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Sim, P. G.; Boyd, R. K.; Pleasance, S.

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METHODS FOR ANALYSIS OF ANTISAPSTAIN AGENTS AND OF MARKER COMPOUNDS FROM BLEACHED KRAFT MILL EFFLUENTS IN TISSUES OF EXPOSED FINFISH

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Report prepared for:

Dr. G. Kruzynski,
West Vancouver Laboratory,
Fisheries and Oceans Canada,
4160 Marine Drive,
West Vancouver, B.C. V7V 1N6.

Report written by : **P.G. Sim and R.K. Boyd,**

based on experimental data and information supplied by :

S. Pleasance* and P.G. Sim,
Analytical Chemistry Group,
Institute for Marine Biosciences,
National Research Council,
1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1.

*** Under contract from SCIEX, 55 Glencameron Road, Thornhill, Ontario L3T 1P2.**

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INTRODUCTION

The practice of wood preservation through application of chlorophenols has led to wide concern due to the toxicity of these compounds. Thus tetra- and pentachlorophenols have been widely used both in oil solution for long-term timber preservation, and as sodium salts in an aqueous formulation for surface treatment of green lumber against sapstain and mould fungi. The problem of finding suitable replacements, which are selectively toxic to fungi but not to other organisms, has engaged world-wide attention. Amongst many fungicidal compounds, considered as replacement wood preservatives, 2-(thiocyanomethylthio)-benzothiazole (TCMTB) was at one time considered to be the most promising candidate, and in fact was used extensively for this purpose in British Columbia for some years. TCMTB is in wide use as the active ingredient in a number of proprietary fungicide formulations [1] used in the leather industry in the treatment of "wet-blue" hides [2]. Analytical procedures based on high-performance liquid chromatography (HPLC) have been developed for TCMTB in technical formulations and in aqueous samples [3], in extracts of treated lumber [4] [5], in wet-blue hides [6] and in leather process liquors [7], and for degradation products of TCMTB in river water [8]. Concern with TCMTB and its degradation products in aqueous environments and associated biota led to development of extraction and clean-up procedures for HPLC-UV analyses of water samples which had been in contact with treated lumber, and for these compounds in fish bile [9,10]. All of these HPLC methods [3-10] used UV detection. One objective of the present work, as described below, was to develop HPLC methods with mass spectrometric (MS) detection, and to

apply these more diagnostic LC-MS techniques to analysis of TCMTB and its degradation products and metabolites in fish.

Another major problem for the forest industry arises from xenobiotic compounds produced in bleached Kraft mill effluent. An introductory summary of the chemistry involved is available [11], and an authoritative account of the distribution and fate of the organochlorine compounds, formed during production of bleached pulp, has been published recently [12]. Chlorinated phenolic compounds, which may contain methylated phenolic functionalities and/or aromatic aldehyde groups, have been identified as among the more toxic of these byproducts of pulp bleaching. An early application of gas chromatography with mass spectrometric detection (GC/MS) characterized the chlorophenols in spent bleach liquors as their ethyl derivatives [13]. An alternative strategy to GC determination of these chlorophenols in bleach liquor involves conversion to their acetyl derivatives [14-16]. An adaptation of the latter approach to determination of tetra- and pentachlorophenols in topsoil and earthworm samples, collected from sawmill environments, has been described [17]. In exposed fish, chlorophenols were found in the bile only as glucuronide or sulfate conjugates, but in the blood plasma at most 70% of these compounds were conjugates [18]. It is possible to detect the unconjugated chlorophenols in fish by GC/MS and by GC with detection *via* odour using the human nose, with correlation of the chlorophenol content thus determined with flavour impairment tests [19]. Extracts of the wood pulp product itself have been analyzed for chlorophenols by GC/MS [20]. The great sensitivity of

the electron capture detector (ECD) to chlorinated organics has lent itself to analysis of pulp-mill effluents for chlorophenols by GC-ECD, following derivatization with diazomethane, acetic anhydride, heptafluorobutyric anhydride, or silylating reagents [21-23]. However, none of these GC methods is well suited to analysis of these chlorophenolic compounds, and their glucuronide and sulfate conjugates, in tissues of exposed biota. Therefore, a second objective of the present work (also enlarged upon below) was to investigate the applicability of LC-MS techniques to this purpose.

Other toxic constituents of effluents, from pulp mills which use softwood species, are the diterpene tricylic "resin acids" [24] and their chlorinated forms [25] from bleach mills. Procedures have been described [26] for the extraction of resin (and fatty) acids from pulp mill effluents, followed by derivatization by diazomethane and GC analysis with flame ionization detection. However, HPLC with UV detection was used in studies of the biotransformation of one of the resin acids (dehydroabietic acid) [27] and of one of its chlorinated forms [28]. Also, HPLC with detection by UV [29] or fluorescence [30] spectroscopy has been used to analyze resin acids (including dehydroabietic acid) in pulp mill effluents. However, it has been shown [31] that resin acids in exposed rainbow trout are entirely present in the bile as conjugates (mostly glucuronides), though appreciable levels of the free acids were found in the blood plasma. Here again, the unique advantages of modern LC-MS techniques are likely to be well suited to this analytical problem.

The foregoing provides a necessarily incomplete summary of the context which led to the present work. Fisheries and Oceans Canada, in collaboration with Environment Canada, contracted NRC's Institute for Marine Biosciences (IMB) *via* a Financial Arrangement (see Appendix A), to investigate the possibility of applying modern mass spectrometric and LC/MS techniques to analysis of fish tissue (bile, bile ducts, plasma and urine) for the above-mentioned xenobiotics, and their degradation products and metabolites. The compounds selected for study in the anti-sapstain part of this work were 2-(thiocyanomethylthio)-benzothiazole (TCMTB) and a series of possible degradation products. These included mercaptobenzothiazole (MBT), 2-(methylthio)-benzothiazole (MTBT), 2-methylbenzothiazole (MeBT) and benzothiazole (BT). The other xenobiotic compounds initially selected were a group of three previously shown to be markers for bleached Kraft mill effluent (BKME) contamination. These compounds were 3,4,5-trichloroguaiacol (345-TCG), tetrachloroguaiacol (TeCG) and dehydroabietic acid (DHA). At a later stage of the project, 4,5,6-trichloroguaiacol (456-TCG) was added to the list of target analytes.The structures of these target compounds are shown in Figure 1.

Under the terms of the original agreement, the objectives of the study were:

1. To develop combined chromatographic-mass spectrometric methods for the analysis of TCMTB and its degradation products at trace levels;

2. to apply techniques thus developed to the analysis of TCMTB and its degradation products in complex matrices such as bile, plasma and urine obtained from exposed fish;

3. to develop extraction techniques to analyze TCMTB and its degradation compounds in other complex matrices, such as the liver, brain, kidney and muscle from exposed fish, and;

4. to develop and apply chromatographic-mass spectrometric methods to the analysis of selected chlorophenolic compounds and wood extractives (345-TCG, TeCG, and DHA) in bile samples of exposed fish.

Briefly, the phases and timing of the project were originally planned to be as outlined below (see Appendix A):

Phase 1 (August 1, 1990 - October 31, 1990):

Develop reliable methods for analysis of TCMTB, MBT and MTBT, using Fast Atom Bombardment and HPLC-Ionspray MS-MS techniques; conduct a preliminary investigation of the feasibility of determining MBT-glucuronide and -sulfate conjugates by these techniques. Pure conjugate standards were to be supplied by Environment Canada in Burlington.

Phase 2 (November 1, 1990 - March 31, 1991):

Apply methods developed in Phase 1 to the analysis of TCMTB and its degradation products in up to 25 samples of bile, plasma and urine from fish exposed to TCMTB under laboratory conditions or exposed to waters near lumber mill run-off. Initial experiments to be performed on archived samples provided to NRC by Environment Canada in Burlington; other samples to be provided by DFO.

Phase 3 (April 1, 1991 - January 15, 1992):

Apply these same methods to samples of other complex matrices from exposed fish. Develop methods for extracting the analytes of interest from the various matrices. Up to 50 samples from DFO to be analyzed.

Phase 4 (August 1, 1991 - January 15, 1992):

(4 i) *(August 1, 1990 - March 31, 1991)* Develop chromatographic-mass spectrometric techniques for the detection and analysis of DHA, 345-TCG and TeCG in bile and apply these techniques to the analysis of bile samples obtained from fish exposed under laboratory conditions. Environment Canada to supply pure standard compounds. NRC to also purchase compounds for analysis as necessary; DFO to supply samples from freshly exposed fish.

(4 ii) (April 1, 1991 - January 15, 1992) Analyze up to 50 DFO samples of bile from fish exposed under field or laboratory conditions.

However, it was necessary to amend the workplan since Enviroment Canada was unable to synthesize the MBT conjugates necessary for completion of Phases 1 and 4. Although methods were developed which permitted HPLC-MS analysis of samples for TCMTB and its possible degradation products, in the absence of authentic standards it was impossible to determine the concentrations of the conjugates in the samples provided. With the verbal approval of Dr. Kruzynski, it was decided to divert effort into attempts to synthesize these compounds, and to continue with the other phases of the project as far as possible.

A number of bile, plasma and urine samples and fractions were provided by Fisheries and Oceans Canada and Environment Canada. The provenance of the samples provided to NRC is as follows:

The first batch, received in November 1990, contained:

1(a,b) Two 1 mL brown vials, labelled 'Bile extract fr#2, in acetonitrile, Nov 23/89' and 'Bile extract fr#4, in acetonitrile, Nov 23/89'

2 1 resealable plastic envelope, labelled 'Control rainbow bile, urine and plasma' containing one vial of each of the three matrices.

3,4 2 resealable plastic envelopes, each labelled '10ppb Rainbow FW(?) Plasma' and each containing three vials, labelled bile, plasma and urine.

5 1 resealable plastic bag, labelled '20 ppb Rainbow #1 FW (?)', again containing three vials, labelled bile, plasma and urine.

6 1 resealable plastic bag, labelled ' 20 ppb Rainbow Fish #2 FW', also containing vials of all three matrices.

7 1 resealable plastic bag, labelled '10ppb Coho Salmon' containing vials of bile and plasma only.

The second batch of samples, received in April 1991, contained:

8 1 jar, labelled 'Bile 1, Harmac 26 Apr 89', containing one bile duct.

9-11 3 scintillation vials, each containing 1 bile duct and labelled '901424 #109 ratfish bile, Aug 13/90', '901424 #110 ratfish bile, Aug 13/90' and '901424 106 Hake-bile, Aug 13/90' respectively.

12 1 container, labelled 'April 9/91 Chinook DHA exposed', containing three vials, labelled (a) '25 bile', (b) 100 bile' and (c) '400 bile', respectively.

The third batch, received in July 1991, contained:

13 1 container, labelled '21 June '91, Bile DHA 10 ppb'.

The fourth batch, also received in July 1991, contained:

14 4 plasma samples (a) - (d), labelled '10,25,100,400 ppb' respectively; 4 urine samples (e) - (h), labelled '10,25,100,400 ppb' respectively, from the same salmon that yielded the samples of 12, above.

The fifth and final batch, received in January 1992, contained:

15 4 sets of samples, each comprising 1 sample each of bile, plasma and urine, from salmon exposed respectively to 250 ppb of (a) 345-TCG, (b) 456-TCG, (c) TeCG, and (d) DHA.

All other descriptions of chemicals and materials used, methods and techniques developed, *etc.*, are included below at the appropriate points in the report.

B. TCMTB, 2-(thiocyanomethylthio)-benzothiazole

Mercaptobenzothiazole (MBT), 2-methylbenzothiazole (MeBT), 2-(methylthio)-benzothiazole (MTBT), and benzothiazole (BT) were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. TCMTB and a further sample of MBT were provided by Dr. G. Kruzynski. All materials were used as supplied.

B.1 Mass spectrometry of standards.

In view of the thermal lability of these analytes, room-temperature ionization techniques were used. Fast atom bombardment (FAB) ionization was employed using a VG Analytical ZAB-EQ double-focussing mass spectrometer (Fisons VG Analytical, Manchester, UK). The ion source was operated at 8kV and the primary beam was provided by a Cs^+ ion gun operated at 30 kV. Matrices employed were glycerol, 3-nitrobenzylalcohol and "magic bullet" (a mixture of dithioerythritol and dithiothreitol). Samples were added to the matrix on the tip of a standard FAB direct insertion probe. Figure 2 shows positive-ion FAB spectra of MBT, MTBT and TCMTB. In each case intense $(M + H)^+$ ions dominate the spectrum though, in the case of TCMTB, fragment ions (discussed in B.2 below) are also significant. In view of the relatively low sensitivity of the FAB ionization technique in this case, and also in view of the difficulty involved in coupling to HPLC (continuous-flow FAB), these experiments were not pursued further except as a means of generating ions to be subjected to tandem mass

spectrometry (MS/MS) experiments (section B.2).

Most of the mass spectrometry experiments reported here were performed using ionspray ionization. The pure standards, as methanol or acetonitrile solutions, were introduced into the SCIEX API III IonSpray system (SCIEX, Thornhill, Ont., Canada) by either of two techniques, *viz.* flow injection (FIA) or *via* an HPLC column (HPLC-MS). In both cases the mobile phase was 1:1 acetonitrile:water, containing 0.1% trifluoroacetic acid (TFA). In the FIA experiments the flow rate was 50μL/min, while in the preliminary HPLC-MS experiments it was 200μL/min with a 1:3 post column split. Thus, the total flow to the mass spectrometer was the same in both cases. The mobile phase stream was pumped by an HP 1090 HPLC (Hewlett-Packard, Avondale, PA, USA) equipped with a binary DR5 solvent delivery system. The coupling to the mass spectrometer was *via* a SCIEX IonSpray series 2 HPLC-MS interface. In the preliminary LC-MS experiments 5 μL of a 1 mg/mL solution of sample were injected, *i.e.* 5 μg of sample. In the case of the FIA experiments all of the injected sample reached the ionspray needle, but under HPLC-MS conditions the post-column split delivered only 25% of the sample to the mass spectrometer.

Generally, the ionspray mass spectra (Figure 3) are simpler than the corresponding FAB spectra (Figure 2), although the spectrum of TCMTB is complicated by significant intensity of the acetonitrile adduct (m/z 280) of the protonated molecule. The persistence of this proton-bound adduct reflects the conditions in the sampling orifice-skimmer region of the atmospheric pressure ionization (API) source (the "cluster-

buster" in SCIEX terminology). This aspect is discussed in more detail below, in the context of ionspray sensitivity for TCMTB.

Instrumental detection limits for these compounds were determined by FIA triplicate analyses of solutions obtained by serial dilutions of a 1 mg/mL stock solution. Typical results for each compound are shown in Figures 4-8. In each of these Figures, the trace was obtained by replicate FIA injections of 1μL. Quantities of compound injected are noted on the Figures. It is apparent that instrumental detection limits for MeBT and MTBT were in the 50-100pg range, and for BT about 500pg, while those for MBT and TCMTB were in the 1-5ng range.

The sensitivity for TCMTB was a little lower than that obtained in previous work [32]. The reasons for this were investigated and found to lie in the conditions under which the API source interface is operated. Specifically, the orifice-skimmer potential difference, which is a measure of the declustering energy, had to be lowered from 60V to 45V to obtain good transmission of the protonated TCMTB molecule (*i.e.* $(M + H)^+$ ion). The original setting [32] of 60V was chosen from a series of initial experiments, where MeBT was used to optimize the interface conditions. However, the ionization and declustering characteristics of the two compounds are sufficiently different to make this 60 V value less suitable for the determination of TCMTB. In fact, the best value of the orifice-skimmer potential drop for TCMTB is a particularly delicate balance between declustering and fragmentation of the $(M + H)^+$ ion. The effect of varying this

voltage on the relative intensities of the $(M + H)^+$ ion (m/z 239) and the principal fragment ion (m/z 180) is shown in Figure 9.

While these instrumental detection limits for pure standards, introduced by FIA, are indeed relevant parameters for the present work, much more important in practice are the calibration curves obtained for LC-MS analysis. These are described in Section B.2 below.

B.2 LC-MS analysis of standards.

In the course of this work, a variety of HPLC separations was examined. Chronologically, the separation schemes employed were:

1. Isocratic, 1:1 acetonitrile(ACN) : water (0.1% TFA), using a Vydac 218TP52 column. This method was employed in the preliminary survey work, solely to establish that the HPLC-Ionspray MS technique could be used to analyze this class of compound.

2. Gradient; 20-100% ACN in water (0.1% TFA) over 30 min., also using a Vydac 218TP52 column. This method was used to determine whether gradient elution could improve both the separation efficiency and the peak shapes found in the first series of experiments.

3. For some preliminary HPLC-UV experiments, the 1:1 isocratic mobile phase of 1. above was used with a Supelco LC-18 column.

4. In the first round of HPLC-MS experiments, two further isocratic separation methods were developed, using different columns in an attempt to improve chromatographic peak shapes. A mobile phase of 55% ACN in water (0.1%TFA) was used, in conjunction with a Vydac 201TP52 or a Zorbax Rx-C8 column. The conclusions of this work were that the best peak shapes, which still achieved adequate separations, were obtained using the Zorbax column, but that isocratic elution would have to be replaced by a gradient in order to achieve useful separations. This led to the development of the final separation schemes employed in the balance of the work.

5. Two gradient schemes were finally developed for real-world samples, depending on the application. For the analysis of "as received" samples of body fluids, where a separation of possible conjugates both from the solvent front and from any unconjugated material was desired, a gradient of 20-80% ACN in water (0.1% TFA) over 20 min was used with the Zorbax Rx-C8 column (4.6 mm x 25 cm). In cases where the detection of conjugates was not desired, such as in the analysis of enzyme digests of the body fluids, a shorter gradient of 40-80% ACN in water (0.1% TFA) over 15 min was used, also with the Zorbax Rx-C8 column. In all cases the mobile phase flowrate was 1 mL/min,

with a 1:20 post-column split delivering 50 μL/min to the mass spectrometer. Injection volumes were 20 μL.

The results of an LC-MS analysis of a mixture of the five substituted benzothiazole standards are summarized in Figure 10. These chromatograms were obtained by selected ion monitoring (SIM) of the $(M + H)^+$ ions for each species. The separation is incomplete, but the molecular weight information is sufficient to permit quantification of all species of interest. These mass chromatograms were all obtained using the 40-80% ACN in water gradient described in method 5 above.

Calibration curves for quantitative analysis were also obtained for LC-MS conditions described in method 5. In all cases the peak areas in the SIM mass chromatograms were measured relative to that for MeBT, added as an internal standard of fixed concentration to the solutions prepared by serial dilution of stock solutions of each individual analyte (MTBT, MBT and TCMTB). The response curve for MTBT (Figure 11) is linear over only a limited range, but does include the origin to within the statistical uncertainty in the y-intercept. The corresponding curves (Figure 12) for TCMTB, and particularly for MBT, are linear over a much wider range, and also include the origin. Detection limits for all three analytes under these LC-MS conditions were all of the order of 1-2 ng injected on-column, *i.e.* 50-100 pg reaching the mass spectrometer. For a 20 μL injection volume this corresponds to a concentration detection limit of about 50 ng/mL, which is comparable with that determined for LC-UV analyses (data not shown). It is not clear why these LC-MS detection limits should be

so different (apart from that for MTBT) from those determined by FIA (section B.1).

B.3 Tandem mass spectrometry of standards

A series of tandem mass spectrometry experiments was performed, using the ZAB-EQ instrument with FAB ionization, with the aim of determining the analytical usefulness of collisionally induced dissociation (CID) for these compounds. This type of experiment may be performed to several different ends: it may be used to deduce structural information about the selected precursor ions, or to search for species which yield a common fragment ion, or to search for characteristic collisionally induced reactions (m/z of both precursor and fragment ions specified) and thereby gain greater specificity of analysis. In the current work, experiments of the first kind were performed to determine whether any analytically useful reactions occurred during CID of ions formed from the target compounds, which would allow experiments of the third kind to be employed on real world samples. The advantage of performing such multiple reaction monitoring (MRM) experiments is the increase in specificity of detection thereby attained, though usually at the expense of a significant loss in absolute signal levels. However, increased specificity can lead to an increase in signal/noise in cases where chemical noise is the dominant contribution, and also to a reduced need for sample clean-up and hence a reduced risk of analyte losses or contamination.

In Figure 13, the CID spectra of the three most intense ions in the FAB mass

spectrum of TCMTB (Figure 2), and of the $(M + H)^+$ ion of MTBT, are shown. Unlike the other members of the series, TCMTB has a rich fragment ion spectrum revealing much structural information. The dominant feature is the loss of thiocyanic acid (HSCN, 59 Da) from the protonated molecular ion (m/z 239) to yield the ion at m/z 180. This ion then undergoes a further loss of 44 Da (presumably CS), to yield an ion at m/z 136 formulated as $C_7H_6NS^+$. A competing pathway involves initial loss of 27 Da (HCN) to give an ion at m/z 212, which subsequently loses 44 Da (again presumably CS) resulting in an ion at m/z 168. The other compounds have less complex, and much less intense, fragmentation spectra. Thus, the $(M + H)^+$ ion of MTBT (m/z 182) appears to undergo loss of a methyl radical from the even-electron $(M + H)^+$ ion to yield an odd-electron ion at m/z 167, but at a relative intensity of only 2% (Figure 13). In similar experiments, the fragmentation behaviour of the other members of the set was examined. The resulting spectra (not shown) contained only weak signals under the low-energy CID conditions (few tens of eV) used in the tandem quadrupole section of the ZAB-EQ instrument. However, a fragment ion at m/z 109 was consistently observed at moderate intensity, *e.g.* the $(M + H)^+$ ion of the parent compound BT lost 27 Da, presumably HCN (or HNC), to form this fragment. Loss of RCN from molecular radical cations $M^{+\cdot}$ of substituted benzothiazoles is characteristic [33] [34] of such compounds, and some analogous reaction of the corresponding even-electron $(M + H)^+$ ions appears to lead to the m/z 109 ion which is formally a thia-analogue (S replacing CH_2) of the well-known isomeric benzyl-tropylium ions $C_7H_7^+$.

Although these MS/MS experiments were conducted using FAB ionization and the ZAB-EQ instrument, the low-energy collision conditions were closely similar to those pertinent to the SCIEX API III instrument used subsequently, and the conclusions drawn here are applicable in at least a semi-quantitative manner.

B.4 Sample preparation.

In some preliminary experiments conducted by Environment Canada [10], bile from Coho salmon and rainbow trout which had been exposed to TCMTB was subjected to an elaborate and meticulous clean-up and fractionation procedure. This lengthy procedure was necessary since the analysis step was conducted [10] using LC-UV, a method of little specificity subject to extensive interferences from endogenous compounds. Control experiments, using bile from fish which had not been exposed to TCMTB, yielded LC-UV chromatograms free of interferences in the retention time windows appropriate to MBT and TCMTB. However, much simpler sample preparation procedures were found to be adequate for the much more diagnostic analyses afforded by the LC-MS technique.

The bile ducts were allowed to thaw at room temperature, and a small quantity (*ca.* 25 µL) of bile was removed with a clean syringe. Other body fluids, including the samples of bile supplied already extracted from the ducts, were prepared for HPLC injection as follows:

Bile: dilute 1:10 in methanol, add internal standard spike (MeBT), and filter (0.22μm).
Plasma and urine: dilute 1:10 in methanol, add internal standard spike (MeBT), and filter (0.22 μm, then 10,000MW).

The 10,000MW filtration step is quite time consuming, even with a 0.22μm step beforehand. However, it was essential to remove as much high molecular weight material as possible before the chromatography, since otherwise proteins or other large molecules present in the plasma and urine caused the pre-column to block after only one or two injections. The bile samples appear to be free of such material, since no signs of column blockage were observed from them despite the omission of the 10000 MW cut-off filtration step.

In many cases it was found that an enzymatic digestion was necessary for reliable assessment of contamination by the target analytes. Thus, samples were digested with β-glucuronidase to liberate the target compounds from their glucuronides, and separately incubated with a sulfatase to decompose sulfo-conjugates. The procedures used for these treatments were as follows:

Glucuronidase:

Bile: a 25 μL sample was mixed with 100 μL of a solution of the enzyme (β-glucuronidase, Sigma, St. Louis, MO), 10 μL of the internal standard solution (100 ng/mL) were added, and the mixture was incubated at 37°C for 1 hr, filtered (0.22 μm), and made up to a final volume of 250 μL.

Plasma: a 50 µL sample was mixed with 200 µL of the enzyme solution, plus 20 µL of the internal standard solution, incubated at 37°C for 1 hr, , filtered (0.22 µm, then 10,000MW), and made up to a final volume of 300 µL.

Urine: these samples were treated as for the plasma, except that all volumes were reduced by 50% because of the small sample sizes available.

Sulfatase:

All matrices were treated as for the glucuronidase digestion procedures described above, except for substitution of the different enzyme (Sulfatase Type VI, Sigma, St. Louis, MO. This enzyme preparation has no detectable β-glucuronidase activity at pH 7).

B.5 Analysis of fish bile samples.

Sample LC/MS chromatograms of bile from control fish and from fish exposed to TCMTB are shown in Figures 14 and 15. These chromatograms are from experiments designed to detect MBT and its glucuronide, the latter presumably corresponding to esterification of the MBT thiol group by glururonic acid [OHC $(CHOH)_4$ COOH]. Accordingly the m/z values monitored were 150 (MeBT, internal standard), 168 (MBT) and 344 (MBT glucuronide). In the control bile (Figure 14) the m/z 150 trace shows one well-defined peak at *ca.* 7.8 min corresponding to the MeBT internal standard, while the trace for m/z 168 shows no evidence for the presence of MBT and the m/z

344 trace shows the presence of two late-eluting components. These two components are, however, too non-polar to be glucuronides under these reversed-phase LC conditions, and must be endogenous compounds found in the bile. This chromatogram contrasts with that shown in Figure 15, which shows the corresponding traces for bile from a fish exposed to 20 ppb TCMTB. As before the internal standard elutes at *ca.* 6.8 min, but the other two traces are completely different from Figure 14, each showing a relatively intense peak at *ca.* 3.2 min. Since MBT itself elutes about 0.2 - 0.4 min earlier than the internal standard under these conditions, it was assumed that the m/z 168 peak in Figure 15b arose from decomposition of the $(M + H)^+$ ion of the glucuronide (m/z 344) during passage through the API interface.

This hypothesis was tested in two ways. In the first experiment, tandem mass spectrometry was used in an MRM experiment which simultaneously monitored the 150→109 transition of the MeBT internal standard and the 344→168 reaction of the (postulated) MBT glucuronide. The resulting MRM chromatograms are shown in Figure 16, together with the fragment ion spectrum of m/z 334 (from a different LC-MS/MS experiment) acquired at the crest of the chromatographic peak at 3.2 min (Figure 15c). These results strongly support the hypothesized presence of a glucuronide of MBT in the bile sample. Further confirmation was obtained from experiments in which the bile was digested with the enzyme β-glucuronidase. A similar series of MRM chromatograms is shown for the enzyme-digested bile sample in Figure 17. In contrast to Figure 15, the most notable features are the absence of an intense peak at 3.2 min in the m/z 344 and m/z 168 chromatograms, and the

appearance of a peak at *ca.* 6.9 min in the m/z 168 chromatogram. (The peak at *ca.* 2.2 min in the m/z 344 chromatogram appears to arise from the enzyme itself, since it was observed in all samples treated with the glucuronidase.) In this case all of the m/z 168 signal is found in a single chromatographic peak with the same retention time as that of an authentic standard of MBT. Because of the relative ease of integrating the area of this peak, and because of the unavailability of a standard for the glucuronide conjugate, it was decided to use enzyme digests for quantification of MBT in the samples. The calibration curves for MBT (Figure 12) were used to generate the quantitative results listed in Table 1. No free MBT was found in any of the bile samples. In a similar fashion, the samples were analyzed for the presence of sulfo-conjugates, through the use of the sulfatase enzyme digestion, and by conducting LC-MS/MS runs in which the mass spectrometer was set to monitor possible conjugate ions arising from both C-sulfation and O-sulfation (addition of SO_4 or of SO_3 respectively, involving possible hydroxy metabolites in the latter case). No evidence was found for the presence of sulfo-conjugates in the bile samples.

B.6 Analysis of fish plasma samples.

The plasma samples were treated in a fashion similar to that described above for the bile samples, but including the additional clean-up procedure (10,000 MW filter) described in B.4. Typical mass chromatograms obtained by an LC-MS analysis of plasma from a fish exposed to 20 ppb TCMTB, are shown in Figures 18-20 for

untreated, glucuronidase treated, and sulfatase treated plasma, respectively. It will be helpful in interpreting these chromatograms to bear in mind that, under the chromatographic conditions employed, MBT (m/z 168) elutes 0.4 - 0.6 min before MeBT (m/z 150), while MTBT (m/z 182) elutes 2.0 - 2.2 min after the internal standard. The polar glucuronide and sulfate conjugates are expected to elute very early (reversed - phase LC conditions).

The LC-MS analysis of the untreated plasma (Figure 18) shows no evidence for free MBT (m/z 168). However, the peak at 9.2 min in the m/z 182 chromatogram *may* indicate the presence of free MTBT, although this retention time is a little long relative to the usual range for MTBT (2.0 - 2.2 min after MeBT, which in this experiment eluted at 6.8 min). As for the bile samples, the m/z 344 chromatogram was acquired in order to search for the glucuronide of MBT. In contrast, the corresponding LC-MS analysis of the glucuronidase digest of the same plasma sample (Figure 19) shows good evidence for free MBT liberated from its glucuronide. Similarly, an intense signal within the retention time window for MTBT in the m/z 182 chromatogram strongly suggests that this plasma sample contained significant levels of MTBT glucuronide. This conclusion is a little surprising, and the identification should be considered suspect until enough signal strength can be generated to permit acquisition of a full scan spectrum (and possibly a full-scan precursor-ion LC-MS/MS analysis for selected fragment ions at m/z 182), to allow unambiguous identification. Such a positive identification will require at least a 5-10-fold concentration of the sample, and thus a

more complex clean-up technique will have to be employed. The sample sizes available in the present work made such an extension impossible. The concentrations of MTBT glucuronide, listed in Table 1, are based upon this incomplete evidence.

Similar comments apply to the results of LC-MS analysis of the sulfatase-treated plasma (Figure 20). No MBT-sulfate was detected, but again a significant peak was observed in the m/z 182 chromatogram, just outside the expected retention-time window for MTBT. (The additional peak at about 4.4 min in this chromatogram has not been identified). As for the glucuronide analysis, this tentative indentification of an MTBT-sulfate conjugate should be confirmed, if more sample becomes available. The concentrations listed in Table 2 are again based upon these tentative identifications.

B.7 LC-MS analysis of urine samples.

The urine samples were treated in the same way as the plasma samples, except that all volumes were halved due to the small sample sizes. Results of the LC-MS analyses of untreated urine, and of the digests resulting from treatment by glucuronidase and by sulfatase, are summarized in Figures 21-23, respectively. There is no evidence for free MBT in the m/z 168 chromatogram, but a possible candidate peak for MTBT is observed in the m/z 182 chromatogram (Figure 21) somewhat similar to that observed (Figure 18) in the corresponding plasma sample. (The unknown peak at 4.4 min, noted in Figure 20, is observed here also). Upon digestion

with glucuronidase, no evidence for liberated MBT or MTBT was found (Figure 22). However, sulfatase digestion of this urine sample did liberate some MBT and also MTBT (Figure 23), though the qualitative identification of the latter is subject to the same uncertainties as for the plasma samples (section B.6). Again, the concentrations listed for the MTBT-sulfate in Table 2 assume that this tentative identification is valid.

C. MARKER COMPOUNDS FOR BLEACHED KRAFT MILL EFFLUENT; chloroguaiacols and dehydroabietic acid.

Dehydroabietic acid (DHA) was initially supplied by Dr. G. Kruzynski, but later samples were purchased from Helix Biotech Corp., Richmond, BC, who also supplied the chloroguaiacol standards.

C.1 Mass spectrometry of standards.

Some effort was expended in developing a suitable LC-MS Method for analysis of these compounds. The IonSpray interface was found to give very poor performance, in both positive and negative ion modes. The reasons for this are unclear, but the sensitivity was very poor and the mass spectra obtained were also of poor quality. In positive ion experiments attempts were made to vary the strength of the acid in the solution by changing the TFA content from 0.1 to 1%, and then to 3%, but with no observable improvement in signal. In negative ion mode experiments were conducted with both pre- and post-column addition of basic buffers (ammonia and tributylamine), in an attempt to increase the degree of ionization of the compounds in solution. Again, these attempts failed to yield a workable solution.

It was then decided to investigate the feasibility of using the atmospheric pressure chemical ionization (APCI) interface. This technique bears some resemblance to the

frequently employed thermospray technique, in that the HPLC effluent is passed through a nebulization and heated volatilization zone, with the product gas stream being passed on into the mass spectrometer source. Unlike thermospray, however, the heated nebulizer APCI interface used in this work does not require the addition of chemical buffers, and the ionization occurs at atmospheric pressure and moderate temperatures (measured gas temperatures up to 120°C). Volatilization of the extremely small particles of analyte, remaining after removal of the solvent, proceeds much more readily than from the bulk solid. The vaporized analyte is then flowed past a corona discharge needle, and is ionized by well-established APCI mechanisms [35].

Positive-ion APCI yielded very poor response for these target compounds, but negative-ion mode was found to be highly sensitive. The major reactant ions in the APCI plasma formed in moist air are the superoxide anion $O_2^{\cdot-}$, its hydrates $O_2\ (H_2O)_n^{\cdot-}$, and clusters $O_2\ (O_2)_n^{\cdot-}$, which ionize the sample molecule M by electron transfer or by proton abstraction to form the molecular radical anion $M^{\cdot-}$ or the even-electron ion $(M - H)^-$, respectively [35]. The negative-ion APCI spectra of 3, 4, 5-trichloroguaicol (345-TCG), tetrachloroguaicol (TeCG) and dehydroabietic acid (DHA) obtained using FIA, are shown in Figure 24. Under the conditions used in the present work all of the chloroguaiacols and DHA were ionized by proton abstraction to yield the $(M - H)^-$ ions. This result was anticipated for DHA, but it was not clear *a priori* whether the much more electronegative chloroguaiacols would be ionized by electron attachment or by proton abstraction. The only real surprise in the mass spectra shown in Figure 24 is

the facile expulsion of a methyl radical from the even-electron $(M - H)^-$ ions.

Production of odd-electron fragments from even-electron precursors is highly unusual, and must imply that the required homolytic bond dissociation energy is compensated for by a considerable stabilization of the dissociation products. In the present case the $(M - H - CH_3)^{-\cdot}$ ions from chloroguaiacols contain two oxygen atom substituents *ortho* to one another on the phenyl ring. One has a complete 8-electron shell and bears a formal negative charge, while the other has only 7 electrons in its valence shell and is a neutral radical site. Stabilization by resonance exchange of these two characteristics between the two oxygen atoms probably contributes to the driving force for the methyl radical expulsion.

It should be mentioned that initially it was difficult to calibrate the m/z scale of the mass spectrometer in negative ion APCI mode. Eventually, a reliable procedure was found involving calibration in negative ion IonSpray mode using cesium nitrate, as described previously [36]. This calibration was then found to be applicable to the spectra obtained on switching to the heated nebulizer APCI interface. In the present example, this was checked by obtaining spectra of pure compounds whose negative-ion APCI could be predicted with confidence. These compounds included saturated fatty acids, tetraglycine, and small perfluorinated organic acids, all of which yielded $(M - H)^-$ ions as anticipated. There was thus no doubt that the chloroguaiacols also formed $(M - H)^-$ ions under the APCI conditions used here.

The DHA spectrum (Figure 24) also contains a peak at m/z 303, which was found (see below) to correspond to a chromatographically separable impurity in the DHA sample. The degree of fragmentation observed in these spectra was again sensitive to the potential difference maintained between the sampling orifice and the skimmer in the API source interface. The spectra shown in Figure 24 were obtained using a potential difference of 60V, representing a compromise between efficient declustering of solvent molecules from the analyte ions on the one hand, and ion fragmentation on the other.

C.2 LC-MS analysis of standards.

In view of the choice of negative-ion mass spectrometric detection, the LC conditions had to be chosen with some care. It was not possible to use trifluoroacetic acid (TFA) as mobile-phase modifer, since even traces of this highly electronegative compound can overwhelm the ionizing power of the APCI plasma.

Even less electronegative modifiers, such as formic and acetic acids, are not compatible with negative-ion detection for LC-MS operation. Accordingly the HPLC conditions had to be devised without the benefit of acidic modifiers. The preferred column was a Zorbax Rx-C8 (25 cm x 4.6 mm I.D.). Also tried were a (20 cm x 2.1 mm I.D.) version of the same column type, and an Rx - C18 (25 cm x 2.1 mm I.D.) column. Two solvent systems were used. In the initial experiments an isocratic

mobile phase (80% acetonitrile in water) gave adequate performance. However, when analyzing fish body fluid samples for polar conjugates of the target analytes, a linear gradient (typically 25 to 100% acetonitrile in 20 min) was employed. Injection volumes were generally 1 µL for isocratic elution and 20 µL for gradient elution.

As for the work described in section B above, a Hewlett Packard 1090L liquid chromatograph, equipped with a binary DR5 solvent delivery system, was employed. For some of the later work an HP 79847A autosampler-autoinjector was also used.

Figures 25 and 26 show the results of LC-MS analyses of solutions of each of the three standard compounds. The selected ion monitoring (SIM) chromatogram for 345-TCG (m/z 226), shown in Figure 25, indicates the presence of a low-level impurity eluting before the main component. The LC peak for the latter is much less sharp than that for TeCG, also shown in Figure 25. The 345-TCG showed unusually strong affinity for the Zorbax C8 column, as demonstrated for example by desorption of some of this compound when the column was recycled back to initial conditions following a gradient elution. This phenomenon sometimes gave rise to broad peaks at long retention times in SIM chromatograms for TCG (m/z 226), and resulted in lower sensitivity for TCG than for TeCG. The SIM chromatogram for DHA (m/z 299, Figure 26) shows no sign of impurities in this standard but, as shown in its mass spectrum (Figure 24) it does appear to contain an impurity with molecular weight 4 Da higher. The SIM chromatogram for m/z 303 (Figure 26) shows that this impurity is

chromatographically resolved from DHA, and probably corresponds to dihydroabietic acid.

Because the negative ion APCI system gave good sensitivity for these compounds, it was decided to determine the instrumental limits of detection attainable. A series of experiments was performed on serially diluted standard solutions. An initial set of three solutions, each approximately 1 mg/mL in one of the standard compounds, was diluted in methanol. A succession of 1/10 dilutions was performed, with the final stage being 1/100,000 of the original (*ca.* 10ng/mL concentration). These diluted solutions were then successively injected *via* the HPLC column, starting with a methanol blank, then going up in concentration from the most dilute. A methanol blank was also injected after each standard. In this way the detection limits given in Table 3 were obtained. By way of illustration a mass chromatogram, obtained under SIM conditions for an injection of approximately 125 fg of DHA on-column, is shown in Figure 28. The superior sensitivity for DHA is due, at least in part, to the fact that essentially all of the ion current is carried by the $(M - H)^-$ ion, whereas considerable ion fragmentation occurs for the chloroguaiacols (Figure 24). It should be emphasized that these limits were determined under optimum conditions, with pure standard compounds dissolved in clean solvents. For real world samples the detection limits could be expected to be one or two orders of magnitude higher. The results of LC-MS analysis of a mixed solution of the three targer compounds are shown in Figure 27.

Part way through this work, it was suggested that an additional compound, 4,5,6-trichloroguaiacol (456-TCG), be added to the target list. Unfortunately, it was found that the isomeric TCG compounds coelute under the LC conditions employed here. Since the LC-MS response factors for the isomers were very close to one another, the TCG levels reported below are to be interpreted as total trichloroguaiacol concentrations. The only exceptions involve samples from fish exposed under laboratory conditions to one or other of the TCG isomers.

C.3. Sample preparation.

The simple clean-up procedures, for body fluids from fish exposed to chloroguaiacols and/or DHA, were essentially the same as those used for the benzothiazole contaminants (see B.4, above), although in the present case an internal standard (trichlorosyringol, 2,6-dimethoxy-3,4,5-trichlorophenol) was added to the untreated sample prior to cleanup. Briefly, 25 µL of raw body fluid were diluted 1:10 with methanol, and 25 µL of a solution of the internal standard, at a concentration which gave comparable concentrations of internal standard and analyte in the final mixture, were added. The mixture was then filtered through a 0.22 µm filter, and then through a 10,000 MW cutoff filter in the case of plasma and urine (but not bile). The total volume was adjusted to 500 µL with methanol, *i.e.* the concentration in the raw body fluid was 20 times greater than that determined in the final mixture analyzed by LC-MS.

In those cases where the body fluids were enzymatically digested prior to LC-MS analysis, the procedures used were the same as those described in B.4, the sole difference being addition of the internal standard prior to digestion.

As a final check on the overall procedure, a bile sample from one of the control fish used for the work in Section B, above, was spiked with a mixture of the 345-TCG, TeCG and DHA, taken through the sample preparation procedure, and analyzed by LC-MS using the same conditions as those used for the clean solution of mixed standards (Figure 27). The results of this analysis are shown in Figures 29 and 30 which compare the unspiked and spiked control bile samples, respectively. The target analytes are observed free from endogenous interferences in their respective mass chromatograms. Excellent signal/noise ratios were obtained (Figure 30) for 365 pg 345-TCG, 540 pg TeCG and 360 pg of DHA injected on-column.

C.4 Samples from salmon exposed to dehydroabietic acid.

As an example, the results of the LC-MS analysis of a bile sample exposed to 100 ppb of DHA (sample 12b) are summarized in Figure 31. Negative-ion SIM chromatograms were acquired for m/z 299 (DHA), 379 (O-sulfate of DHA, *i.e.* SO_3 moiety rather than SO_4 characteristic of C-sulfates), and 475 (DHA glucuronide conjugate, presumably corresponding to esterification of one of the hydroxyl groups of glucuronic acid by the DHA carboxyl group). Note that the gradient elution scheme

described in C.2 was used, so retention times are appreciably longer (about 15 min for DHA) than for the isocratic elution conditions used to obtain Figures 28-30. No peaks attributable to a sulfate are evident in the m/z 379 chromatogram, but there are intense co-eluting peaks at about 5 min in the m/z 299 and 475 chromatograms. As for the MTB glucuronide discussed in B.5 above, this observation is most readily explained in terms of the fragility towards collision induced dissociation of the DHA-glucuronide anion in the sampling orifice-skimmer region. This interpretation receives support from the full-scan mass spectrum (Figure 32), obtained at the crest of this chromatographic peak in a separate experiment. This spectrum contains an intense mass peak at m/z 299, corresponding to the $(M - H)^-$ ion of DHA, and a much less intense but still well-defined peak at m/z 475, the value predicted for the $(M - H)^-$ ion of DHA glucuronide. This interpretation of Figures 31 and 32 is further confirmed by the results of analyzing the glucuronidase digest of the same bile sample. Figure 33 shows that the LC peaks at 5 min in the m/z 299 and 475 chromatograms (Figure 31) were entirely removed by this enzymatic digestion, and replaced by an extremely intense peak at about 15 min in the m/z 299 chromatogram. Indeed this peak was so intense that it overloaded the pulse-counting detector, giving rise to the fragmented shape of the LC peak. Indeed, this digested bile extract had to be extensively diluted in order for the m/z 299 chromatogram to remain on-scale. Figure 34 shows the results of LC-MS analysis of the same digested bile extract, but diluted by a factor of 10^4. The peak at 15 min in the m/z 299 chromatogram is now well-defined and on-scale, while that at 5 min is reduced to a vestige of its former intensity. Similar

experiments on a sulfatase digest of this bile sample (data not shown) showed no evidence for detectable levels of DHA sulfate.

Analogous analyses of the other body fluids indicated that the plasma contained only the DHA sulfate, while the urine contained mostly sulfate plus (perhaps surprisingly) some glucuronide. Table 4 summarizes the estimates of DHA concentrations, present as the appropriate conjugates, in the three body fluids of four salmon exposed to different levels of DHA. As for the benzothiazoles discussed in Section B, the unavailability of pure standards of the DHA conjugates implies that these measurements of levels of DHA liberated by enzymatic digestion must be treated as semi-quantitative only. This *caveat* is further emphasized by the fact that the values obtained for the bile and urine samples were not highly reproducible, while those for the plasma samples were much more so. The reasons for this difference are unknown. There was some indication [37] that the length of time for which the samples remained in solution in acetonitrile may have had some effect on the analytical results. This observation is discussed further below. Each of the total concentration estimates, given in Table 4, represents the mean of 3 or 4 separate analyses conducted on separate occasions. It is interesting that the plasma and urine contain comparable levels of DHA conjugates (mostly sulfates), while the bile contains much lower levels (as glucuronides). These relative levels in the three body fluids roughly parallel those listed for the benzothiazoles in Tables 1 and 2.

C.5 Samples from salmon exposed to chloroguaiacols.

Figures 35-37 summarize results of LC-MS analyses of body fluids from a salmon exposed to 250 ppb of TeCG (sample set 15c). The most notable feature of these results is the high level of free chloroguaiacol, contrasting sharply with the case of DHA (see C.4 above) which was liberated from its glucuronide and sulfate conjugates only by appropriate enzymatic digestion. It was discovered at this time in the project that the free chloroguaiacol was liberated from its conjugates on standing in solution in aqueous acetonitrile. Presumably the chloroguaiacol glucuronide involved an ester linkage between the phenolic hydroxyl and the carboxyl group of glucuronic acid which, for some reason not explored here, was more susceptible to hydrolysis by the aqueous acetonitrile than the DHA glucuronide (which must involve a hydroxyl group of glucuronic acid and the carboxyl of the DHA). The O-sulfate conjugate of the chloroguaiacol was also succeptible to this purely chemical hydrolysis. Although it was therefore impossible to measure the concentration of free chloroguaiacol in the body fluid prior to its first contact with acetonitrile, this facile chemical hydrolysis provided a simple means of liberating the analyte from its conjugates without the requirement for enzymatic digestion. A few hours in aqueous acetonitrile at room temperature was all that was required. A few samples were also digested enzymatically, and no significant differences were detected in the levels of free chloroguaiacol thus liberated. The data shown in Figures 35-37 represent analyses where this hydrolysis was not complete in all cases, *e.g.* the O-sulfate in the urine

sample (Figure 36) was only about 50% hydrolyzed.

Table 5 lists the quantitative results of these analyses of sample sets 15 (a) - (d), *i.e.* 3 body fluids from each of 4 salmon, each fish exposed to 250 ppb of one of 345-TCG, 456-TCG, TeCG or DHA. The only results to cause concern are those for TeCG, for which the reported value in the bile is 3-5 times larger than those in plasma and urine. These relative values are the inverse of those observed for all other analytes, including the TCGs. It seems unlikely that this result can be valid, but no acceptable explanation for the discrepancy can be provided at this time. The results for DHA in Table 5 are consistent with those reported for different fish in the earlier work (Table 4).

C.6. Bile samples from feral fish.

The final stage of the analytical work consisted of the analysis of bile samples from four bile ducts, collected from fish caught in the wild near pulp mills. A set of chromatograms, from a Harmac bile sample (bile duct sample 8) treated with glucuronidase, is shown in Figure 38. Since the samples as received were all in the form of intact bile ducts, from which it was necessary to extract a portion of the bile, it was decided to use an enzyme hydrolysis treatment (glucuronidase) in case the method of extracting the bile was in some way different from that used by DFO to extract the other bile samples analyzed in this work. In Figure 38 there is evidence for

the presence of a little TCG, but no DHA in this case. Moreover, TeCG could not be detected in this, nor in any of the other bile ducts (samples 9-11) submitted, which were all treated in the same way. All of the mass chromatograms obtained for these feral fish indicated the presence of many endogenous compounds which interfered to some extent with the determinations. These compounds, which were not noted in the bile samples from salmon exposed under laboratory conditions, may arise from the more complex nature of the medium to which the fish were exposed, or may simply reflect species differences. The other bile duct samples were all treated the same way. The results, summarized in Table 6, are subject to an even greater degree of uncertainty than those in Tables 1-5 due to the lower levels and the interferences.

Within the limitations of the determinations, as discussed above, the results do not appear to be to remarkable except for the absence of measurable TeCG from all the samples, and of DHA from the Harmac bile. These negative results may be a consequence of the interferences by endogenous material, implying the need for improved clean-up techniques.

D. ATTEMPTS TO SYNTHESIZE CONJUGATES OF THE TARGET COMPOUNDS.

Attempts to synthesize glucuronide and sulfate conjugates of the target analytes were undertaken with a view to their use as standards for quantitative analyses. Excellent reviews of relevant synthetic methods are available [38] [39].

Glucuronides:

The major method used for this work is based on that of Bollenback *et al.* [40]. In this approach glucuronolactone, which is commercially available, is transformed to methyl tetra-O-acetyl glucopyranuronate, then to methyl tri-O-acetyl α-D-glucopyranosyl bromide. This compound may then be used in a number of synthetic schemes.

In one of several attempts to synthesize the glucuronide of MBT, the following procedure was used:

1. MBT (0.07 mol) was dissolved in anhydrous methanol and the solution cooled to -78°C. 0.05 mol of potassium was added and allowed to dissolve, then 0.1 mol of the glucopyranosyl bromide.

2. The flask was then allowed to warm slowly to room temperature, to dissolve all the bromo compound. After 2.5 hr at room temperature, the solution was

decolorized with carbon and then concentrated to a syrup under reduced pressure.

3. The syrup was extracted with 30 mL of pyridine, and the extract then cooled to -20°C and reacted with 30 mL of acetic anhydride. The resulting mixture was then allowed to stand in an ice-water bath overnight.

4. After a number of clean-up steps, the (presumed) triacetyl glucurono ester of MBT was crystallized from methanol.

The same product was also prepared using an alternative synthesis [41] based on the use of cadmium carbonate as catalyst.

These methods were applied to all of the BKME target compounds. In all cases the acetylated glucuronide appeared to be formed, in greater or lesser yield. All attempts to go further and deacylate these compounds to the true glucuronides failed. We were unable to find a base sufficiently strong to remove the acetyl groups, and yet weak enough to preserve the ester linkage. This is particularly true for MBT, where the linkage seems to be very sensitive to base.

That the O-acetyl glucuronides can be prepared was demonstrated by the ionspray mass spectrum of the reaction product of MBT. $(M + H)^+$ ions of the MBT glucuronide

with 1-4 acetylated hydroxyls were observed. All attempts to prepare the de-acetylated glucuronide from this compound yielded products with uninterpretable mass spectra.

Sulfates:

The methods employed in these attempts were based on that developed by Raudsepp and Mikkal [42], rather than on more recent methods [43]. A typical procedure was as follows:

1. 10 mg TeCG were dissolved in 80 mL of a solution of 30% N,N-dimethylaniline in chloroform. 40 mL of a 20% solution of chlorosulfonic acid in chloroform were added slowly, with frequent cooling. The resulting mixture was left at room temperature for 1 hr.

2. The solution was then titrated to pH 7 with 10% NaOH, by adding the base in small portions with frequent stirring.

3. After neutralization the organic layer was separated, and the aqueous layer extracted with 15 mL chloroform. The combined organic layers were extracted twice with 1 mL of 10% NaOH. The alkaline extracts were combined with the aqueous layer, the combined extract neutralized with HCl, then acidified by the addition of 10 mL of concentrated HCl. The mixture was then allowed to stand at room temperature overnight.

4. The solution was then heated on a steam bath for 1 hr., cooled, and extracted twice with 8 mL portions of diethyl ether. The combined ether extracts were washed with 2mL of water, and dried over anhydrous sodium sulfate. The ether was then evaporated to dryness in a gentle stream of nitrogen and the residue redissolved in anhydrous ethanol and recrystallized.

Similar preparations were tried for all the chloroguaiacols, DHA and MBT. In all cases a crystalline product could be obtained, but none of these was very water soluble indicating that the products may not be sulfo-conjugates. In some preliminary HPLC-MS experiments, it was found that these products do not elute at retention times expected for highly polar substances, and they do not show the expected masses (*e.g* DHA sulfate should show peaks at m/z 379 (O-sulfate) or m/z 395 (C-sulfate) as well as m/z 299).

E. Summary and Conclusions.

Objectives 1 and 2 of the present work (see Introduction) were largely accomplished. A simple gradient elution LC method was developed which was suitable for use with on-line ionspray mass spectrometry for detection of TCMTB and its degradation products, and their metabolites (glucuronide and sulfate conjugates), in body fluids from exposed fish. No TCMTB was detected in any of the samples supplied, neither free nor conjugated. The most clearcut evidence for TCMTB contamination was obtained in analyses for metabolites of MTB in bile and blood plasma (glucuronide) and in urine (sulfate). These analyses involved liberation of free MTB by digestion with appropriate enzymes. Evidence was also obtained which can be interpreted as demonstrating the presence of MTBT as its glucuronide in plasma and as its sulfate in urine (and possibly also in plasma), but it is felt that the evidence for qualitative identification of the MTBT conjugates is not as strong as for the MTB counterparts. Unfortunately, limitations of available sample size, particularly for the urine samples, precluded testing these tentative identifications *via* appropriate LC-MS/MS experiments.

Quantitative analyses of the body fluids were hampered by the unavailability of standards of the glucuronide and sulfate conjugates. Since nothing is known about the efficiencies of transformation of the conjugates of *e.g.* MBT, to free MBT which can be successfully analyzed, the concentrations given in Tables 1 and 2 should be

regarded as semi-quantitative estimates.

Objective 3 (see Introduction) involved analysis of more complex fish tissues (liver, kidney, brain, muscle) for the same analytes derived from TCMTB. Since no such samples were supplied by our collaborators, no work on this aspect was possible. However, since the use of TCMTB by the Canadian lumber industry has now been discontinued, the problem is no longer one of high priority.

The sensitivity and selectivity of the LC-MS techniques developed in this work for DHA and the chloroguaiacols are more than adequate for detection and quantitation of these analytes in body fluids of salmon exposed under laboratory conditions, despite the very simple clean-up procedures (filtering) used in this work. Equally simple hydrolysis prodecures, either enzymatic or chemical (aqueous acetonitrile, mechanism unknown), successfully liberated the analytes from their glucuronide and sulfate conjugates. The results thus obtained for laboratory-exposed fish (Tables 4 and 5) seem reasonable and self-consistent with the exception of the results for TeCG, as discussed in C.5 above.

However, these techniques do not appear to be adequate for bile from feral fish captured from waters exposed to bleached Kraft mill effluent. Although traces of TCG and DHA could be identified qualitatively, the quantitative data presented in Table 6 are particularly unreliable due not only to the low levels but also to interferences from

endogenous compounds in the SIM chromatograms. In turn, these interferences probably reflect the much greater chemical complexity of the polluted water to which these feral fish were exposed. This suggests that an improved clean-up procedure will be required for successful analysis of real-world samples for the BKME marker compounds. It is believed that the sensitivity of the LC-MS technique described here should be more than adequate, although an improved HPLC technique capable of resolving the TCG isomers, but still compatible with APCI mass spectrometry (negative ion mode), would be advantageous.

In summary, Objective 4 (see Introduction) called for development of analytical techniques for the three marker compounds in bile of exposed fish. This objective was achieved, with the reservations noted above, and was extended to plasma and urine.

It is highly disappointing that all of the effort devoted to synthesis of these conjugates failed to produce the desired results. The efforts of Environment Canada towards this end appear to have led to a similarly disappointing outcome. In any future work, it seems advisable to assign this task to research groups specialized in synthetic work of this kind.

Table 1

Glucuronidase Treated Samples of body fluids from fish exposed to TCMTB

Sample	MBT(µg/mL)	MTBT(µg/mL)
BILE		
Control (Sample 2)	n.d.	n.d.
10ppb Rainbow #1 (Sample 3)	0.41	n.d.
10ppb Rainbow #2 (Sample 4)	0.36	n.d.
20ppb Rainbow #1 (Sample 5)	1.06	n.d.
20ppb Rainbow #2 (Sample 6)	1.15	n.d.
10ppb Coho (Sample 7)	n.d.	n.d.
PLASMA		
Control (Sample 2)	n.d.	n.d.
10ppb Rainbow #1 (Sample 3)	1.68	n.d.

Table 2

Sulfatase treated samples of body fluids from fish exposed to TCMTB

Sample	MBT(μg/mL)	MTBT(μg/mL)
BILE		
Control (Sample 2)	n.d.	n.d.
10ppb Rainbow #1 (Sample 3)	n.d.	n.d.
10ppb Rainbow #2 (Sample 4)	n.d.	n.d.
20ppb Rainbow #1 (Sample 5)	n.d.	n.d.
20ppb Rainbow #2 (Sample 6)	n.d.	n.d.
10ppb Coho (Sample 7)	n.d.	n.d.
PLASMA		
Control (Sample 2)	n.d.	n.d.
10ppb Rainbow #1 (Sample 3)	n.d.	n.d.
10ppb Rainbow #2 (Sample 4)	n.d.	n.d.
20ppb Rainbow #1 (Sample 5)	n.d.	0.16
20ppb Rainbow #2 (Sample 6)	n.d.	0.24
10ppb Coho (Sample 7)	n.d.	n.d.
URINE		
Control (Sample 2)	n.d.	n.d.
10ppb Rainbow #1 (Sample 3)	1.4	0.04
10ppb Rainbow #2 (Sample 4)	0.9	0.11
20ppb Rainbow #1 (Sample 5)	4.5	2.11
20ppb Rainbow #2 (Sample 6)	4.1	1.14

Table 3

LC-MS Detection Limits for the Target BKME Compounds; APCI, negative-ion mode.

Compound	SIM Detection limit
3,4,5-trichloroguaiacol	20 pg
tetrachloroguaiacol	10 pg
dehydroabietic acid	200 fg

Table 4

Results from analyses of body fluids from salmon exposed to DHA

Bile samples contained only glucuronide, plasma contained sulfate only, while urine contained mostly sulfate with some glucuronide.

Sample I.D.	DHA (ng/mL), as conjugates
10 ppb bile (Sample 13)	5.3
25 ppb bile (Sample 12a)	14
100 ppb bile (Sample 12b)	120
400 ppb bile (Sample 12c)	852
10 ppb plasma (Sample 14a)	24
25 ppb plasma (Sample 14b)	98
100 ppb plasma (Sample 14c)	606
400 ppb plasma (Sample 14d)	6650
10 ppb urine (Sample 14e)	15
25 ppb urine (Sample 14f)	112
100 ppb urine (Sample 14g)	580
400 ppb urine (Sample 14h)	7520

Table 5

Results of analyses of body three body fluids from each of four salmon (sample sets 15 (a) - (d)). Each fish was exposed to 250 ppb of a single compound.

(All results reported in ng/mL)

Compound	Bile	Urine	Plasma
345-TCG	58	236	208
456-TCG	73	293	247
TeCG	954	284	186
DHA	206	1875	1723

Table 6

Results from the analysis of bile samples from feral fish (samples 8-11).

Sample	TCGs (ng/mL)	DHA (ng/mL)
Bile 1, Harmac 26 Apr 89	23	n.d.
901424 #109 ratfish bile, Aug 13/90	15	27
901424 #110 ratfish bile, Aug 13/90	12	24
901424 106 Hake bile, Aug 13/90	21	33

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CAPTIONS FOR FIGURES

Figure 1. Structures of the target analytes.

BT: benzothiazole; MBT: mercaptobenzothiazole;

MeBT: 2-methylbenzothiazole;

MTBT: 2-(methylthio)-benzothiazole;

TCMTB: 2-(thiocyanomethylthio)-benzothiazole;

DHA: dehydroabietic acid; TeCG, tetrachloroguaiacol;

456TCG: 4,5,6-trichloroguaiacol; 345TCG: 3,4,5-trichloroguaiacol.

Figure 2. FAB ionization positive ion mass spectra of MBT, MTBT and TCMTB. In each case 2 μg of the compound were applied to a film of "magic bullet" matrix on the tip of a standard FAB direct insertion probe.

Figure 3. Positive-ion ionspray mass spectra of MBT, MTBT and TCMTB, in a 1:1 acetonitrile-water solvent stream containing 0.1% triflurooacetic acid. The compounds were introduced by flow injection into a solvent stream of 50 μL/min, all of which was transported to the ionspray needle maintained at 4.8 kV. Air was used as nebulizing gas. The potential difference between the sampling orifice and the skimmer, in the atmospheric pressure ionization source interface, was maintained at 45V.

Figure 4. Instrumental response, flow-injection ionspray mass spectrometry, for BT (triplicate injections, serial dilutions).

Figure 5. Instrumental response, flow-injection ionspray mass spectrometry, for MeBT (triplicate injections, serial dilutions).

Figure 6. Instrumental response, flow-injection ionspray mass spectrometry, for MBT (triplicate injections, serial dilutions).

Figure 7. Instrumental response, flow-injection ionspray mass spectrometry, for MTBT (triplicate injections, serial dilutions).

Figure 8. Instrumental response, flow-injection ionspray mass spectrometry, for TCMTB (triplicate injections, serial dilutions).

Figure 9. Effects of varying the sampling orifice-skimmer potential on the relative intensities of ions produced by ionspray ionization of TCMTB (flow injection).

(a) Fragment ion, m/z 180

(b) $(M + H)^+$ ion, m/z 239

Figure 10. Mass chromatograms, obtained by LC-MS analysis using selected ion monitoring, of a mixture of five benzothiazole standards. Chromatographic conditions were those described as method 5 (see text). The ions monitored were the $(M + H)^+$ ions of BT (m/z 136), MeBT (m/z 150), MBT (m/z 168), MTBT (m/z 182) and TCMTB (m/z 239).

Figure 11. Response curve for LC-MS analysis (SIM) of MTBT standard solutions containing a fixed concentration of MeBT as internal standard. Peak area ratios, for the $(M + H)^+$ ions of analyte and internal standard, are plotted as a function of the amount of MTBT injected on-column (1:20 split to the mass spectrometer). Chromatographic conditions as for Figure 10.

Figure 12. Response curves for LC-MS analyses (SIM) of MBT and of TCMTB standard solutions (separate series of experiments), each containing a fixed concentration of MeBT as internal standard. Peak area ratios, for the $(M + H)^+$ ions of analyte and internal standard, are plotted as a function of the amount of analyte injected on-colum (1:20 split to the mass spectrometer). Chromatographic conditions as for Figure 10. the lower curve for MBT used a different concentration of internal standard, permitting the wider dynamic range to be explored.

Figure 13. Fragment ion spectra observed in low-energy CID of precursor ions formed by FAB ionization of TCMTB and of MTBT. The precursor ions were:

(a) TCMTB, $(M + H)^+$ ion, m/z 239.

(b) TCMTB, fragment ion, m/z 212.

(c) TCMTB, fragment ion, m/z 180.

(d) MTBT, $(M + H)^+$ ion, m/z 182.

Figure 14. Analysis by LC-MS (SIM) of a bile sample, from a control rainbow trout (sample #2), for MBT (m/z 168) and its glucuronide (m/z 344). The internal standard MeBT was monitored at m/z 150.

Figure 15. Analysis by LC-MS (SIM) of bile from a rainbow trout exposed to 20 ppb TCMTB (sample # 3). The chromatograms for m/z 150, 168 and 344 monitor the $(M + H)^+$ ions of the internal standard MeBT, of MBT, and of MBT-glucuronide, respectively.

Figure 16. Analysis by LC-MS/MS of bile from a rainbow trout exposed to 20 ppb TCMTB (sample # 3).

(a) MRM chromatogram for the fragmentation (m/z 150 → 109), monitoring the internal standard MeBT.

(b) MRM chromatogram for the fragmentation (m/z 344 → 168), monitoring the MBT-glucuronide.

(c) Full-scan fragment spectrum of the precursor ion m/z 344, acquired at the crest of the LC peak at 3.2 min.

Figure 17. Analysis by LC-MS (SIM) of the ß-glucuronidase digest of the bile from a rainbow trout exposed to 20 ppb TCMTB. The chromatograms for m/z 150, 168 and 344 monitor the $(M + H)^+$ ions of the internal standard MeBT, of MBT, and of the MBT-glucuronide, respectively.

Figure 18. Analysis by LC-MS (SIM) of blood plasma taken from a rainbow trout exposed to 20 ppb of TCMTB. The chromatograms for m/z 150, 168, 182 and 344 monitor the $(M + H)^+$ ions of MeBT (internal standard), MBT, MTBT and MBT-glucuronide, respecitely.

Figure 19. Analysis by LC-MS (SIM) of blood plasma, taken from a rainbow trout exposed to 20 ppb of TCMTB, and subsequently treated with β-glucuronidase. The chromatograms for m/z 150, 168 and 182 monitor the $(M + H)^+$ ions of MeBT (internal standard), MBT and MTBT, respectively.

Figure 20. Analysis by LC-MS (SIM) of blood plasma, taken from a rainbow trout exposed to 20 ppb of TCMTB, and subsequently treated with sulfatase. The chromatograms for m/z 150, 168 and 182 monitor the $(M + H)^+$ ions of MeBT (internal standard), MBT and MTBT, respectively.

Figure 21. Analysis by LC-MS (SIM) of urine taken from a rainbow trout exposed to 20 ppb of TCMTB. The chromatograms for m/z 150, 168, 182 and 344 monitor the $(M + H)^+$ ions of MeBT (internal standard), MBT, MTBT and MBT-glucuronide, respectively.

Figure 22. Analysis by LC-MS (SIM) of urine taken from a rainbow trout exposed to 20 ppb of TCMTB, and subsequently treated with ß-glucuronidase. The chromatograms for m/z 150, 168 and 182 monitor the $(M + H)^+$ ions of MeBT (internal standard), MBT and MTBT, respectively.

Figure 23. Analysis by LC-MS (SIM) of urine taken from a rainbow trout exposed to 20 ppb of TCMTB, and subsequently treated with sulfatase. The chromatograms for m/z 150, 168 and 182 monitor the $(M + H)^+$ ions of MeBT (internal standard), MBT and MTBT, respectively.

Figure 24. Negative-ion APCI mass spectra of three marker compounds for BKME. The compounds were dissolved in methanol at concentrations of 500 µg/mL, and 1 µL of each solution was injected into a 1 mL/min flow of 50% aqueous acetonitrile. This entire liquid flow (no split) was delivered to the heater-nebulizer APCI source. The potential difference between sample orifice and the first skimmer was maintained at 60V (orifice more negative). The controlled temperature on the nebulizer shroud heater was 320°C (the resulting gas temperature inside the shroud was much lower).

Figure 25. LC-MS analyses of each of the standard samples of 345-TCG (m/z 225) and TeCG (m/z 259), using APCI mass spectrometry in negative ion mode, with selected ion monitoring (SIM). Isocratic elution in 80% acetonitrile/water, with no acid modifiers, was used at a flowrate of 1 mL/min which was delivered without splitting to the heated nebulizer APCI interface.

Figure 26. LC-MS analysis of a standard sample of DHA (m/z 299), using APCI mass spectrometry in negative ion mode (SIM). Isocratic elution in 80% acetonitrile/water, with no acid modifiers, was used at a flowrate of 1 mL/min, which was delivered without splitting to the heated nebulizer APCI interface. The SIM chromatogram for m/z 303 was obtained in order to characterize the impurity detected in the mass spectrum of this sample (Figure 24).

Figure 27. LC-MS analysis of a mixture of the three standard samples 345-TCG (m/z 225), TeCG (m/z 259) and DHA (m/z 299). Chromatographic and mass spectrometric conditions were the same as those used to obtain Figures 25 and 26.

Figure 28. LC-MS analysis (negative ion SIM mode) of a serially diluted stock solution of DHA. Chromatographic and mass spectrometric conditions were the same as those used to obtain Figures 25-27. Approximately 125 fg of DHA were injected on-column.

Figure 29. Analysis of a control rainbow trout bile sample for 345-TCG (m/z 225), TeCG (m/z 259) and DHA (m/z 299). The LC-MS conditions were the same as those used to obtain Figure 27.

Figure 30. Analysis of a control rainbow trout bile sample spiked with a mixture of 345-TCG, TeCG plus DHA. The LC-MS conditions were the same as those used to obtain Figure 27. Calculated quantities injected on-column were 365 pg of 345-TCG, 540 pg of TeCG, and 360 pg of DHA.

Figure 31. LC-MS analysis of the bile from a salmon exposed to 100 ppb of DHA. The mass spectrometric conditions were identical to those used to obtain Figures 29 and 30 (negative ion APCI), but in this case the LC conditions involved gradient elution, 25 to 100% acetonitrile in 20 min. The SIM chromatograms correspond to the $(M - H)^-$ ions of DHA (m/z 299), DHA O-sulfate (m/z 379) and DHA glucuronide (m/z 475).

Figure 32. Negative ion mass spectrum obtained at the crest of the LC peak at 5 min in Figure 31. LC-MS conditions were the same as those used to obtain Figure 31, except that a spectral scan (m/z 100 to 500) was used instead of SIM.

Figure 33. LC-MS analysis of the glucuronidase digest of the bile sample examined in Figure 31. LC-MS conditions were identical to those used to obtain Figure 31.

Figure 34. Repeat of the LC-MS experiment which produced the results summarized in Figure 33, except that the digested bile was diluted with methanol by a factor of 10^4.

Figure 35. LC-MS analysis of bile from a salmon exposed to 250 ppb of TeCG. The bile was mixed with aqueous acetonitrile and allowed to stand at room temperature for several hours, thus hydrolyzing the TeCG conjugates. The HPLC conditions used gradient elution (20 - 100% acetonitrile in 20 min), and negative-ion APCI mass spectrometry was used in conjunction with a heated nebulizer interface. The SIM chromatograms for m/z 259, 261 and 435 monitor the $(M - H)^-$ ions of TeCG ($^{35}Cl_4$), TeCG ($^{35}Cl_3\ ^{37}Cl$) and the glucuronide of TeCG ($^{35}Cl_4$), respectively.

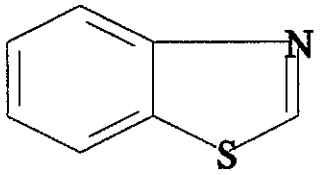
Figure 36. LC-MS analysis of urine from a salmon exposed to 250 ppb of TeCG. All experimental conditions were identical to those used to obtain Figure 34. The SIM chromatograms for m/z 259, 339 and 355 monitor the $(M - H)^-$ ions of TeCG ($^{35}Cl_4$), its O-sulfate and its C-sulfate, respectively.

Figure 37. LC-MS analysis of plasma from a salmon exposed to 250 ppb of TeCG. All experimental conditions were identical to those used to obtain Figure 35. The SIM chromatograms for m/z 259 and 435 correspond to the $(M - H)^-$ ions of TeCG ($^{35}Cl_4$) and of its glucuronide, respectively.

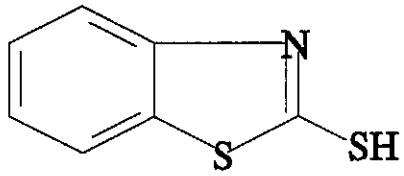
Figure 38. LC-MS analysis of a feral Harmac captured from waters polluted with bleached Kraft mill effluent. All experimental conditions were identical to those used to obtain Figure 35, except that the bile was subjected to digestion by glucuronidase. The SIM chromatograms for m/z 225, 259 and 299 monitor the $(M - H)^-$ ions of TCG, TeCG and DHA, respectively, whose retention times are (approximately) 12.5, 12.5 and 15.2 min, respectively.

Figure 1

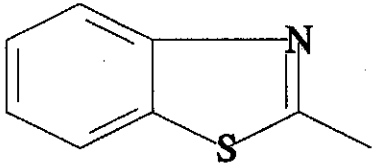
Structures of the target analytes



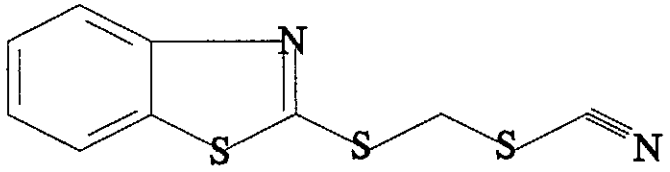
BT



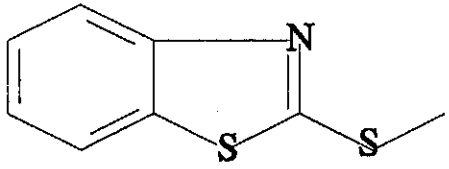
MBT



MeBT



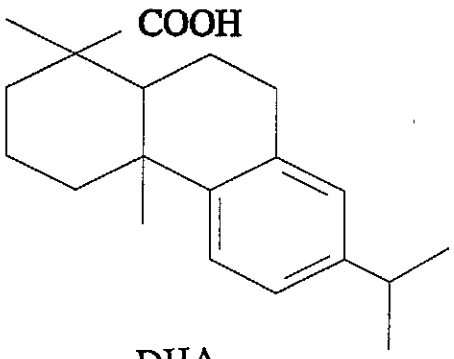
TCMTB



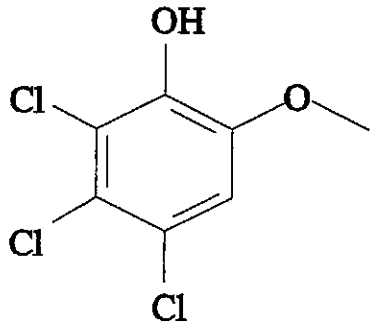
MTBT

Figure 1

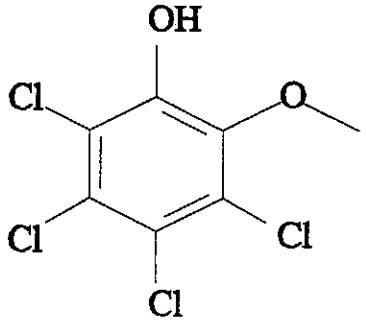
Structures of the target analytes



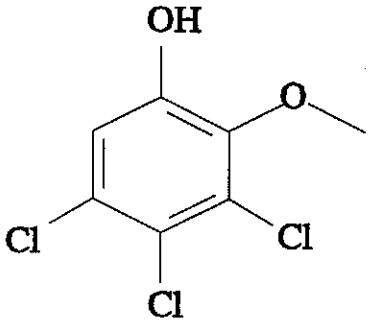
DHA



456-TCG



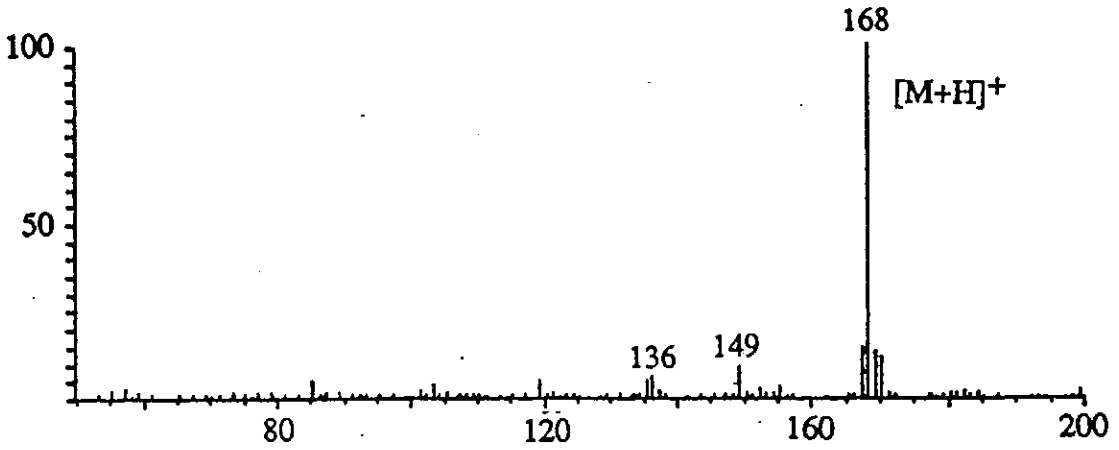
TeCG



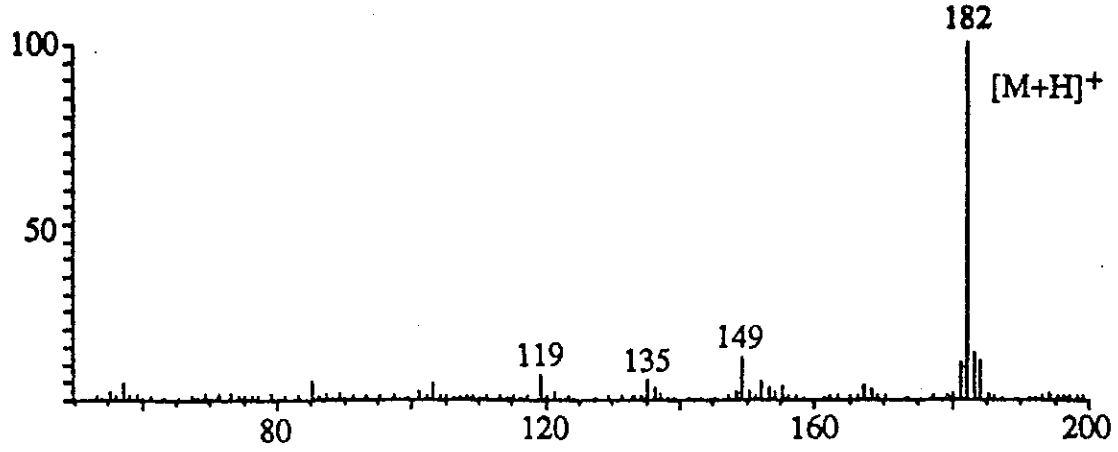
345-TCG

Figure 2

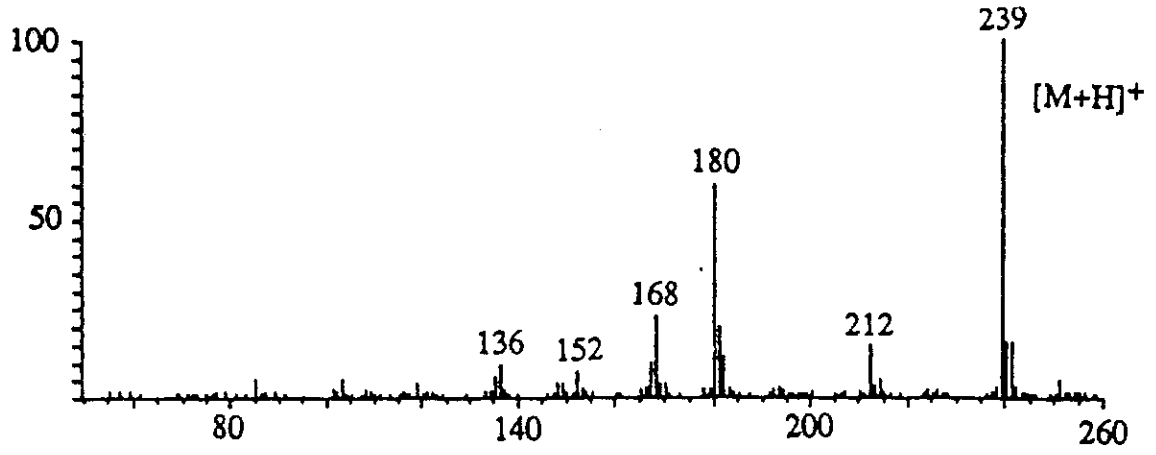
FAB-MS mass spectra of (a) MBT, (b) MTBT and (c) TCMBT



Positive ion FAB mass spectrum of MBT.



Positive ion FAB mass spectrum of MTBT.



Positive ion FAB mass spectrum of TCMTB.

Figure 3

ISP-MS mass spectra of (a) MBT, (b) MTBT and (c) TCMTB

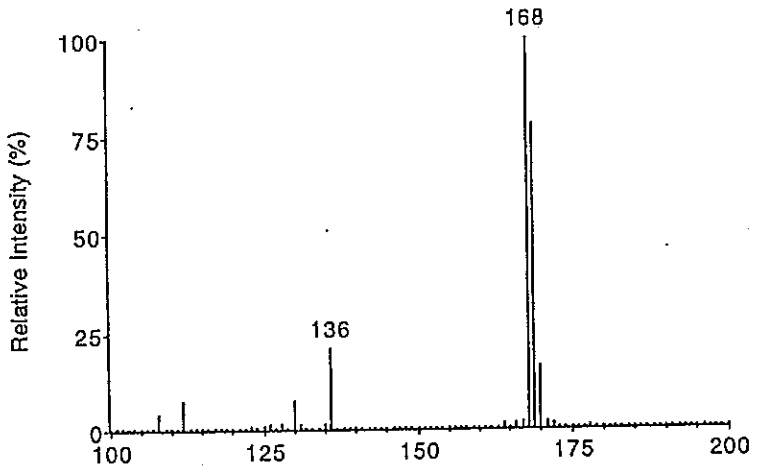
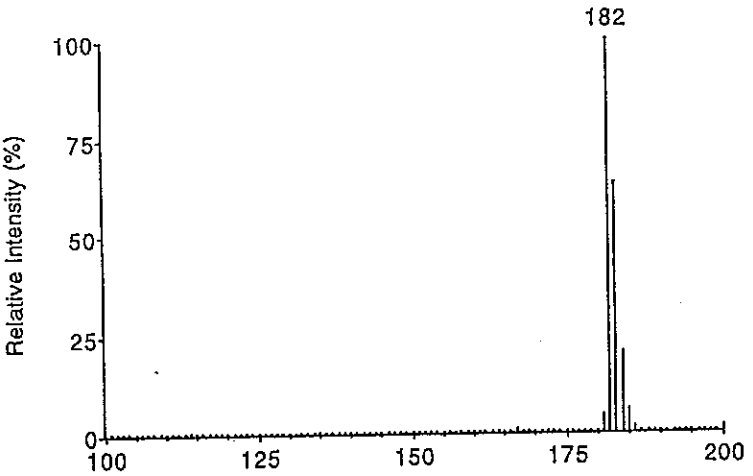
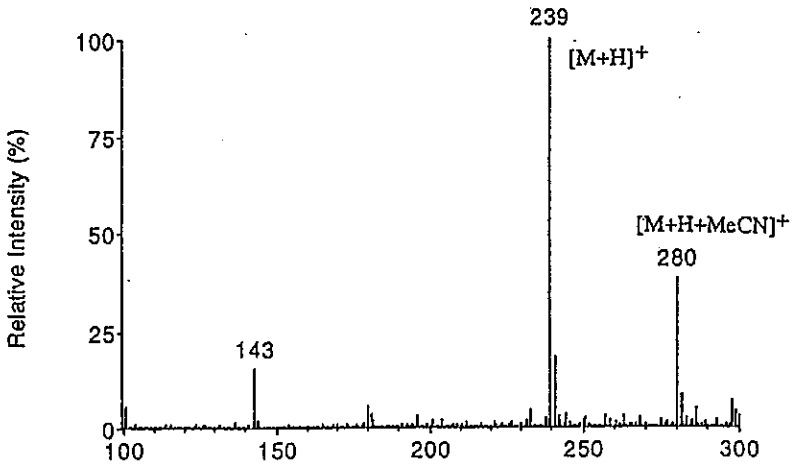


Figure 10 Background subtracted ISP LC-MS mass spectrum of MBT.



Background subtracted ISP LC-MS mass spectrum of MTBT.



Background subtracted ISP LC-MS mass spectrum of TCMTB.

Figure 4

FIA-ISP-MS replicate injections of BT

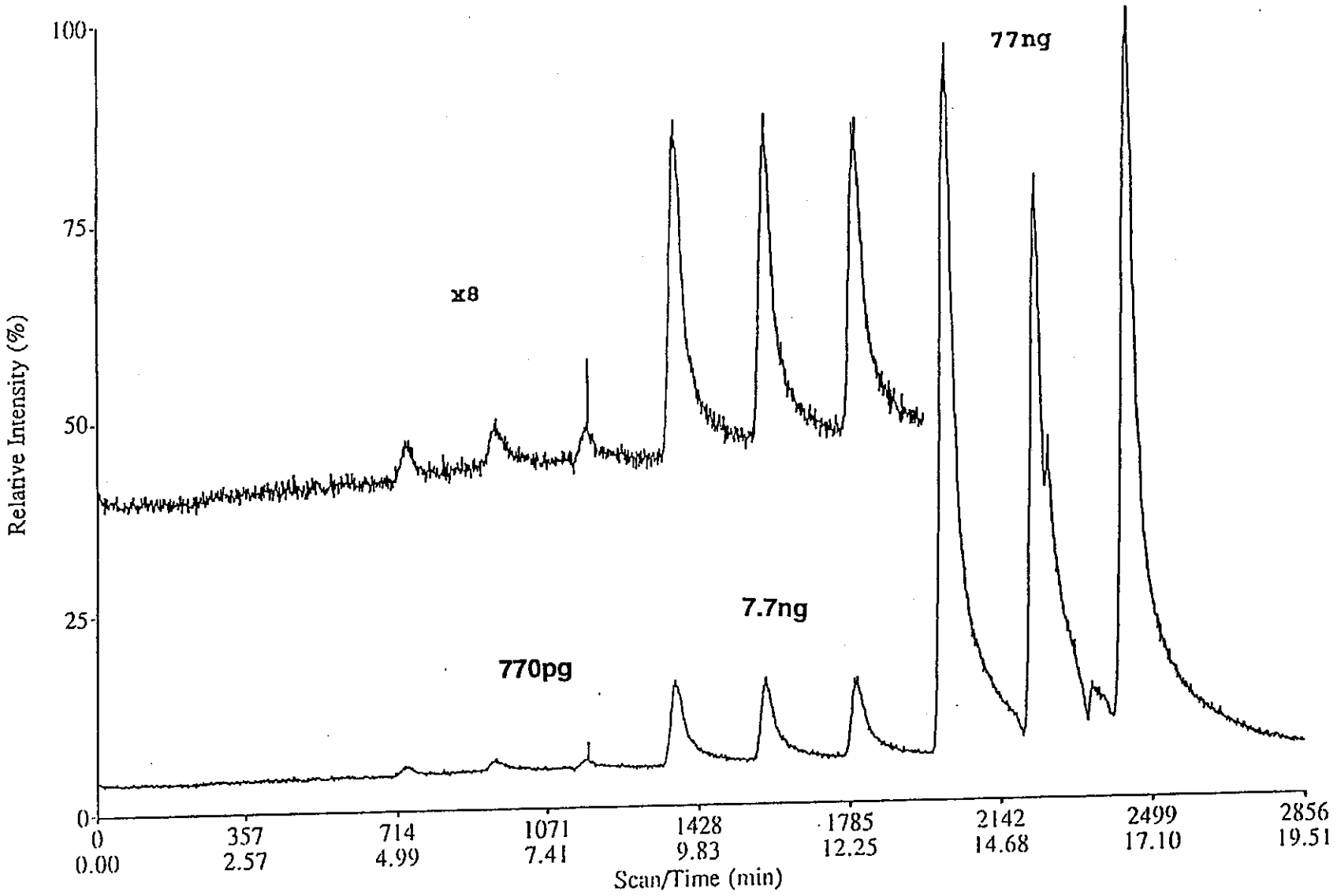


Figure 5

FIA-ISP-MS replicate injections of MeBT

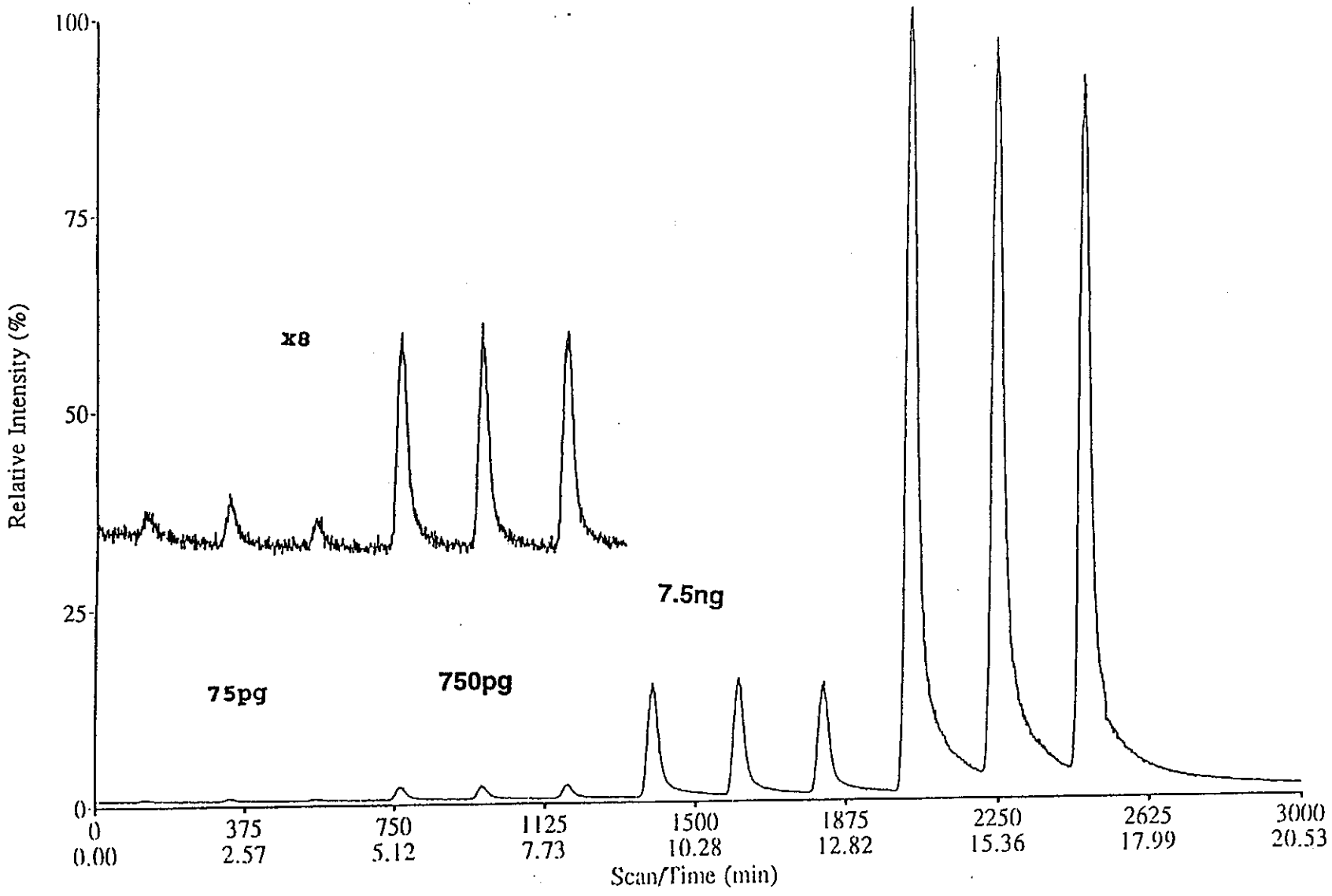


Figure 6

FIA-ISP-MS replicate injections of MBT

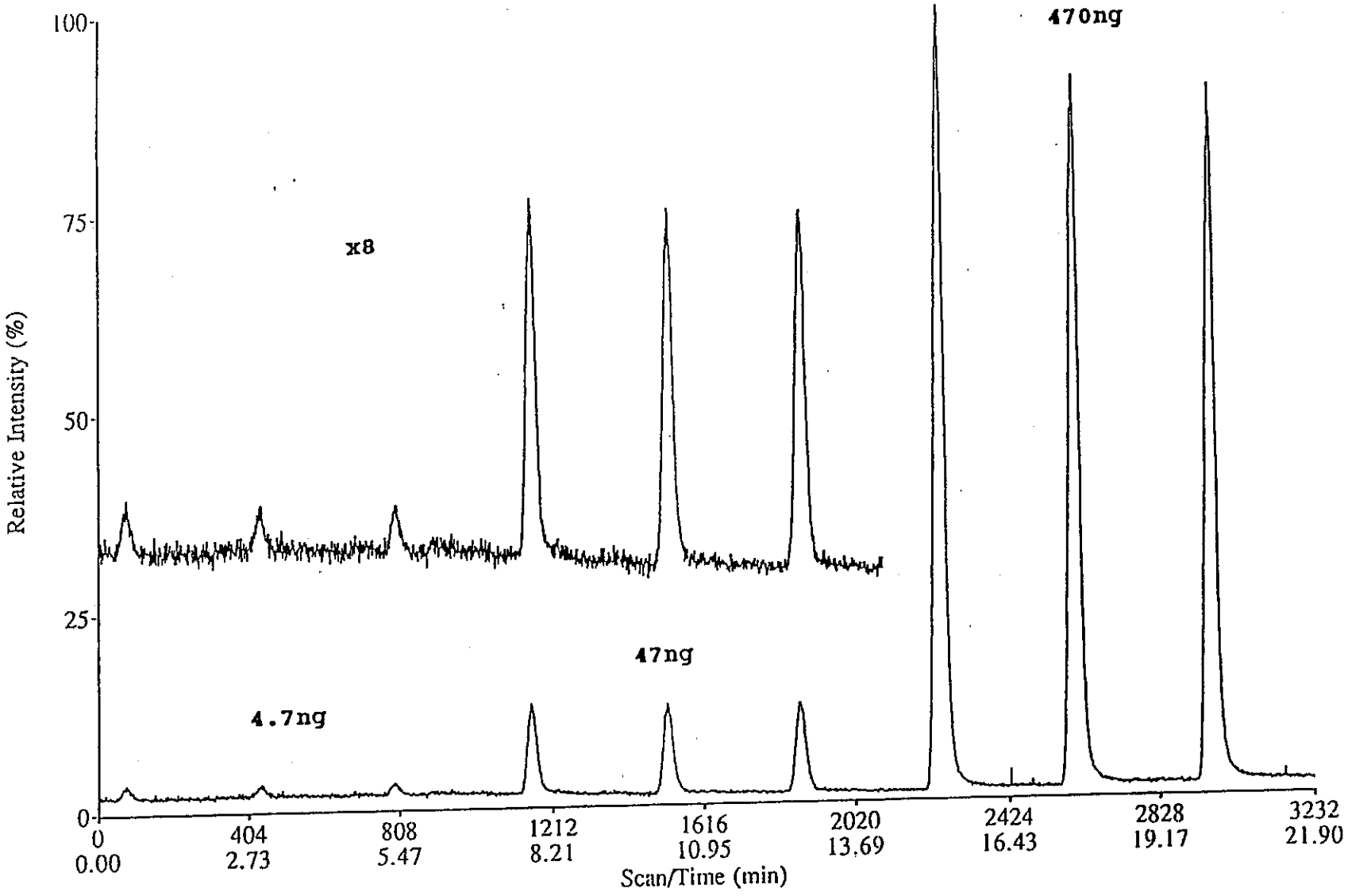


Figure 7

FIA-ISP-MS replicate injections of MTBT

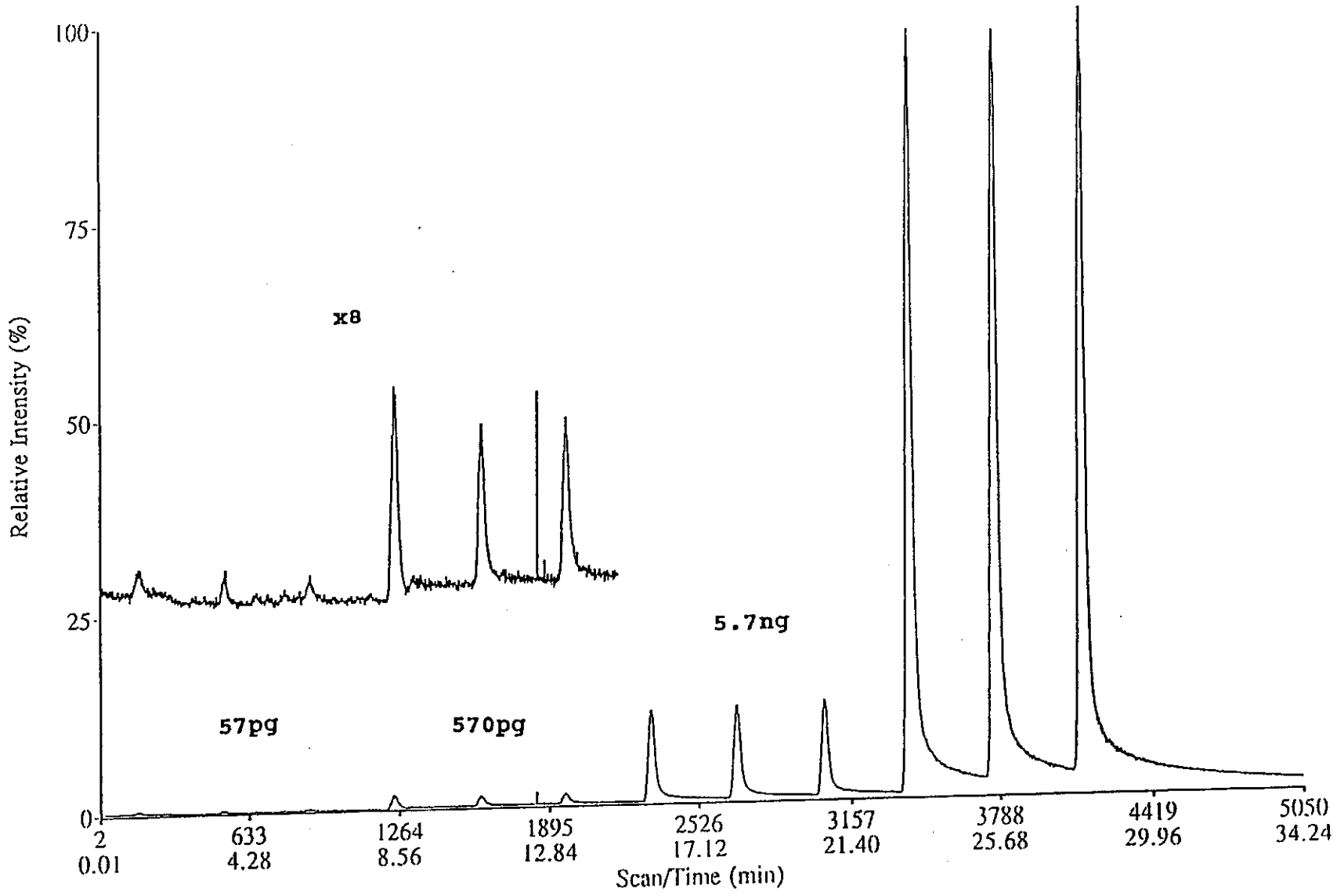


Figure 8

FIA-ISP-MS replicate injections of TCMTB

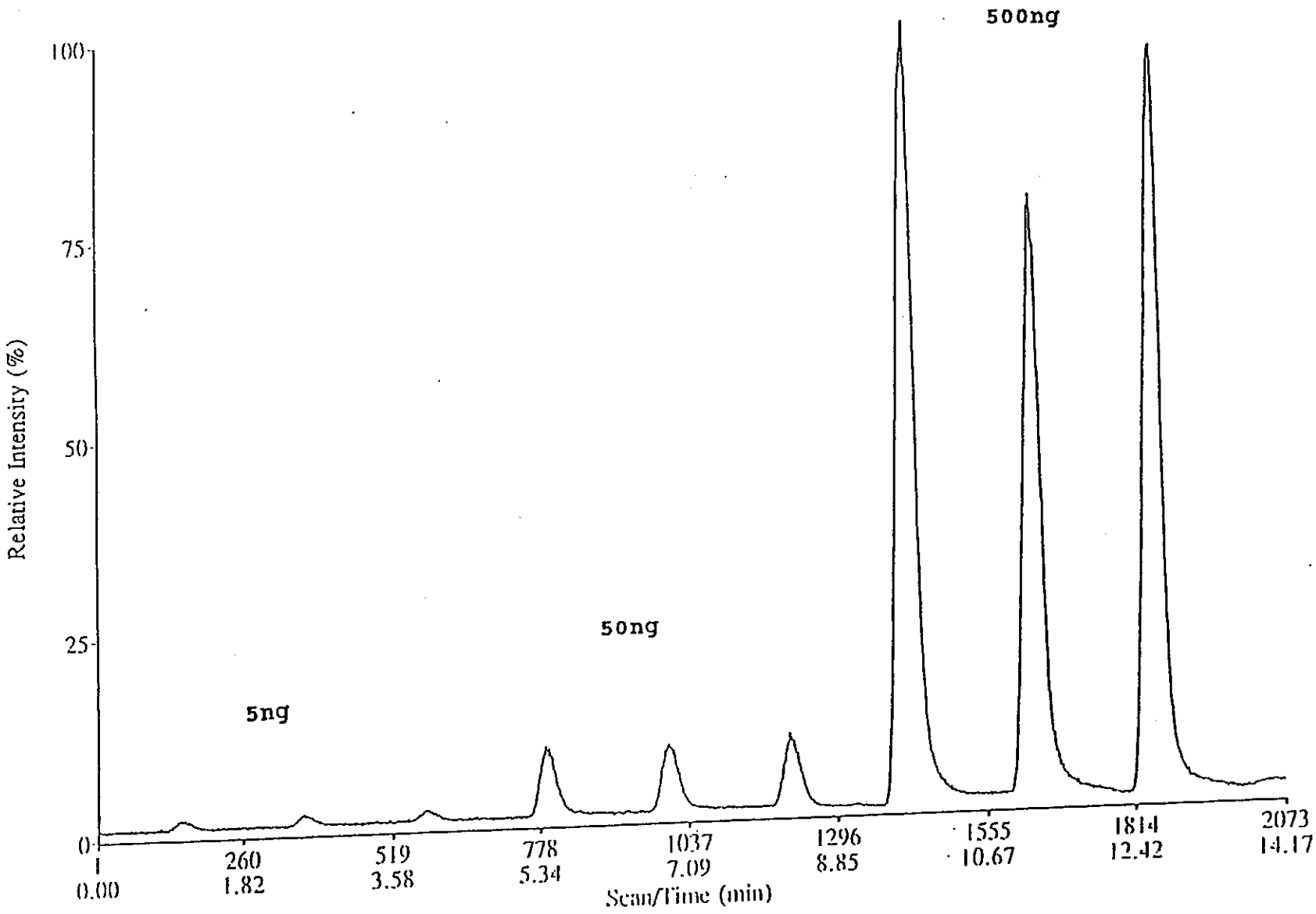


Figure 9

Effect of varying orifice voltage on the relative intensities of the m/z 239 and m/z 180 peaks in the ISP-MS mass spectrum of TCMTB

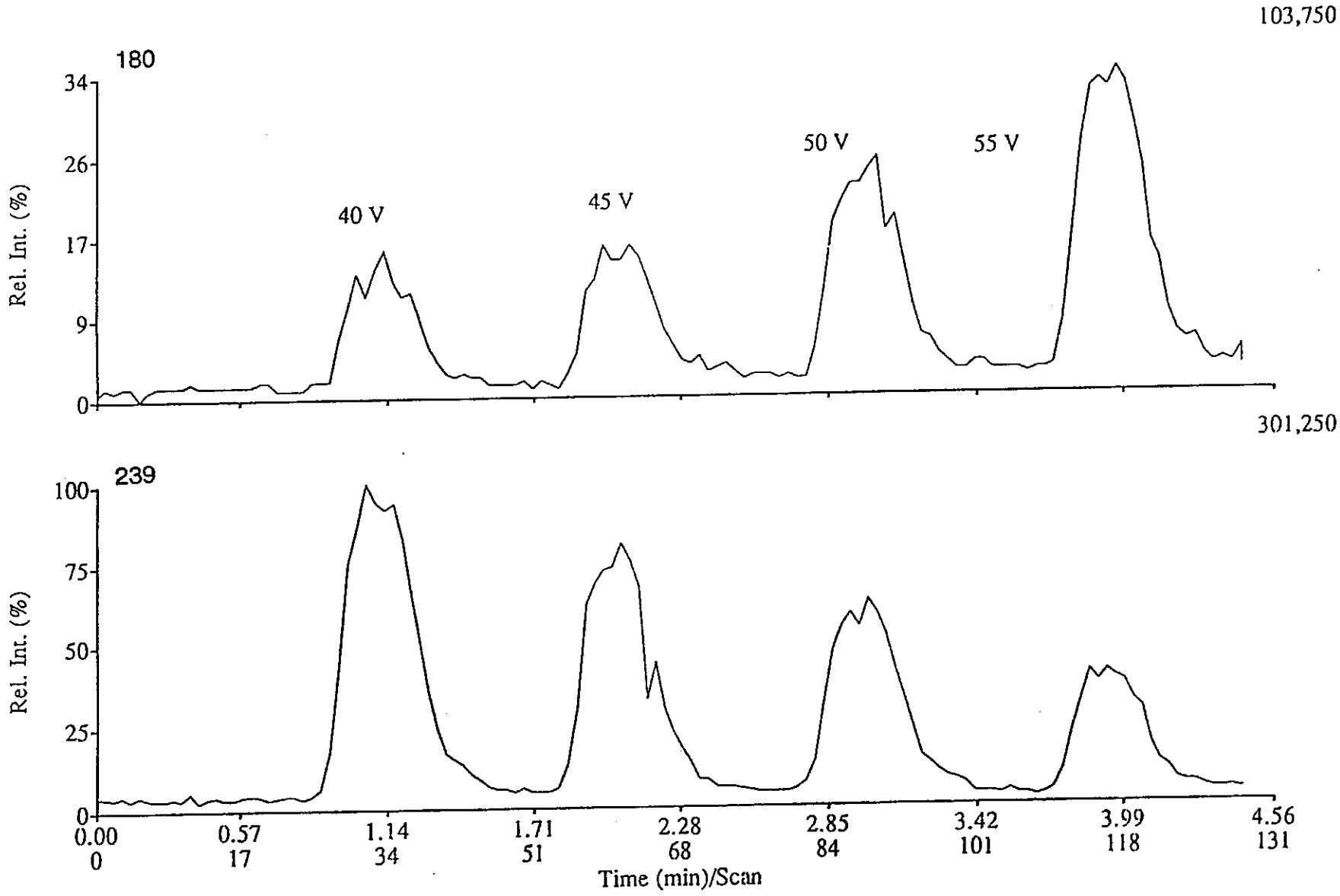


Figure 10

HPLC-ISP-MS mass chromatograms of a separation of a mix of the five benzothiazole compounds

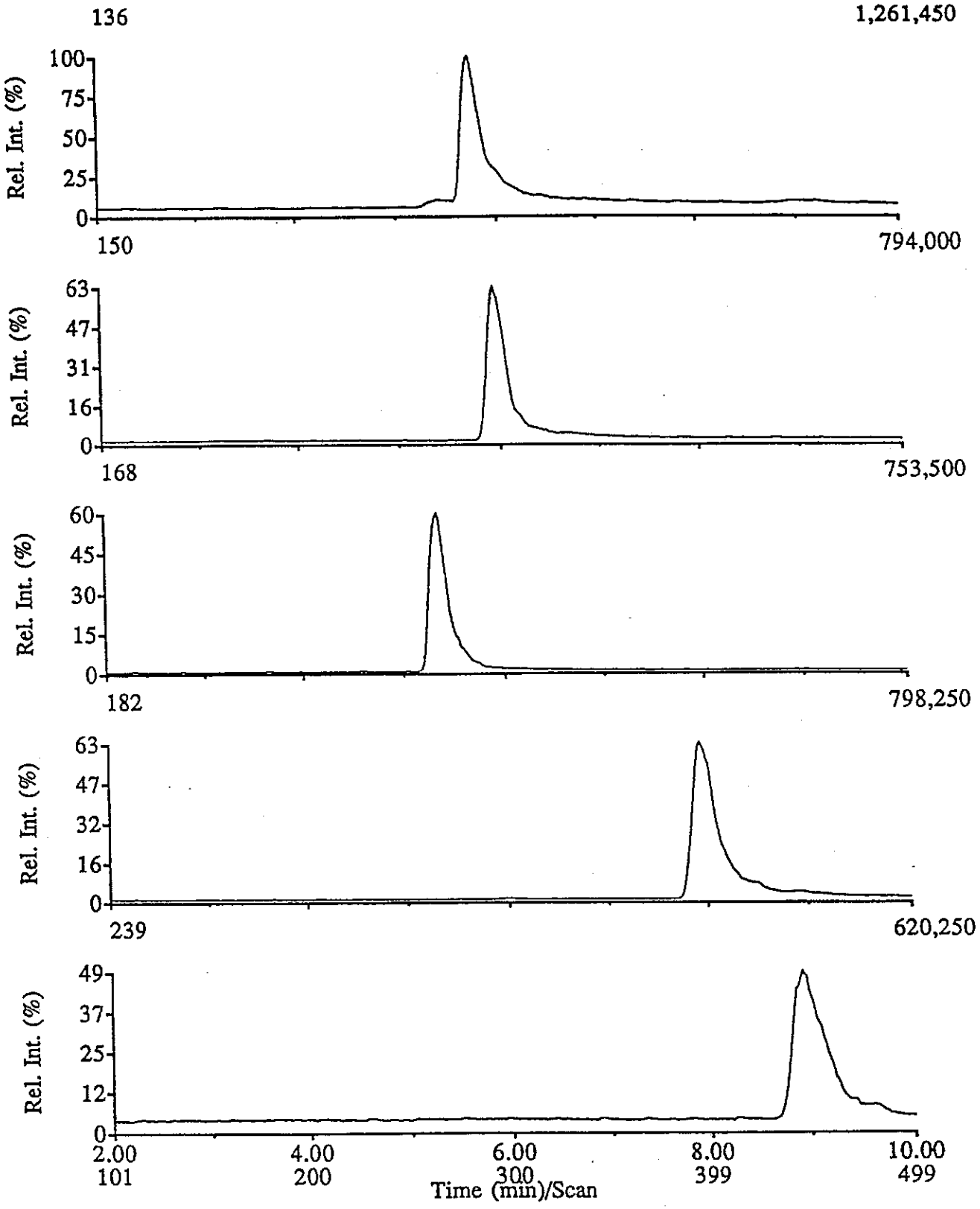


Figure 11

ISP-MS calibration for MTBT

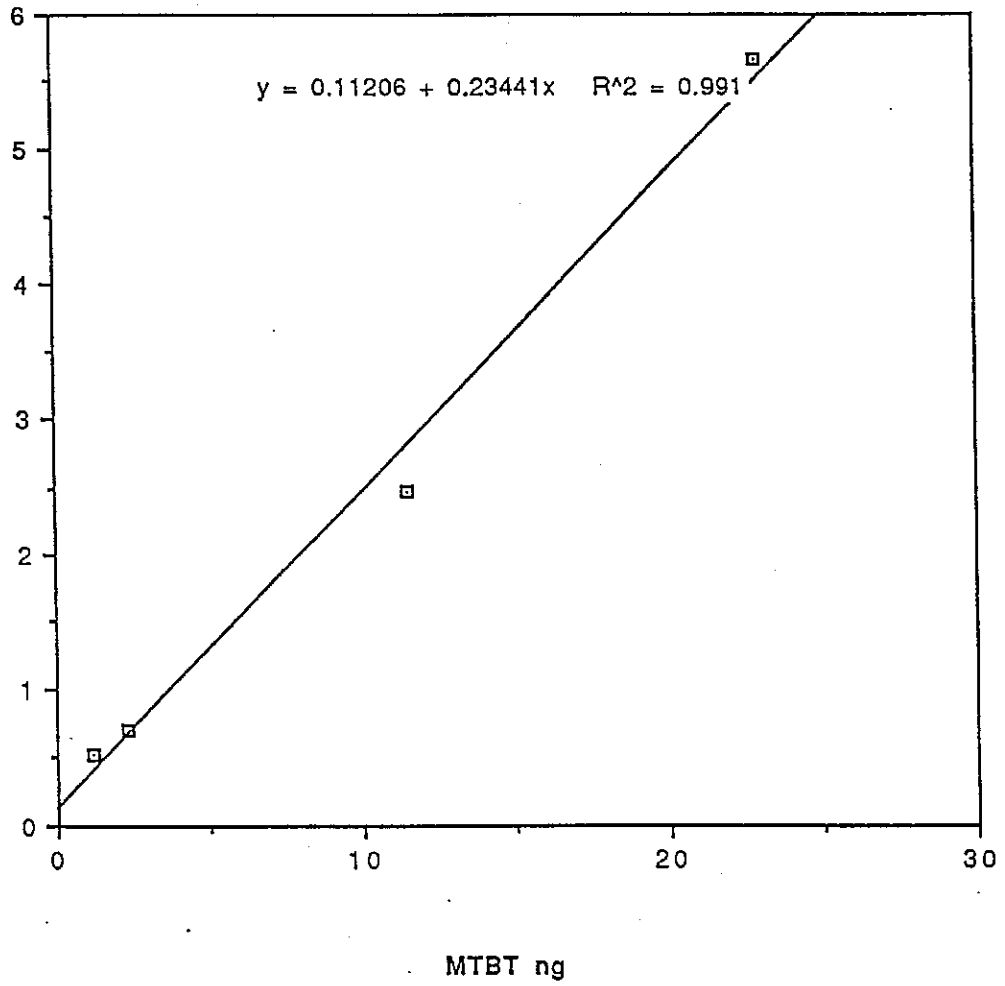
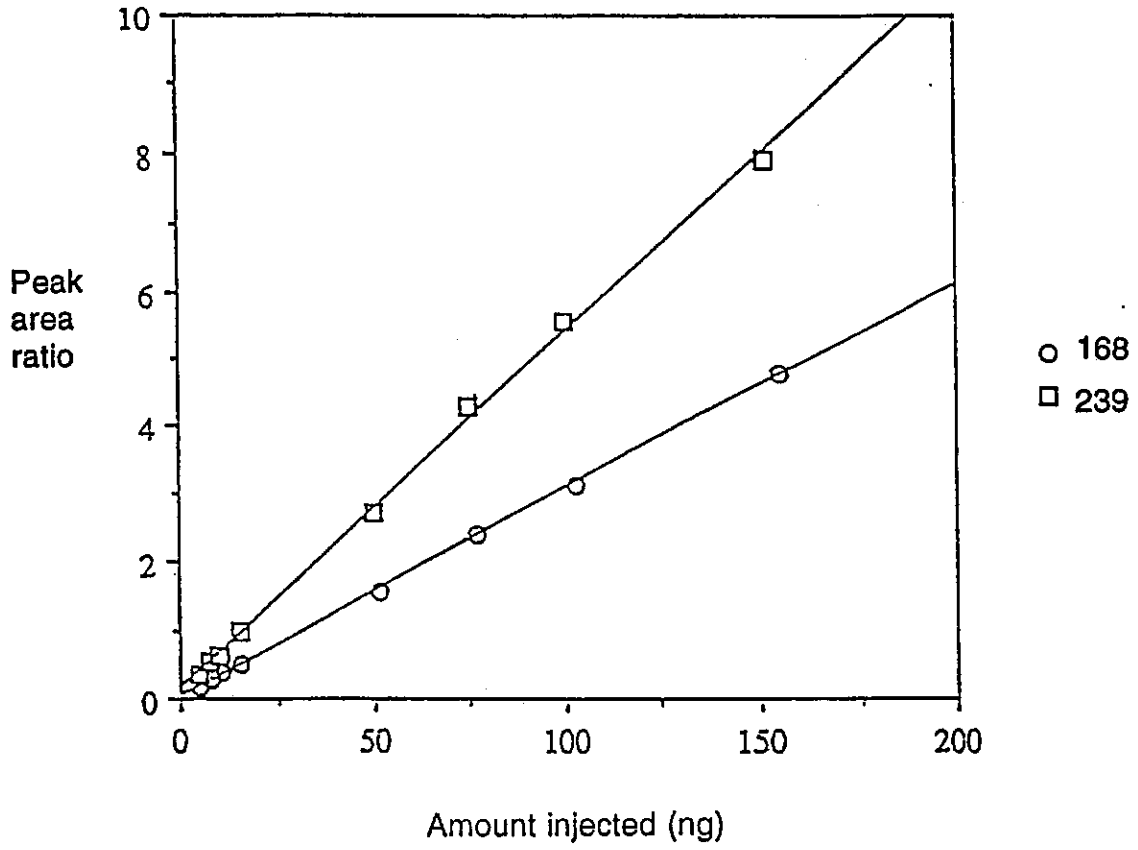


Figure 12

Calibration curves for LC-MS (SIM) analysis of MBT (m/z 168) and TCMTB (m/z 239)

The lower curve for MBT covers a range 5 times larger than the upper curve.



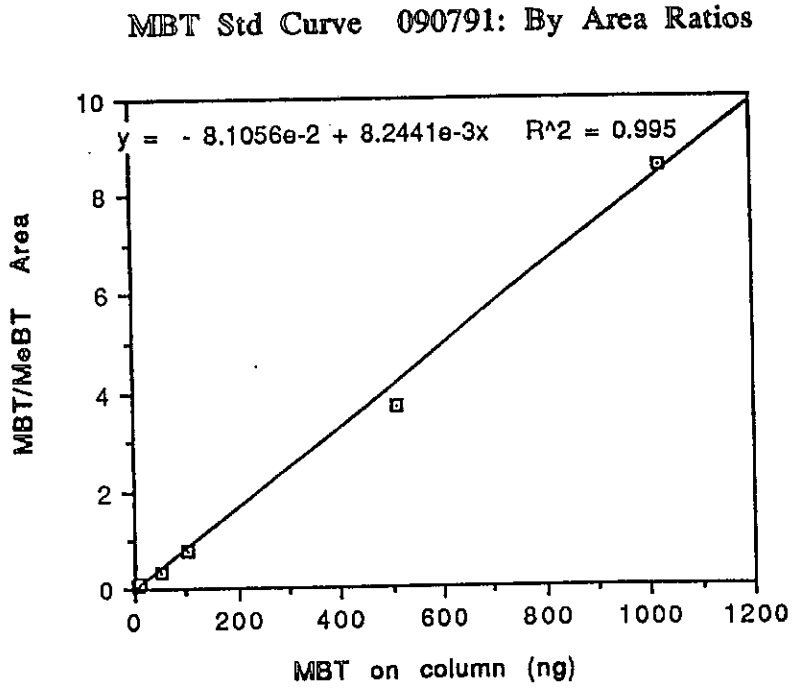


Figure 13

Tandem mass spectra of ((a) m/z 239, (b) m/z 212 and (c) m/z 180) from TCMTB and of (d) m/z 182 from MTBT

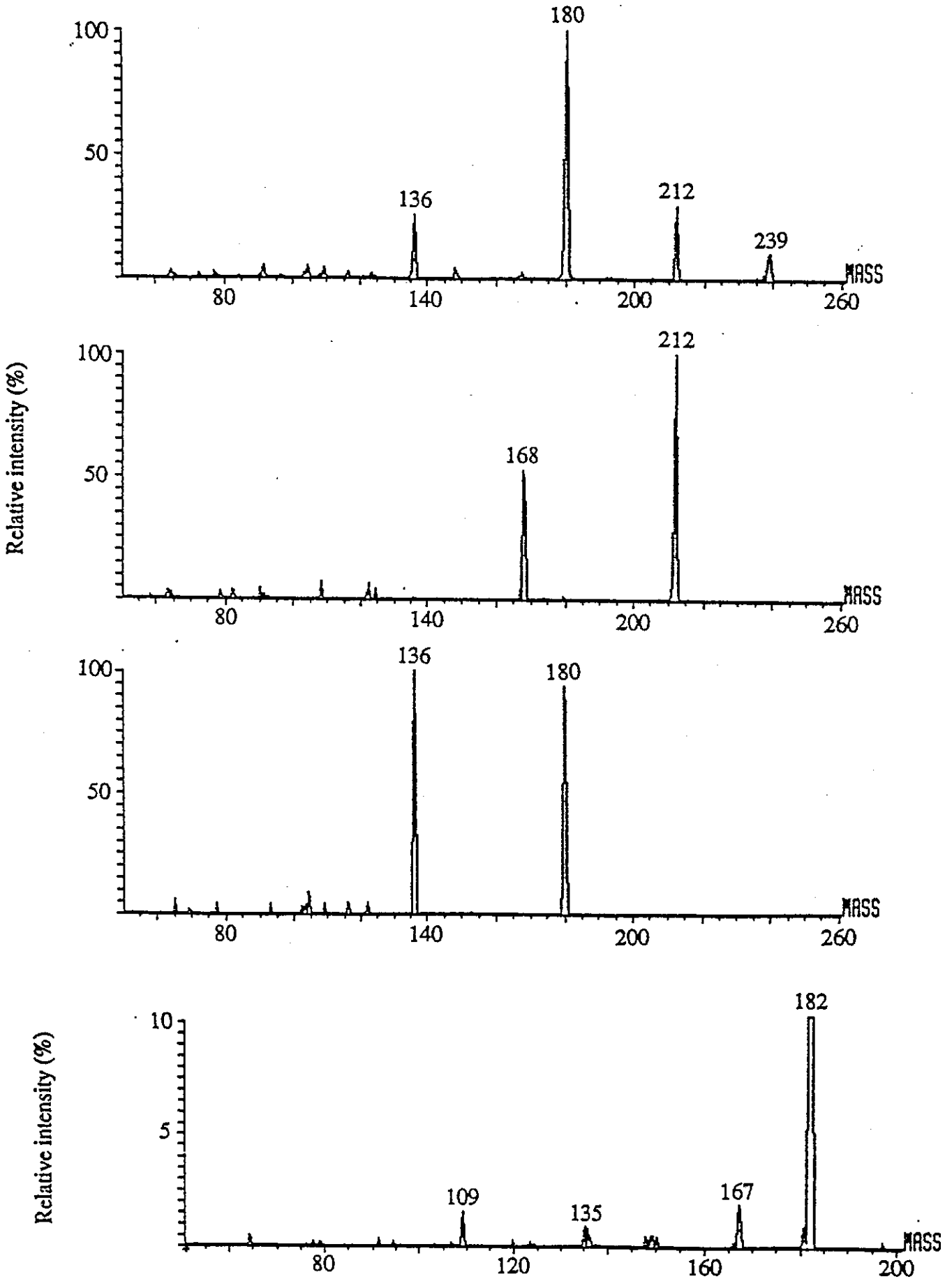


Figure 14

HPLC-ISP-MS mass chromatograms of control bile

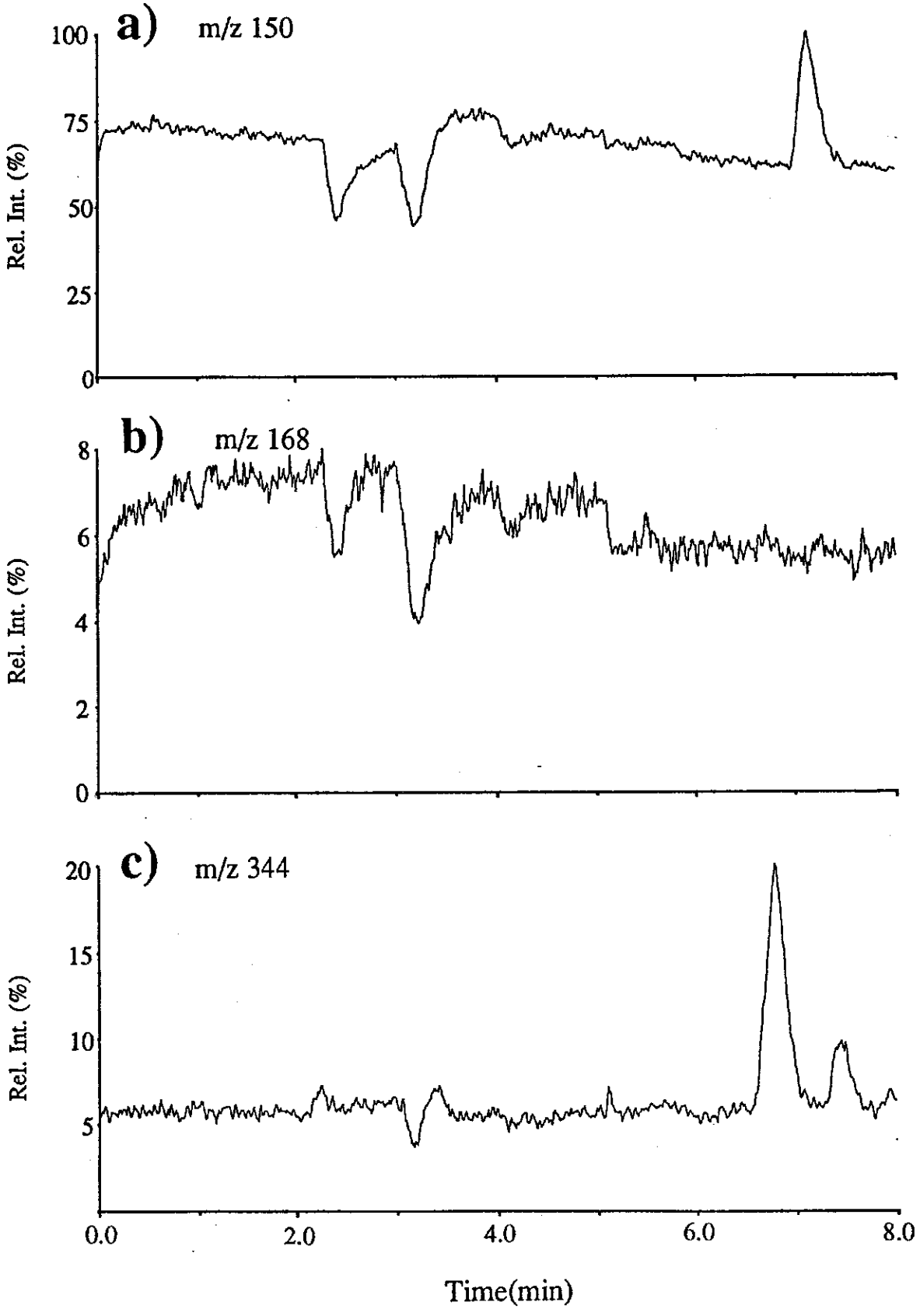


Figure 15

HPLC-ISP-MS mass chromatograms of bile from fish exposed to 20 · ppb TCMTB

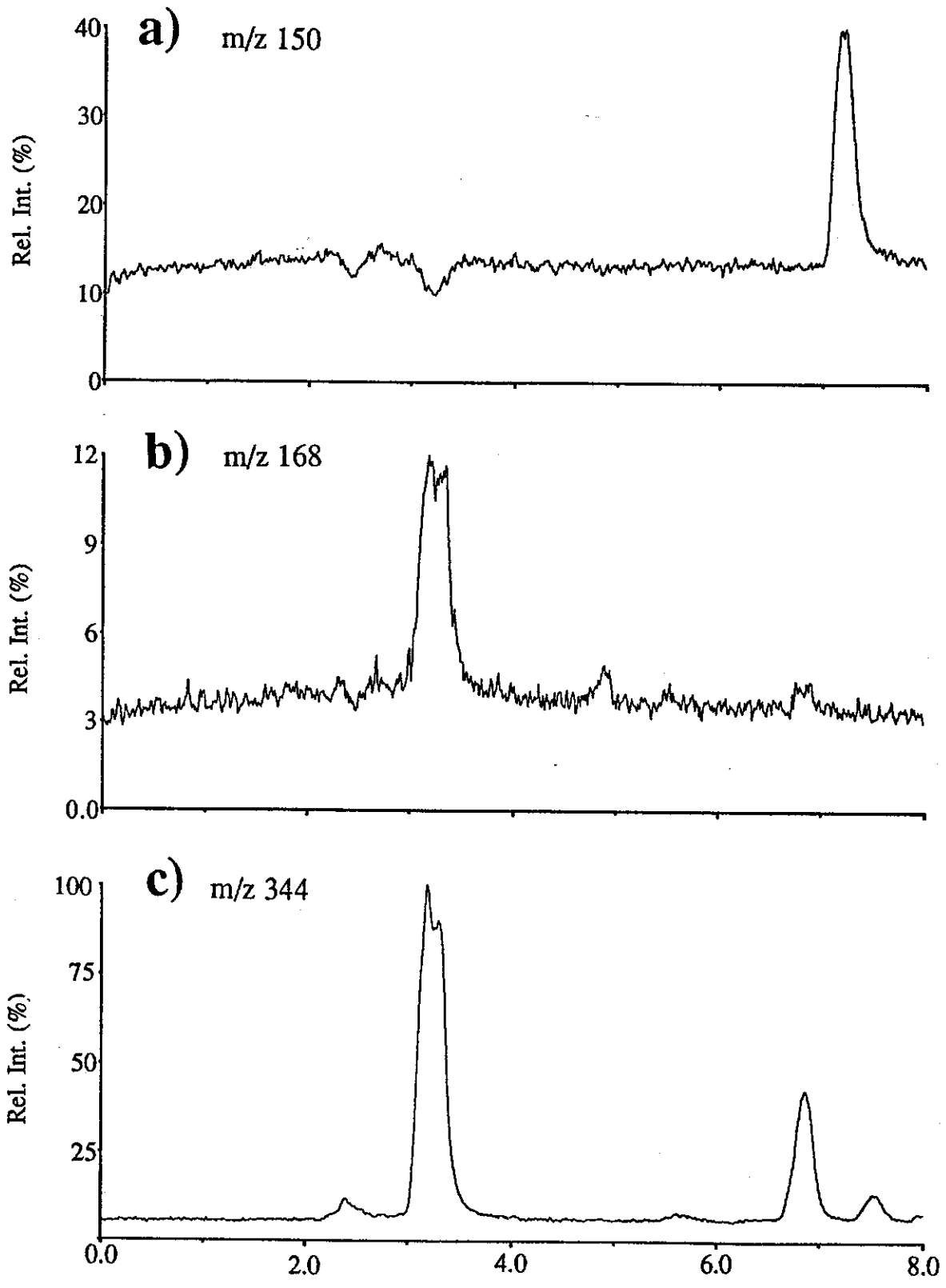


Figure 16

HPLC-ISP-MRM chromatograms of bile from fish exposed to 20 ppb TCMTB

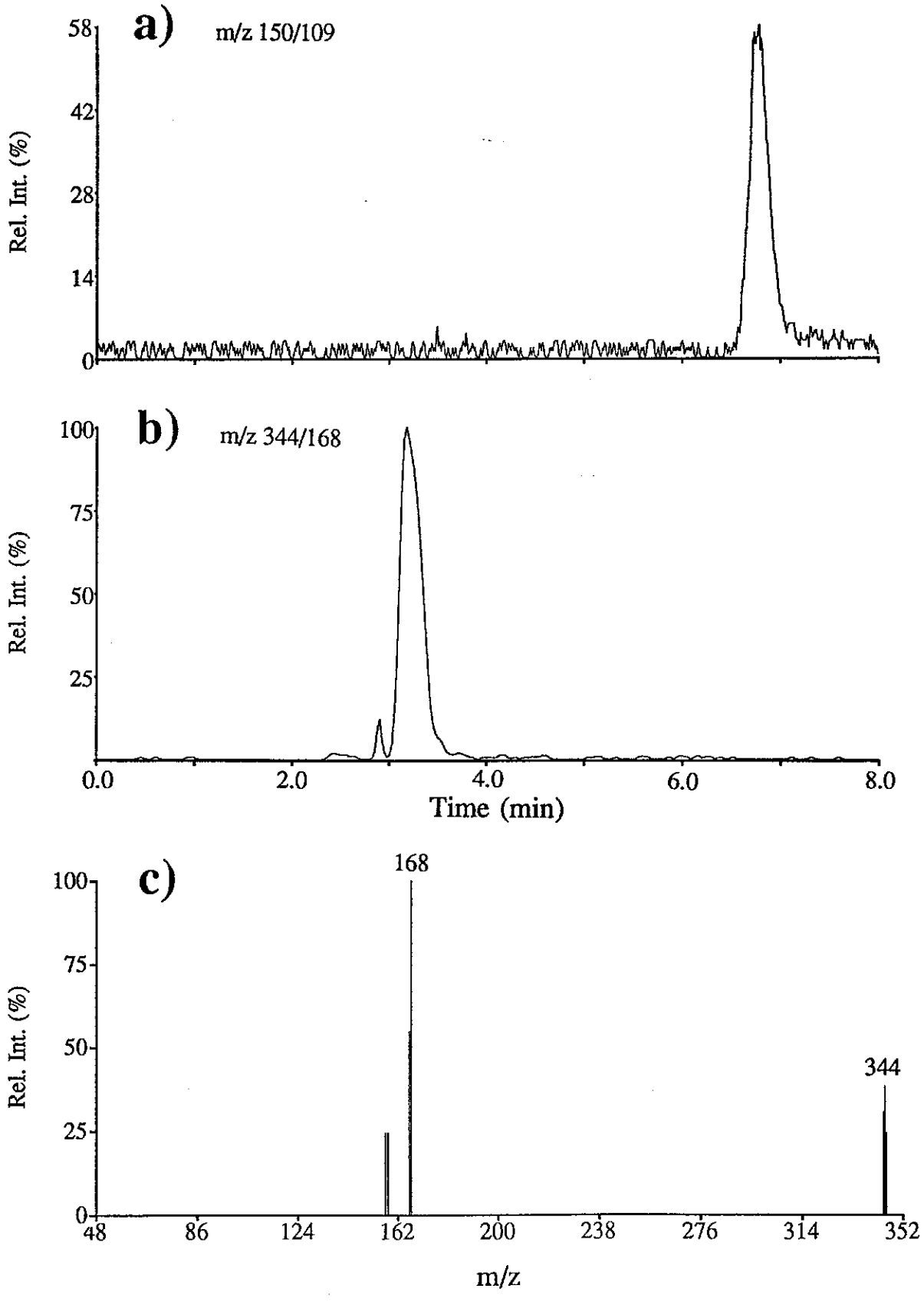


Figure 17

HPLC-ISP-MS mass chromatograms from an enzymatic digest of the bile from fish exposed to 20 ppb TCMTB

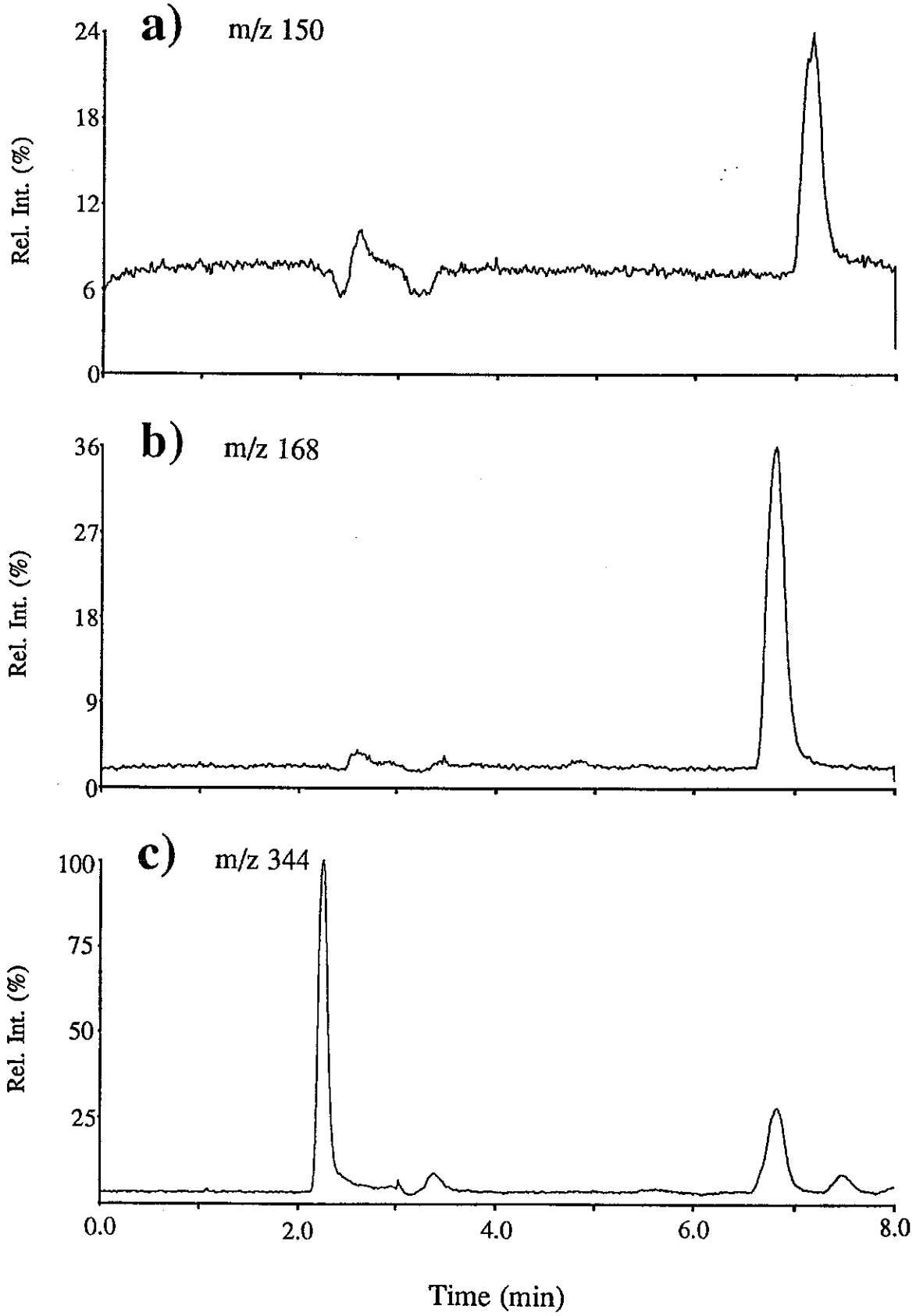


Figure 18

HPLC-ISP-MS mass chromatograms from plasma of fish exposed to 20 ppb TCMTB

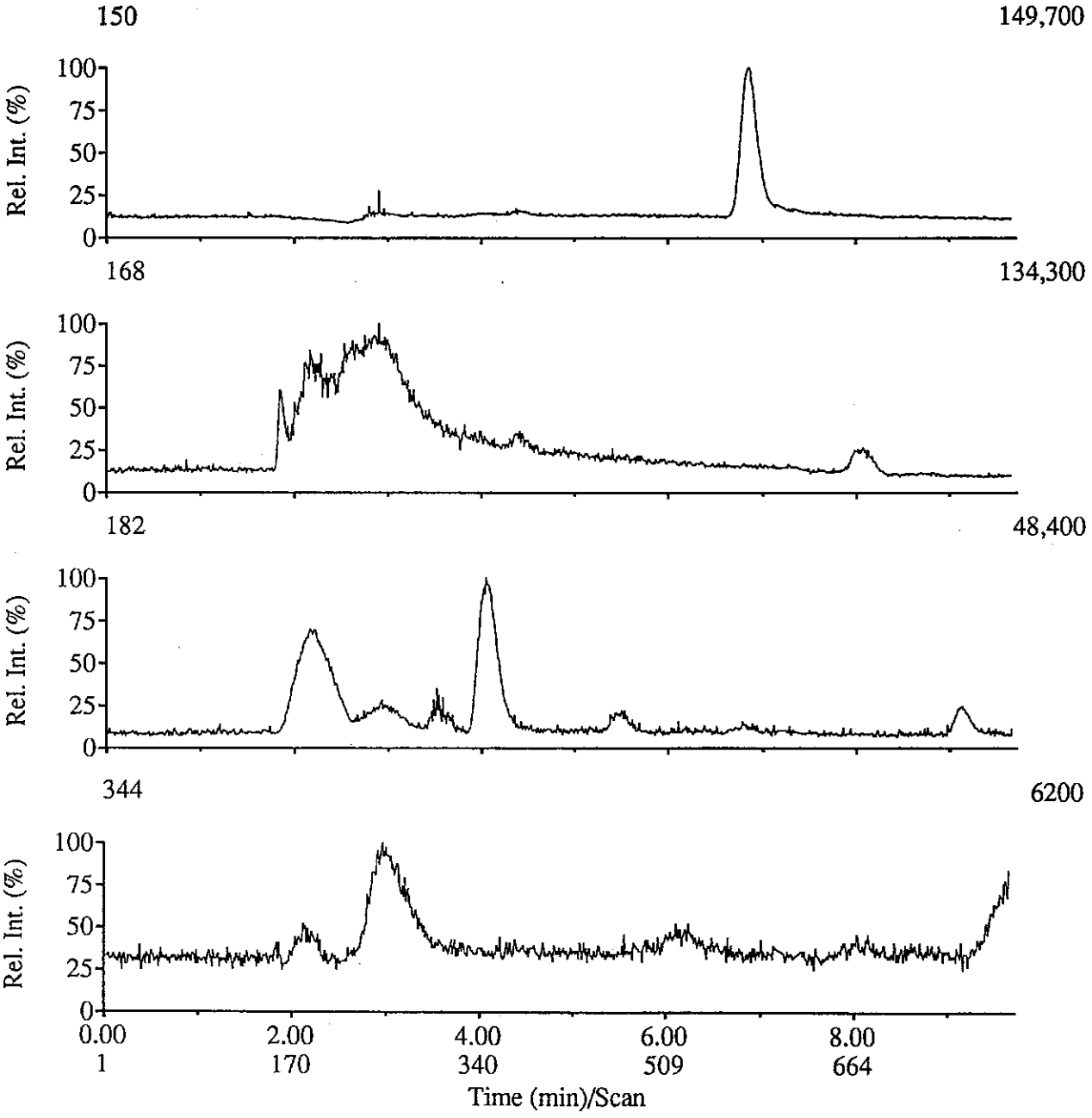


Figure 19

HPLC-ISP-MS mass chromatograms from β-glucuronidase digest of plasma of fish exposed to 20 ppb TCMTB

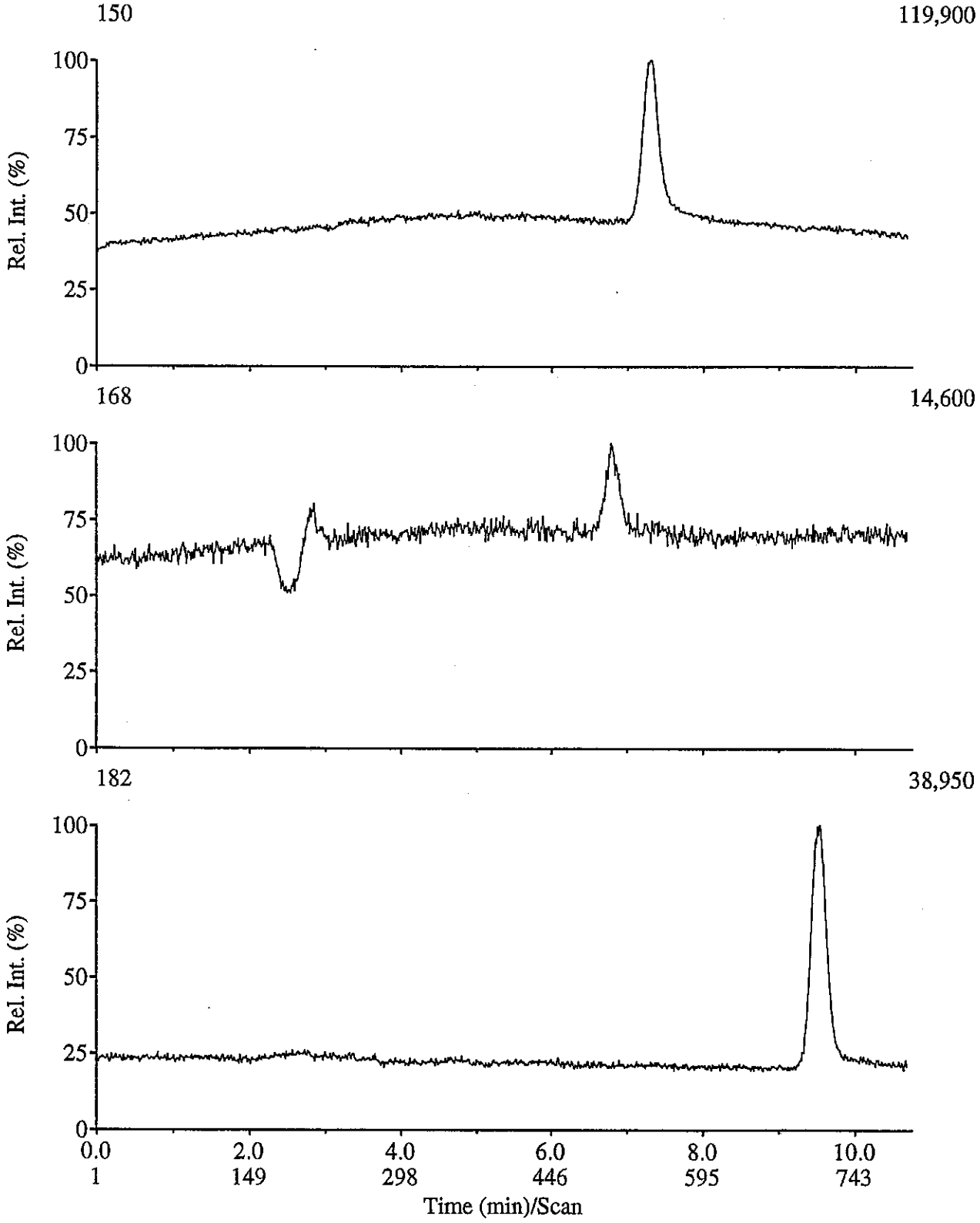


Figure 20

HPLC-ISP-MS mass chromatograms from sulfatase digest of plasma from fish exposed to 20 ppb TCMTB

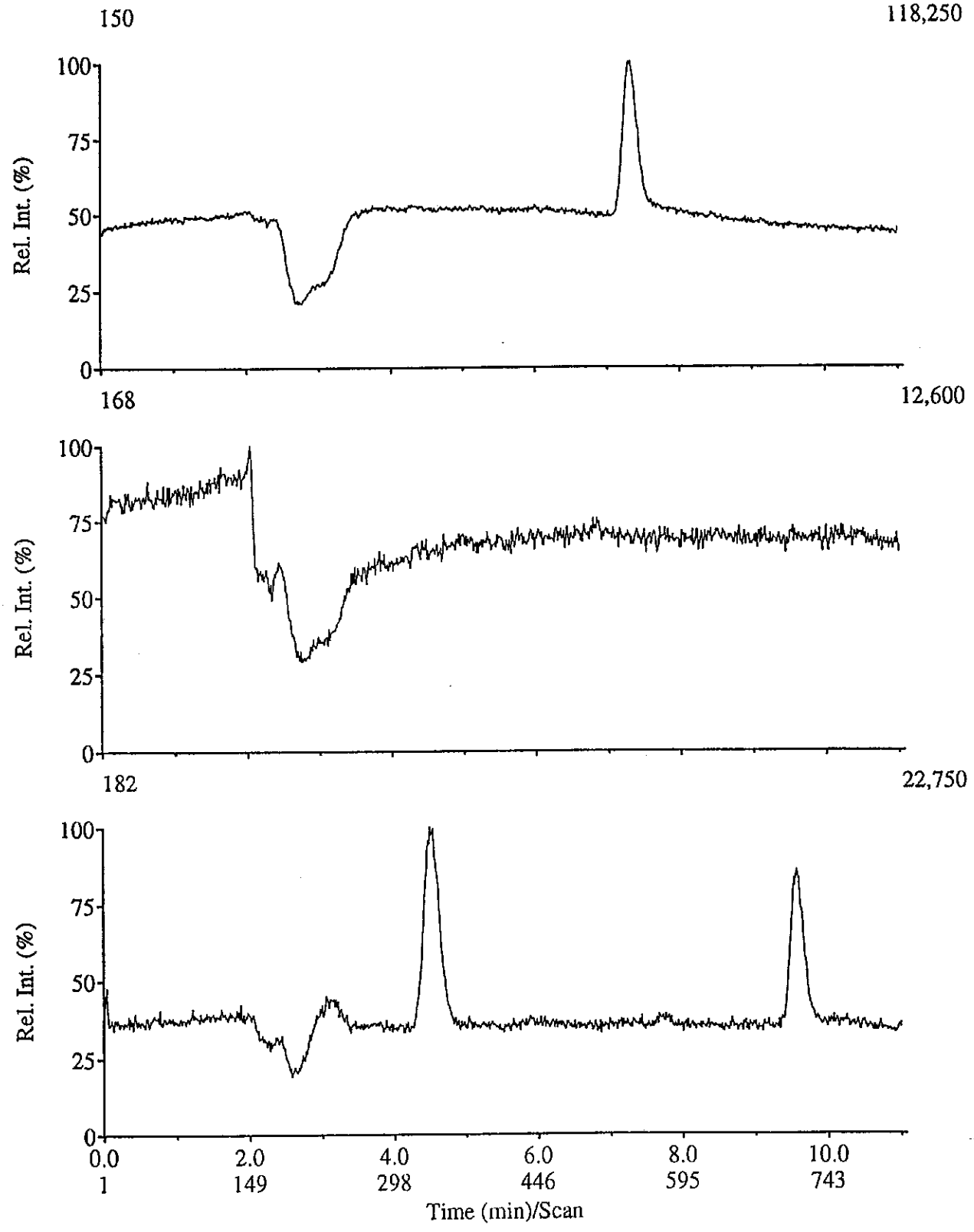


Figure 21

HPLC-ISP-MS mass chromatograms from urine of fish exposed to 20 ppb TCMTB

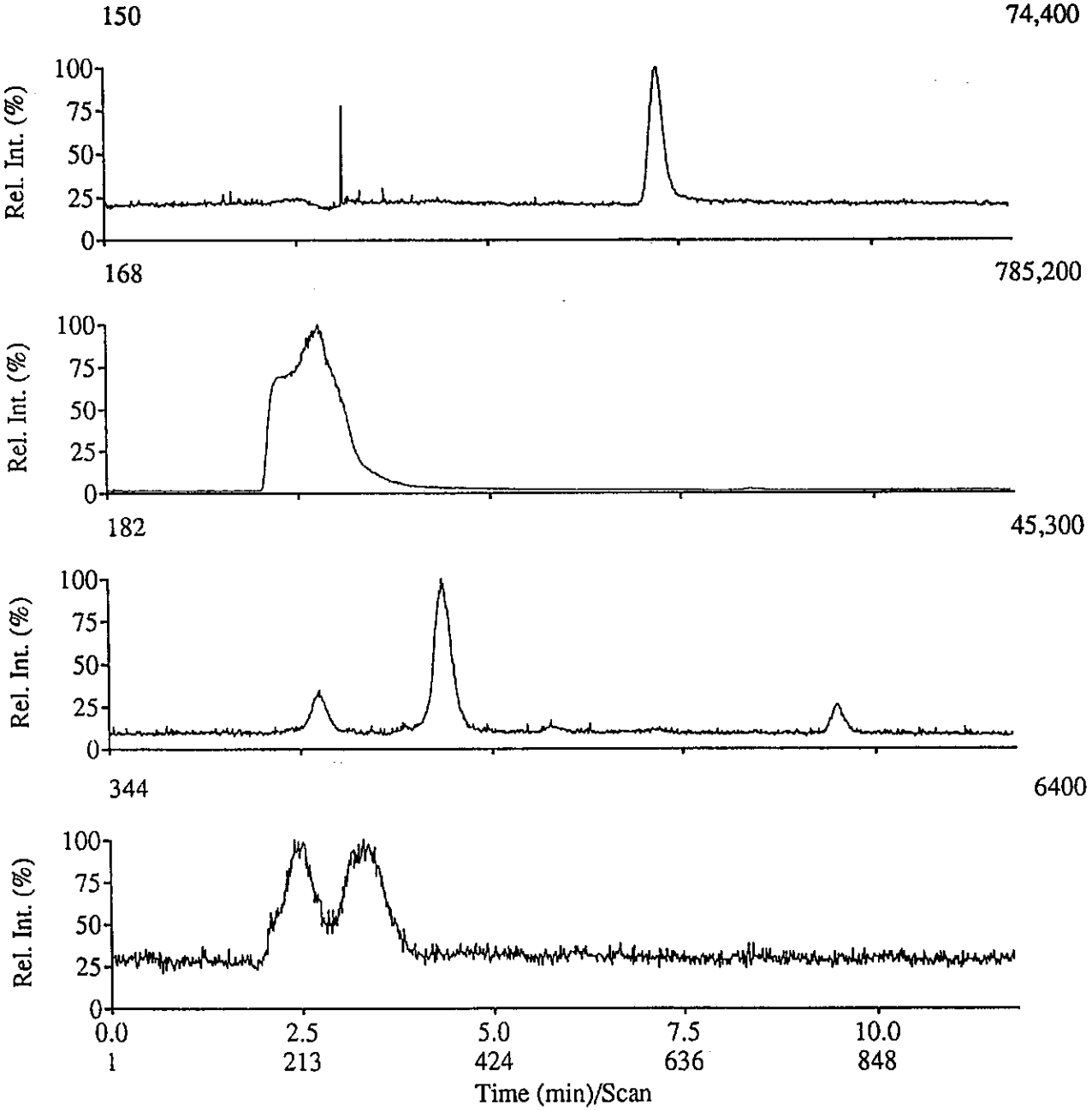


Figure 22

HPLC-ISP-MS mass chromatograms from β-glucuronidase digest of urine of fish exposed to 20 ppb TCMTB

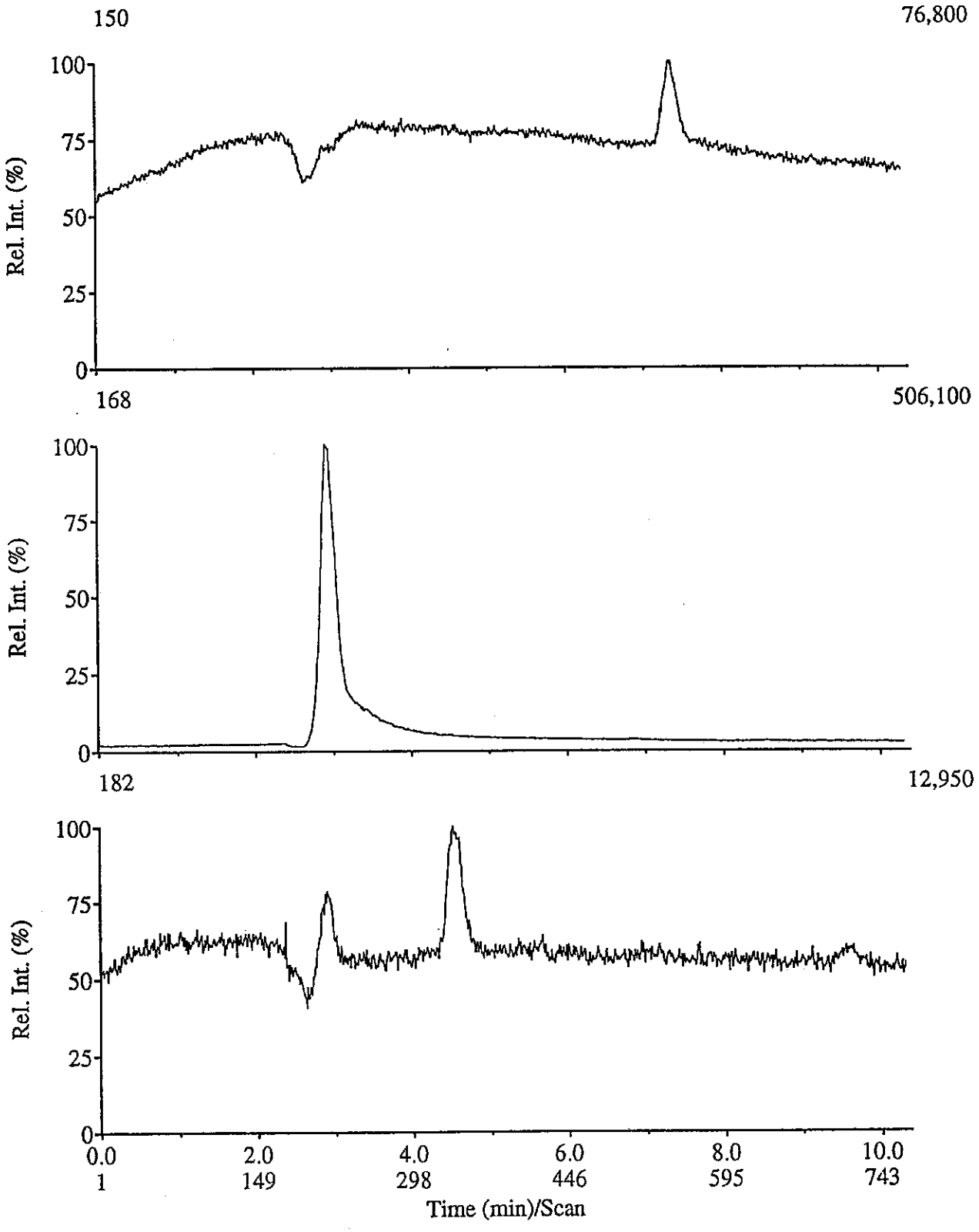


Figure 23

HPLC-ISP-MS mass chromatograms from sulfatase digest of urine from fish exposed to 20 ppb TCMTB

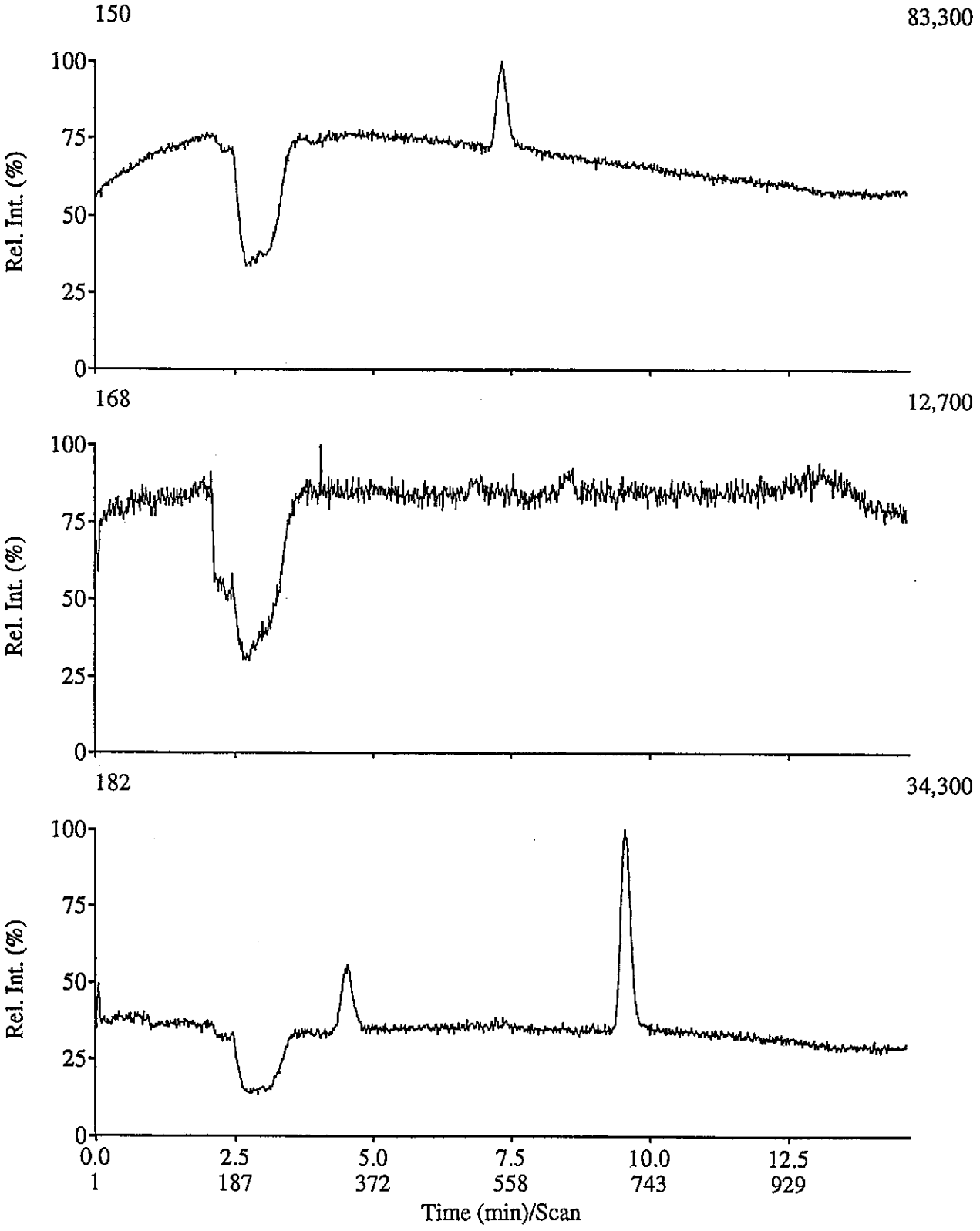


Figure 24

APCI-MS Mass Spectra of BKME Target Compounds

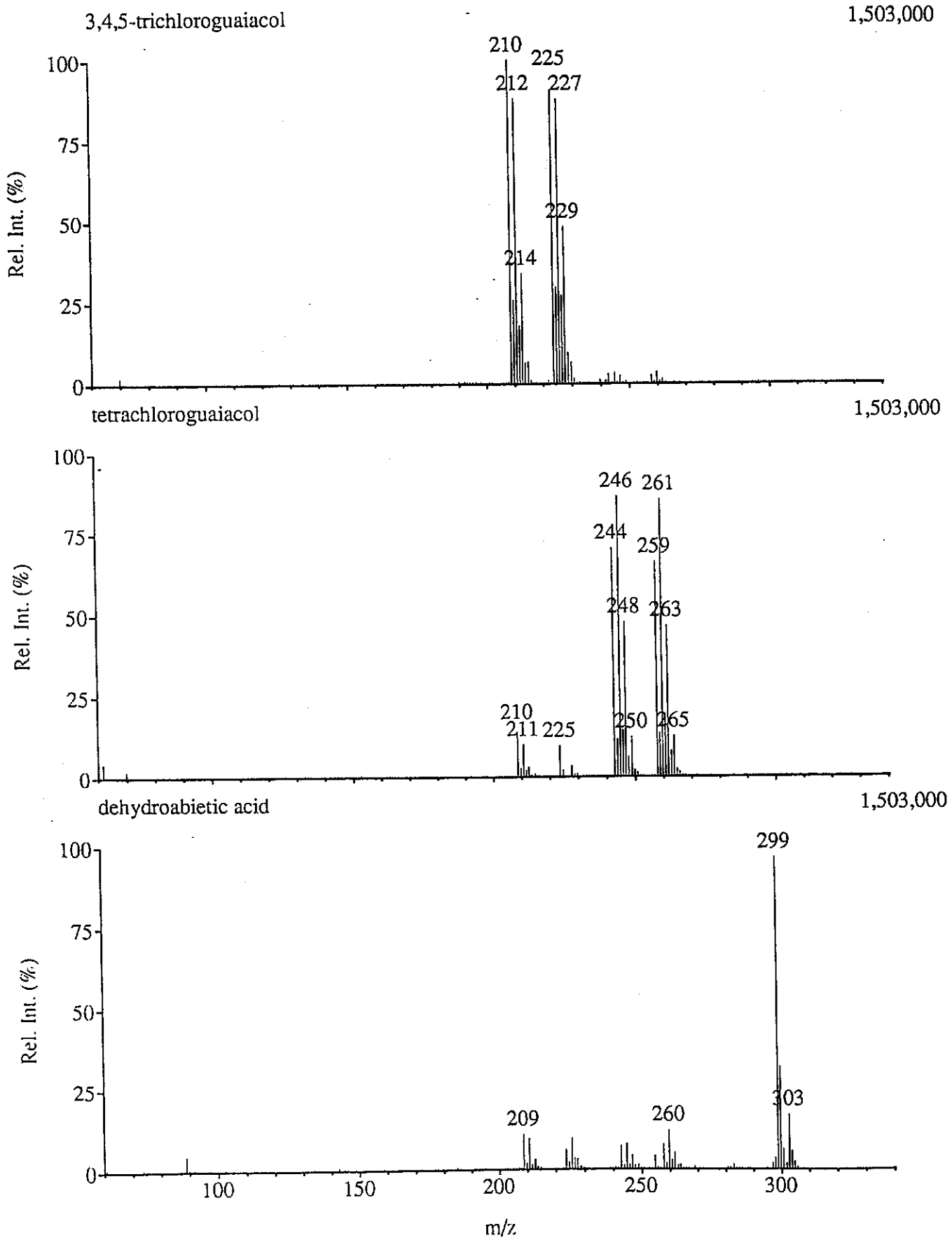
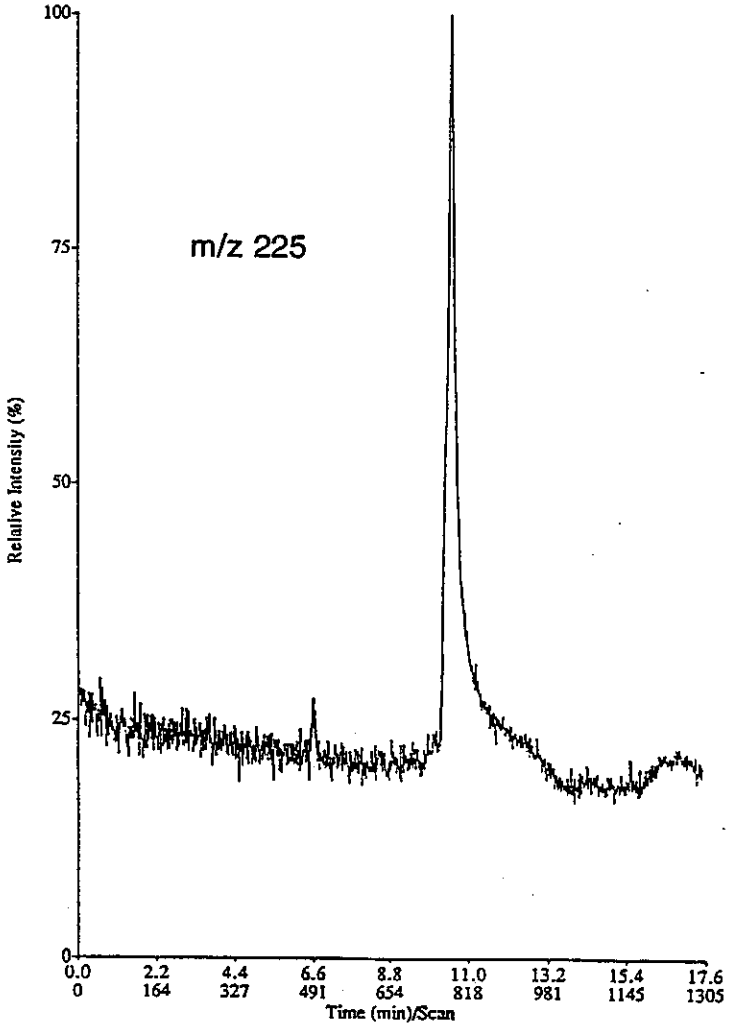


Figure 25

LC-MS Analyses of Solutions of Standards of 3,4,5-trichloroguaiacol (m/z 225) and of tetrachloroguaiacol (m/z 259).



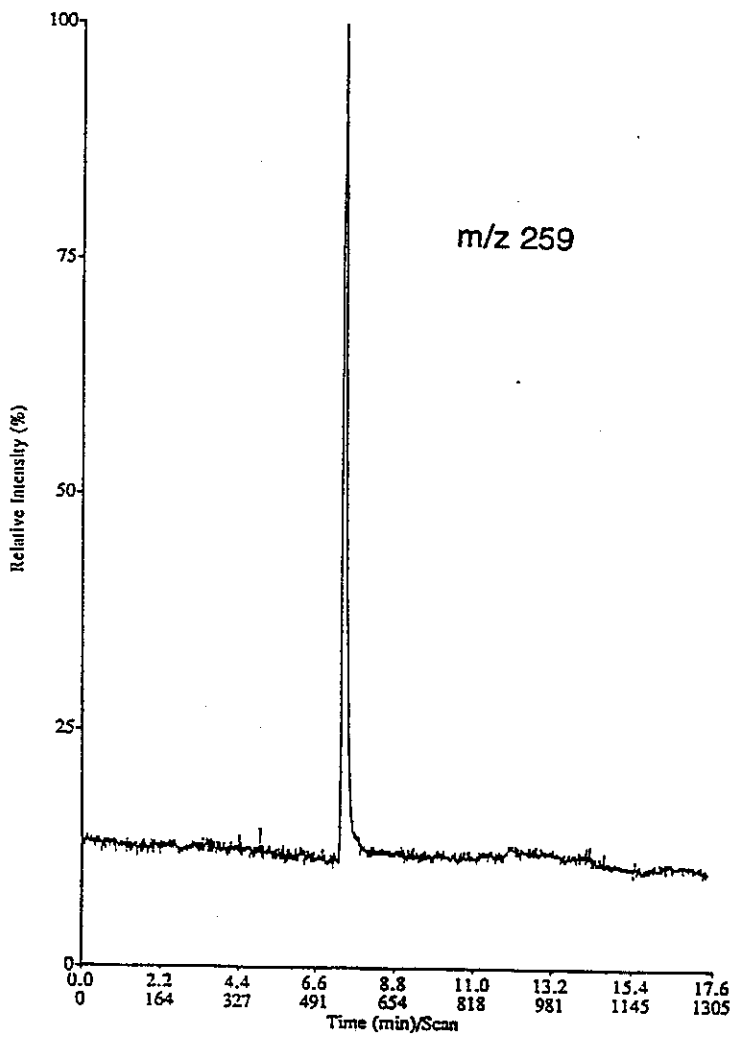


Figure 26

LC-MS Analysis of a Solution of the Standard Sample of Dihydroabietic Acid.

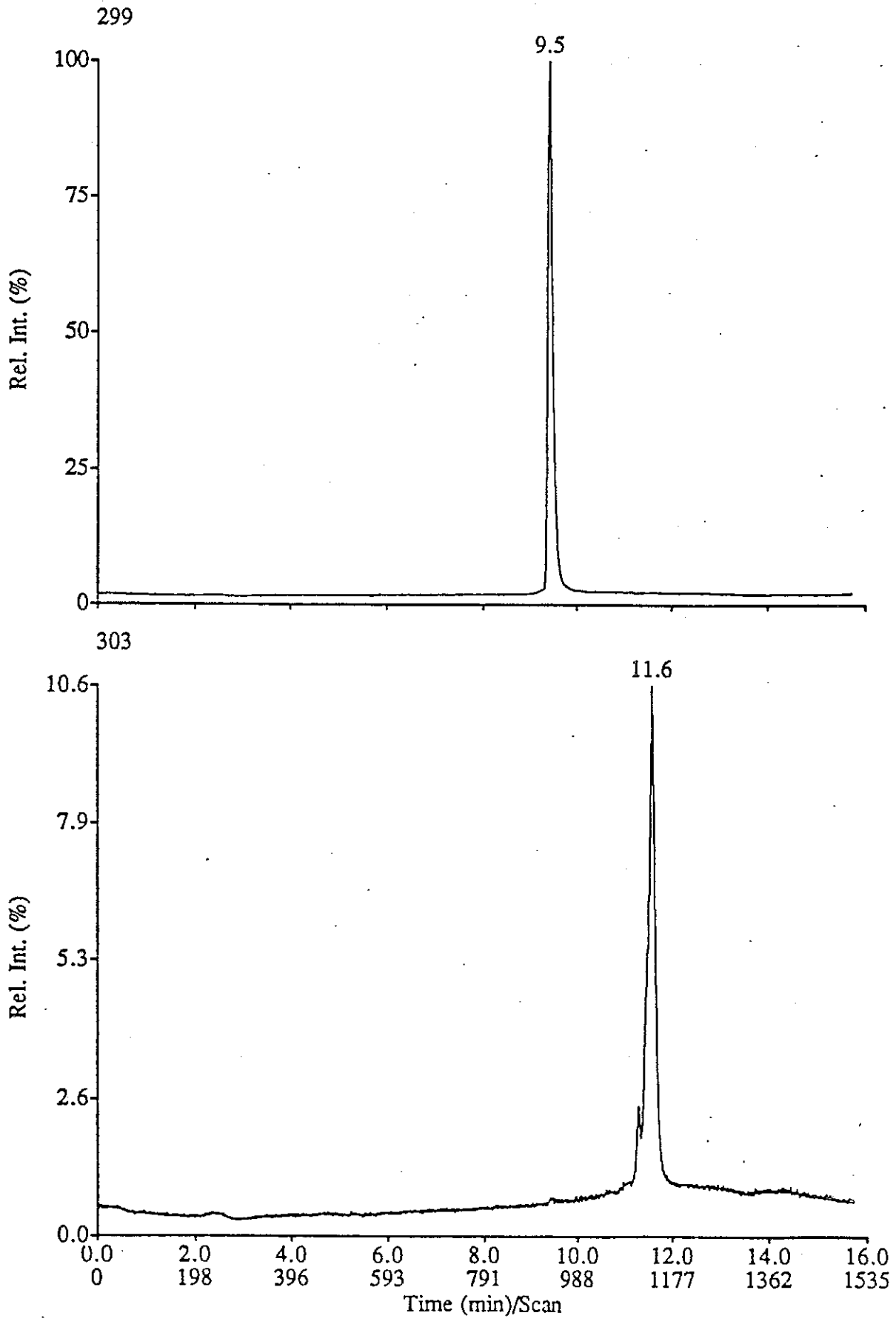


Figure 27

LC-MS Analysis of a Mixture of 345-TCG (m/z 225), TeCG (m/z 259), and DHA (m/z 299).

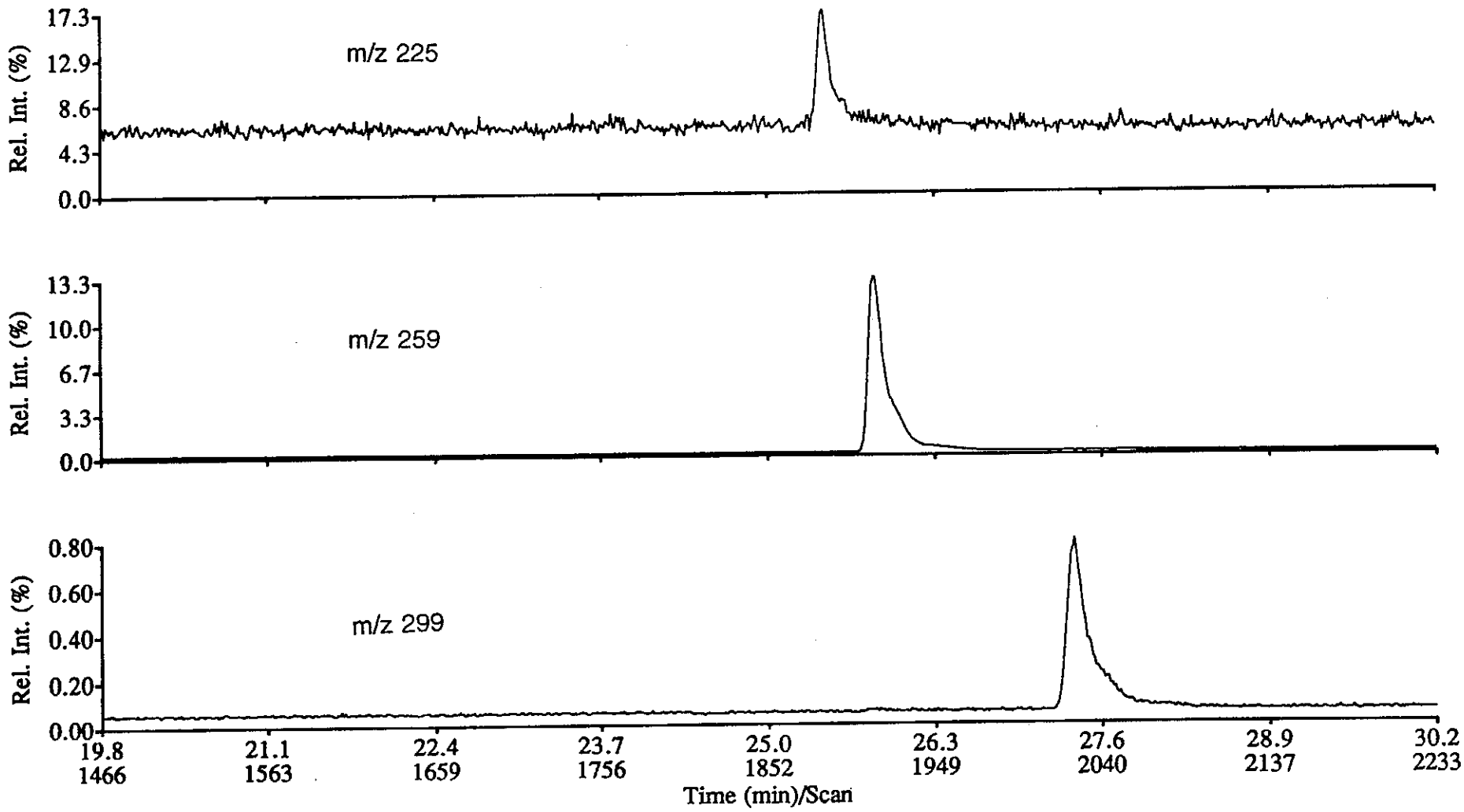


Figure 28

LC-MS Analysis (negative ion APCI, SIM m/z 299) of 125 fg of DHA injected on-column.

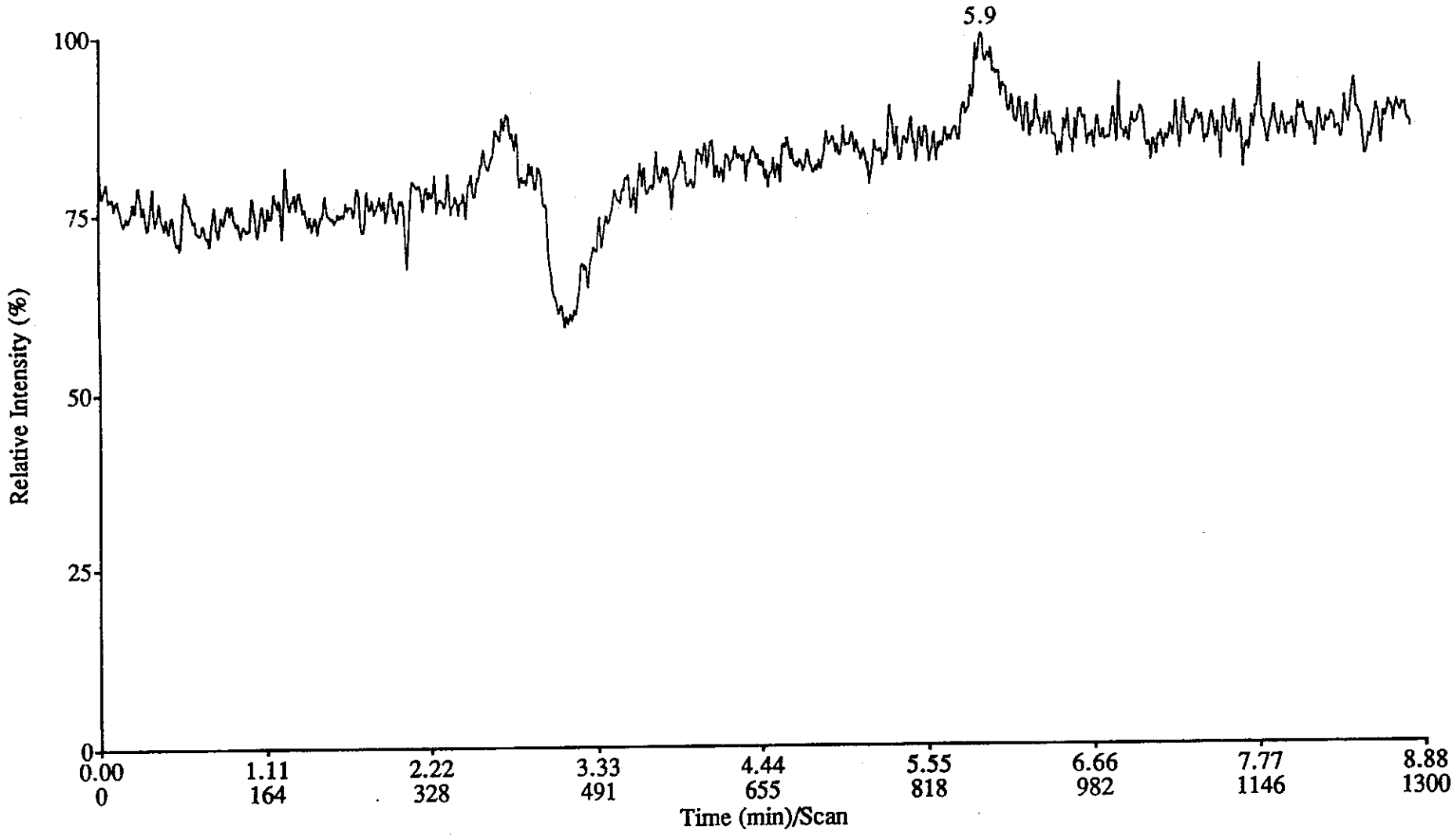


Figure 29

LC-MS analysis of a control fish bile sample for BKME marker compounds

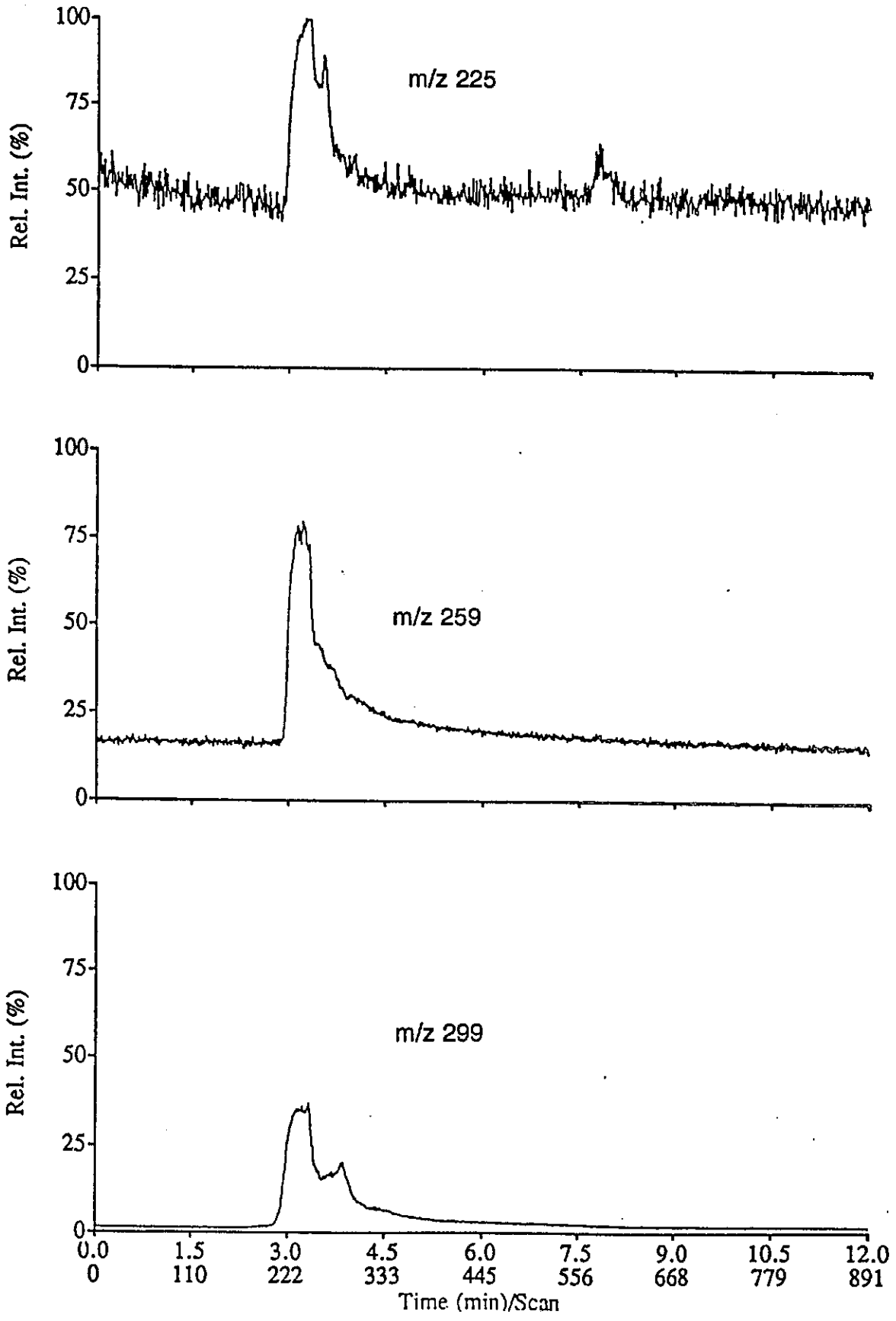


Figure 30

LC-MS analysis of a control fish bile sample spiked with a mix of three BKME marker compounds, 345-TCG (m/z 225), TeCG (m/z 259) and DHA (m/z 299)

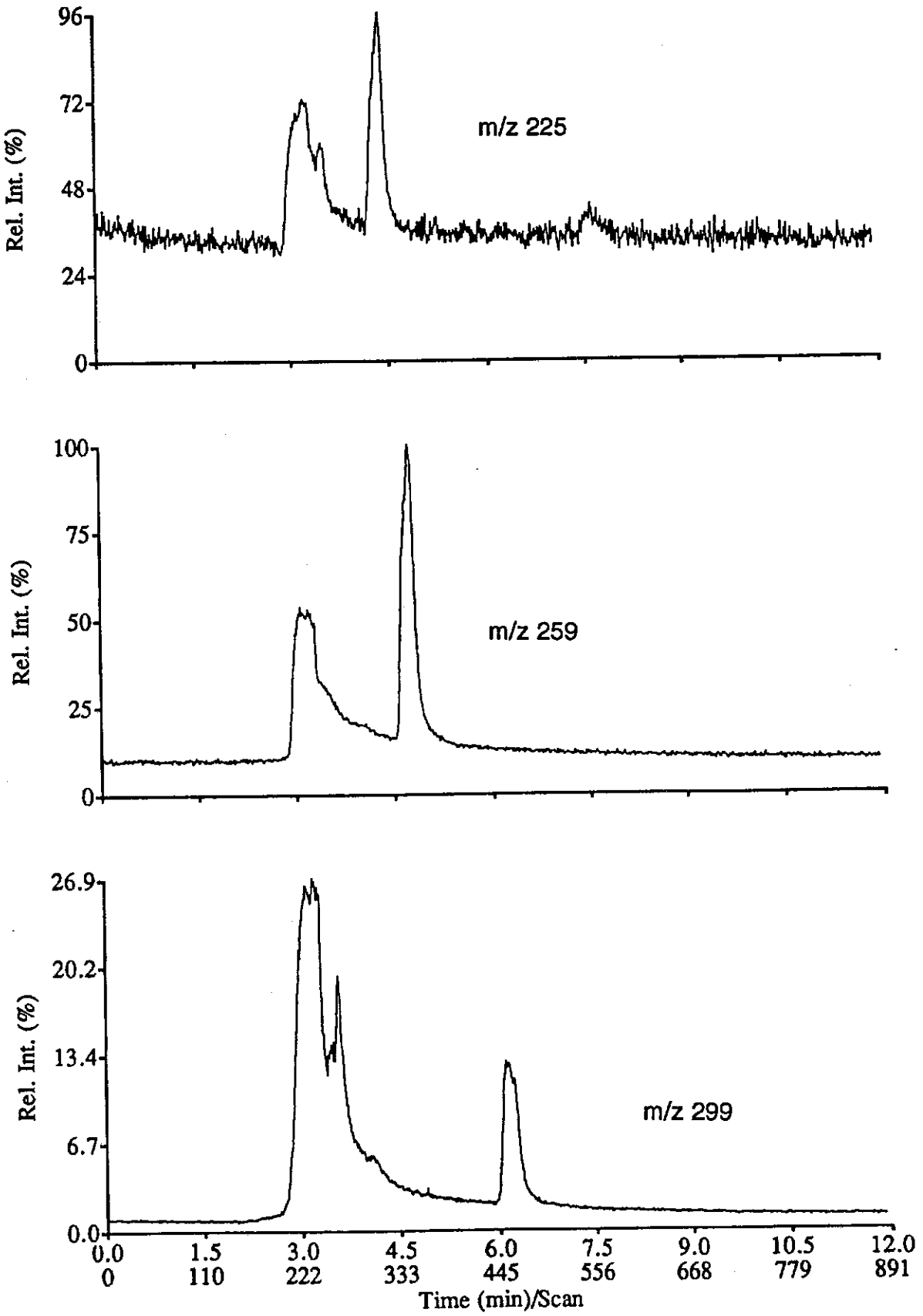


Figure 31

HPLC-APCI-MS mass chromatograms from bile of fish exposed to 100 ppb of DHA

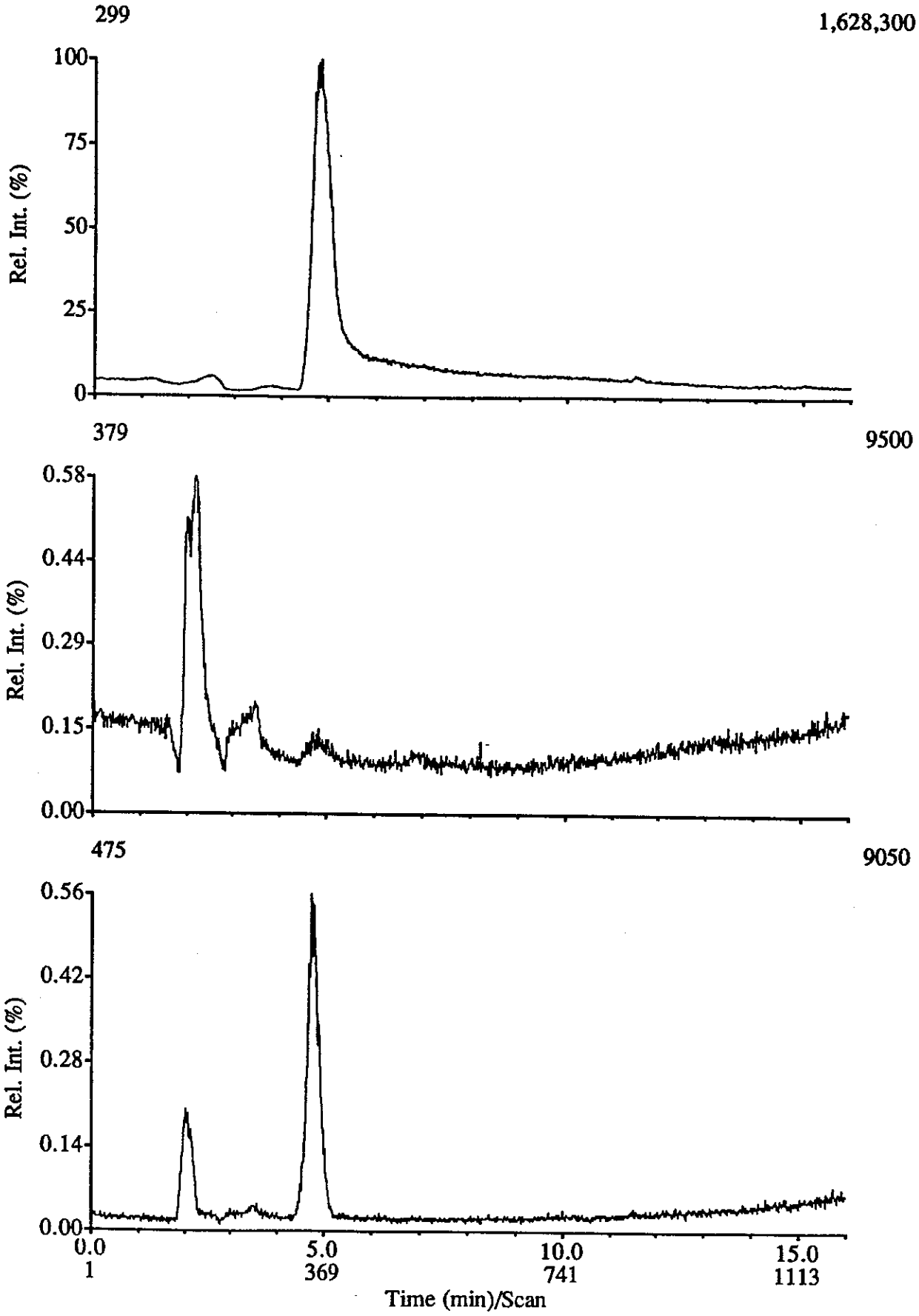


Figure 32

Mass spectrum of possible DHA glucuronide

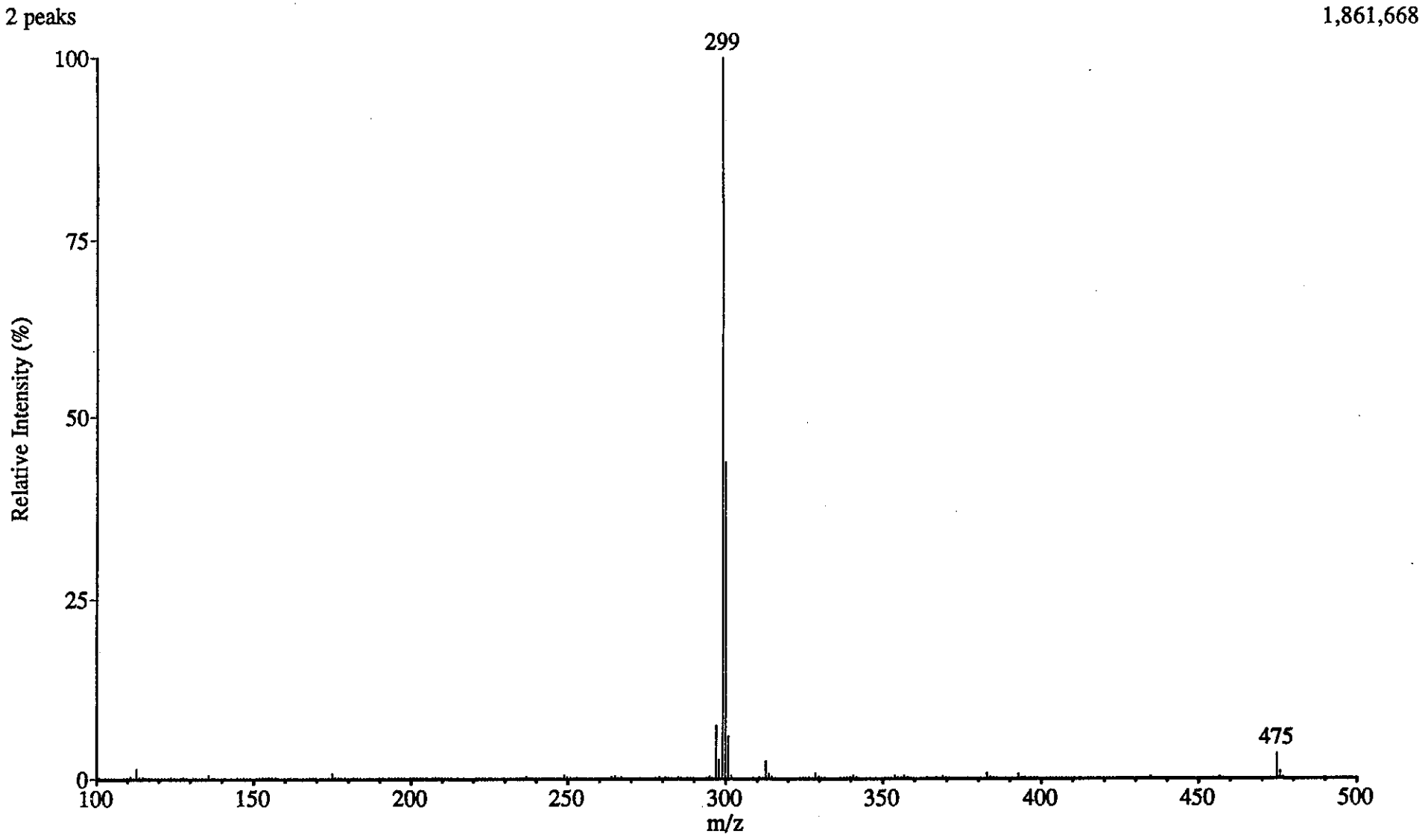


Figure 33

HPLC-APCI-MS mass chromatograms from β-glucuronidase digest of bile from fish exposed to 100 ppb DHA

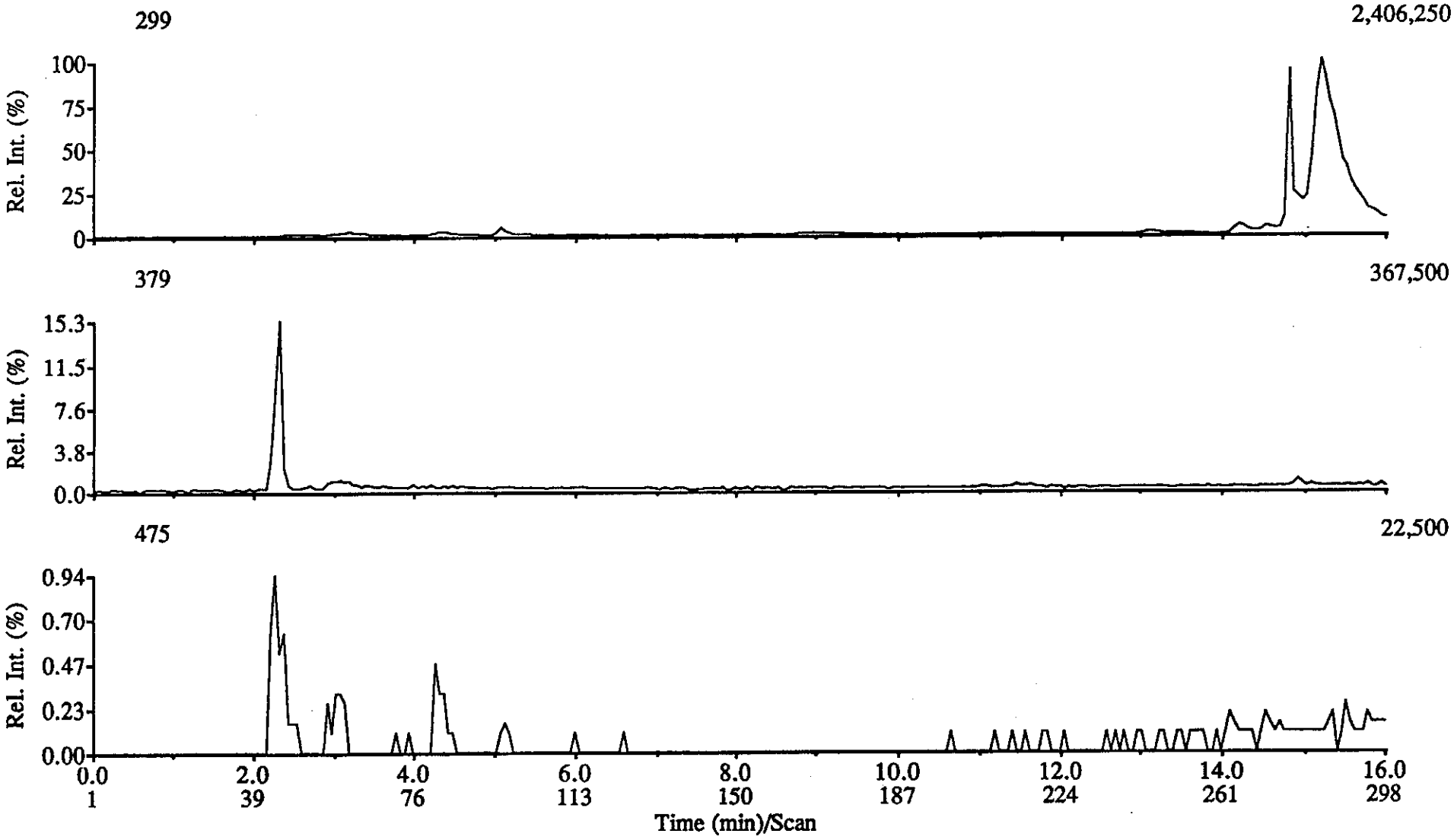


Figure 34

HPLC-APCI-MS mass chromatograms from 1/10,000 dilution of β-glucuronidase digest of bile from fish exposed to 100 ppb DHA

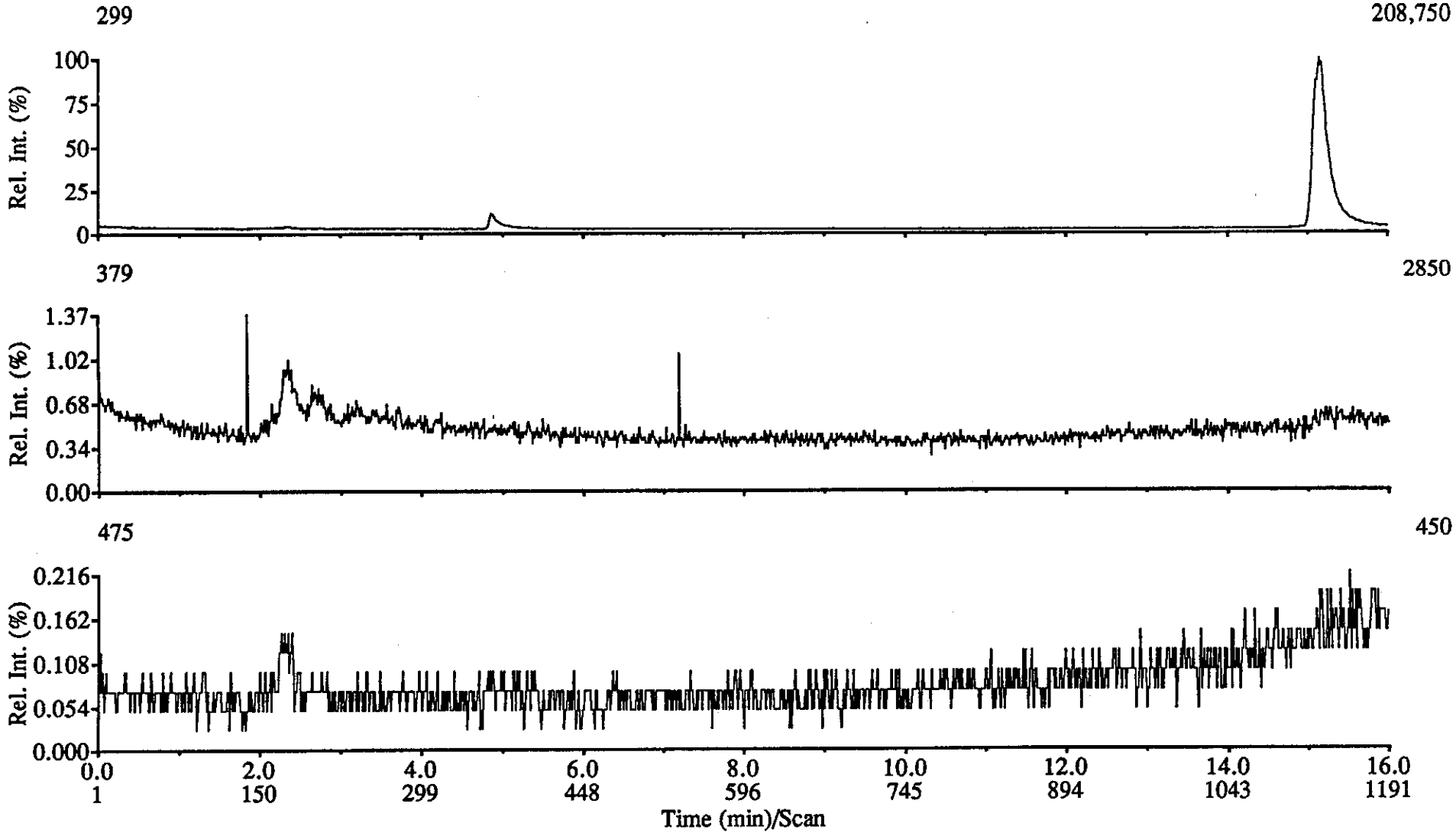


Figure 35

LC-MS analysis of bile from fish exposed to 250 ppb of TeCG (m/z 259 and 263)

The SIM chromatogram for m/z 435 monitors the glucuronide of TeCG

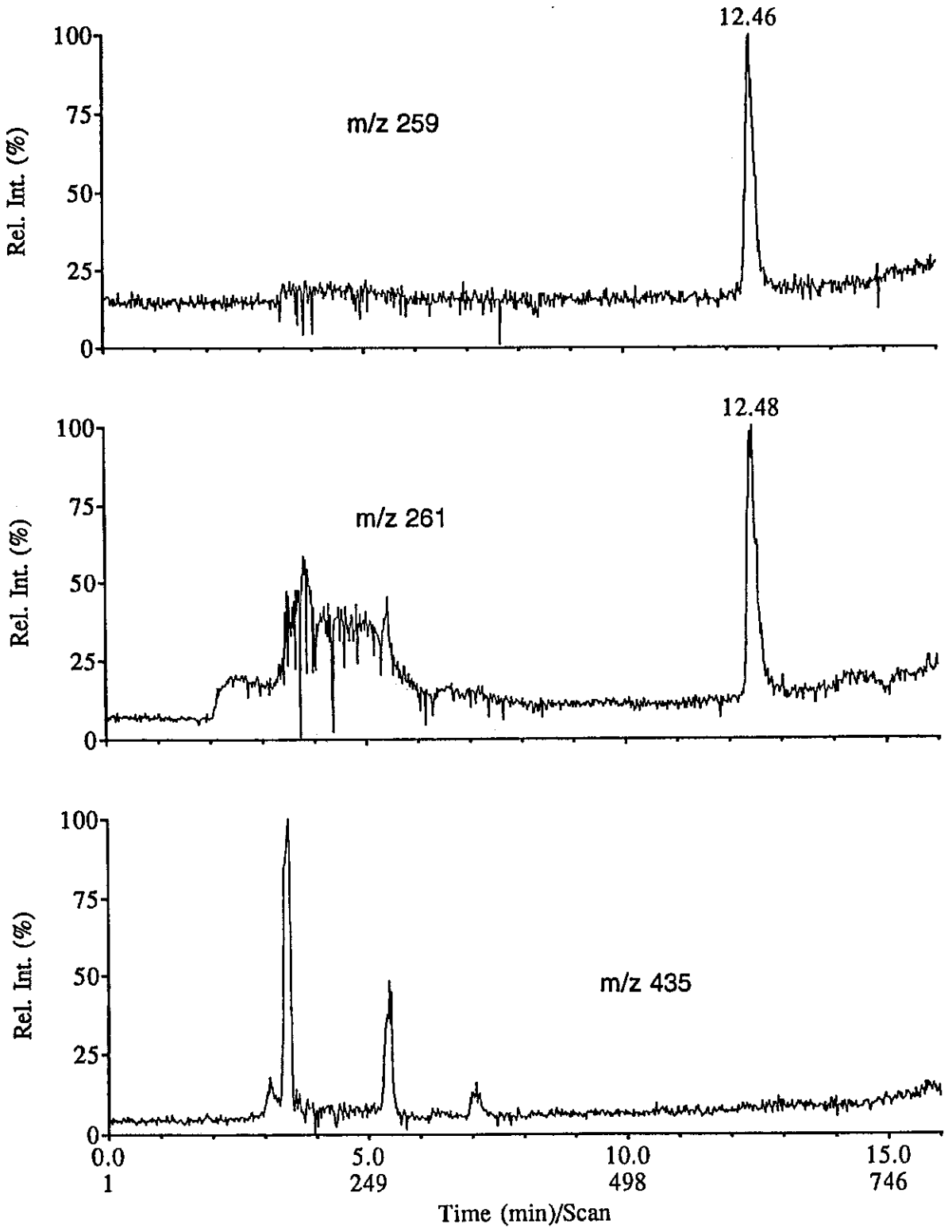


Figure 36

LC-MS analysis of urine from fish exposed to 250 ppb of TeCG (m/z 259)

SIM chromatograms for m/z 339 and 355 monitor the O-sulfate and C-sulfate of TeCG

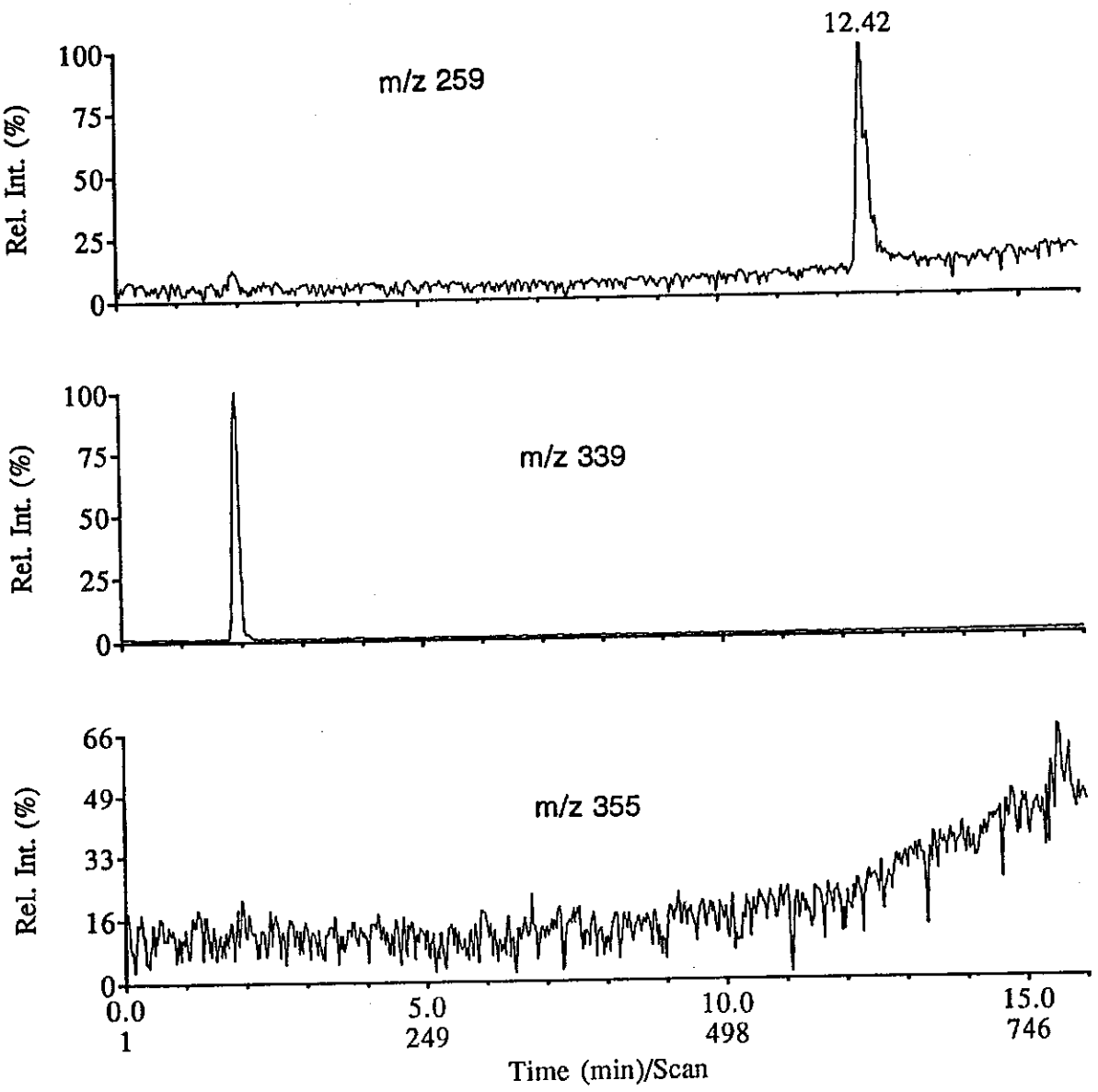


Figure 37

LC-MS analysis of plasma from fish exposed to 250 ppb of TeCG (m/z 259)

The SIM chromatogram for m/z 435 monitors the glucuronide of TeCG

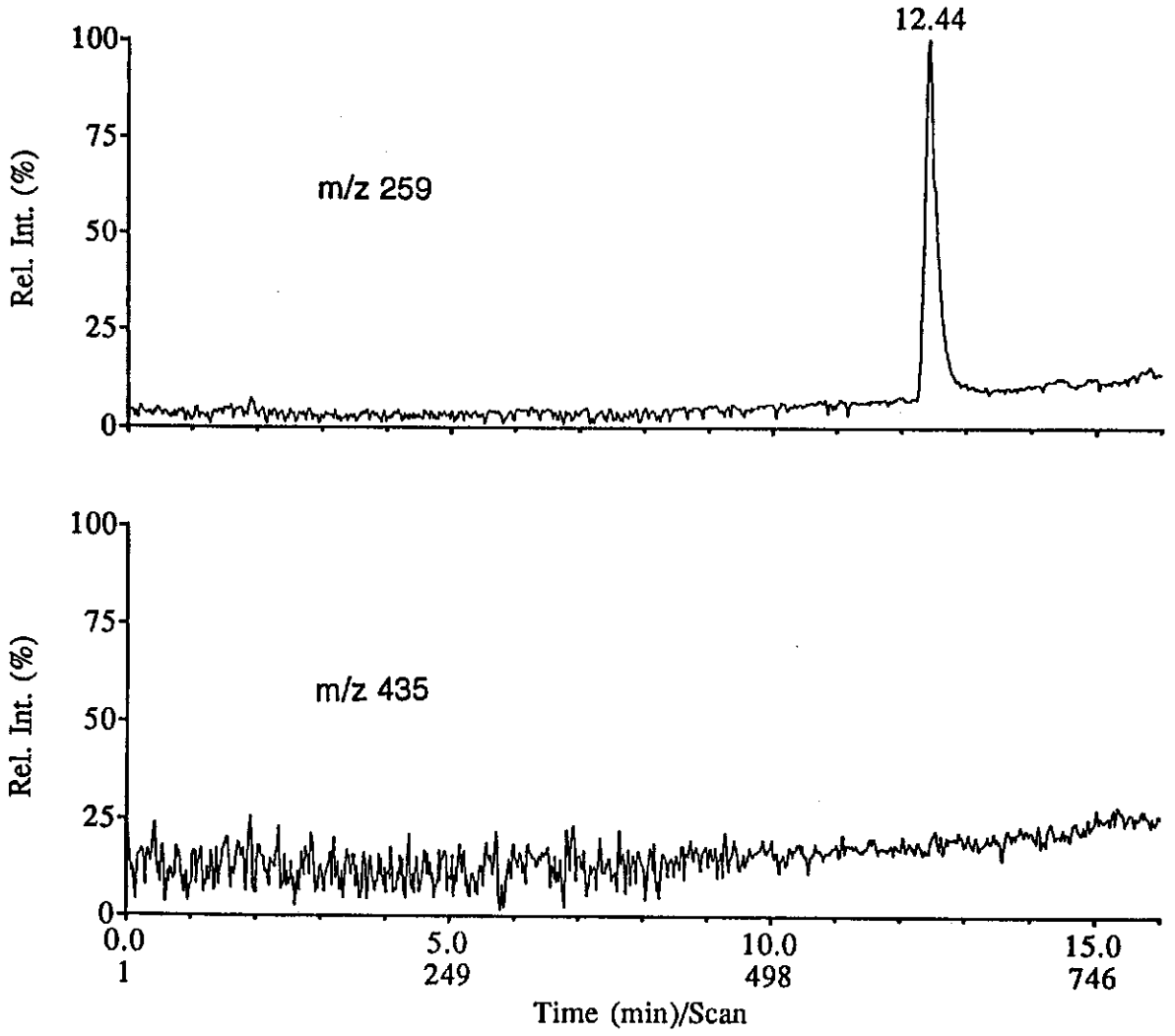
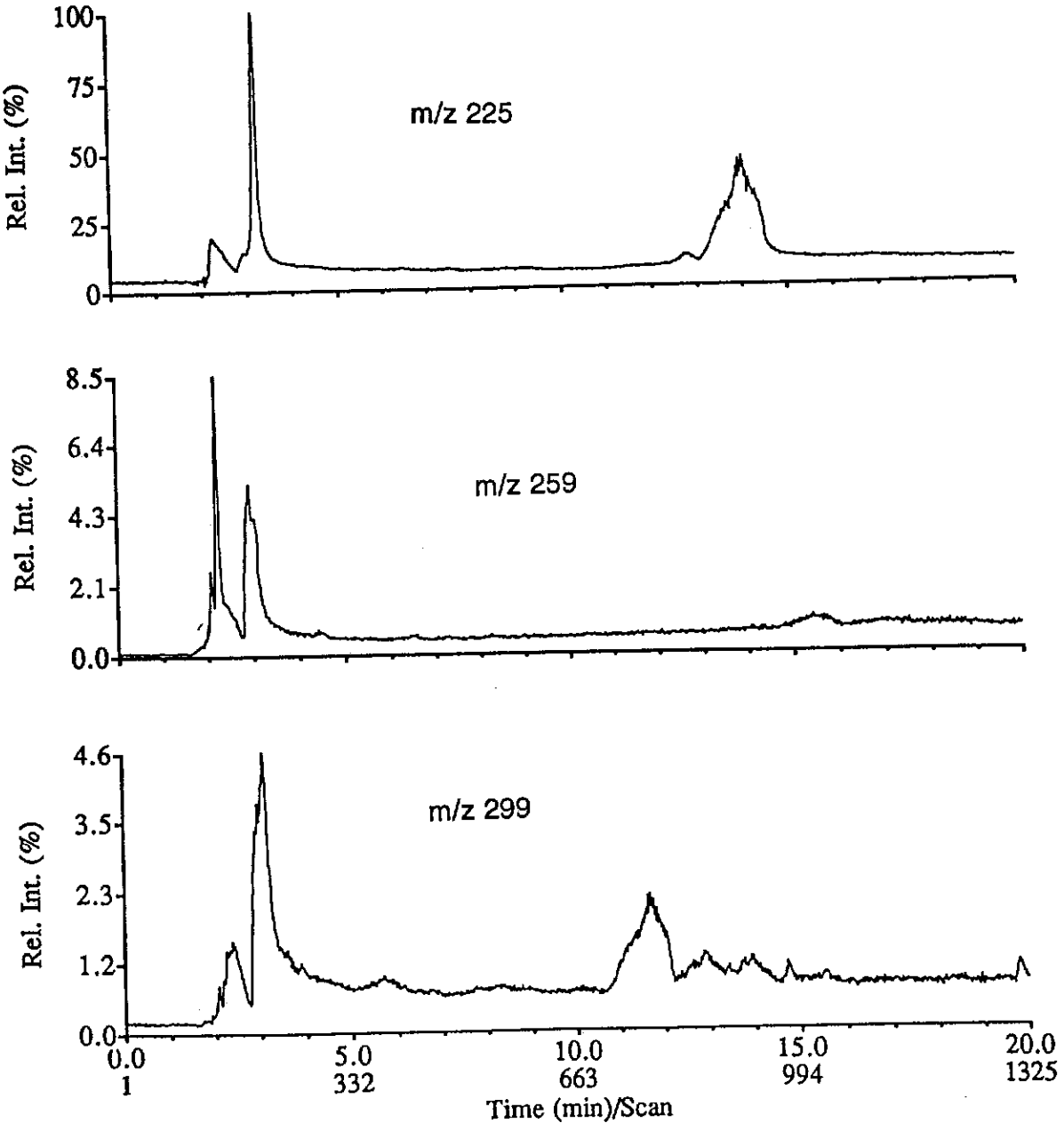


Figure 38

LC-MS analysis of the glucuronidase digest of bile from a feral fish captured in waters polluted by bleached Kraft mill effluent

The SIM chromatograms for m/z 225, 259 and 299 monitor TCG, TeCG and DHA



Financial Arrangement Between the Department of Fisheries and Oceans and the National Research Council

TITLE:

- Development and Application of Chromatographic and Advanced Mass Spectrometric Methods for the Analysis of Anti-sapstain Fungicides and for Selected Xenobiotics Originating from Pulp Mill Effluents.

OBJECTIVES:

1. To develop chromatographic and mass spectrometric methods for the detection and analysis of 2-(Thiocyanomethylthio) benzothiazole (TCMTB) and its degradation compounds at trace levels;

2. To apply the techniques developed in (1) above to the analysis of TCMTB and its degradation compounds in complex matrices such as bile, plasma, and urine obtained from exposed fish;

3. To develop extraction techniques to analyze TCMTB and its degradation compounds from other complex matrices, such as the liver, brain, kidney, and muscle samples of exposed fish; and

4. To apply chromatographic and mass spectrometric methods to the analysis of selected chlorophenolic compounds and wood extractives in bile samples of exposed fish.

BACKGROUND:

- Chlorophenol-based anti-sapstain fungicides are used extensively in Canada for the preservation of lumber. Concerns have arisen, however, over the aquatic toxicity and other environmental impacts of these compounds. For these reasons, alternate fungicides are being proposed. One leading contender to replace the chlorophenols contains the active ingredient 2-(thiocyanomethylthio)benzothiazole (TCMTB). Although Agriculture Canada approved the use of TCMTB in lumber, there is little published information about the fate and toxicology of this chemical and its degradation products.

- DFO is undertaking a collaborative research program with Environment Canada (West Vancouver laboratories and NWRI in Burlington) and the University of Lethbridge to study the fate and effects of TCMTB in the Fraser River. This team is: developing new analytical methods for monitoring TCMTB in water, sediment and biota; determining the contamination of stormwater by TCMTB and the fate of this chemical once in the river system; and applying these results to the study of the effects of TCMTB on salmon behaviour and physiology.

- The Institute for Marine Biosciences (IMB) of the National Research Council has recently acquired a SCIEX LC/MS/MS mass spectrometer system. This system represents a breakthrough in MS technology and allows the mass spectrometric analysis of many previously intractable families of compounds. Under this agreement, IMB will assist DFO in developing analytical methods for TCMTB and its primary degradation compounds using the SCIEX and other mass spectrometric and chromatographic techniques. Preliminary investigations by IMB into the application of the SCIEX LC/MS/MS system to TCMTB gave very promising results.

- In addition to the study of TCMTB, IMB will also assist DFO in analyzing for compounds produced as a result of wood pulp manufacture. The major types of these compounds which have been identified in pulp mill effluent are various: chloro-phenols; -guaiacols; -catechols; -syringols; and -vanillins. Other compounds, such as abietic acid, syringealdehydes and veratroles may also be produced. Studies are underway to monitor a number of these compounds in effluents and to determine their paths through the food chain. DFO and Environment Canada have identified three compounds : dehydroabietic acid; 3,4,5 - trichloroguaiacol; and tetrachloroguaiacol, as being of particular interest. As part of this agreement, IMB will attempt to develop analytical methods for detecting these three compounds, and some of their major metabolites, in environmental and biological matrices using advanced analytical methods including the SCIEX LC/MS/MS system.

STATEMENT OF WORK:

- It is proposed that the Institute for Marine Biosciences, under a financial arrangement and in close consultation with the Department of Fisheries and Oceans, arrange to have developed chromatographic and mass spectrometric methods for the detection, identification and quantitation of TCMTB and its degradation compounds. In addition, IMB will arrange to have developed, or will develop itself as part of its own research program, chromatographic and mass spectrometric methods for the analysis of three compounds derived from wood pulp processing.

- This study will be conducted in phases over a period of 18 months as outlined below:

Phase I: (August 1, 1990 – October 31, 1990)

- Development of reliable methods for determining TCMTB and its degradation products:

  2-Mercaptobenzothiazole (MBT); and
  2-(Methylthio) benzothiazole (MTBT),

  using Fast Atom Bombardment (FAB) mass spectrometric and LC/MS/MS IonSpray techniques.

- Preliminary investigation of MBT-glucuronides and MBT-sulfates using the mass spectrometric techniques mentioned above. Samples will be provided to NRC by DFO's collaborator Environment Canada in Burlington.

Phase II: (November 1, 1990 – March 31, 1991)

- Application of methods developed in Phase 1 for the analysis of TCMTB and its degradation products in samples of bile, plasma and urine taken from fish exposed to TCMTB under laboratory conditions and samples of bile taken from fish exposed to waters near lumber-mill run-off. Initial experiments will be performed on archived samples provided to NRC by DFO's collaborator Environment Canada in Burlington. If necessary, DFO will supply NRC with samples from freshly exposed fish. NRC recognizes that it will take DFO at least one month to obtain these fresh samples.

- Up to 25 samples will be analyzed.

Phase III: (April 1, 1991 – January 15, 1992)

- Application of the analytical techniques developed in Phases I and II to the analysis of TCMTB and its degradation products in the liver, brain, kidney and muscles of

exposed fish. NRC will be required to develop techniques for extracting these compounds from the complex matrix of interest. DFO will supply NRC with samples from freshly exposed fish. NRC recognizes that it will take DFO at least one month to obtain these fresh samples.

- Up to 50 samples will be analyzed.

Phase IV: (August 1, 1990 – January 15, 1992. In both Parts, NRC will be required to develop techniques for extracting: dehydroabietic acid; 3,4,5 - trichloroguaiacol; and tetrachloroguaiacol, and their major metabolites from bile).

(i) Part I (August 1, 1990 - March 31, 1991): Development of chromatographic and mass spectrometric methods for the detection and analysis of these compounds and application of these techniques to the analysis of bile samples obtained from fish exposed under laboratory conditions. DFO's collaborator Environment Canada will supply NRC with pure standard compounds. NRC may also purchase compounds for analysis as necessary. In addition, DFO will supply NRC with samples from freshly exposed fish. NRC recognizes that it will take DFO at least one month to obtain these fresh samples.

(ii) Part II (April 1, 1991 - January 15, 1992): DFO will supply NRC with samples of bile obtained from fish exposed under field or laboratory conditions. NRC recognizes that it will take DFO at least 2 months to obtain these samples. Up to 50 samples will be analyzed.

DELIVERABLES:

- IMB will provide DFO with brief (2-3 page) interim progress reports at the end of each Phase.

- IMB will provide DFO with a final report (approximately 15 pages plus data) on or before January 15, 1992. If in the judgement of NRC and DFO, the results of this study merit publication, they shall be published or communicated in a mutually acceptable form.

FUNDING:

DFO will prepay the costs of each Phase at the beginning of each Phase. NRC will, in turn, provide financial statements.

PHASE I:	$ 2,500	
PHASE II:	4,500	
PHASE III:	7,000	
PHASE IV - PART I	13,000	
- PART II	13,000	
TOTAL	$40,000	
Fiscal-Year Funding Profile:	$ 20,000	in 1990-91
	$ 20,000	in 1991-92.

DFO will be responsible for collecting contributions from participating departments and organizations, including approximately $7,500 from Environment Canada in 1990-91.

REPRESENTATIVES:

IMB Representative:

Dr. Greig Sim
Institute for Marine Biosciences
National Research Council
1411 Oxford Street
Halifax, N.S.
B3H 3Z1
(902) 426-8281

DFO Scientific Authority:

Dr. George Kruzynski
West Vancouver Laboratory
Department of Fisheries and Oceans
4160 Marine Drive
West Vancouver, B.C.
V7V 1N6
(604) 666-7913

* *

For: Department of Fisheries and Oceans

Title: G Kruzynski / Research Biologist

Date: 11 July, 1990.

C D Levings
Sec Hd, Salmon
Habitat

11/7/90

10/07/90

For: National Research Council

Title: Chief Executive Officer, Director-General

Date: 17 July 1990