Identification of bound nitro musk-protein adduct in fish liver by gas chromatography-mass spectrometry: Biotransformation, dose-response and toxicokinetics of nitro musk metabolites protein adducts in trout liver as biomarker of exposure

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Abstract

Ubiquitous occurrences of synthetic nitro musks are evident in the literature. The *In vivo* analysis of musk xylene (MX) and musk ketone (MK) - protein adducts in trout liver have been performed by gas chromatography-mass spectrometry using selected ion monitoring (GC-SIM-MS). Biotransformation, dose-response and toxicokinetics studies of 2-amino-MX (2-AMX), 2-amino-MK (2-AMK) and 4-amino-MX (4-AMX) metabolites, covalently bound to cysteine amino acids in proteins in fish liver, formed by enzymatic nitro-reduction of MX and MK, have been described. Trout were exposed to single exposures of 0.010, 0.030, 0.10, and 0.30 mg/g MX and/or MK. Forty-two fish liver samples were collected from exposed- and control- fish subsequent to exposure intervals of 1 day, 3 days, and 7 days and were composited as per exposure schedules and times. Alkaline hydrolysis released bound metabolites from exposed liver composites that were extracted into *n*-hexane and then concentrated and analyzed by GC-SIM-MS. The presence of the metabolites in liver extracts was confirmed based on agreement of similar mass spectral properties and retention times with standards. In the dose-response study, the maximum adduct formation was 492.0 ng/g for 2-AMX, 505.5 ng/g for 2-AMK and 12588.5 ng/g for 4-AMX in liver at 0.03 mg/g MX and MK fish in 1 day after exposure. For toxicokinetics investigation, the highest amount of the target metabolites was found to be the same concentration as observed in the dose-response study for 1 day after exposure with 0.03 mg/g MX and MK fish and the half-lives of the metabolites were estimated to be 2 to 9 days

based on assumption of first-order kinetics. Average recoveries exceeded 95% with a relative standard deviation (RSD) around 9%, and the limit of detection (LOD) ranged from 0.91 to 3.8 ng/g based on a signal to noise ratio of 10 (S/N= 10) could be achieved for the metabolites. No metabolites were detected in the controls and exposed non-hydrolyzed liver extracts. This is the first report on dose-response and toxicokinetics of nitro musk-cysteine-protein adducts in fish liver.

Keywords: Nitro musk-proteins adduct, biotransformation, dose-response and toxicokinetics biomarker of exposure, fish liver, GC-MS.

Introduction:

Synthetic musk (SM) is often used in fragrances, soaps, lotions and other perfumed household personal care products as substitutes for the more expensive natural musk. Musk xylene (1,3-dimethyl-2,4,6-trinitro-5-tert-butylbenzene, MX) and musk ketone (1-tert-butyl-3,5-dimethyl-2,6-dinitro-4-acetylbenzene, MK) belong to the common group of nitro musk compounds with an estimated annual production of about 6,100 metric tons (Rimkus, 1999). The main environmental exposure of nitro musk occurs after sewage introduction and their ingredients enter into the environment and reach detectable and potential harmful concentration levels because most are not regulated environmental contaminants (Bethesda, 2006). The ubiquitous occurrence and fate of nitro musks and their metabolites as pollutants and biomarkers in the environment have led many scientists to research these emerging compounds (Daughton and Ternes, 1999, Kelly et al., 2007).

SMs are not readily biodegradable and have high bioaccumulation potentials (Müller et al., 1996). SMs have been determined as environmental pollutants in water (Gatermann et al., 1998), sewage treatment effluent (Osemwengie and Steinberg, 2001), air samples (Kallenborn et al., 1999), aquatic and terrestrial organisms (Oost et al., 2003), human tissues (Liebl and Ehrenstofer, 1993), human adipose tissue and breast milk (Rimkus et al., 1994), mussels and shrimp (Rimkus and Wolf, 1995), fish (Ramirez et al, 2009), and human blood (Hu et al. 2010) samples. Metabolites of the nitro musks have been characterized and quantified in river waters, domestic, industrial sewage sludge (Gatermann et al., 1998, Berset et al., 2000), homogenized whole fish (Osemwengie and Steinberg, 2003), and carp blood (Mottaleb et al. 2004) samples.

Some studies have documented ecotoxicity of nitro musk and their metabolites (Giddings et al., 2000). The acute toxicity of nitro musk, such as MX and MK, is low, though a nondose-dependent increase in the incidence of liver tumors was observed in male and female mice after long-term administration of MX in the diet (Maekawa et al., 1990). Several comparative studies of genotoxicity of nitro musk compounds suggested that MX and or MK were not genotoxic (Emig et al. 1996; Api et al. 1995; Putman et al. 1993). The fact that MX was identified as an inducer of hepatic cytochrome P450 2B enzymes suggested that non-genotoxic mechanisms, such as increased cell proliferation, might be responsible for the increase in liver tumors, analogous to phenobarbital (Lehman-McKeeman et al., 1997). From a toxicological view point, MX or MK were known as co-mutagenic substances for their similarity to the great number of polycyclic aromatic compounds and aromatic amines. The biotransformation of MX was studied in rats and was identified as an inducer of detoxifying enzymes in rat liver, and a half-life for elimination of MX and its metabolite of less than a few days was reported (Minegishi et al., 1991). MK is a cytochrome P450 1A1 and 1A2 isoenzymes inducer (Mersch-Sundermann et al., 1996). The toxicokinetics of MX in human blood plasma yielded about a 70-day half-life for the 4-animo musk xylene metabolite (Riedel and Dekant, 1999). The toxicokinetic (half-lives) of the nitro musk metabolites originating from trout hemoglobin were determined as 1 to 1.6 days (Mottaleb et al., 2005). Recently, Bravo et al. 2011 exposed juvenile trout to high molecular weight polycyclic aromatic hydrocarbons (PAHs) and demonstrated by the use of bile fluorescence the presence of aromatic compounds as biomarkers of exposure, consistent with PAH exposure over 50 days.

The bound metabolites obtained from liver proteins may be used as indicators of internal exposure to chemical carcinogens. The metabolites of nitro musks or other related nitroarenes, bound to the cysteine sulfhydryl group (- SH) of proteins in liver as biomarkers of exposure, could potentially be used to assess continuous exposure over a longer time range, and thus, may be better suited for risk assessment than quantitation of urinary metabolites (Farmer et al., 1987). Nitroarenes are enzymatically reduced to nitroso reactive intermediates, nitrosoarenes, capable of covalently binding with the - SH group of cysteine amino acids in proteins to form an acid/base labile sulfonamide adducts that hydrolyzes to aromatic amines in the presence of aqueous base (Sabbioni, 1994). The biological transformation process of MX and MK to their corresponding amine metabolites, with cysteine containing proteins in the liver results in adduct formation, as

shown in Figure 1. The aromatic amines were considered to be good dosimeters for the target tissue dose of the ultimate carcinogenic metabolites of the amine (Skipper and Tannenbaum, 1994).

The consumption of fish and water as well as the use of certain households and personal care products such as body perfumes could lead to an ingestion of nitro musk substances in humans. Although the acute oral and dermal toxicity of MX or MK is low, some hint for the carcinogenic potential of the compounds was found in animal experiments. These findings associated with stability against biological and chemical degradation raise significant attention. Thus, biological monitoring and the toxicology of the nitro musks are of considerable interest to the scientific community. This present study describes the biotransformation, dose-response and toxicokinetic assessments of the nitro musk-protein adducts in liver of trout exposed to MX and MK compounds. Metabolites of MX and MK bound to cysteine amino acids containing proteins in fish liver, formed by enzymatic nitro-reduction in MX or MK, are detected, characterized, and quantified by gas chromatography – mass spectrometry using the selected ion monitoring (GC-SIM-MS) mode. To the authors' knowledge, this is the first report on dose–response and toxicokinetics of nitro musk liver protein adducts from rainbow trout exposed to MX and MK and therefore this data could be used as a pilot for exposure research of aquatic and other living organisms.

2. Experimental

2.1 Chemicals, standards and solvents

Solid sodium hydroxide pellets, acetone (HPLC grade), n-hexane (purity 95%), granular anhydrous sodium sulfate (purity 99.3%) were purchased from Fisher Scientific, Pittsburg, PA, USA. Sodium dodecyl sulfate (SDS) powder was purchased from Bio-Rad, Hercules, CA, USA. The internal standard (IS) phenanthrene-d10 (purity 98%) and surrogate naphthalene-d8 (purity 99%) were purchased from ISOTEC, Sigma-Aldrich, St. Louis, MO, USA. The nitro musk standards musk xylene (MX), musk ketone (MK) and their metabolites 2- amino-MX (2-AMX), 4-amino-MX (4-AMX) and 2-amino-MK (2-AMK) were synthesized and provided by one of the authors (L.I.O). Tricane methane sulfonate (MS 222) was obtained from Sigma-Aldrich. De-ionized nanopure water was used in all preparations.

2.2 Fish exposure to nitro musk compounds

Trout exposure experiments were performed at the Department of Environmental & Molecular Toxicology, Oregon State University (OSU) (Corvallis, OR) for the sampling periods of 24 hr (1 day), 72 hr (3 days), and 168 hr (7 days), under supervision of Professor Larry Curtis. A series of standard test solutions containing 10, 30, 100, and 300 mg/mL MX or MK were prepared in salmon oil (pharmaceutical grade, Yukon Nutritional Co**.,** Lake Wales, FL, USA) as the delivery vehicle for trout exposure to MX and MK. At the higher intended concentrations (100 or 300 mg/mL), neither the MX nor the MK dissolved completely in the oil, but instead formed an emulsion. Well-shaken standard solutions were injected intraperitoneally into fish that were anesthetized in an aqueous MS 222 (75 mg/L) solution in a 15-L tank. The anesthetized trout were weighed before injecting the standard solutions into the fish. The mean standard deviation (± SD) value of the wet weight for the fish used in this study is 220 (± 44) g.

In total, 45 trout were exposed to MX and MK solutions by injection, using salmon oil as the delivery vehicle. In the dose–response study, three trout were exposed to one of four MX and/or MK concentrations for 1 day. For the toxicokinetics examination, three trout were exposed to a single concentration of MX and/or MK for a 3-day or 7-day period. As controls, nine fish were exposed to the salmon oil (with no MX and/or MK) for the same sampling periods. After that, fish were returned to the labeled tanks with circulating water at 13^{o}C. Table 1 summarizes the *in vivo* fish exposure experiments.

Following exposure, no food was given to the fish, which were closely monitored in the labeled circulating water tank. In the dose–response study, two trout exposed to the 30 mg/mL MX solution were observed to be sick (losing equilibrium) in the tank on day 1 and day 7 groups. One fish exposed to 10 mg/mL MK died on 1-day. Liver samples were not used from dead or sick fish in this study.

2.3 Collection of fish liver samples

Liver samples were collected from the fish after 1-, 3- and 7- days exposure schedule. Before collecting the liver samples, trout were anaesthetized with MS 222 (250 mg/L) and this concentration was high enough to kill the trout. Anaesthetized fish were washed with water and dissected along the belly line on a clean dissection tray, using a small sharp scissor. The intestine

and liver were seen inside the fish and the liver was carefully removed using cleaned tweezers. The liver samples were weighed and transferred to a scintillation glass vial (20 ml volume) for each fish. Each vial was identified with a number based on exposure level and exposure time. The specimens were stored in - 80^{o}C and shipped to Northwest Missouri State University, Maryville, Missouri via EPA, Las Vegas, NV by overnight delivery under frozen conditions. Prior to dissection of another fish, the tray and other associated accessories were cleaned with deionized water, acetone and hexane after each fish procedure to avoid cross contamination. Blood samples from the same fish were also collected and analyzed (Mottaleb *et al*., 2005).

2.4 Preparation of the liver composite and homogenization

From different sizes of trout, the weights of liver ranged from 0.6 to 1.5 g. Due to the small sample size, liver specimens were composited taking three livers, with exception of the livers from the sick or dead fish, for each of the exposure levels of 10, 30, 100, and 300 mg/mL MX and/or MK, with exposure times of 1-day, 3-days and 7-days. The nine control liver specimens were combined and prepared as one composite. In total, 13 composites were prepared and homogenized separately, using a Power Gen 125 (Fisher Scientific) tissue homogenizer set to rotate at 30,000 rpm, and stored at -80°C prior to analysis. The control composite was used to develop the methodology before analysis of the exposed samples.

2.5 Liberation of bound amino metabolites from liver composite specimens

In our earlier investigation, an alkaline hydrolysis, extraction, and preconcentration procedure for liberation of the bound amino metabolites from carp hemoglobin was reported (Mottaleb et al. 2004). An almost similar procedure was employed here. Briefly, approximately 0.35 to 0.60 g of homogenized exposed liver composite specimen was taken into a 20 mL glass scintillation vial, where 10 mL of 0.5% SDS, 1 mL of 10 N sodium hydroxide and 400 ng (100 µL of 4 ng/µL) of surrogate naphthalene-d8 were added. The mixture was then extracted for 1 hr by a Tube Rotator (Fisher Scientific) at 50 rpm in an ambient temperature. Following extraction, samples were rinsed into a 50 mL centrifuge tube using 2 mL nanopure water and centrifuged at 13,000 rpm for 1 hr at 22^{o}C. The supernatant was decanted into a 50 mL Kimax borosilicates glass tube (Fisher Scientific) and then extracted 4 times with 10 mL n-hexane at room temperature, while stirring for 5 min. The extraction was performed slowly at 7 rpm to minimize

the formation of the emulsion that developed during stirring. Sonicating the tubes for about 2 min broke the emulsion. Pressure developed inside the tube and was released by opening the cap. The tube was placed in a refrigerator for about an hour to freeze the sample. A clear hexane layer was obtained as an extract on the top of the aqueous layer in the tube. The extract was transferred into a conical flask by disposable Pasteur pipette. The extract was then passed through a column containing granular anhydrous Na_2SO_4 to remove the residual water. Prior to passing the extract, about 8 ml of *n*-hexane was run through the drying column to activate the Na_2SO_4 granules in the column. The solvent was evaporated almost to dryness, under a gentle stream of nitrogen at room temperature. Subsequently, the sample was reconstituted in 180 uL of n-hexane where 50 ng (25 µL of 2 ng/µL) IS (phenanthrene-d10) was added. The solution was sealed in GC vials and analyzed by GC-SIM-MS.

2.6 Spike recovery and limit of detection (LOD) analysis

Control composite specimens were used for recovery and LOD studies. In the case of the recovery analysis, four replicate measurements were performed taking 0.350 ± 0.025 g of composite. The specimens were spiked with 300 µL of 1 ng/µL of each target 2-AMX, 2-AMK and 4-AMX metabolites from a mixture of standard solution and 400 ng of surrogate naphthalene-d8. For the LOD study, about 0.42 g of control liver composite was spiked with 50 µL of 0.1 ng/µL of each target metabolite with the same amount of surrogate. The extraction procedure described in section 2.5 was then used.

2.7 Test of unbound amine metabolites

To determine the presence of unbound amino metabolites in the control and exposed trout liver composites, a non-hydrolyzed experiment with control and exposed composite was performed. In these experiments, except for the NaOH, all chemicals and solvents were added to the control (0.44 g) and exposed (0.53 g) composite specimens. The same extraction and preconcentration procedures were followed as described in section 2.5.

2.8 Gas chromatography and mass spectrometry (GC-MS)

A bench top Varian gas chromatograph (Model 450-GC) equipped with a Varian mass spectrometer (Model 320-MS TQ) connected to a Varian autosampler (Model CP 8400) was

used. Separations were carried out on a BPX50 (SGC Analytical Science) fused silica capillary column (30 m long x 0.25 mm i.d. x 0.25 µm film thickness) with helium gas at constant flow rate of 1.2 ml/min (linear velocity 40.3 cm/s), using splitless injection mode. The autosampler injected 1 µL volume of sample extract or standard solutions into the GC system with gradient oven temperature starting at 55^{o}C for 1 min, to 150^{o}C at 10^{o}C/min, to 250^{o}C at 8^{o}C/min, and to 300^{o}C at 10^{o}C/min, and holding that final temperature for 6 min. The injector and transfer line temperature were 240^{o}C and 275^{o}C, respectively. The ion source temperature was 240^{o}C and operated in positive electron ionization (EI) 70 eV mode.

Initially, known concentrations (approximately 3 to 5 ng/µL) of pure individual surrogate, internal standard and standard metabolites solutions were scanned between 50 and 600 *m/z* using the full-scan mode of GC-MS. By selecting the base peak/quantitation and confirmation/qualifier ions from the surrogate, internal standard and authentic metabolites standards, the mass spectral acquisition was performed with selected ion monitoring (SIM) using the Varian MS workstation version 6.9.3, with filament delay set at 8 min. Base peak ion *m/z* 136 (molecular ion) and qualifier ions *m/z* 108 and 54 were selected for the surrogate (naphthalene-d8), while a quantitation ion *m/z* 188 (molecular ion) and qualifier ions *m/z* 184, and 160 were selected for the internal standard (phenanthrene-d10). The ions monitored for the metabolites are given in Table 2.

2.9 Calibration curve, quality assurance/quality control (QA/QC)

Calibration standards were prepared by dissolving known concentrations of metabolites and surrogates in acetone. Calibration plots were constructed for each analyte by plotting the ratio of peak areas (analyte area divided by internal standard area) versus the ratio of 2-AMX, 2-AMK, and 4-AMX concentrations to the internal standard concentration, resulting in at least a 7-point calibration curve. A linear unweighted regression, forced through the origin, was performed for each analyte, resulting in correlation coefficients (R^2) of 0.99967 for 2-AMX, 0.9999 for 2-AMK, and 0.9996 for 4-AMX. The resulting equation of the line provided response factor (RF) relative standard deviation (RSD) < 6.5% and was used to calculate the concentrations of the metabolites. Each group of samples to be analyzed was bracketed before and after by a representative standard/internal standard QC sample to establish adherence to the calibration curve equation and agreement with the retention time and RF RSD of the standard. Deviations

from the calibration curve of ±15% RF RSD would cause rerunning of the standards, and construction of a new calibration curve, or replacement of the capillary GC injector liner. Retention time variations were generally ± 5 s, and peak widths at half-height were about 3 s. On the day of analysis, a sequence of injection: solvent blank, standard QC sample, solvent blank, sample extract, solvent blank, standard QC sample was used. No carryover of the metabolites was detected. In all the analyses of control and exposed liver composite specimens, the surrogate recovery was observed to be between 56 and 59%.

3. Results and discussion

All extracts derived from control and exposed trout liver composite samples were analyzed by the GC-SIM-MS method. The extracts examined were obtained from alkaline hydrolysis or non-hydrolysis experiments of the control composite. They did not show any characteristic mass signals of the target 2-AMX, 2-AMK and 4-AMX metabolites. The control composite matrix was subsequently used to determine the methodology and method performance for analysis of nitro musk protein adducts in fish liver specimens. In the case of non-hydrolyzed extraction, no metabolites were found to be detected in any of the exposed composite samples. Thus, it was established that the metabolites, formed in the nitro musk exposed fish, and released from the alkaline hydrolysis extraction, were originally bound to the exposed fish liver.

3.1 Analytical method performance

The analytical performance of the method was evaluated through percent recovery and limit of detection studies. Table 2 illustrates the analytical method performance together with retention times and ions monitored for the metabolites. Average (n=4) spike recoveries of the 2-AMX, 2-AMK and 4-AMX metabolites ranged from 95 to 114% with relative standard deviations (RSD) of 4 to 9%. The limit of detection (LOD) for the metabolites was calculated based on a signal to noise ratio of 10:1 (S/N=10). The LODs for 2-AMX, 2-AMK and 4-AMX metabolites were estimated to be about 0.24, 0.49 and 0.91 pg/µL, at the lowest level of the calibration used. These values corresponded to detection limits in the liver samples of approximately 0.91, 3.80, and 1.11 ng/g (based on 0.4241 g control liver composite per 200 µL final volume of extract), respectively (Table 2). Figure 2 (A) shows an illustration of the ion chromatogram observed for the LOD measurement of the 2-AMK metabolite. The SIM- mass spectrum in Fig. 2 (B) for 2-

AMK, constructed by selecting the peak that eluted at 24.1 min, provided a good agreement, approximately matching over 77% of mass signals/features of base peak and confirming ions between the composite sample extract and the lowest level of the calibration standard. Ion chromatograms and mass spectra for LODs estimation of 2-AMX and 4-AMX metabolites are not shown.

3.2 Detection, identification and characterization protocols for bound nitro musks-protein adducts in fish liver

GC-MS in the SIM mode was used to detect the bound nitro musk-protein adduct metabolites in the trout liver samples. Mass spectral properties and retention times, given by the protein adducts from the composite extracts, were employed to characterize the metabolites, by comparing with the authentic standards. Figure 3 illustrates the typical SIM ion chromatograms for (A) a standard solution containing, 50 pg/μL of 4-AMX metabolite and (B) a liver composite extract for the 1-day exposure study with dosing level of 0.01 mg/g of MX in trout. These chromatograms show an excellent similarity of the retention time of the 4-AMX metabolite eluted from the capillary column at 24.97 min with an increment of 0.01 min in liver samples. A variation of retention time (± 0.01 min) was also observed for 2-AMX and 2-AMK metabolites (not shown), and peak widths at half-height were about 3 s. The deviations of retention behavior were expected because of interferences from the matrix which co-extracted with the target metabolites from the liver samples. The surrogate naphthalene-d8, IS phenanthrene-d10 and 2-AMX were eluted from the capillary column at 10.20, 19.78 and 23.76 min, respectively (not shown).

To characterize the individual identity of the bound metabolites in the liver composites, the ion relative abundance ratios between the base peak and the confirming peak ions were used. The presence of the metabolites in the liver samples was confirmed when the difference of the relative abundance ratio was less than or equal to approximately ± 20%, or an agreement of the relative abundance ratio of about 80%. Figure 4 compares the mass spectra derived from the ion chromatogram (Fig. 3) for (A) standard 4-AMX metabolite and (B) liver sample extract solution. These exhibit an excellent agreement of the mass spectral features/signals with a variation of about ± 20.0%. A similar agreement of ion relative abundance ratio was observed for characterization of other metabolites in the different liver composites.

3.3 Concentration of bound protein adducts in fish liver composites.

Internal standard based calibration curves were used to quantify the concentration of the bound adducts in the trout liver. Table 3 summarizes the concentration of 2-AMX, 2-AMK and 4-AMX metabolites formed in the trout liver for MX or MK exposure over the period of 1 day, 3 days and 7 days. Maximum adduct formation for the 2-AMX, 2-AMK and 4-AMX metabolites was found to be present as 492.0, 505.5 and 12588.5 ng/g, respectively, for 1-day with the exposure dosing level of 0.03 mg/g. Moreover, a predominant formation of about 20 to 29 times more of 4-AMX over 2-AMX occurred for all of the exposure periods and dosing levels. This was consistent with explanation that that the *para*- position of nitro group in MX (Fig. 1) underwent faster enzymatic reduction to the nitroso reactive intermediate that covalently binds to cysteine in the liver proteins than *ortho*-position of the nitro moiety in the compound. Moreover this justifies the work of another scientist (Sabbioni, 1994) who demonstrated that the *para*-position nitro compound formed the highest concentration of cysteine-hemoglobin adducts in female Wistar rats than *ortho*-position of nitro aromatics. Moreover, variations of the observed metabolite concentration were likely due to the fact that the different trout had their own individual metabolic activity. Thus, the observed concentration differences in the 2-AMX and 2-AMK formation in fish liver were reasonable.

3.4 Dose-response and toxicokinetics consideration of the nitro musk-protein adducts

The trend of adduct formation as a function of concentration of nitro musks dosing to fish was investigated. Figure 5 shows the logarithmic plot of dose-response relationship of the bound nitro musk adducts formed in the trout liver at 1 day after exposure. In general, it provided evidence that the initial dose-response was linear but fell off at higher dosages. This suggested that binding of MX and or MK as an adduct to the cysteine groups of liver proteins increased in a dose-dependent fashion and that maximum formation occurred at 0.03 mg/g dosage. Adduct formation was found to drop at dose levels of 0.1 and 0.3 mg/g. We experienced solubility issues for the MX and or MK in preparation of 100 mg/mL and 300 mg/mL solutions in the vehicle, salmon oil (see section 2.2), for dose levels of 0.1 and 0.3 mg MX or MK /g fish, respectively. These two dose levels were not in proper solutions; rather, they were thick emulsions that did not dissolve all of the solutes. And well-shaken standard clear solutions/ emulsions (100 mg/mL and 300 mg/mL MX or MK) were used to expose the fish. The rate of

formation of adduct may have experienced a drop due to the presence of the MX or MK as suspensions and, thus active ingredients of the nitro musks were not readily available for absorption or transportation into intracellular system. Therefore, the starting materials may have affected the formation of the metabolites.

For toxicokinetic study, total eighteen fish exposed with a single dose of 0.03 mg/g MX or MK over the period of 1-day, 3-days and 7-days and nine control fish exposed with salmon oil over same exposure period were used (Table 1). At the administered dose level, the parent MX and or MK compounds dissolved completely in salmon oil carrier. The results were obtained with the actual dose of 0.03 mg/g, not with the higher doses of 0.1 or 0.3 mg/g. Figure 6 shows the graphical representation of the toxicokinetic assessment of the 2-AMX, 2-AMK and 4-AMX metabolites. This plot evaluated the natural logarithm (ln) of the concentration of the metabolites versus sampling times for the dosing level of 0.03 mg/g. The time dependence of the adduct development for each of the metabolites indicated a maximum formation at 1 day (24 hr) after exposure. The kinetics of the elimination of the protein adducts showed the negative slope linear relationship curve, as expected. We estimate the half-lives of 2 to 9 days for the metabolites in the trout liver based on assumption of first-order kinetics. The lack of more sampling points precludes a more definitive analysis. Table 4 depicts the individual values of the elimination rate constants and the half-lives of the 2-AMX, 2- AMK and 4-AMX metabolites in the fish liver. A variation of rate constant of the metabolites suggested that the toxicokinetics are more complex than a simple first-order reaction because additional internal biological processes transpire in the living organisms. Researchers revealed that nitro musks (MX and or MK) were identified as inducers of hepatic cytochrome P450 2B enzymes and P450 1A1 and 1A2 isoenzymes (Minegishi et al., 1991; Mersch-Sundermann et al., 1996) and are non-genotoxic (Emig et al. 1996; Api et al. 1995; Putman et al. 1993). Although the non-genotoxic carcinogenesis is not fully understood, it is believed that the non-genotoxic mechanism, such as increased cell proliferation, might be responsible for the increase in the liver tumors (Shaw and Jones, 1994; Lehman-McKeeman et al., 1997). In the present investigation, the MX and or MK- cysteine - protein adducts in fish liver were used to monitor nitro musks hazards as biomarker of exposure. This is an initial study of nitro musk-cysteine amino acids of the liver proteins adduct in fish exposure and provides a basis for more precise kinetics and other studies in aquatic organisms exposed to hazardous compounds.

Conclusion

A suitable GC-SIM-MS protocol was proposed and employed for analysis of nitro musk binding with HS- group of cysteine containing proteins in fish liver. The bioavailability of 2-AMX, 2-AMK, and 4-AMX metabolites in fish liver was explored after a single administration of MX and/or MK dosing with salmon oil in 45 trout over exposure periods of 1, 3, and 7 days. The *p*- and *o*- positions nitro groups of MX and *o*- position nitro group of MK were enzymatically metabolized and reduced to a nitroso reactive intermediate, capable of forming adducts with cysteine containing proteins in the liver that subsequently yielded an aromatic amines. Those amines could be suitable as biochemical end-points in monitoring, and for assessing risk, as biomarkers of exposure to nitro musk hazards. The 2-AMX, 2-AMK, and 4-AMX were identified and quantified as metabolites bound to cysteine containing proteins in the liver of trout exposed to MX or MK, by GC-SIM-MS. The half-lives of the metabolites were determined to be about 2 to 9 days. This was a first study of nitro musk-cysteine-protein adducts in liver in fish exposure. More sampling data points are necessary for a more accurate examination of the toxicokinetics.

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Figure legends

Figure 1. A possible biological transformation pathway of nitro musks (MX or MK) to corresponding metabolite amino compounds illustrating formation of nitroso adduct with cysteine containing proteins in the fish liver. Scheme 1: musk xylene (A) to 4-amino- musk xylene (B) and to 2-amino- musk xylene (C). Scheme 2: musk ketone (D) to 2-amino- musk ketone (E).

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Figure 3. GC-SIM-MS ion chromatogram for (A) a standard solution containing, 50 pg/uL of 4-AMX metabolite and (B) a liver composite extract for the 1-day exposure dosing with 0.01 mg/g of MX in trout. The GC-MS operating conditions are given in section 2.8.

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Figure 5. Dose-response relationship graphs produced as natural log (ln) of concentration of the amino metabolites obtained as nitro musk cysteine-proteins adduct in the fish liver for 1 day exposure versus dose of MX or MK injected into fish.

Figure 6. Toxicokinetic plot prepared as natural log (ln) of concentration of the amino metabolites obtained as nitro musk cysteine containing proteins adduct in the fish liver versus nitro musk exposure period for the dosing level of 0.03 mg/g of MX or MK in fish. The elimination rate constant and half-lives of the metabolites were estimated from this graph.

Table(s)

Table 1. *In vivo* trout exposure dosing schedule with nitro musk compounds and salmon oil vehicle.

Exposure time, Day	MX exposure					MK exposure				
	MX conc. (mg/mL)	Fish wet weight, (g)	Liver wet weight, (g)	MX dose /trout, (mg)	Average dosing level, (mg/g)	MK conc. (mg/mL)	Fish wet weight, (g)	Liver wet weight, (g)	MK dose /trout, (mg)	Average dosing level, (mg/g)
1- Day	10	202	0.75	2.0	0.01	10	257	0.88	2.6	0.01
		256	0.87	2.5			237	0.77	2.4	
		165	0.50	1.6			222†	NC	2.2	
	30	180	0.75	5.4	0.03	30	199	0.70	6.0	0.03
		256	0.92	7.5			230	0.76	6.9	
		280‡	1.41	8.4			212	0.69	6.3	
	100	236	0.76	24.0	0.10	100	272	1.26	27.0	0.10
		264	0.92	26.0			271	1.26	27.0	
		204	0.74	20.0			197	0.78	20.0	
	300	250	0.88	75.0	0.30	300	190	0.70	57.0	0.30
		310	1.52	90.0			270	1.18	81.0	
		227	0.71	69.0			250	1.00	75.0	
	Control	206	0.76	0.20 mL, exposed with salmon oil only						
		304	1.52	0.30 mL, exposed with salmon oil only						
		184	0.70	0.18 mL, exposed with salmon oil only						
3-Days	30	208	0.79	6.3	0.03	30	278	1.22	8.4	0.03
		244	0.81	7.2			156	0.46	4.5	
		193	0.71	6.0			196	0.68	6.0	
	Control	253	0.89	0.25 mL, exposed with salmon oil only						
		272	1.23	0.27 mL, exposed with salmon oil only						
		233	0.75	0.23 mL, exposed with salmon oil only						
7-Days	30	212‡	0.71	6.3	0.03	30	121	0.30	3.6	0.03
		230	0.76	6.9			241	0.88	7.2	
		204	0.74	6.0			167	0.53	5.1	
	Control	273	1.12	0.27 mL, exposed with salmon oil only						
		305	1.45	0.30 mL, exposed with salmon oil only						
		250	0.89	0.25 mL, exposed with salmon oil only						

†Trout was found dead and the liver sample was not collected (NC). ‡Trout was found sick (not equilibrium condition) and the collected liver specimen was not used in this study for composite preparation. All control liver samples were mixed together to make one control composite specimen. The collected MX and or MK exposed liver samples were composited mixing three liver for each dosing level with exception of sick or dead fish liver.

Table 2: Analytical method performance and ions monitored for target metabolites

Target metabolites	Retention time (min)	Ions monitored (m/z)	Spike analysis		
			LOD† (ng/g)	% Recovery (n=4) ± SD‡	RSD (%)
2- AMX	23.7	**267**, 252, 175	0.91	95 ± 8.5	8.9
2-AMK	24.1	**264**, 215, 191	3.80	114 ± 6.2	5.4
4-AMX	24.9	**252**, 267, 218	1.11	100 ± 4.2	4.2

† represents limit of detection (LOD), ‡ represents standard deviation (SD), RSD is relative standard deviation and bold face ions monitored are used as the quantitation ion and the other ions as confirming of the metabolites.

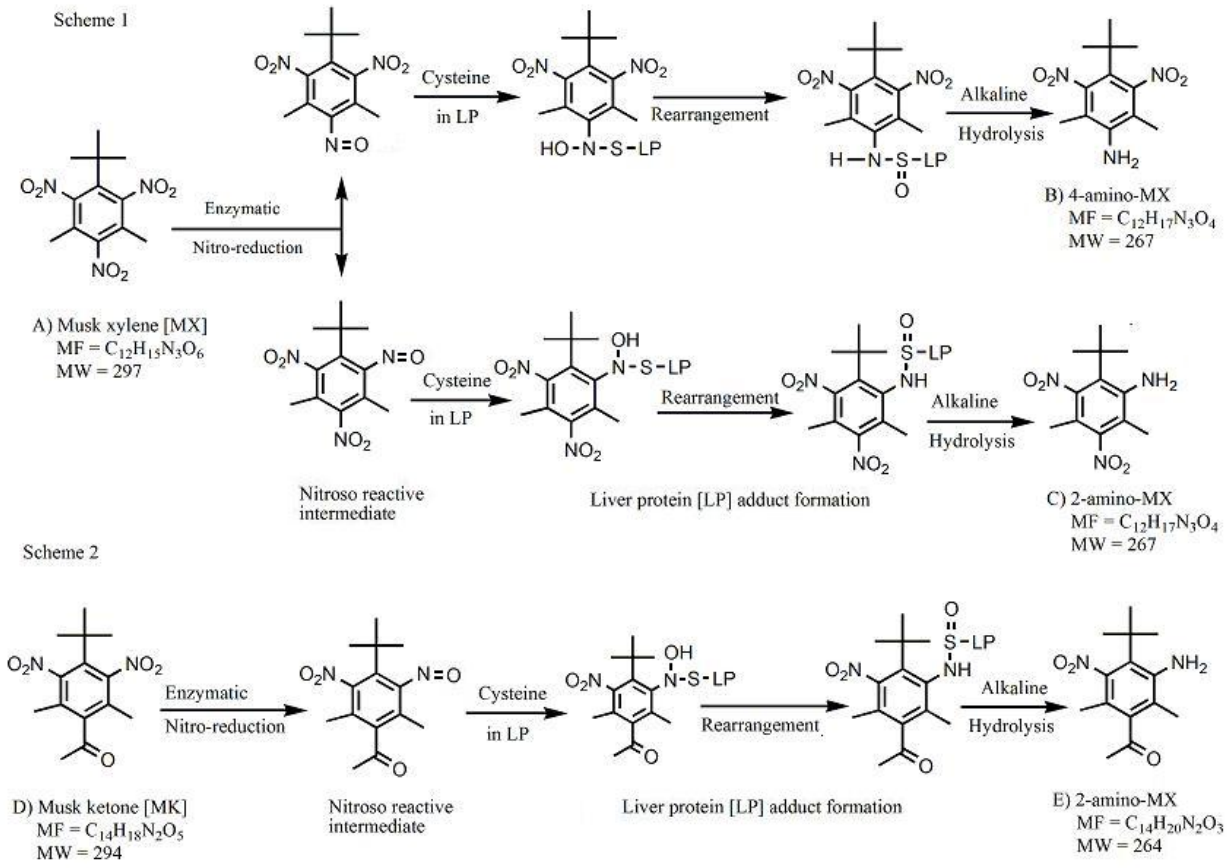
Table 3: Concentration of nitro musk metabolites in trout liver samples using hydrolyzed extraction

Exposure period (Day)	Exposure level MX or MK (mg/g)	Nitro musk metabolites (ng/g)		
		2-AMX	4-AMX	2-AMK
1-Day	0.01	94.0	2404.4	115.4
	0.03	492.0	12588.5	505.5
	0.10	444.1	10325.9	426.6
	0.30	259.1	5147.3	396.1
3-Days	0.03	213.6	6097.6	357.8
7-Days	0.03	113.5	2988.3	298.0
Controls	None	Not detected		

Table 4. Elimination rate constant (k) and half-life ($t_{1/2}$) of bound metabolites in trout liver on first order kinetics.

Target metabolites	ln of concentration of metabolites, (ng/g)			Elimination rate constant (k), (Day^{-1})		Half-life ($t_{1/2}$), (Day)	
	Day 1	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
2-AMX	6.2	5.4	4.7	0.40	0.25	2.0	3.0
4-AMX	9.4	8.7	8.0	0.35	0.23	2.0	3.0
2-AMK	6.2	5.9	5.7	0.15	0.08	5.0	9.0

Figure(s)



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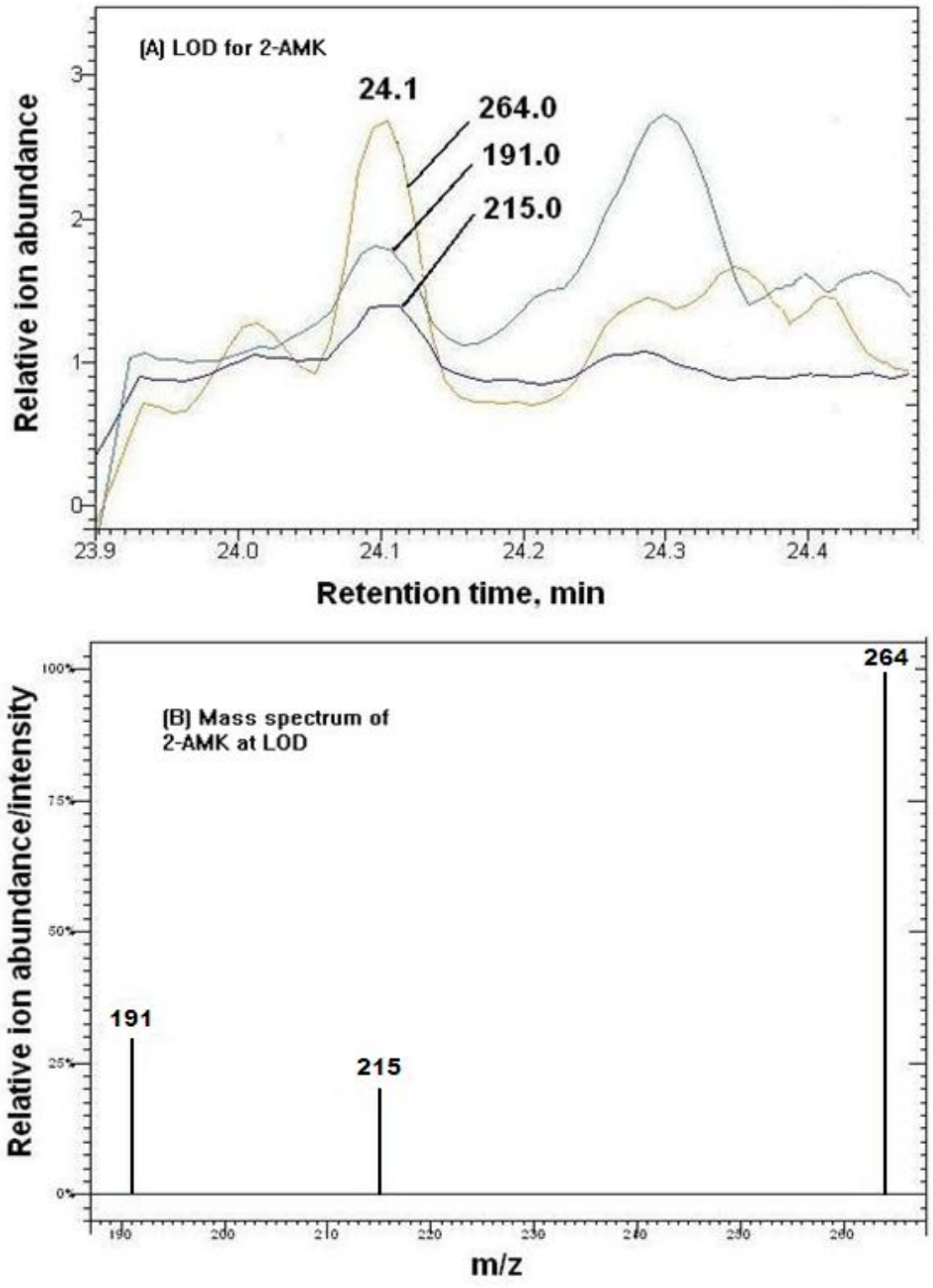

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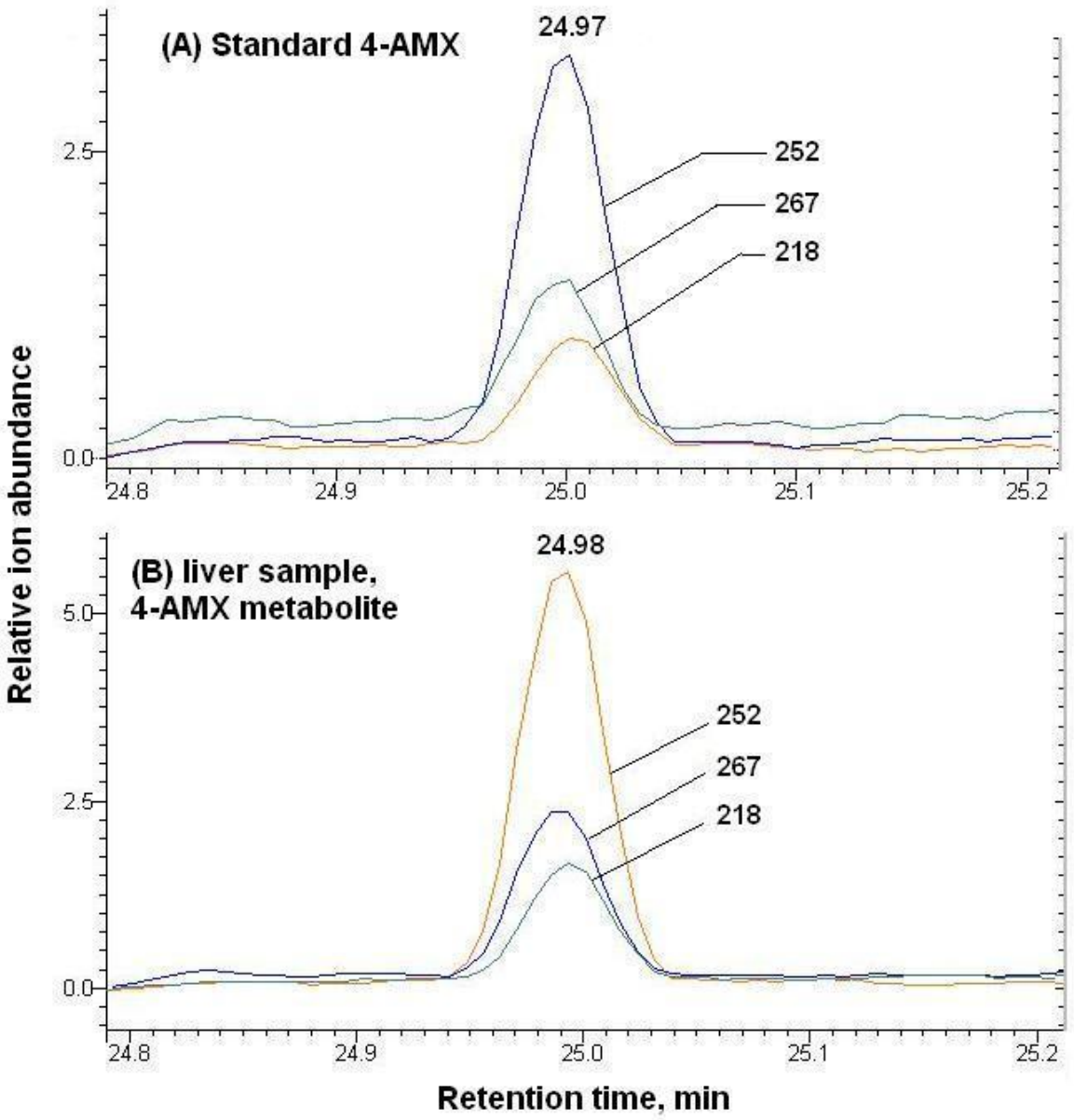

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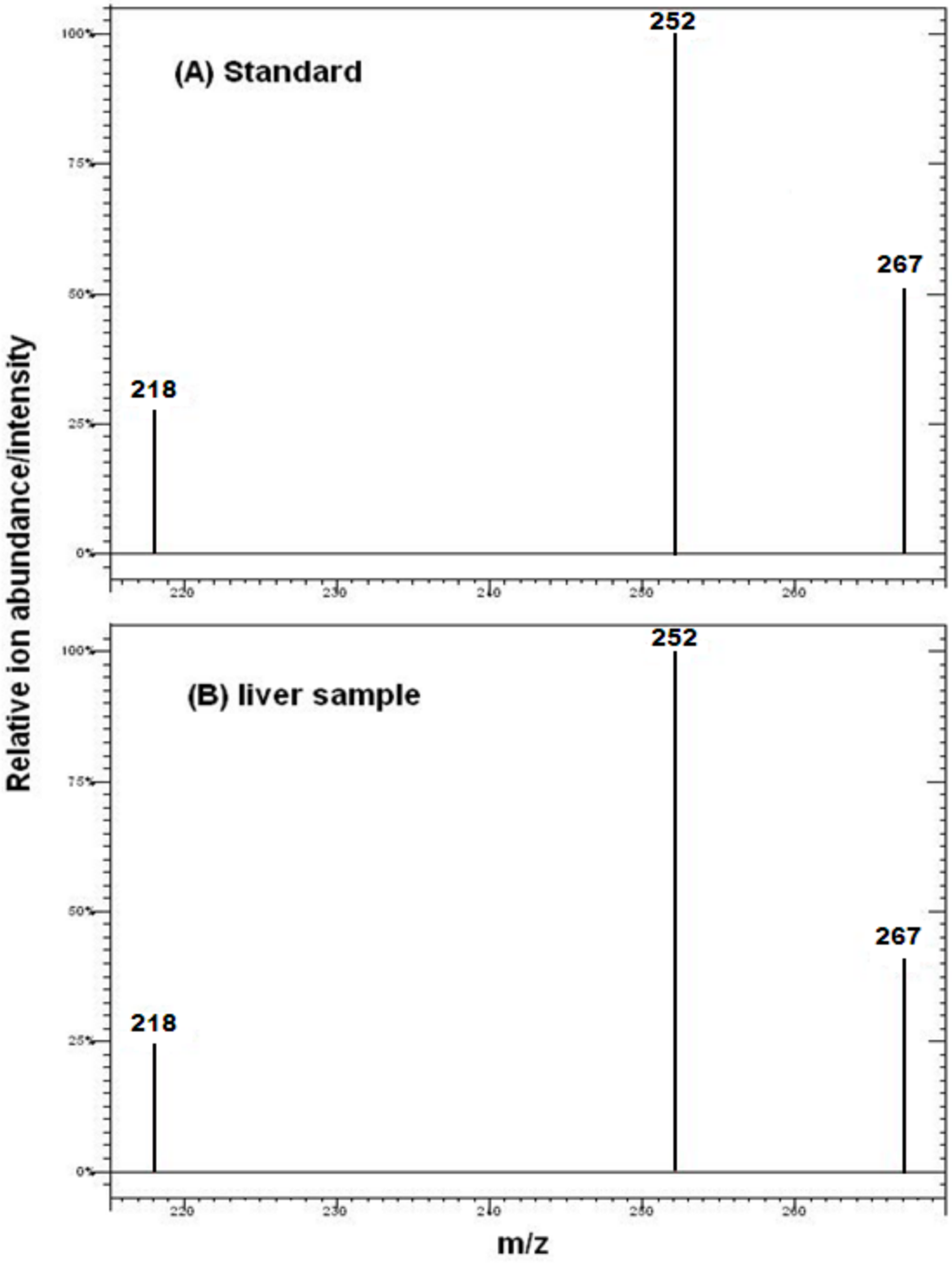

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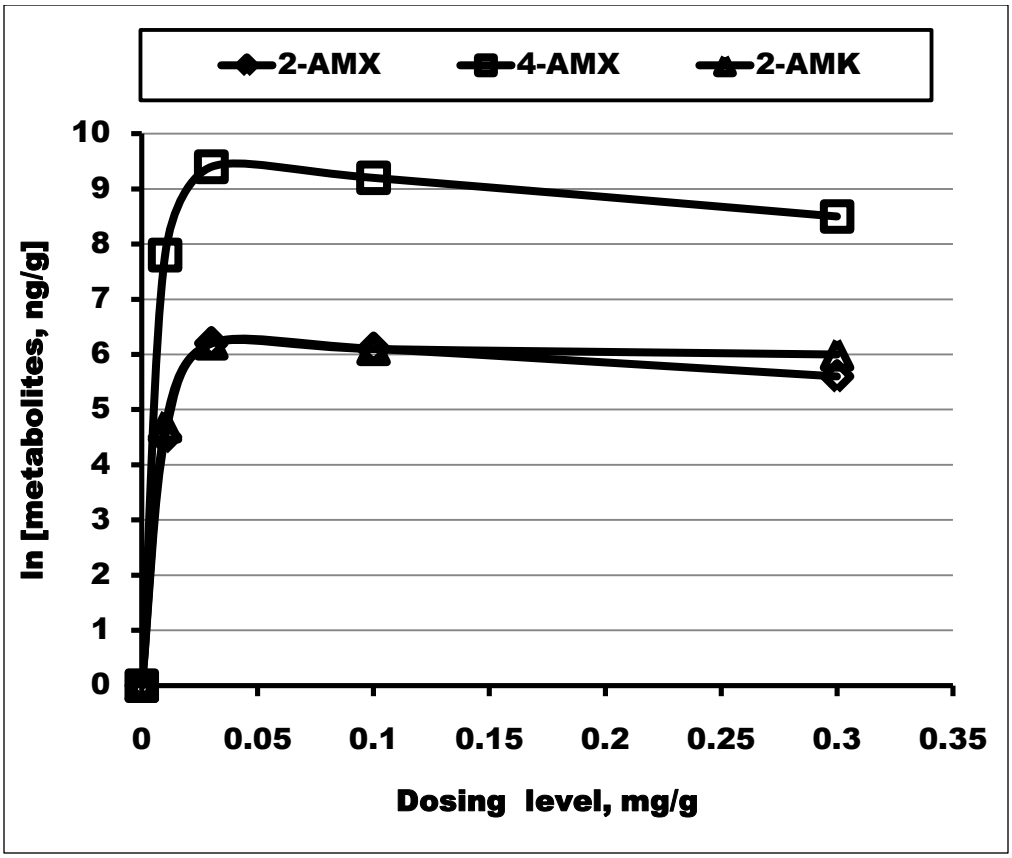

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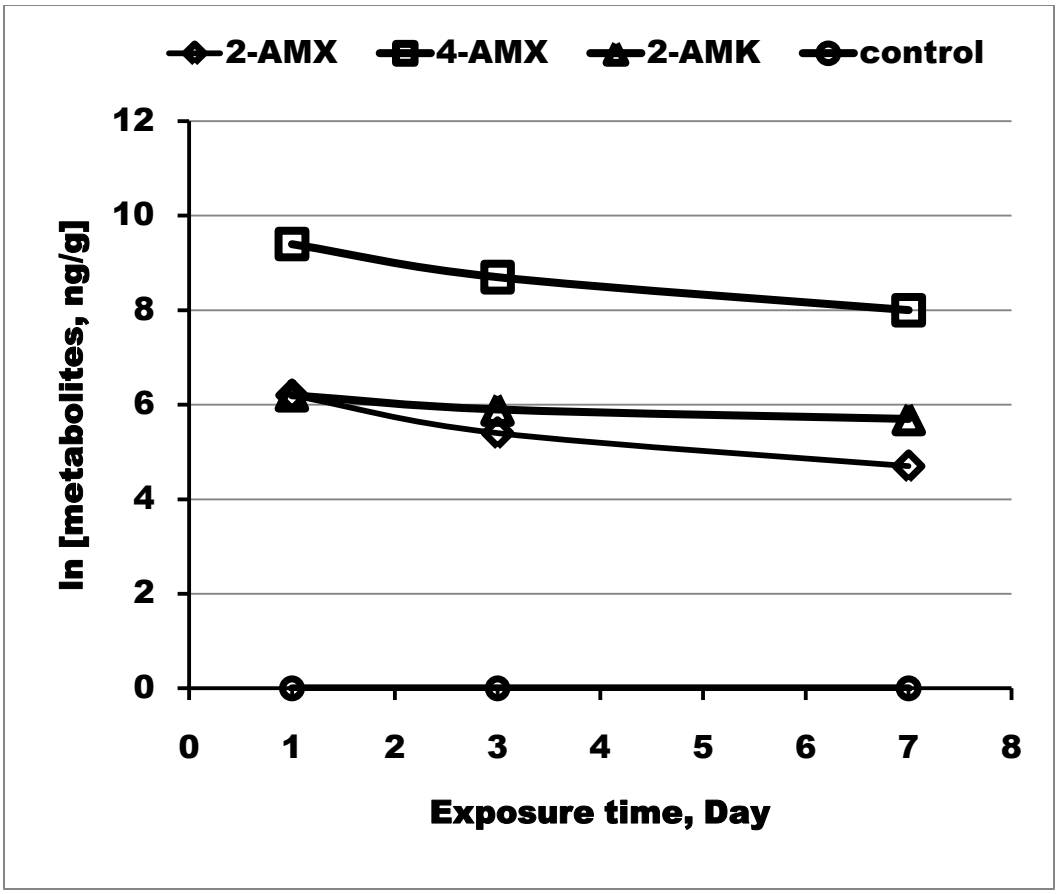

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Research Highlights

• Bound nitro musk-protein adducts in fish liver, by gas chromatography-mass spectrometry, have been explored after a single administration of MX and or MK in 45 trout exposed over a period of 1, 3, and 7 days.

• Biotransformation, dose-response and toxicokinetics assessments of 2-AMX, 2- AMK and 4-AMX metabolites, covalently bound to cysteine amino acids in proteins in fish liver, formed by enzymatic nitro-reduction of MX and or MK have been demonstrated. The *para*- position of nitro group underwent faster enzymatic reduction and yielded the most quantity of 4-AMX metabolite in the liver specimen than *ortho*-position of the nitro moiety in the MX compound.

• Dose-response study explained the bound nitro musk adducts formed to cysteine groups of liver proteins increased in a dose-dependent fashion and the maximum formation occurred at 0.03 mg/g, but fell off at higher dosages. The maximum adduct formation was 492.0 ng/g for 2-AMX, 505.5 ng/g for 2-AMK and 12588.5 ng/g for 4-AMX in the liver.

• Toxicokinetics examination associated with time dependence of the adduct development indicated the highest formation at 1 day (24 hr) after exposure. The half-lives of the metabolites were estimated to be 2 to 9 days based on assumption of first-order kinetics.

• Average recoveries achieved for the metabolites exceeded 95% and the limit of detection (LOD) ranged from 0.91 to 3.8 ng/g based on a signal to noise ratio of 10 (S/N= 10).

• This is a first study of nitro musk-cysteine amino acids of the liver proteins adduct in fish exposure and provides a basis for more precise kinetics and other studies in aquatic organisms exposed to hazardous compounds.