An overview. Hoechst and AgrEvo Inc., Frankfurt, Germany, June 23, 1995.

Servos, M.R., L.M. Hewitt, I. Scott, K.R. Munkittrick and G.J. Van Der Kraak. Isolation and identification of chemicals responsible for physiological and reproductive disruptions in fish from complex mixtures. Society of Environmental Toxicology and Chemistry - EUROPE Congress, Copenhagen, June 25-30, 1995.

Servos, M.R. The toxicity and behaviour of nitro-trifluoromethyl-chloro substituted dioxins in TFM formulations. Natural Resources Department, Bad River Band, Ashland Wisconsin, June 19, 1995.

Servos, M.R., L.M. Hewitt, I. Scott, K.R. Munkittrick and G.J. Van Der Kraak. MFO induction in TFM formulations. Great Lakes Fishery Commission Annual Meeting, Toronto, June 5-7, 1995.

Hewitt, L.M., M.R. Servos, J.H. Carey, G.J. Van Der Kraak and K.R. Munkittrick. Isolation and characterization of contaminants in a lampricide formulation associated with MFO induction in rainbow trout. International Association of Great Lakes Research, East Lansing, MI., May 28-June 1, 1995.

Hewitt, L.M., Servos, M.R., I. Scott, G.J. Van Der Kraak and K.R. Munkittrick. Development of TIEs for complex mixtures using physiological responses in fish. Fifth Symposium on Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment, American Society for Testing and Materials, Denver, CO. April 3-5, 1995.

Servos, M.R. and M.L. Hewitt. Overview of the contaminants in TFM formulations. Expert Panel: Great Lakes Fishery Commission, Romulus, MI., March 2, 1995.

Servos, M.R., M.L. Hewitt, G. Van Der Kraak, J.C. Carey and K.R. Munkittrick. Effect of TFM on MFO induction in stream resident fish. Annual Meeting of the Great Lakes Sea Lamprey Control Agents, Traverse City, MI, Jan. 18-19, 1995.

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USE OF AN MFO-DIRECTED TOXICITY IDENTIFICATION EVALUATION TO ISOLATE AND CHARACTERIZE BIOACTIVE IMPURITIES FROM A LAMPRICIDE FORMULATION

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Abstract—Recently, a field formulation of the lampricide containing 3-trifluoromethyl-4-nitrophenol (TFM) was identified as a potent inducer of mixed-function oxygenase (MFO) detoxification enzymes in fish. It was further shown that induction was not associated with primary formulation ingredients. Using a toxicity identification evaluation (TIE) approach based on rainbow trout hepatic MFO activity, the TFM field formulation was investigated to isolate the compound(s) responsible for induction. Solid phase extraction and reverse-phase high-pressure liquid chromatography (HPLC) isolated activity in two distinct fractions, which were profiled by gas chromatography–high-resolution mass spectrometry. The major constituents in both fractions were confirmed by synthesis as nitro-, trifluoromethyl-, and/or chloro-substituted diphenyl ethers. However, fish exposures to the pure compounds failed to cause MFO induction. After further fractionations by HPLC, induction was determined in three new subfractions. Confident identification of a chloro-nitro-trifluoromethyl-substituted dibenzo-*p*-dioxin has been made in two of these fractions. Although the specific chemicals responsible for induction have not been confirmed, a suite of impurities including chloro-, and/or nitro-, and/or trifluoromethyl-substituted phenols, diphenyl ethers, and dibenzo-*p*-dioxins have been identified in the formulation. It is likely that these materials originate during industrial synthesis of TFM. These findings suggest that additional structurally related impurities are also present in this formulation.

Keywords—TFM MFO Diphenyl ether Dibenzo-*p*-dioxin TIE

INTRODUCTION

The predation of sea lamprey (*Petromyzon marinus*) on native salmonid populations in the Great Lakes Basin was first noted in the late 1800s [1]. Since the early 1950s, the Great Lakes Fishery Commission has conducted an extensive control program in an effort to protect salmonids from expanding sea lamprey populations. The application of 3-trifluoromethyl-4-nitrophenol (TFM) to nursery streams has been the primary means of control for sea lamprey ammocoetes since the late 1950s. Streams are treated on a 3- or 4-year cycle and current usage approximates 50,000 kg per year of TFM. Treatment concentrations vary from 1.0 mg/L to 14 mg/L of TFM, depending on pH and hardness [2–4]. In some cases, treatments have been supplemented with the addition of 2′,5-dichloro-4′-nitrosalicylanilide (Bayer 73) [2,5].

Mixed-function oxygenase (MFO) activity has recently been associated with use of lampricides [6]. Mixed-function oxygenase enzymes are a class of detoxification enzymes and induction of P450IA enzyme activity in fish has been associated with exposure to compounds such as polychlorinated biphenyls (PCBs) [7], polychlorinated dibenzo-*p*-dioxins and furans (PCDD/PCDFs) [8], and polyaromatic hydrocarbons [9]. Induction of MFOs was initially observed in white sucker (*Catostomus commersoni*) caged during a lampricide treatment of the Nipigon River in August 1992 and later replicated in static laboratory exposures using rainbow trout (*Oncorhynchus mykiss*) [6]. Attempts to isolate the responsible compound(s) showed that induction was associated with the TFM formulation and not Bayer 73. Further experimentation showed that MFO induction was associated with the field formulation and not TFM itself [6]. Formulation concentrations of known MFO-inducing chemicals, including polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and polyaromatic hydrocarbons were below detection limits.

Although MFO induction and steroid hormone disruptions were associated with exposure to the formulation in the previous laboratory experiments [6], it is not possible to evaluate the significance of the contamination without identifying the contaminants in the formulation that are responsible for the biochemical responses. A full understanding of the significance of the contamination would require information on contaminant structure, levels in the formulation, environmental fate, and a perspective on historical levels of release over the last four decades. The first step in identifying the unknown chemicals would be to isolate them from the formulation. The objective of this study was to develop and apply an MFO-directed toxicity identification evaluation (TIE) to isolate the biologically active chemical(s).

MATERIALS AND METHODS

The methods described are modifications of the toxicity identification and evaluation (TIE) approach [10–12]. Fractionations were directed by rainbow trout hepatic MFO activity as the biological endpoint. Solid phase extraction was employed to isolate bioactive formulation impurities from primary formulation ingredients that had been previously shown to not exhibit

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the ability to induce MFO activity [6]. Subsequent fractionations were performed using reverse-phase preparative high-pressure liquid chromatography (HPLC). Bioactive fractions were characterized by gas chromatography–high-resolution mass spectrometry (GC-HRMS). Fractionations and exposures were conducted on the 1990-batch #2 field formulation manufactured by Hoescht Chemical (provided by Sea Lamprey Treatment Centre, Sault Ste. Marie, ON, Canada). The formulation is 37% (w/v) TFM in a solution of isopropanol and aqueous sodium hydroxide.

Fish exposures

Juvenile rainbow trout (*Oncorhynchus mykiss*; 3–5 g; Rainbow Springs Hatchery, Thamesford, Ontario, Canada) exposures were conducted in glass aquaria in darkness at a loading density of 2.5 g/L (n = 6). Fish were acclimated to 13°C and fed (Martin's Feed Mill, Elmira ON, Canada) ad libitum until 6 d prior to exposures and were not fed during exposures. Exposures were for 72 h to 150 μl of filtered (<0.5 μm) field formulation (one formulation equivalent) in 12 L water used for holding (dechlorinated Burlington city tap water; pH 7.5–8.0, hardness 128–133 mg/L). This resulted in a nominal TFM concentration of 4.6 mg/L, which is within the range of concentration used in field treatments and optimized the MFO response while avoiding acute toxicity [13]. Exposures were run with two negative controls, which included reference fish (unexposed) and fish exposed to the maximum amount of solvents associated with the exposed fractions. One exposure to filtered (<1 μm) formulation served as a positive control for each experiment. To determine if induction potential was affected during HPLC separations, the eluent from the entire run of each HPLC separation was collected, concentrated under reduced pressure to 5 ml, and exposed to fish. Treatments were conducted singly within each experiment and experiments were repeated separately. After exposures, fish were euthanized by concussion and spinal severance, and livers were excised for immediate determination of MFO activity.

Rainbow trout hepatic MFO activity

Mixed-function oxygenase activities were determined as ethoxyresorufin-*O*-deethylase (EROD) activity. Excised livers were placed in cryovials and immediately homogenized using a microhomogenizer (Kontes; Baxter Diagnostics Corp.) in 1 ml of cold HEPES-KCl (HEPES, Sigma Chemical Company, St. Louis, MO; KCl, BDH Chemical Company, Toronto, ON, Canada) buffer (0.02 M HEPES, 0.15 M KCl, pH 7.5). Determinations of EROD activity (pmol/min/mg) were conducted using the spectrofluorometric method described by McMaster et al. [14]. A simplified method was used to determine EROD activity in the TIE procedures. Spectrofluorometric assays were performed using 300 μl fresh liver homogenates; EROD activities are expressed in fluorescence units (FU). In addition to increasing sensitivity, this modification significantly reduced analysis time, facilitating the optimization of TIE method development. A high correlation (r^2 = 0.89) between homogenate and postmitochondrial supernatant activities normalized for protein was shown for fish exposed to a range of formulation concentrations [13]. Duplicate analyses were performed for each fish and one blank was determined for each aquaria.

Solid phase extraction

Solid phase extraction (SPE) was initially employed to isolate bioactive formulation impurities from primary formulation ingredients. Cartridge phase, packing size, and elution solvents were optimized to isolate bioactivity. All SPE fractionations were performed on a preparative scale using 150 μl of formulation (one formulation equivalent); fish exposures were conducted directly with the fractions generated. Cartridges (C_{18}, 500 mg; 6 ml, J.T. Baker Chemical Co.) were conditioned with four bed volumes of methanol (DIG grade, Caledon Laboratories, Georgetown, Ontario, Canada) followed by four bed volumes of 40:60 (%) methanol : tris (Canlab, Mississauga, ON, Canada) buffer (pH 8, 0.2 M). Formulation aliquots were applied directly to the cartridges and elutions were performed under 5" Hg vacuum using a cartridge manifold (Chromatographic Specialties). Elution of TFM was accomplished with 9 × 1 ml additions of methanol : tris buffer (SPE-1). Inducing contaminants were recovered in 3 × 500 μl methanol (SPE-2).

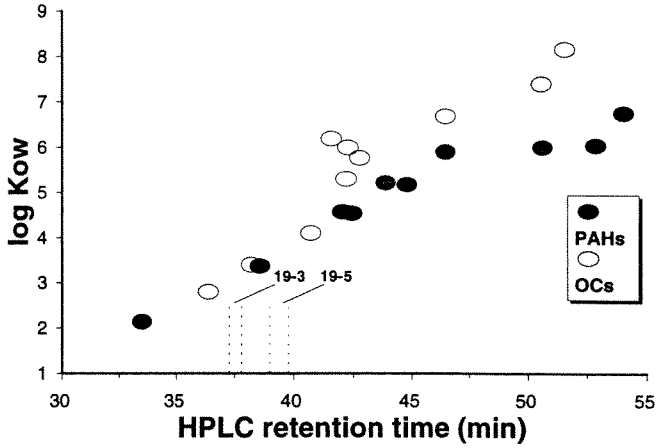

Fig. 1. Relationship between log K_{ow} and HPLC retention time under primary fractionation chromatographic conditions.

HPLC fractionations

Extracts from SPE-2 that contained the inducing contaminants were fractionated directly using preparative HPLC. A Waters (Millipore Corp., Milford, MA, USA) system consisting of a 717 autosampler, a 600E system controller, a 610 valve station, and a 481 spectrophotometer UV detector set at 254 nm (Millipore Corp.) was utilized. Separations were achieved with a 500 mm × 9.4 mm i.d. reverse-phase Partisil 10 ODS 2 column (Watman, Clifton, NJ) under the following conditions: flow rate 4 ml/min, column preconditioning and an initial 2 min hold of 10:90 (%) methanol : 0.2 M pH 4 acetate (Caledon Laboratories) buffer, linear gradient to 100% methanol at 34 min, and hold for 21 min. The HPLC retention times using these conditions were determined for a variety of polyaromatic hydrocarbons and organochlorine compounds (PCBs, benzenes, and insecticides). The relationship between reported log K_{ow} [15–20] and HPLC retention time was used to estimate the log K_{ow} of the bioactive fractions (Fig. 1). To maintain a consistent fraction classification system, fractions are identified according to the experiment in which they were derived and the order in which they were collected within that experiment (e.g., 16-1: experiment 16, fraction number 1).

Primary HPLC conditions were optimized to resolve SPE-2 into as many components visible at 254 nm as possible. To achieve maximal resolution of the components within the primary bioactive fractions, secondary fractionations utilized different elution programs and fractions were concentrated threefold. Solvent programming for 19-3 separations was as follows:

column preconditioning and an initial 2-min hold of 10:90 (v/v) methanol : acetate buffer, a linear gradient to 70:30 (v-v) methanol : acetate buffer at 21 min, followed by a linear gradient to 100% methanol at 55 min, which was held for 15 min. The solvent program for the 19-5 subfractionation was identical to the 19-3 program except that the final linear gradient to 100% methanol was extended to 65 min.

Extraction and GC-HRMS characterizations

High-pressure liquid chromatography fractions associated with MFO induction were frequently a mixture of methanol and acetate buffer; solvent exchange was necessary for GC-HRMS analysis. Extraction of bioactive fractions was optimized to recover biological activity. High-pressure liquid chromatography fractions associated with induction were diluted to 50 ml with purified water (Millipore Corp.), and vigorously stirred with 10 ml toluene (DIG grade, Caledon Laboratories) for 1 h. Toluene extracts were quantitatively recovered with a pasteur pipette, concentrated under a gentle stream of nitrogen (ultrahigh-purity carrier grade, CANOX, Mississauga, Ontario, Canada) to dryness, and dissolved in methanol for fish exposures.

High-resolution gas chromatography–high-resolution mass spectrometry analyses of toluene extracts were performed with a Hewlett Packard 5890 GC equipped with a retention gap consisting of 1 m × 0.53 mm i.d. deactivated fused silica gel connected to a 2.5 m × 0.25 mm i.d. section of deactivated fused silica. The retention gap was fitted to a 60 m × 0.25 mm i.d. DB-5 (J & W Scientific, Folsom, CA, USA) column bonded to 0.25-μm phase thickness. The GC was interfaced at 280°C to a VG Autospec-Q mass spectrometer (Fisons, VG Analytical, Manchester, UK). Injections were 2 μl on-column with a helium (ultrahigh-purity carrier grade, CANOX) carrier. Gas chromatography oven and injector temperatures were initially held at 80°C for 0.1 min then programmed at 4°C/min to 280°C, held for 2 min, programmed at 4°C/min to 290°C, and held for 10 min. Positive-ion electron-impact (EI) mass spectra were obtained using both full-scan and selection ion monitoring (SIM). Prior to chromatographic sequences, the instrument was calibrated using high-purity perfluorokerosene. During SIM acquisitions, small amounts were continually bled into the source and a lockmass within the range of target ions was acquired during each scan. Full-scan analyses were conducted at 1,000 resolution from 40 to 450 amu, a scan rate of 1.00 scans/s, a source temperature of 270°C, an electron energy of 70 eV, an accelerating voltage of 8,000 V, and filament current of 4.04 A. Unknown spectra were compared with computer searches of the NIST library. SIM analyses of exact masses were acquired at 10,000 resolution with a scan rate of 700 ms.

Syntheses and purification of diphenyl ethers

All diphenyl ether syntheses were carried out under identical conditions and, because each product contained a TFM moiety, the reactions differed only in one of the starting materials; 1-bromo-3-trifluoromethyl-4-nitrobenzene (Aldrich, Milwaukee, WI, USA) was used in each case. Equimolar amounts (0.001 moles) of this compound were combined with *p*-chlorophenol (Aldrich) in the formation of 3-trifluoromethyl-4-nitro-4′-chlorodiphenyl ether (compound A), *o*-chlorophenol (Aldrich) in the preparation of 2-chloro-3′-trifluoromethyl-4′-nitrodiphenyl ether (compound B), and pure TFM (Aldrich) for the synthesis of 3,3′-bis(trifluoromethyl)-4,4′-dinitrodiphenyl ether (compound C). For each reaction, the reagents were dissolved with 10 g potassium carbonate (Caledon Laboratories) in 300 ml acetone (DIG grade, Caledon Laboratories) contained in a round-bottomed flask. Boiling chips were added and the mixture was refluxed overnight. Purified products were obtained by preparative HPLC separations of crude reaction mixtures. The solvent program used in the SPE-2 fractionations was sufficient in purifying compounds A and C but a modified elution program was necessary to isolate compound B. After preconditioning the column with 10:90 (v/v) methanol : acetate buffer and holding for 2 min, the mobile phase was linearly programmed to 70:30 methanol : acetate buffer at 5 min, then linearly programmed to 100% methanol at 40 min. Purified products were dissolved in toluene and analyzed by full-scan GC-HRMS. Confirmation of identity was obtained when the GC retention time and mass spectra of the tentatively identified constituents matched those for the purified synthetic materials [21]. Additional confirmation for compounds A and C was obtained through HPLC purification; HPLC product retention times were within the retention ranges of the corresponding fractions in which these materials had been tentatively identified. Analytical standards for quantification of compounds A, B, and C were prepared by recrystallizing the products obtained from HPLC purifications twice with toluene.

Fish exposures to synthesized products

After identity confirmation, each compound was tested for the potential to cause MFO induction. Purified synthetic products were prepared for fish exposures using preparative HPLC as described previously. Using UV detector responses, enough of each purified product was collected to expose fish to a range of formulation equivalents. Because compounds A and B were found in the same fraction, a separate exposure to a combination of 10 equivalents each was performed. Mixed-function oxygenase activities associated with each compound or mixture were compared to their respective fractions, exposed concurrently.

Statistics

Data were checked for normality using SYSTAT software [22] and had to be log transformed. One-way analyses of variance (ANOVA) were conducted for individual experiments. Tukey's HSD pairwise comparisons and post hoc contrasts were used to compare differences between treatments. Data are presented untransformed. Bars on all figures represent ±1 standard error of the mean.

RESULTS

Solid phase extraction

Based on the conclusions of Munkittrick et al. [6], we hypothesized that the inducers within field formulations were contaminants less polar than TFM. Solid phase extraction was employed to separate inducing formulation contaminants from TFM because of the large chromatographic interference and the acute toxicity associated with TFM; the isolation of bioactive formulation impurities would be facilitated after TFM removal. After optimizing the solvent/buffer systems and cartridge size, >99% of TFM was eluted in the first SPE fraction (SPE-1), whereas induction was recovered in SPE-2 ($p < 0.001$) (Fig. 2).

Primary HPLC fractionations

High-performance liquid chromatography fractionations of SPE-2 were optimized to achieve maximum resolution of constituents visible at 254 nm. The first experiment showed that,

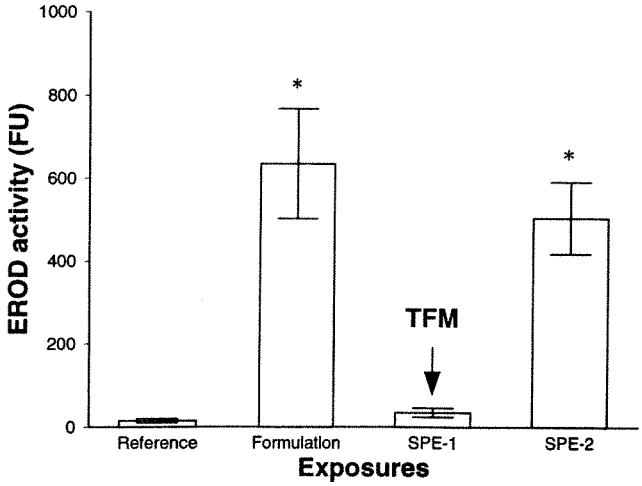

Fig. 2. Rainbow trout hepatic EROD activity for fish exposed to fractions generated from SPE of the TFM formulation. No significant induction was associated with SPE-1 ($p = 0.254$). Bars with asterisks are not significantly different from each other ($p < 0.001$).

relative to the whole formulation, MFO activity was not reduced after passage through the HPLC ($p = 0.707$) and that induction was recovered with the chromatographic region showing peaks at 254 nm ($p = 0.912$). After several series of fractionations and exposures, it was determined that two distinct fractions were associated with induction, 19-3 and 19-5 ($p < 0.001$) (Fig. 3). Exposures for the 19-series of fractionations were conducted at three formulation equivalents to ensure complete resolution of activity as these fractions were collected at the separatory limit of the elution program. Although statistically significant ($p < 0.05$) induction was associated with one replicate of 19-4, the induction associated with fraction 19-4 was weak (approximately threefold) and not observed in a repeat of the entire experiment. It is possible that the induction observed for this fraction was a result of carryover during fraction collections. It should also be noted that this induction was observed after exposures to three formulation equivalents and EROD activities were determined on fresh whole liver homogenates, which are

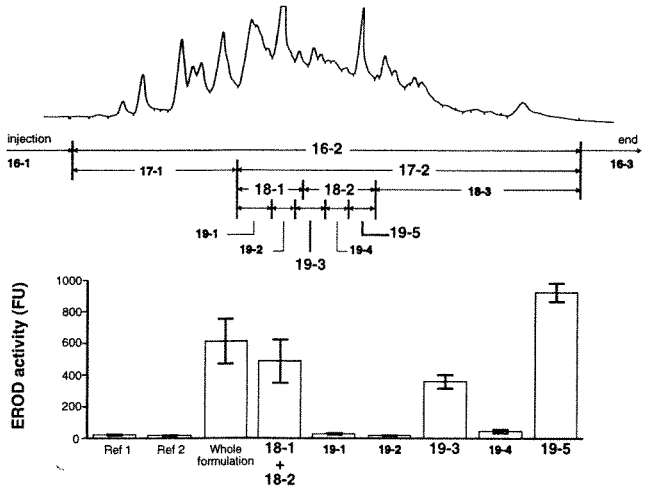

Fig. 3. HPLC fractionation of SPE-2 fraction with detection at 254 nm. Fractions indicated in large type show where MFO activity was observed. The graph depicts rainbow trout hepatic EROD activity associated with the final fractions. Exposures to fractions 19-1 through 19-5 were conducted at three formulation equivalents.

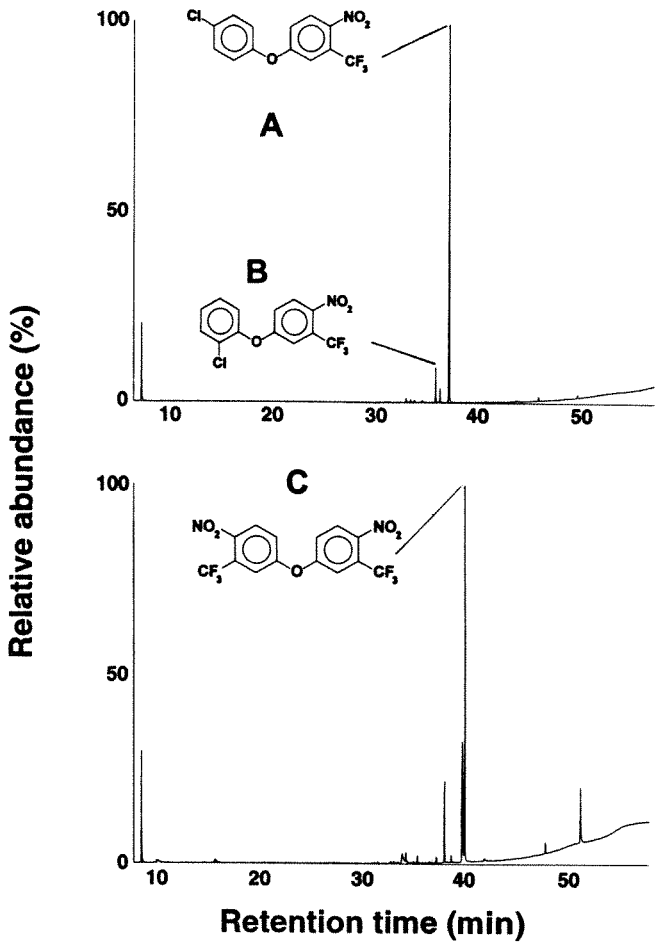

Fig. 4. Total ion chromatograms for the toluene extracts of fractions 19-3 and 19-5. Compounds A, B, and C were confirmed by synthesis.

more sensitive than assays conducted using postmitochondrial supernatants; subsequent experiments focused on fractions 19-3 and 19-5. Relative to fraction 19-3, higher levels of induction were consistently associated with fraction 19-5 ($p < 0.001$).

GC-HRMS characterizations of primary bioactive fractions

Ethoxyresorufin-*O*-deethylase activities associated with the original fractions were not significantly different from the induction recovered from toluene extraction of fractions 19-3 and 19-5 as well as the SPE-2 fraction ($p > 0.282$). Total ion chromatograms of fractions 19-3 and 19-5 are presented in Figure 4. Both fractions are dominated by one or two large constituents (A, B, C), which are also evident in the toluene extract of SPE-2 (not shown). The presence of these compounds in the SPE-2 extract suggested that these compounds were potential inducers. In the following discussion, published guidelines regarding the use of spectroscopic and chromatographic properties for the identification of unknowns will be followed [21]. Identifications are noted as confident when there is no authentic standard available but either the spectral or chromatographic data matches closely published data, including mass spectral databases and retention indices. Compounds are described as confirmed when retention times and mass spectra match those for authentic standards analyzed under identical conditions.

The positive-ion EI mass spectra for compounds A, B, and C are given in Figure 5. The spectrum for compound A shows a strong apparent molecular ion at m/z 317. The isotope ratio

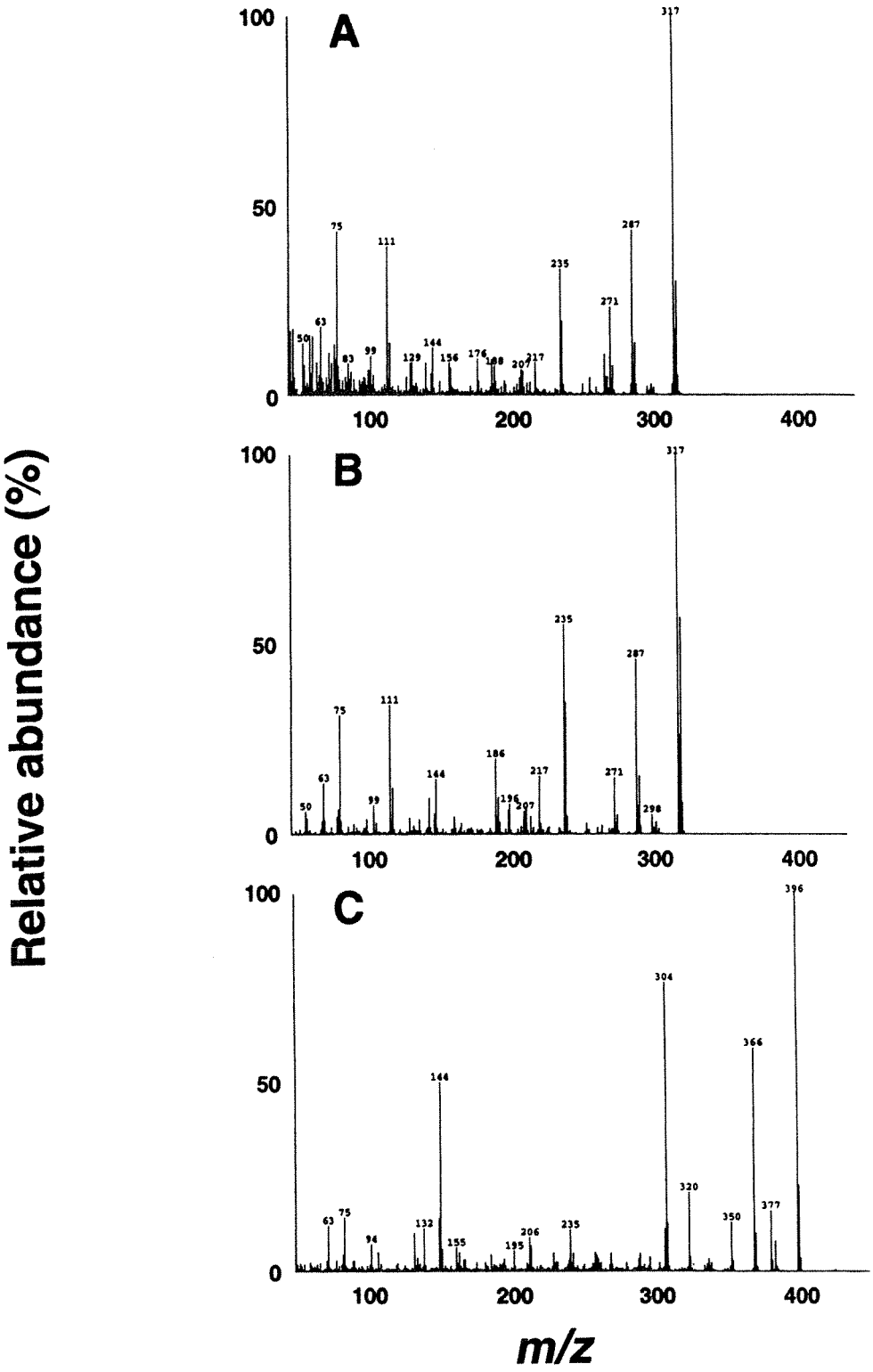

Fig. 5. Electron impact mass spectra obtained from fractions 19-3 and 19-5 for compounds A, B, and C.

of the m/z 319 ion indicated the presence of chlorine substitution, the loss of which is seen from m/z 271 to 235. The losses of 30 and 16 amu from the molecular ion are characteristic of an aromatic nitro group. The relatively strong m/z 75 ion also indicated the presence of electron-withdrawing groups on an aromatic ring [23]. The 110 amu molecular weight difference between the unknown and TFM (207 g/mol) suggested that more than one aromatic ring may be involved. Full-scan analysis of the toluene extract of SPE-2 showed the presence of several single-ringed phenolic derivatives. The presence of mono-chlorophenol was confirmed with an authentic standard. Based on mass spectral interpretation and retention times relative to chlorophenol, three isomers of chloro-trifluoromethylphenol, trifluoromethylphenol, and two isomers of TFM have been confidently identified. A diphenyl ether configuration combining TFM and chlorophenol with the loss of water has a molecular

Table 1. Concentrations of selected impurities in the TFM formulation

	Formulation impurity	Concentration ± SE (mg/L formulation)
Compound A	3-Trifluoromethyl-4-nitro-4′-chlorodiphenyl ether	66.6 ± 3.3
Compound B	2-Chloro-3′-trifluoromethyl-4′-nitrodiphenyl ether	1.1 ± 0.1
Compound C	3,3′-Bis(trifluoromethyl)-4,4′-dinitrodiphenyl ether	23.5 ± 1.4
		Estimated concentrations (mg/L formulation)
Compound D	Chloro-hydroxyl-nitro-trifluoromethyldiphenyl ether	18
Compound E	Dichloro-hydroxyl-trifluoromethyldiphenyl ether	0.1

weight of 317. The toluene extract of 19-5 was reanalyzed at 10,000 resolution under SIM, scanning for the exact masses of the molecular ion as well as several fragment ions of the proposed diphenyl ether. The analysis showed the presence of these ions at the retention time of the unknown. The structure with the chlorine located *para* to the ether linkage was later confirmed by synthesis (compound A).

The spectrum of the earlier-eluting unknown was almost identical. It was hypothesized that the *ortho*-substituted analogue would elute earlier, and this was also confirmed by synthesis (compound B).

The major peak in fraction 19-3 showed an apparent molecular ion at m/z 396 amu with no visible chlorine isotope cluster. The losses of 30 and 16 amu and the prominent ions at m/z 366, 320, and 304 indicate consecutive losses of aromatic nitro groups. The ions at m/z 63 and 75 indicate the presence of aromatic electronegative substituents. A diphenyl ether structure combining two TFM molecules with the elimination of water has a molecular weight of 396. Further evidence of this structure is provided by the strong m/z 144 ion, which represents cleavage of the ether to yield single rings, each containing a trifluoromethyl group. The molecular formulae of these ions were confirmed using SIM. The structure with the trifluoromethyl and nitro groups oriented *meta* and *para*, respectively, to the ether linkage was verified by synthesis (compound C). Formulation concentrations of these materials are presented in Table 1.

MFO induction associated with diphenyl ethers

Rainbow trout were exposed to a range of formulation equivalents of each compound. No significant induction was associated with compounds A and B, exposed singly at several concentrations, or together at 10 equivalents each ($p < 0.001$). No induction was associated with compound C at any of the concentrations exposed ($p < 0.001$). Because both bioactive fractions were collected at the separatory limit of the primary HPLC fractionation, further fractionations were investigated by reinjection of 19-3 and 19-5 on the HPLC.

HPLC subfractionation of primary bioactive fractions

After further separations of both fractions were optimized, successive fractionation experiments proceeded as with the first series of HPLC fractionations. For both fractions it was shown that induction was recovered after injection and passage through the HPLC system and that induction was again associated with the chromatographic region containing peaks visible at 254 nm ($p < 0.001$) (Figs. 6 and 7). Continued fractionations of the visible peaks' portion of the 19-3 profile showed that induction was confined within a region containing approximately three unresolved peaks of relatively low intensity, designated 30-3 (Fig. 6). Subfractionation of the 19-5 fraction eventually demonstrated that most of the induction of this fraction (and the formulation) was associated with a relatively small peak (fraction 34-2) and that a low level of activity was consistently associated with the dominant peak of the chromatogram (fraction 31-3), which contained compounds A and B ($p < 0.05$) (Fig. 7).

GC-HRMS analysis of active subfractions

Analysis of the toluene extract of 30-3 showed a single unique peak and the mass spectrum is presented in Figure 8 (compound D). An apparent molecular ion at m/z 333 has an isotope cluster indicating monochloro substitution. The neutral losses of 30 and 16 amu indicate the presence of an aromatic nitro substituent. Nitro substitution further indicated the presence of a TFM moiety, but to obtain a molecular mass of 333 another ring system is likely. The presence of chlorine substitution suggested that chlorophenol was again involved. A diphenyl ether similar to compound C without the elimination of water has a molecular weight of 333. The molecular formula of the tentative structure was later verified by high-resolution scans.

Analyses of the toluene extract of 31-3 also showed one unique peak and its mass spectrum of presented in Figure 8 (compound E). The unknown has an apparent molecular ion of m/z 322. The isotope cluster indicates dichloro substitution, and the loss of both chlorines is reflected by the absence of a cluster at m/z 252. A dichlorinated diphenyl ether containing a trifluoromethyl group without the elimination of water has a molecular weight of 322. The molecular formula of this structure was also verified by SIM. The presence of hydroxy diphenyl ethers in the base-insoluble extracts of pentachlorophenol has been noted previously [24]. Substituent locations on these structures would require synthesis for confirmation and are depicted as such based on the precursors identified in SPE-2. Approximate concentrations for compounds D and E have been proposed using the response factors from compounds A, B and C (Table 1).

Initial full-scan analyses were unable to detect any unique peaks in the fraction with the highest potency, 34-2. Because sensitivity is compromised when acquiring mass spectra by full scan, greater quantities of 34-2 were generated and the extracts were combined and concentrated for reanalysis. A highly concentrated extract of 34-2 showed trace levels of one unique constituent and its mass spectrum is presented in Figure 9. The unknown has an apparent molecular ion at m/z 331, with an isotope cluster indicating monochloro substitution. The presence of m/z 301 and 285 indicate loss of an aromatic nitro group, which suggests the presence of a TFM moiety. Chlorine substitution indicates that chlorophenol is again involved, especially considering the relatively high molecular weight. A dibenzo-

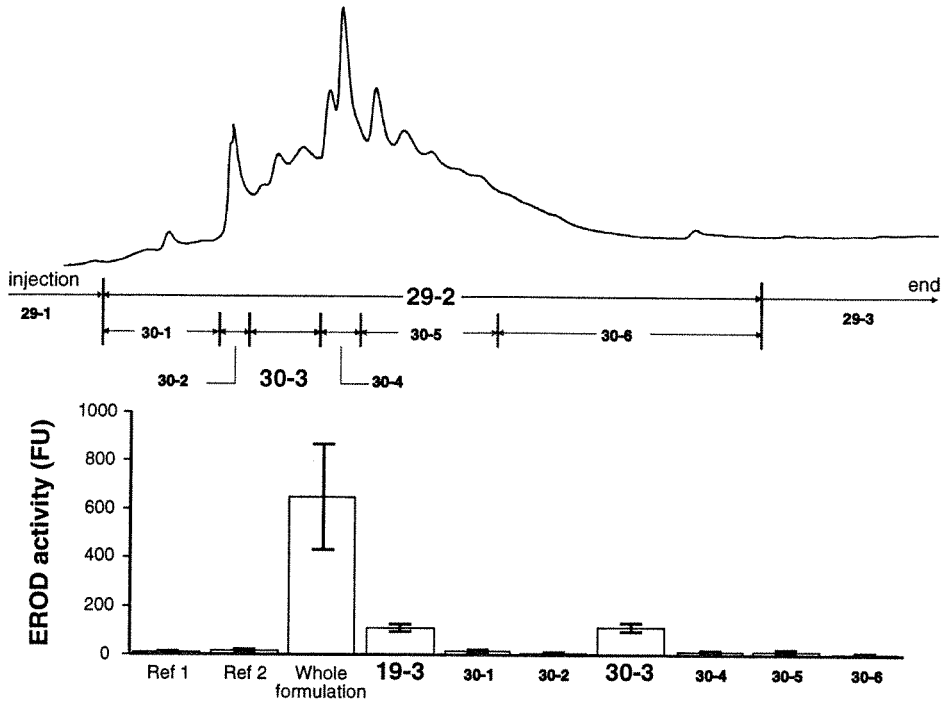

Fig. 6. HPLC fractionation of fraction 19-3 with detection at 254 nm. Fractions indicated in large type show where MFO activity was observed. The graph depicts rainbow trout hepatic EROD activity associated with the final fractions.

p-dioxin condensation of compounds A or B gives a molecular weight of 331 and is proposed as the structure (compound F). In addition to chlorine losses, chlorinated dioxins typically lose a neutral COCl fragment during EI ionization [25]. A COCl fragmentation in the unknown is indicated by the loss of 63 amu from m/z 285 to m/z 222. Additional evidence for this structure was gained after the molecular formulae and ratios of these ions were corroborated by SIM. Selection ion monitoring analyses of the SPE-2 fraction also showed the presence of this compound and analysis of 31-3 detected low levels of three apparent isomers.

Further evidence for the existence of this compound was received after several closely related structural analogues were obtained (S. Safe, Texas A & M University, College Station, TX, USA). Standards of 2,3,7-trichloro-8-trifluoromethyldibenzo-*p*-dioxin, 2,3,7-trichloro,8-nitrodibenzo-*p*-dioxin, and 2,3-dichlo-

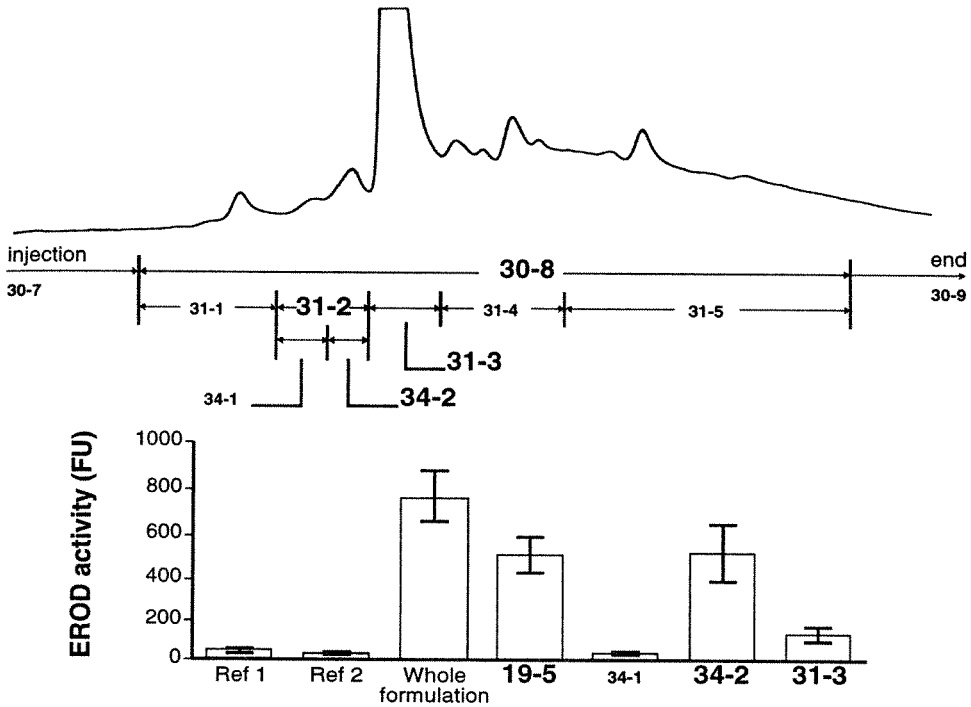

Fig. 7. HPLC fractionation of fraction 19-5 with detection at 254 nm. Fractions indicated in large type show where MFO activity was observed. The graph depicts rainbow trout hepatic EROD activity associated with the final fractions.

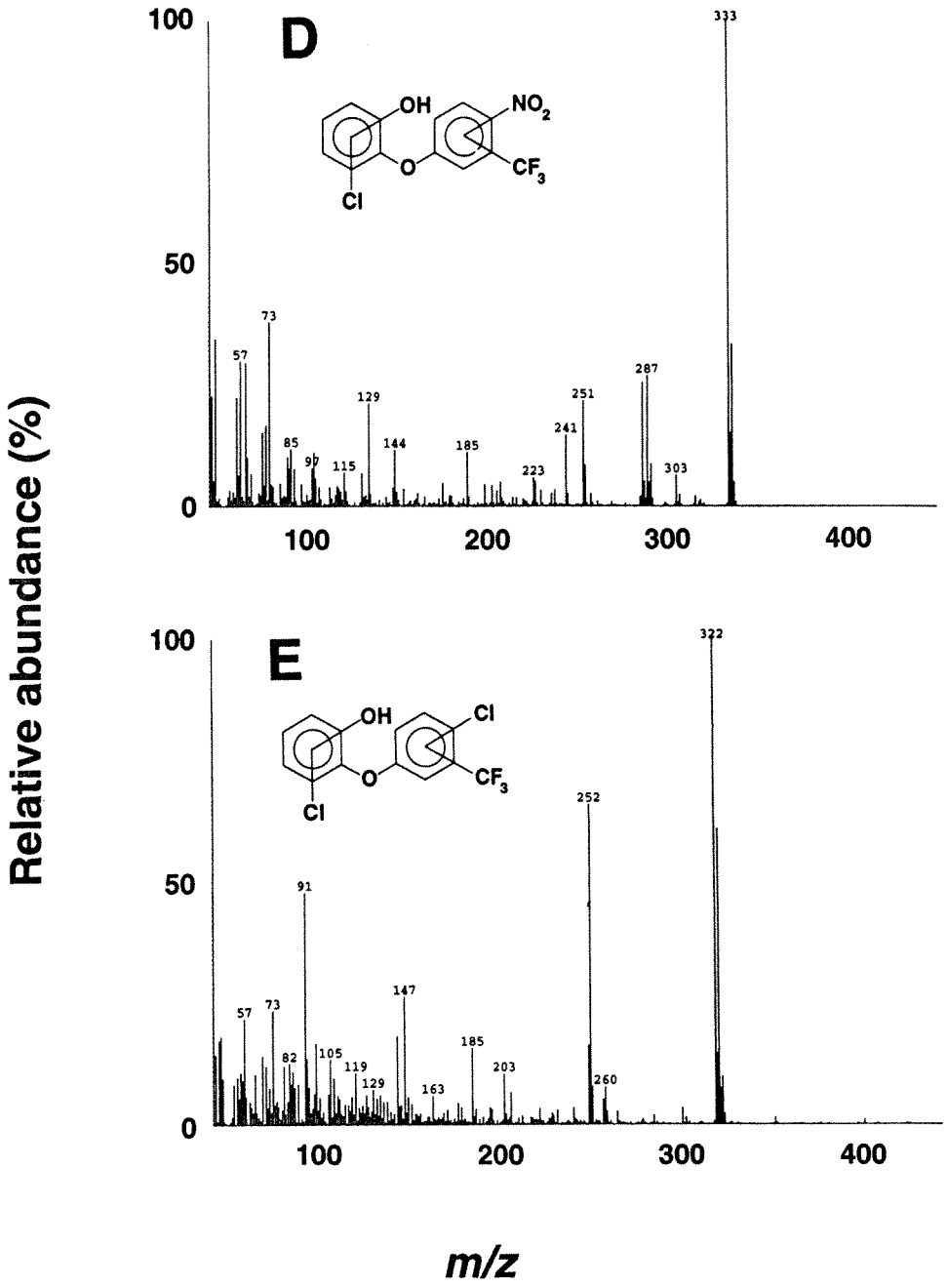

Fig. 8. Electron-impact mass spectra and tentatively assigned hydroxylated diphenyl ether structures for unknowns in fractions 30-3 (compound D) and 31-3 (compound E).

ro-7-trifluoromethyldibenzo-*p*-dioxin were prepared and analyzed by GC-HRMS. Electron impact mass spectra for these compounds are consistent with spectra for compounds A, B, and C as well as for compound F. Neutral losses of COCl are also observed for the dioxins. With these compounds, the tetra-substituted dioxins eluted after the trisubstituted congener, as is expected with chlorinated dioxins and furans on columns of this polarity. Substitution of chlorine with nitro- and trifluoromethyl-substituents appears to increase and decrease, respectively, GC retention times by approximately equal factors, within the tetra-substituted congener series. During these elutions oven temperatures are being programmed at 4°C/min and have not reached their final holding temperature. 2,3,7-Trichloro-8-trifluoromethyldibenzo-*p*-dioxin eluted at 39.6 min and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) elutes at 45.0 min, a difference of 5.4 min. Substitution of chlorine with a nitro-substituent increased retention time by 5.3 min, as 2,3,7-trichloro-8-nitrodibenzo-*p*-dioxin eluted at 50.3 min. 2,3-Dichloro-7-trifluoromethyldibenzo-*p*-dioxin eluted at 35.5 min and one would predict a retention time approximating 41 min for a corresponding chloro-nitro-trifluoromethyl-substituted dioxin. Compound F elutes at 43.5 min; the discrepancy may be due to several factors, including substituent orientation. Based on response factors from these compounds, the formulation concentration of compound F is estimated in the low parts per billion range.

In addition to the diphenyl ethers and single-ringed precursors previously noted in the toluene extract of SPE-2, numerous

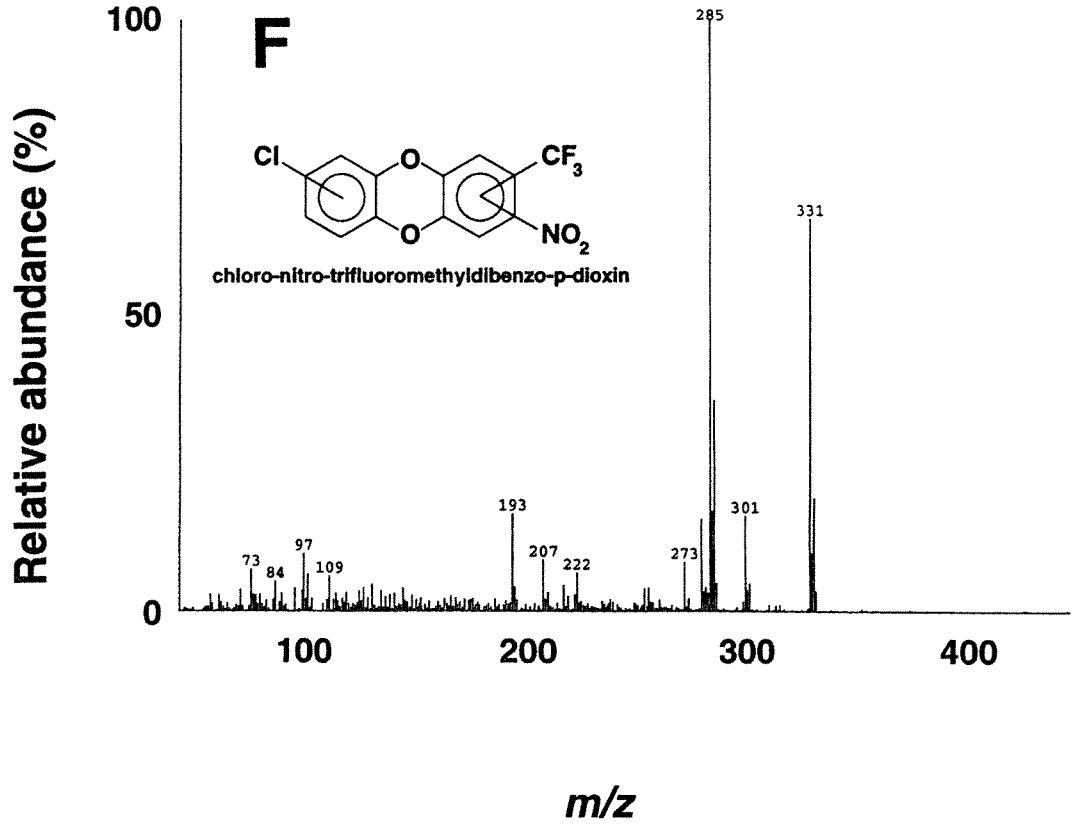

Fig. 9. Electron-impact mass spectra and molecular structure proposed for compound F, identified in fraction 34-2.

impurities possessing chloro- and/or nitro-functionalities with apparent molecular ions > 300 amu have been observed. The impurities identified in the bioactive fractions as well as the presence of compound F suggested that other dioxins with similar substitutions may also be present. Toluene extracts of SPE-2 were monitored at 10,000 resolution for the molecular ions of several suspected tri- and tetra-substituted dioxins, furans, and diphenyl ether structures. The molecular ions and corresponding ^{37}Cl isotopes in their proper ratios have been detected for several compounds and the retention times are consistent with the synthesized diphenyl ethers and the related dioxins obtained in this study. Rough approximations based on the response factors of related compounds place the suspected dioxin and furan formulation concentrations in the low to sub-nanogram per liter range, which would result in low picogram per liter concentrations in river systems during application.

DISCUSSION

The objective of the MFO-directed TIE was to isolate and recover induction from the TFM field formulation. Solid-phase extraction followed by preparative HPLC was successful in isolating impurities that induce fish hepatic EROD activity from the formulation. High-pressure liquid chromatography analysis using UV detection at 254 nm showed the presence of many formulation impurities and bioactivity was isolated within two narrow fractions during primary HPLC separations. Chemical analysis was focused on bioactive fractions in an attempt to identify the responsible chemicals. Solvent extraction of bioactive compounds from each fraction was verified with bioassays prior to analysis by GC-HRMS. Cold on-column injection ensured delivery of bioactive extracts onto the GC column and reduced the potential decomposition of thermally labile compounds.

Mixed-function oxygenase induction was eventually isolated in a total of three HPLC fractions (34-2, 31-3, and 30-3). It is possible that additional inducing compounds are present in fractions that showed no induction because they were below the thresholds associated with the short-term exposures employed. However, the objective of this study was to focus on the chemicals primarily responsible for causing induction observed after short-term (<24-h) field application of the lampricide. The induction associated with fraction 31-3 could be a result of carryover from 34-2 and this hypothesis is supported by the apparent presence of compound F in both fractions. The identity of the compound(s) responsible for the induction associated with fraction 30-3 is presently unknown, but it can be concluded that at least two compounds with the potential to induce MFO activity are present in the formulation.

The presence of three diphenyl ether impurities (compounds A, B, C) was confirmed in the primary bioactive fractions (Fig. 5). Formulation concentrations of these compounds were as high as 66 mg/L (Table 1). Low levels of MFO induction in rats and fish have been associated with exposure to diphenyl ethers [26]. However, several concentrations failed to cause MFO induction in exposed fish. Two additional hydroxylated diphenyl ethers were also confidently identified in the final bioactive fractions 30-3 and 31-3 (Fig. 8). Based on the previous exposures to structurally similar compounds (compounds A, B, C), it is unlikely that MFO induction is associated with these materials.

All of the diphenyl ethers identified contain at least one TFM moiety and all but one are chlorinated to some degree. Carey et al. [27] reported the presence of several TFM impurities in

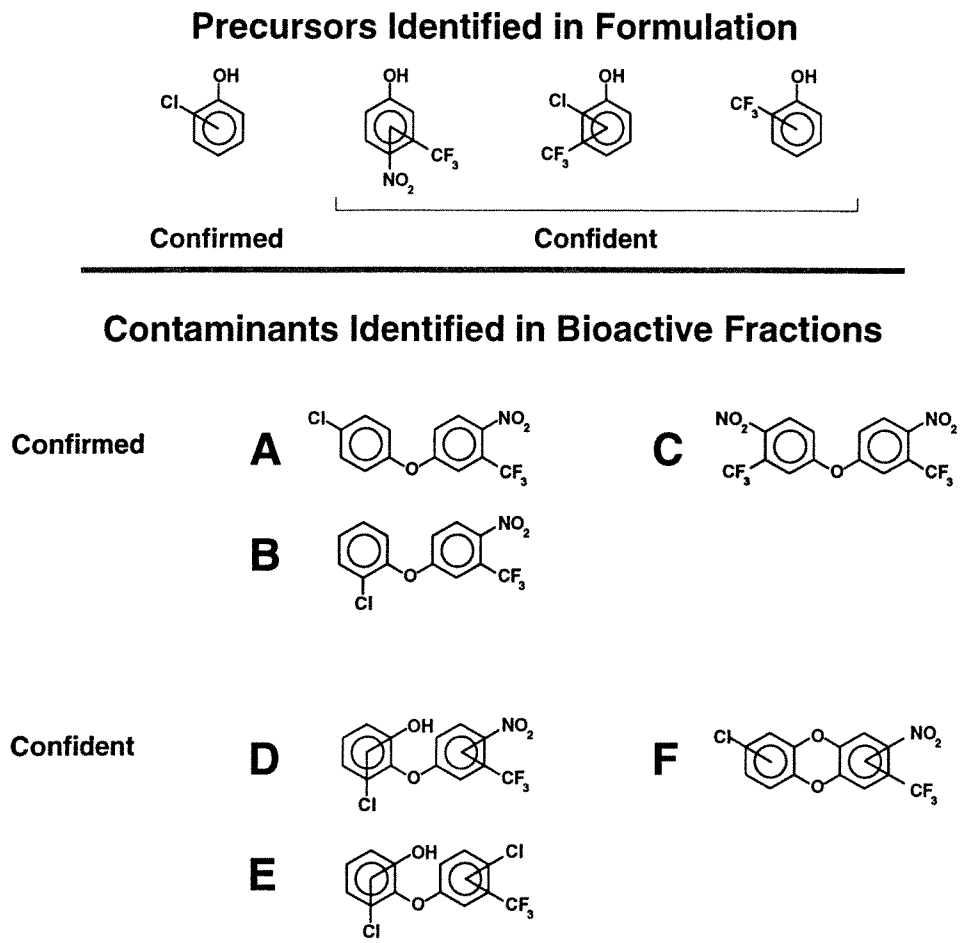

Fig. 10. Summary of the chemical structures detected and proposed in the TFM formulation.

samples collected downstream of an application point at Lynde Creek, Ontario, in May 1981. Four of the impurities were structurally related to TFM and five others were reported to be monochlorinated aromatics of unknown origin. These findings are consistent with the contaminants identified in this study. The formulation applied to Lynde Creek in 1981 and the formulation investigated in this study are from the same manufacturer and presumably prepared using the same industrial process. Although the origins of the chlorinated compounds are not known, they may be contaminants in one of the starting materials, or generated during the industrial synthesis of TFM.

The chloro-nitro-trifluoromethyl-substituted dioxin (compound F) confidently identified in fraction 34-2 also contains TFM and chlorophenol moieties (Fig. 9). Dioxins and furans have been previously shown to form during preparation of industrial phenolics [24,28], and diphenyl ether herbicides [29]. These compounds are potent inducers of fish P450IA activity [7,8], which is mediated by binding to the aryl hydrocarbon (Ah) receptor [30]. Substituent locations on compound F remain to be verified by synthesis. The limited literature relating to the toxicology of dioxins possessing these substituents is restricted to mammalian studies. Using the rat hepatic cytosolic TCDD receptor protein, Romkes et al. [31] examined the competitive Ah receptor binding affinities of various 8-substituted 2,3,7-trichlorodibenzo-*p*-dioxins relative to [^{3}H]-2,3,7,8-TCDD. The study found that the 8-trifluoromethyl-substituted analogue exhibited a higher binding affinity for the receptor than TCDD and caused comparable or greater toxicity (body weight loss, thymic atrophy, respectively). Molecules with nitro-substitution at the 8-position also showed a high affinity for the receptor (binding pEC50 7.45 vs. 8.00 for [^{3}H]-TCDD). Similar binding affinities were also noted in a more recent study using the human placental cytosolic Ah receptor [32] and with PCBs containing these substituents [33]. Substitution in the lateral 2, 3, 7, or 8 positions is generally required for highest Ah receptor affinity. However, even if it is substituted in these positions, compound F is trisubstituted and would be expected to be metabolized and excreted relatively rapidly from fish. Toxic equivalency factors for trichlorinated dioxins are generally considered to be orders of magnitude lower than 2,3,7,8-TCDD [34]. Relative to tetrachlorinated- and higher substituted congeners, the magnitude and duration of MFO induction associated with trichlorinated furans is lower in rainbow trout [35]. Based on the degree of substitution and its estimated concentration, it is likely that other unidentified components are contributing to the MFO induction associated with fraction 34-2. Custom synthesis is required to verify the chemical and toxicological significance of compound F.

Although induction was isolated in distinct HPLC fractions and numerous contaminants have been identified in these fractions using GC-HRMS, the specific chemicals responsible remain unknown. However, it has been established that a minimum of two impurities are responsible for induction and several chemical characteristics are evident from their behavior during the TIE. The K_{ow} of chemicals can be predicted from their chromatographic characteristics on reverse-phase HPLC [18]. Using the retention times (HPLC conditions in the primary separation

method) of compounds from several chemical classes and their reported log K_{ow}s, chemicals within fractions 19-3 and 19-5 are predicted to have a log K_{ow} between 3.3 and 4.4 (Fig. 1). The inducing chemicals were separated from TFM using a solvent/buffer system of pH 8, indicating that they are not readily ionizable and are possibly neutral species. Solvent extraction with toluene was effective in recovering activity for GC-HRMS analyses. The fact that induction was not lost during solvent evaporation under nitrogen or during rotory evaporation of HPLC whole-run collections suggests that these compounds are not readily volatilized. Chloro-nitro-trifluoromethyl-substituted dioxins would be expected to exhibit properties in accordance with these observations. Dioxins are neutral, toluene-soluble, and relatively nonvolatile. Log K_{ow}s for dioxins generally increase with increasing chlorine substitution; the log K_{ow} of trichlorinated dioxin is approximately 6.5 and the log K_{ow} of 2,3,7,8-TCDD is approximately 6.75 [20]. Relative to chlorine, trifluoromethyl substituents would likely contribute similarly towards lipophilicity. Conversely, the nitro- functionalities would increase water solubility, decreasing K_{ow}, but the magnitude of this effect in uncertain. Compound F, a chloro-nitro-trifluoromethyl-substituted dioxin, eluted in fraction 19-5, which has a predicted log K_{ow} of $\leq$ 4.4. This would suggest that a tetra-substituted dioxin containing these functionalities would have a significantly lower K_{ow} relative to its chlorinated analogue. The fact that all of the impurities identified in this study possess these functionalities may be suggestive regarding the substitution of other impurities. A tetra-substituted dioxin is expected to have much greater MFO induction potential and could be responsible for the observed induction in the formulations at extremely low concentrations. It is feasible that tetra-substituted dioxins are present at levels in the bioactive fractions that are below the detection limits associated with acquiring mass spectra by full-scan techniques. Numerous additional impurities that have chloro- and/or nitro-substitution with apparent molecular ions >300 amu have been observed in the toluene extract of SPE-2. This suggests that a number of additional diphenyl ether, dibenzo-*p*-dioxin, and possibly dibenzofuran analogues containing these functionalities are present in the formulation.

SUMMARY

Mixed-function oxygenase-directed TIE methodology was developed to isolate and recover inducing compounds from a TFM formulation. Although the specific chemical(s) responsible for induction have not been confirmed, a variety of organic contaminants, including chloro-, and/or nitro-, and/or trifluoromethyl-substituted phenols, diphenyl ethers, and a dibenzo-*p*-dioxin have been identified in the formulation. It is likely that these contaminants originate during industrial synthesis of the active ingredient, TFM. These findings suggest that a variety of other structurally related impurities are also present in the formulation. It is imperative that the environmental fate of the formulation impurities identified in this study be documented. The toxicological significance of this previously undescribed family of chemicals needs to be evaluated to assess their environmental risk.

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L. Mark Hewitt[1,2], Ian M. Scott[2], Glen J. Van Der Kraak[3], Kelly R. Munkittrick[2], Keith R. Solomon[1], and Mark R. Servos[2],

DEVELOPMENT OF TIE PROCEDURES FOR COMPLEX MIXTURES USING PHYSIOLOGICAL RESPONSES IN FISH

REFERENCE: Hewitt, L. M., Scott, I. M., Van Der Kraak, G. J., Munkittrick, K. R., Solomon, K. R., and Servos, M. R., "**Development of TIE Procedures for Complex Mixtures Using Physiological Responses in Fish,**" *Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment—Fifth Volume, ASTM STP 1306,* David A. Bengtson and Diane S. Henshel, Eds., American Society for Testing and Materials, 1996.

ABSTRACT: Methodology was developed for the isolation and recovery of compounds from a commercial lampricide formulation associated with hepatic mixed function oxygenase (MFO) enzyme induction in fish. MFO induction in laboratory bioassays was characterized and optimized prior to fractionation experiments. Recovery and isolation of MFO activity directed the chemical fractionations. Fractionation techniques were developed on a preparative scale so that fish exposures could be conducted directly. Formulation impurities associated with MFO induction were separated from the active lampricide using solid phase extraction. Subsequent HPLC fractionations isolated activity in distinct fractions, indicating that multiple formulation impurities are associated with the observed MFO induction. Activity was recovered from each fraction using toluene extraction and the extracts were characterized by GC-MS. No activity was associated with several compounds confirmed in the bioactive fractions and other structures are proposed. A preliminary application of these techniques to potential reproductive dysfunctions in fish is discussed.

KEYWORDS: TIE, MFO induction, Lampricide, TFM, Method Development

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Induction of the P450IA1 isosyme of hepatic mixed function oxygenase (MFO enzymes from aquatic exposure to anthropogenic pollutants is well documented for several fish species. The mechanism of induction of this enzymatic system has been intensively studied and characterized. Induction, and in some cases, subsequent toxicity, is mediated by binding of the chemical within the cell to the aryl hydrocarbon (Ah) receptor (Poland et al. 1976; Safe et al. 1985, 1986; Safe 1986). Several classes of compounds which demonstrate a high affinity for the Ah receptor are persistent, bioaccumulative and toxic substances. These compound classes are planar aromatic hydrocarbons such as polychlorinated biphenyls (PCBs) (Safe et al. 1985) and selected polyaromatic hydrocarbons (PAHs) (Klotz et al. 1983). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has one of the highest affinities for the receptor and the potencies of other compounds are frequently expressed relative to TCDD (North Atlantic Treaty Organization 1986, Parrott et al. 1995). Induction of P450IA1 in aquatic species is considered as a biomarker for the presence of these types of compounds and an indication of potential toxicity (Payne et al. 1987). However, in environmental situations induction is often a result of the presence of multiple inducers, known and unknown. It is desirable to identify the responsible chemicals in these cases because of the potential toxicity associated with materials which are known to cause MFO induction. Existing protocols on toxicity identification/evaluations (TIE) for bioactive substances from environmental matrices deal with compounds associated with acute and chronic toxicity (U.S. Environmental Protection Agency, 1991, 1992, 1993a, 1993b) and have not been applied to fractionations directed by indicators of potential toxicity such as MFO induction.

The objective of this research was to develop toxicity identification/evaluation (TIE) fractionation techniques to consistently recover and isolate compounds from complex matrices associated with MFO induction in fish. Methodologies have been developed using a field formulation of the lampricide containing 3-trifluoromethyl-4-nitrophenol (TFM). Periodic TFM treatments of nursery streams have been the primary means of controlling sea lamprey (*Petromyzon marinus*) populations in the Great Lakes Basin since the late 1950s. MFO induction at remote, non-industrialized sites was recently associated with lampricides (Munkittrick et al. 1994). Detailed experiments showed that induction was not associated with TFM itself and was presumably a contaminant in the field formulation. Using the lampricide formulation, this paper describes an approach to isolate and identify the compounds associated with MFO induction from complex matrices.

TIE METHOD DEVELOPMENT

Optimization and Standardization of Fish Exposures

Rainbow trout exposure conditions were modified from the previous study. The objective of the optimization was to generate consistent, maximal MFO induction for tracking during TIE experiments, while minimizing acute toxicity. The acute toxicity of TFM to salmonid species is well documented and is highly dependent on pH (Bills et al. 1988) and dissolved oxygen (Seelye and Scholefield 1990). All TIE

method development experiments were performed on the formulation batch (Hoescht 1990-2; Sea Lamprey Control Centre, Saulte Ste. Marie ON) used in the previous study (Munkittrick et al. 1994). Exposures were conducted using juvenile rainbow trout (3-5 g; Rainbow Springs Hatchery, Thamesford, ON) in darkness and glass aquaria containing 12 L of dechlorinated Burlington city tap water (pH 7.5 - 8.0, hardness 128 - 133 mg/L $CaCO_3$), at a loading density of 2.5 g/L (n = 6). Fish were acclimated to 13°C and fed (Martin's Feed Mill, Elmira ON) *ad libitum* until 6 d prior to exposures and were not fed during exposures.

Data was checked for normality and equal variances using SYSTAT software. One-way analyses of variance (ANOVA, $p<0.001$) were conducted for individual experiments using log transformed data. Tukey's HSD pairwise comparisons and Post Hoc contrasts were used to compare differences between treatments.Bars on all graphs depict ±1 standard error of the mean.

The first experiment determined the optimal concentration of the field formulation for exposures. Dose response exposures showed high levels of MFO induction at 150µL field formulation/12 L water or 4.6 mg/L TFM (Fig. 1) after 72 h static exposures (Fig. 1). High levels of mortality were encountered above this concentration (data not shown). Steep toxicity curves are associated with fish exposure to TFM itself (National Research Council of Canada 1985). This concentration is within the range of treatment concentrations (1.0 - 14.0 mg/L (National Research Council of Canada 1985)) used in the Great Lakes basin.

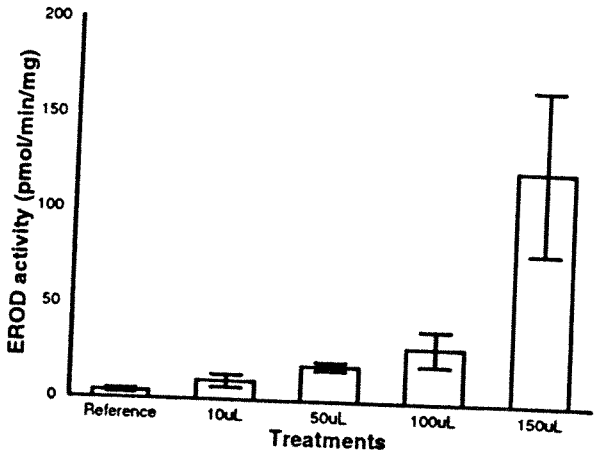

Figure 1. Relationship between rainbow trout hepatic EROD activity and concentration of the TFM field formulation. Formulation aliquots were diluted in 12 L holding water for exposures. EROD activities have been normalized for protein content.

Due to the high throughput of fractions anticipated from the TIE, it was desirable to utilize exposure conditions which minimized labour intensity. Static conditions would be suited for these experiments. Although these conditions do not exactly match field situations (12-18 h continuous, followed by depuration) potentially higher levels of MFO may be necessary for charting induction during detailed TIE experiments. Once identified and obtained in pure form, candidate compounds would then be tested under treatment conditions as part of the confirmation process. Using 4.6 mg/L of the active ingredient, static exposures showed an apparent induction maximum in fish exposed for 72 h (Fig. 2) without acute toxicity.

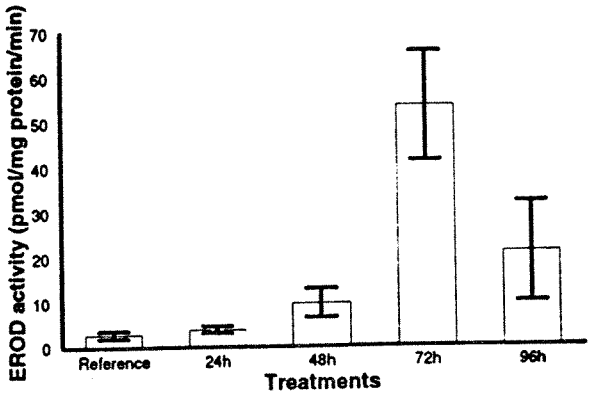

Figure 2. Rainbow trout hepatic EROD activity for static exposures to 4.6 mg/L (active ingredient) TFM formulation after 24, 48, 72 and 96 h.

For each experiment, positive and negative references were conducted concurrently. Positive references consisted of one exposure to a known inducing fraction corresponding to a previous fractionation experiment as well as one exposure to whole filtered (<1 μm) formulation; filtering had previously shown no effect on MFO induction (Munkittrick et al. 1994). Negative reference fish were exposed to the maximum amount of solvents and/or buffer associated with the exposed fractions. Duplicate negative references were performed for each experiment. The development of these exposure conditions enabled the comparative assessment of the potential to induce fish MFO activity of batches from two manufacturers. The batch used for TIE method development (1990-2) was utilized as a positive reference for formulation batch testing (Fig. 3).

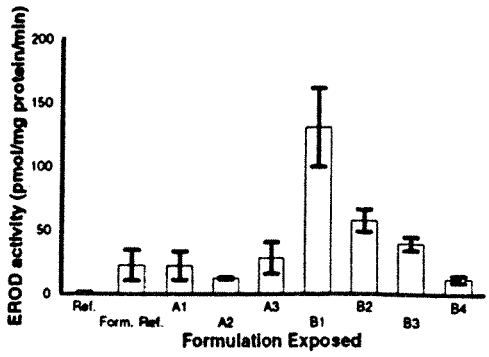

Figure 3. Rainbow trout hepatic EROD activity associated with TFM field formulation batches from two manufacturers. Fish were exposed under conditions developed for TIE experiments. (Form. Ref.) refers to fish exposed to the batch used for TIE method development and as a positive reference for batch testing.

Rainbow Trout Hepatic MFO Activity

The measurement of hepatic MFO activity as ethoxyresorufin-*O*-deethylase (EROD) induction followed the methods of McMaster et al. (1991) but was optimized for TIE exposures by utilizing liver homogenates rather than traditional post mitochondrial supernatants (PMS) containing microsomes. This provided greater sensitivity for detecting MFO induction and reduced analysis time. EROD activities are reported in fluorescence units (FU), corresponding to the amount of substrate produced during the enzymatic assay. A high correlation (FU = 0.569(pmol/mg/min) + 1.28; r^2=0.89) between homogenate and post mitochondrial supernatant (PMS) activities normalized for protein was shown for fish exposed to a range of formulation concentrations (Fig. 4).

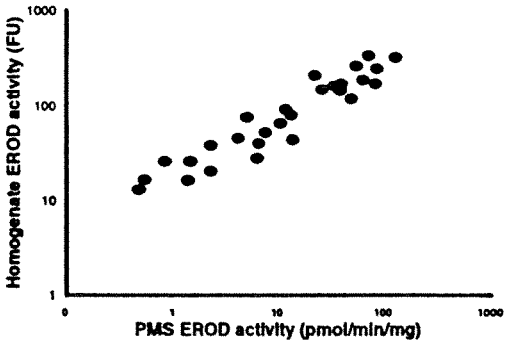

Figure 4. Relationship between EROD activities derived from whole liver homogenates (FU) and post mitochondrial supernatants normalized for protein (pmol/min/mg protein). Both values were derived from the same individual fish.

Under the exposure conditions developed, the relative induction of filtered formulation over negative reference activity was 31 ±6.2 (SE) fold for over 50 separate experiments.

Isolation of Bioactive Formulation Impurities

There are several approaches to the isolation of bioactive compounds, which depend on matrix characteristics as well as the bioassay method. Standard protocols (U.S. Environmental Protection Agency 1991, 1992, 1993a, 1993b) begin with some form of bulk fractionation where separations are based on compound class properties, for example, purgeables. Once isolated, separations can then be initiated within a compound class. A similar approach was adopted for the lampricide formulation but for a different objective. The first objective in the isolation procedure was to separate bioactive impurities from the primary formulation ingredient, TFM. The TFM field formulation is a mixture of TFM (37% w/v), isopropanol and aqueous sodium hydroxide; the pH of the formulation is approximately 9.5. It was desirable to separate bioactive components from TFM because it was demonstrated that TFM was not associated with MFO induction (Munkittrick et al. 1994) and it was suspected that the bioactive compounds are present at low levels in the formulation. In order to isolate trace amounts of bioactive impurities from the mixture of contaminants presumably present, it is necessary to concentrate the mixture. Exposures to higher concentrations of the contaminants would not be possible with TFM present because of the acute toxicity encountered above 4.6 mg/L.. Chromatographic separations would also be facilitated once the large TFM interference was removed.

Solid phase extraction experiments

Previous HPLC separations using C_{18} columns demonstrated that TFM could be separated from bioactive components using this stationary phase (Munkittrick et al. 1994). However, formulation characteristics (namely pH) caused a rapid deterioration in column efficiency. It was hypothesized that the same separations could be achieved using solid phase extraction (SPE) C_{18} cartridges. It was speculated that the inducing contaminants were less polar than TFM, and following the methods of Burkhard et al. (1991) it should be possible to selectively elute TFM from the cartridge, leaving bioactive formulation impurities adsorbed to the packing. Bioactive impurities could then be recovered in a separate, less polar solvent elution. All fractionations were performed on a preparative scale using 150 uL formulation so that the fractions generated were exposed directly using the standardized bioassay. The presence of TFM in each fraction was monitored visually and at its absorbance maximum, 290 nm. The first experiment used a 100 mg C_{18} cartridge and employed solvent conditions similar to those used previously for HPLC separations (Munkittrick et al. 1994). Fig. 5 shows the EROD activity associated with these fractions.

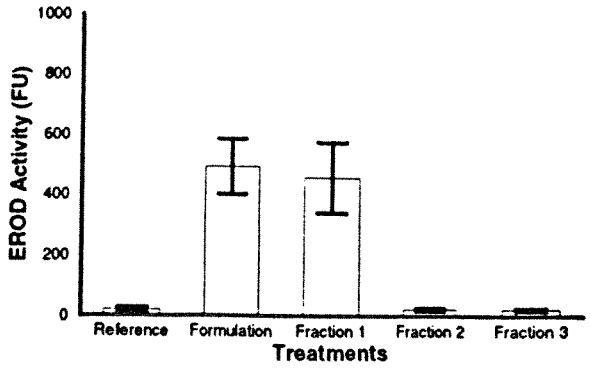

Figure 5. Rainbow trout hepatic EROD activity associated with fractions generated from solid phase extraction of the TFM formulation using 100 mg C_{18} cartridges.

Fraction 1 was collected with 1 mL 25:75 methanol:pH 4 0.2M acetate buffer, fraction 2 with a subsequent 2 mL 85:15 methanol:buffer and fraction 3 with 2 mL 100% methanol. Formulation TFM was distributed between fractions 1 and 2 and separation from induction was unsuccessful. A slightly improved separation was seen when the polarity of the first solvent/ buffer mixture was increased to 10:90 methanol:buffer (Fig. 6).

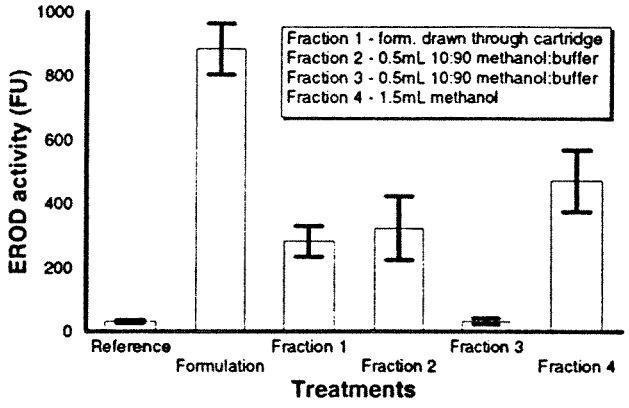

Figure 6. Rainbow trout hepatic EROD activity associated with fractions generated from solid phase extraction of the TFM formulation using 100 mg C_{18} cartridges and a more polar initial elution solvent mixture.

It was hypothesized that the incomplete separation of TFM from inducing contaminants may be due to insufficient chromatographic capacity of the 100 mg SPE cartridges. Under slightly modified elution conditions, a larger cartridge size (500 mg) showed an enhanced separation of TFM from induction (Fig. 7).

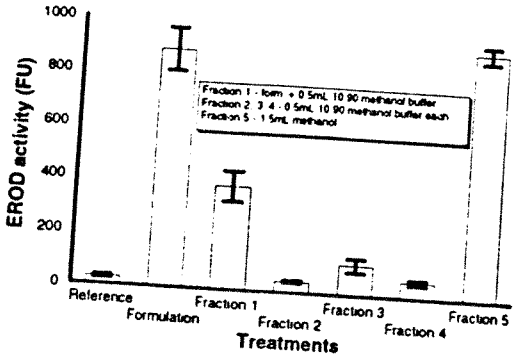

Figure 7. Rainbow trout hepatic EROD activity associated with fractions generated from solid phase extraction of the TFM formulation using 500 mg C_{18} cartridges.

Elution volumes were slightly modified because of the larger packing. Fraction 1 was eluted with the formulation volume plus 0.5 mL of 10:90 methanol:acetate buffer; fractions 2,3 and 4 were eluted with 0.5 mL 10:90 methanol:acetate buffer, and fraction 5 was eluted with 1.5 mL methanol. TFM was absent from fractions 4 and 5. These observations suggested that the capacity of the smaller cartridges may have been exceeded. Further experiments utilized 500 mg SPE cartridges.

Methanol fractions after 500 mg SPE fractionations were profiled by HPLC and showed a significant amount of residual TFM. It was hypothesized that protonation of TFM was occurring during elution with the methanol:pH 4 acetate buffer mixture; the pK_a of TFM is approximately 6.1 (National Research Council of Canada 1985). A buffer with a pH above the pK_a of TFM should maintain TFM dissociation and facilitate its complete elution. Trials using a pH 8 0.2M (trihydroxymethyl)amine buffer with low proportions of methanol demonstrated removal of TFM only after relatively large elution volumes (>10 mL). Increasing the proportion of methanol to 40:60 methanol:tris buffer showed >99.99% removal of TFM after 9 mL (SPE-1). Formulation impurities were visibly removed in a post 1.5 mL methanol elution (SPE-2). Fish exposures to both fractions showed no induction was associated with SPE-1 and that formulation activity was recovered in the methanol fraction (SPE-2) ($p<0.001$) (Hewitt et al. 1996). SPE-2 fractions could now be fractionated directly using preparative HPLC.

HPLC separations

The objective of HPLC fractionation experiments was to isolate activity in a narrow chromatographic window to facilitate chemical characterizations on a minimum number of candidates. HPLC separations were conducted on a preparative scale so that SPE-2 fractions could be fractionated without volume adjustment. The fractions generated from the HPLC could then be directly exposed to fish. HPLC fractionations were carried out using a Waters (Millipore Corp., Milford, MA) system consisting of a 717 autosampler, a 600E system controller, a 610 valve station and a 481 spectrophotometer UV detector set at 254 nm (Millipore Corp.). UV detection at 254 nm provided a crude means of detecting materials containing one or more aromatic rings, a characteristic common to known inducers. It should be emphasized however, that experiments were directed by MFO induction; this detection method provided consistent reference locations within chromatograms for fractionation purposes.

Separations were achieved with a 500 mm x 9.4 mm i.d. reverse phase Partisil 10 ODS 2 column (Watman Inc., Clifton, NJ). Methanol and a pH 4 0.2M acetate buffer were used for HPLC separations as they were previously successful in eluting inducing formulation impurities (Munkittrick et al. 1994). Solvent programming was optimized to achieve maximal resolution of components visible at 254 nm: flow rate 4 mL/min, column preconditioning and an initial 2 min hold of 10:90 (%) methanol:0.2 M pH 4 acetate (Caledon Laboratories) buffer, linear gradient to 100% methanol at 34 min and hold for 21 min.

To ensure recovery of activity from the HPLC system, fish were exposed to the entire run from an injection of SPE-1 (Fig. 8). EROD activity was recovered in the region showing visible peaks at 254nm. Fractions from this region were initially collected during the elution of three main segments. Further collections corresponded to the location of large peaks for reproducibility. Activity was eventually isolated in two distinct narrow regions which demonstrated that a minimum of two formulation impurities were associated with MFO induction (Fig. 8). For complete resolution of activity at the chromatographic limit of the HPLC method, the final collections were exposed at 5 formulation equivalents.

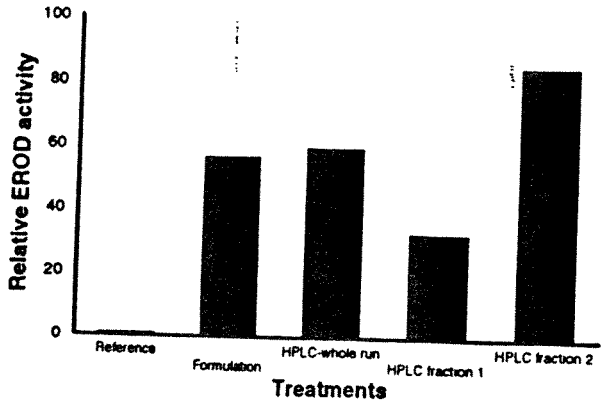

Figure 8. Rainbow trout hepatic EROD activity associated with HPLC fractions generated after solid phase extraction of the TFM formulation. Activities are expressed relative to reference because exposures were performed separately. Both final active fractions were exposed at 5 formulation equivalents.

Chemical Characterizations of Bioactive Fractions

The bioactive fractions generated by HPLC fractionation of SPE-2 were characterized by GC-MS. GC-MS analyses were performed using a Hewlett Packard 5890 GC equipped with a retention gap consisting of 1 m x 0.53 mm i.d. deactivated fused silica gel connected to a 2.5 m x 0.25 mm i.d. section of deactivated fused silica. The retention gap was fitted to a 60 m x 0.25 mm i.d. DB-5 (J&W Scientific, Folsom, CA) column bonded to 0.25 µm phase thickness. The GC was interfaced at 280°C to a VG Autospec-Q mass spectrometer (Fisons, VG Analytical, Manchester, UK). Injections were 2 µL on-column with a helium (ultra high purity carrier grade, CANOX) carrier. GC injections were cold on-column to ensure complete delivery of bioactive extracts onto the GC column and to reduce the risk of decomposition of thermally labile compounds. Injector temperatures were programmed to follow oven conditions. GC oven temperatures were gradually increased over a wide temperature range to resolve compounds covering a broad range of polarities. Oven and injector temperatures were initially held at 80°C for 0.1 min then programmed at 4°C/min to 280°C, held for 2 min, programmed at 4°C/min to 290°C and held for 10 min. Positive ion electron impact mass spectra were obtained using both full scan and selection ion monitoring techniques. Full scan mass spectra of unknowns are necessary for structure determination. The identifications assigned to components detected by GC-MS in the bioactive fractions should follow published guidelines concerning identification of unknowns (Christman 1984). Tentative identifications are assigned when the postulated structure of an unknown is based solely on interpretation of its mass spectrum. Identifications are noted as confident when there is no authentic standard available but either the spectral or chromatographic data matches closely

published data, including mass spectral data bases and retention indices. Compounds are described as confirmed when retention times and mass spectra match those for authentic standards analyzed under identical conditions.

The bioactive fractions were a mixture of methanol and acetate buffer and required solvent exchange prior to characterization. To determine the recovery of bioactive compounds, each fraction was solvent extracted, the extracts reduced in volume to just dryness and then redissolved in methanol for fish exposures. MFO induction in the resuspended extracts were compared to separate original bioactive fractions. Fish exposures to ethyl ether extracts of both fractions showed partial recovery of activity (71% and 43% respectively) while toluene extractions showed complete recovery of activity in both fractions ($p<0.001$) (Fig 9). This also indicated that the responsible compounds were relatively nonvolatile.

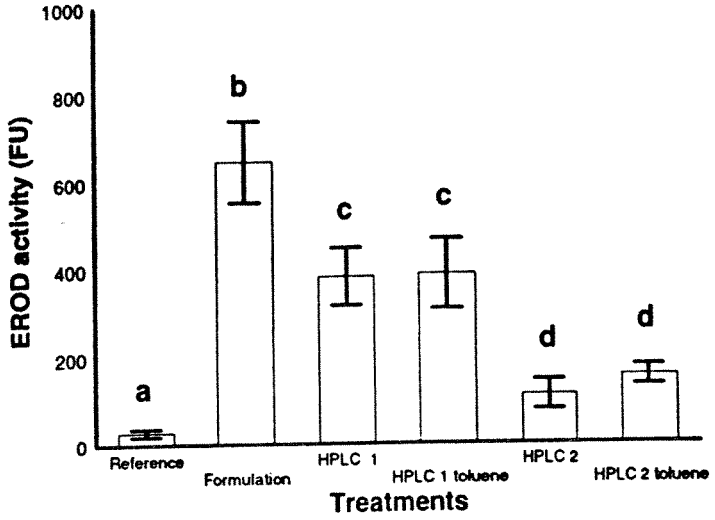

Figure 9. Rainbow trout hepatic EROD activity associated with inducing HPLC fractions and their respective toluene extracts. The fractions and extracts were each exposed at 3 formulation equivalents. Letters above bars correspond to groups which were statistically independent of each other.

The chromatograms of the bioactive fractions were compared in detail with analysis blanks for the determination of unique constituents. The major components of the bioactive fractions were confirmed by synthesis as nitro-, trifluoromethyl-, and/or chloro diphenyl ethers. Fish exposures to the pure materials at a range of formulation equivalent concentrations showed no MFO induction was associated with these compounds (Hewitt et al. 1996). Although the identities of the compounds responsible for induction remained unknown at this juncture in the TIE, it could be concluded that the impurities were not associated with induction. These findings are significant

because low levels of MFO induction have been associated with diphenyl ethers (Chui et al. 1986) and relatively high levels of these materials were present in the formulation (Hewitt et al. 1996). It should be emphasized that even if the TIE is ultimately unsuccessful in that the responsible compounds are not fully characterized, the elimination of materials identified during the course of the TIE that are not associated with the endpoint can yield useful information (Hewitt et al. 1995). Further, properties of the responsible compounds which become evident during the TIE (polarity, volatility) will augment characterizations. Ideally, as in this case, the materials were obtained in pure form from custom synthesis and were exposed in known, accurate concentrations to assess their bioactivity potential.

Further separations were necessary to isolate the responsible compounds. Although not employed here, other techniques such as normal phase HPLC could be applied. For the lampricide formulation being investigated further separations were realized by reinjection onto the reverse phase column under modified elution conditions.

HPLC Subfractionations

The bioactive fractions were concentrated three-fold prior to reinjection to facilitate the resolution of trace materials. Solvent conditions were optimized for each fraction to achieve the greatest resolution of components visible at 254nm. Recovery of activity after passage through the HPLC system was verified for both new elution conditions prior to fractionation experiments ($p<0.001$). Activity from both reinjections were eventually recovered in a total of three new fractions. A trisubstituted dibenzo-*p*-dioxin possessing chloro-, nitro-, and trifluoromethyl substituents was confidently identified in two of these fractions by GC-MS (Hewitt et al. 1996).

Application to Other Physiological Responses

The goal of the development of the TIE techniques described is their application to a variety of physiological responses. In addition to elevated levels of MFO activity, altered levels of circulating sex steroid hormones in both male and female goldfish were associated with waterborne exposures to the lampricide formulation (Munkittrick et al. 1994). The TIE methodology developed using MFO induction was tested using circulating levels of sex steroids in fish as an endpoint. Preliminary experiments were conducted using the SPE-2 fraction of the TFM formulation. Male goldfish were dosed by intraperitoneal injection and basal levels of circulating steroids were measuresed after 4 days using the methods described in Munkittrick et al. (1994). Significantly decreased levels of both testosterone and 11-ketotestosterone were associated with exposure to the SPE-2 fraction (Fig. 10). Using this endpoint, the TIE methodology can be applied to determine the identity of the chemicals responsible for this response. Each step of the TIE must be validated for recovery of the biological activity as was documented here for MFO induction.

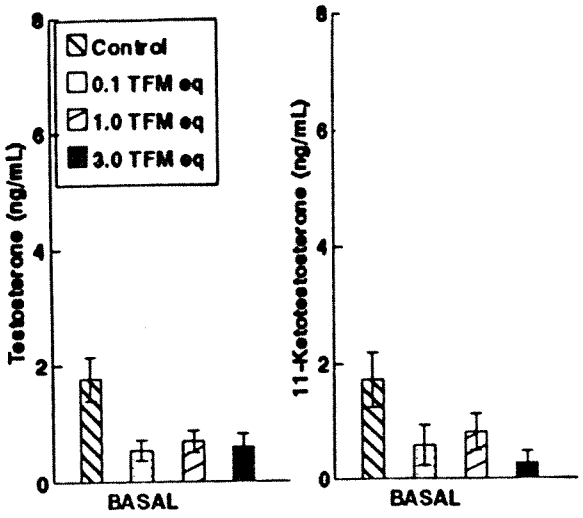

Figure 10. Circulating levels of testosterone and 11-ketotestosterone in male goldfish exposed by intraperitoneal injection to 0.1, 1 and 3 lampricide formulation equivalents of the SPE-2 fraction containing formulation impurities.

SUMMARY

A TFM lampricide formulation was utilized to develop TIE techniques directed by hepatic MFO induction in fish. Prior to fractionation experiments, bioassay conditions were optimized to consistently obtain the highest response while maintaining low toxicity. Hepatic MFO determinations were modified from traditional assays to increase sensitivity and reduce analysis time. Solid phase extraction was successful in separating bioactive formulation impurities from the primary formulation ingredient, TFM. Induction was recovered after solid phase extraction which enabled direct fractionation by preparative HPLC. Activity was not compromised after passage through the HPLC system and was eventually isolated in two distinct fractions. Further fractionations were eventually achieved by reinjection of each fraction onto the HPLC under modified conditions. Bioassays verified that activity was recovered from each fraction by toluene extraction preceding chemical characterizations. Toluene extracts were characterized by cold on-column GC-MS. Characterization of bioactive fractions by GC-MS resulted in the elimination of three nitro-, trifluoromethyl- and/or chloro- diphenyl ethers as inducers. A chloro-, nitro-, trifluoromethyl dibenzo-p-dioxin has also been confidently identified in two active fractions. These techniques have also been initiated for the identification of compounds associated with potential reproductive abnormalities in fish.

ACKNOWLEDGEMENTS

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Characterization of bioactive 3-trifluoromethyl-4-nitrophenol (TFM) lampricide formulation impurities.

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1. Introduction.

The application of lampricides containing 3-trifluoromethyl-4-nitrophenol (TFM) within the Great Lakes basin of North America has been the primary means of control of sea lamprey (*Petromyzon marinus*) since the late 1950s. Tributary streams are treated on a 3 to 4 year cycle, designed to reduce the numbers lamprey ammocoetes that reach the predatory adult phase of their life cycle. Treatment concentrations vary from 1.0 mg/L to 14 mg/L of TFM, and current usage approximates 50,000 kg/year. Induction of hepatic mixed function oxygenase (MFO) detoxification enzymes and altered levels of circulating sex steroids were recently associated with fish exposed to field formulations of TFM[1]. Induction of P450IA enzyme activity in fish has been associated with exposure to planar aromatic compounds such as PCBs[2], PCDD/PCDFs[3], and PAHs[4]. MFO induction was initially observed in white sucker *(Catostomus commersoni)* caged during a lampricide treatment and in static laboratory exposures using rainbow trout *(Oncorhynchus mykiss)*[1]. Attempts to isolate the responsible compound(s) showed that induction was associated with the field formulation and not TFM itself. Formulation concentrations of PCDD/DFs, and PAHs were below detection limits[1].

A full understanding of the significance of the contamination requires information on contaminant structure, levels in the formulation, environmental fate and a perspective on historical levels. The objective of this study has been to develop and apply a Toxicity Identification Evaluation (TIE) directed by biochemical endpoints to isolate the active chemical(s).

2. Methods.

The methods described are modifications of the Toxicity Identification and Evaluation (TIE) approach [5-7]. Fractionations were directed by rainbow trout hepatic MFO induction determined as ethoxyresorufin-*O*-deethylase (EROD) activity. Fish exposure procedures and MFO assays were optimized to reduce fish handling and analysis time and used consistently throughout all TIE

phases[8]. Solid phase extraction was employed to isolate bioactive formulation impurities from primary formulation ingredients which had been previously shown to not exhibit the ability to induce MFO activity[1]. Cartridge phase, packing size and elution solvents were optimized to isolate bioactivity[8]. All SPE fractionations were performed on a preparative scale using 150 µL formulation (1 formulation equivalent), or 4.6 mg/L TFM. Fish exposures were conducted directly with the fractions generated. Subsequent fractionations were performed using reverse phase preparative high pressure liquid chromatography (HPLC). Bioactive fractions were characterized by gas-chromatography-high-resolution-mass-spectrometry (GC-HRMS). Fractionations and exposures were conducted on the 1990-batch #2 field formulation manufactured by Hoescht Chemical (provided by Sea Lamprey Treatment Centre, Sault Ste. Marie, ON Canada). The formulation is 37% (w/v) TFM in a solution of isopropanol and aqueous sodium hydroxide.

3. Results.

SPE was employed to separate inducing formulation contaminants from TFM because of the large chromatographic interference and the acute toxicity associated with TFM; the isolation of bioactive formulation impurities was be facilitated after TFM removal. After optimizing the solvent/buffer systems and cartridge size, >99% of TFM was separated from inducing formulation impurities[9]. Bioassays verified that induction was recovered after SPE and HPLC fractionations and that toluene extractions would recover inducing compounds for GC-HRMS analysis. Induction was initially isolated in two fractions (19-3 and 19-5; Figure 2) and the presence of three diphenyl ethers (A, B, C) in these fractions was confirmed by synthesis (Figure 1).

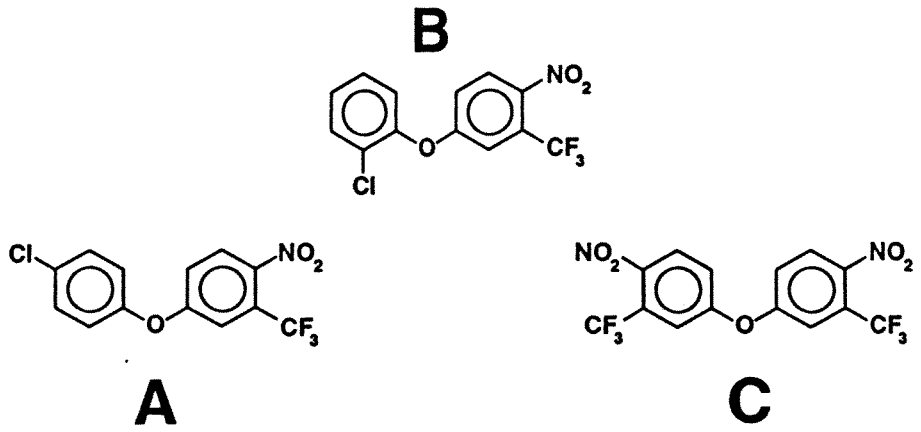

Figure 1. Diphenyl ether impurities confirmed in the TFM lampricide formulation.

However, no induction was observed after exposures to the pure compounds[9]. Induction was eventually isolated in a total of three final sub-fractions (30-3, 34-2, 31-3) depicted in Figure 2. A highly concentrated extract of the fraction with the highest induction potency, 34-2, showed trace levels of one unique constituent; its positive ion electron impact mass spectrum and assigned structure are presented in Figure 3. Additional evidence for this structure was gained after the molecular formulae and ratios of several fragment ions were corroborated by selected ion monitoring (SIM) analyses at 10,000 resolution. SIM analysis of 31-3 detected low levels of three apparent isomers[9].

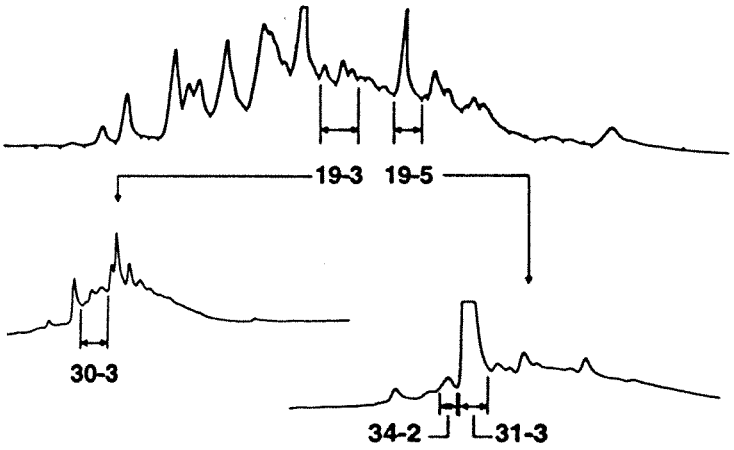

Figure 2. HPLC profiles of formulation impurities and fractions where MFO activity was isolated.

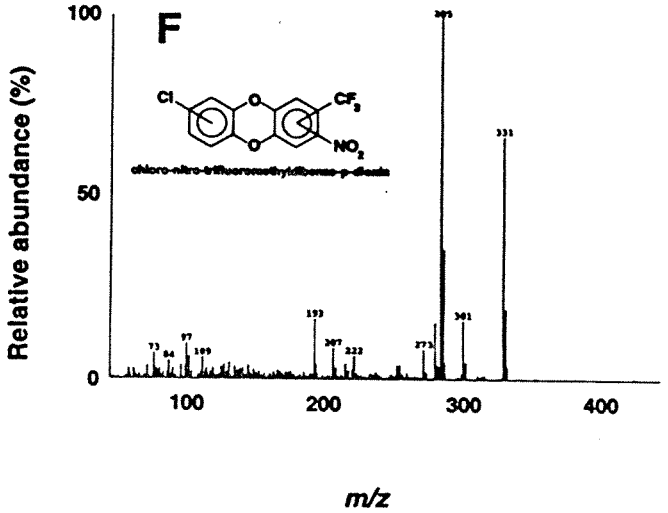

Figure 3. Mass spectrum and assigned structure to compound F, present in the fraction with the highest induction potency, 34-2.

Custom synthesis of the 2,3,7(or 8)-substituted isomer (F1) of this compound was undertaken and analysis of the product by GC-HRMS revealed this compound to be an isomer of the unknown (compound F) in TFM. MFO activities from waterborne trout exposures are presented in Figure 4.

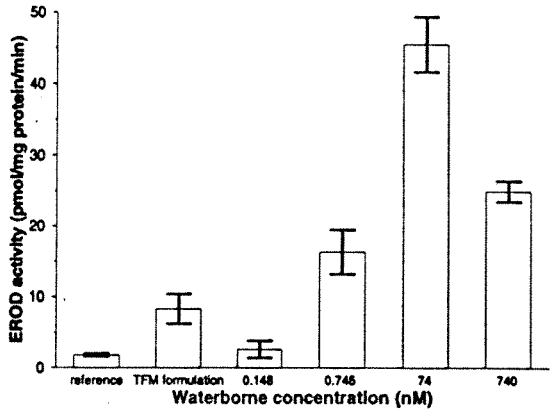

Figure 4. MFO activity determined for rainbow trout exposed waterborne to 2-trifluoromethyl-3-nitro-7(or 8)-chlorodibenzo-*p*-dioxin.

Given that an isomer of the unknown in the bioactive fraction causes induction under the identical, waterborne conditions employed throughout the TIE, custom synthesis of other isomers was undertaken to ascertain the substituent locations. The structures of the compounds synthesized to date are presented in Figure 5.

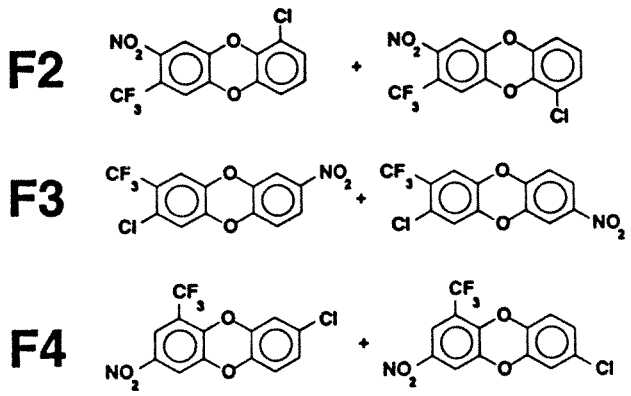

Figure 5. Structures of synthesized tri-substituted dioxins that can be eliminated from the isomer in fraction 34-2.

GC-HRMS analysis has shown that compound F2 is present in the TFM formulation but not in the bioactive fraction 34-2. Compounds F3 and F4 are also not present in the formulation and can be eliminated as possible structures. Once the location of the substituents is verified, a historical perspective and the environmental fate of these impurities can be addressed. Formulation concentrations for the diphenyl ethers in previous batches are presented in Figure 6.

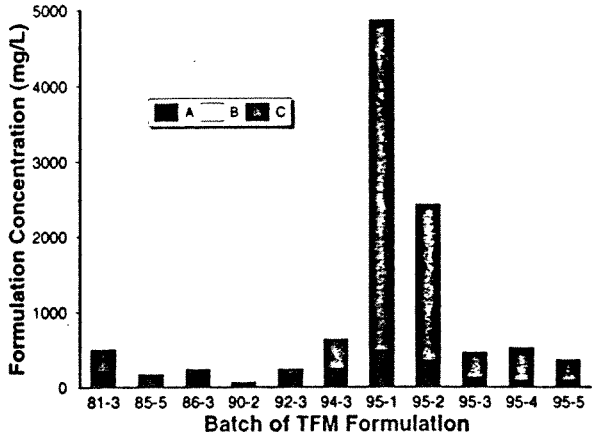

Figure 6. Formulation concentrations of confirmed diphenyl ether impurities from previous batches of TFM field formulations.

4. Conclusions.

TIE methodology directed by hepatic trout MFO induction was successfully developed and applied to the lampricide formulation containing TFM as the active ingredient. A minimum of two chemicals are responsible for induction in the formulation. Isomers of a chloro-nitro-trifluoromethyl-substituted dibenzo-p-dioxin have been identified in two final bioactive fractions. The 2-trifluoromethyl-3-nitro-7(and 8)-chloro- isomer causes *in vivo* induction in fish after waterborne exposures. Several possible isomers have been eliminated as candidates by synthesis.

Relatively high concentrations of other diphenyl ether impurities have been determined in previous batches.

5. References

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Identification of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) as an agonist for the rainbow trout estrogen receptor.

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