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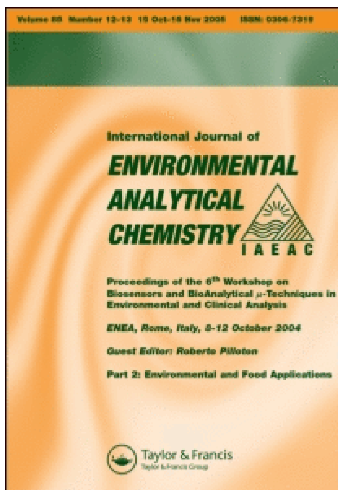

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Nitro musk metabolites bound to carp hemoglobin: determination by GC with two MS detection modes: EIMS versus electron capture negative ion MS

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NITRO MUSK METABOLITES BOUND TO CARP HEMOGLOBIN: DETERMINATION BY GC WITH TWO MS DETECTION MODES: EIMS VERSUS ELECTRON CAPTURE NEGATIVE ION MS

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Nitroaromatic compounds including synthetic nitro musks are important raw materials and intermediates in the synthesis of explosives, dyes, pesticides, and pharmaceutical and personal-care products (PPCPs). The nitro musks such as musk xylene (MX) and musk ketone (MK) are extensively used as fragrance ingredients in PPCPs and other commercial toiletries. Identification and quantification of a bound 4-amino-MX (4-AMX) metabolite as well as a 2-amino-MK (2-AMK) metabolite were carried out by gas chromatography–mass spectrometry (GCMS), with selected ion monitoring (SIM) in both the electron ionization (EIMS) and electron capture (EC) negative ion chemical ionization (NICIMS) modes. Detection of 4-AMX and 2-AMK occurred after the cysteine adducts in carp hemoglobin, derived from the nitroso metabolites, were released by alkaline hydrolysis. The released metabolites were extracted into *n*-hexane. The extract was preconcentrated by evaporation, and analyzed by GC-SIM-MS. A comparison between the EI and EC approaches was made. EC NICIMS detected both metabolites whereas only 4-AMX was detected by EIMS. The EC NICIMS approach exhibited fewer matrix responses and provided a lower detection limit. Quantitation in both approaches was based on an internal standard and a calibration plot.

Keywords: Biomarkers; Synthetic musks; 4-AMX; 2-AMK; Metabolites; Hemoglobin adducts; Carp; Exposure

INTRODUCTION

Nitroaromatic compounds are important raw materials and products of the chemical industry. They are used in the synthesis of a wide range of compounds including explosives, chemical solvents, dyes and dye intermediates, pesticides, and drugs [1]. Their production is estimated to be 225 000 tons/yr for nitrobenzene [2], and 1000 tons/yr for nitro musks, with musk xylene (MX) being the largest contributor [3]. These compounds

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are not readily biodegradable; they persist in the environment and can contaminate ground and surface waters as well as soils near production waste disposal sites [4] and military installations [5]. Humans can potentially be exposed to these compounds by numerous routes, including soil exposure, drinking contaminated water, or ingestion of contaminated food such as fish.

Nitro musks are one of the important classes of nitroaromatic compounds. They are widely used as fragrances in formulating body-care products and household cleaners. The commercial and domestic use and discharge of these compounds into municipal sewage systems has led to their ubiquitous occurrence in the aquatic environment and their presence at various concentrations in some organisms of certain aquatic ecosystems [6]. The low biodegradation rate and high lipophilicity [7] of MX and musk ketone (MK) enable them to bioaccumulate in aquatic and terrestrial organisms and exist as persistent environmental contaminants [8]. MX and MK have been observed in a variety of environmental compartments including the North Sea, rivers and freshwater [2,3], domestic and industrial sewage sludge [9], sewage treatment effluent [10], Norwegian air samples [11], human adipose tissue and breast milk [7,12], developing and adult rats [13], fish, mussels, shrimp [6], and whole fish [14]. Despite the widespread occurrence of MX, only limited knowledge about its toxicology is available. Although the acute toxicity of MX is low, a non-dose-dependent increase in the incidence of liver tumors was reported in mice after long-term administration of MX in the diet [15]. In rats, the half-life for the elimination of MX is less than a few days; however, this contrasts with a slow elimination and a half-life of about 80 days in humans [16]. MX and MK are known as co-mutagenic substances for a great number of polycyclic aromatic compounds and aromatic amines. They were identified as inducers of toxifying enzymes in the rat liver. MK is a cytochrome P450 (CYP) 1A1 and 1A2 isoenzyme inducer, while MX is a CYP 1A2 isoenzyme inducer [17].

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The presence of a biomarker indicates that exposure to a chemical (e.g., a xenobiotic) has occurred. Biomarkers are usually sought in biological fluids such as urine or blood, for a number of reasons including ease of sampling, least invasive approach, and representativeness. In the case of nitroaromatics, the biomarker is a hemoglobin (Hb) adduct in the blood. Thus, Hb adducts of nitroaromatics may be used, in principle, to assess cumulative exposure over the lifetime of red blood cells in contrast to the more transient existence of urinary metabolites [18,19].

Nitroarenes must be activated by reduction to nitrosoarenes, or to other related reduced moieties, before they can react with protein or DNA. In the case of Hb, the activated compound reacts with cysteine residues of Hb to form a sulfinamide that can be hydrolyzed to an aromatic amine in the presence of aqueous base. Hence, the Hb adduct should be a possible dosimeter of the target tissue dose of a possible ultimate agent of toxicity or impaired function [19]. In the case of MX, biotransformation and toxicokinetics in human Hb have been reported [20,21]. In one of our previous studies we detected the 4-amino musk xylene (4-AMX) metabolite from carp Hb for the purpose of ecological assessment of MX exposures [22]. The present study describes the comparison of EI and EC NICIMS in the determination of bound 4-AMX and 2-AMK metabolites, formed by enzymatic reduction of MX and MK, followed by adduct formation with carp Hb. Samples were collected from the Las Vegas Wash (LVW) and Lake Mead (LM), Nevada. The Hb adducts were detected and quantified by gas chromatography–mass spectrometry (GCMS) with selected ion monitoring (SIM) employed for both methods of ionization.

EXPERIMENTAL

Reagents and Chemicals

Sodium dodecyl sulfate (SDS), sodium hydroxide pellets, and *n*-hexane (HPLC grade) were obtained from Sigma-Aldrich, Fisher Scientific, and J.T. Baker, respectively. The internal standard (IS) for EI work, naphthalene-d_8, was purchased from Absolute Standard Inc., CT and the IS for EC NICIMS, pentafluorobenzophenone, was purchased from Aldrich Chemical Co., Milwaukee, WI. The 4-AMX and 2-AMK metabolites were synthesized and quantitative solutions were provided by Dr. L.I. Osemwengie, U.S. Environmental Protection Agency, Las Vegas, Nevada [10,14]. Solutions with known amounts of metabolite and IS were used to prepare calibration curves to quantify the results. De-ionized water was used for all preparations where necessary.

Collection of Carp Blood Samples

Live carp were obtained from downstream (about 100-m distance) of a publicly owned wash, called Las Vegas Wash, and from LM, Nevada. The domestic and sewage water treated by public sanitation treatment plants (STP) flowed down through a wash to LM. The distance between the LVW and LM sample collection points was about 10 river miles. About 6.5 to 8.0 mL of fresh blood samples were collected in whole blood tubes containing heparin solution (Daigger and Company Inc., IL). Blood was obtained from the carp by two methods, (i) severing the caudal peduncle (SCP; used for smaller carp) and/or (ii) using cardiac puncture. After collection of blood, the tubes were placed on ice.

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Isolation of Hemoglobin

Fresh, heparinized blood samples were centrifuged at $3000 \times g$ for 10 min at 4°C and the red blood cells (RBC) were separated from the plasma. The RBC were suspended and washed twice with equal volumes of freshly prepared 0.9% NaCl solution. The cells were then lysed by the addition of two volumes of distilled water, and the solutions were centrifuged again at $3000 \times g$ for 10 min at 4°C. The cellular debris was discarded after centrifugation. The Hb solutions were dialyzed for 72 h to remove small molecules and then placed in a freezer to solidify the Hb solution. A freeze-drying procedure was employed to eliminate water from the frozen Hb by a Sentry Microprocessor Control, Freezemobile and Benchtop Freeze-dryer (The VirTis Company, Inc. NY). The dried Hb was then placed in a freezer for later analysis of the nitro musk metabolite.

Alkaline Hydrolysis

To release the bound amino metabolite from the carp Hb, alkaline hydrolysis was performed. Detailed descriptions of basic hydrolysis, extraction, and preconcentration procedures were described in our earlier work [23]. Briefly, about 13 to 76 mg of dried Hb were placed in cleaned and dried tubes, followed by 9 mL of 0.5% SDS solution and 1 mL of 10 N NaOH solution. The mixture was then stirred for 1 h at room temperature and extracted three times with 10 mL of *n*-hexane. The tube was placed in a refrigerator for about 45 min to freeze the aqueous sample. A clear hexane layer was obtained as an

extract on top of the aqueous layer in the tube. The residual water from the extract was removed by passing the extract through a drying column containing granular anhydrous Na_2SO_4. The dried extract was then concentrated by evaporation under a stream of nitrogen. The IS was added, the solution sealed in GC-vials, and the sample analyzed by GC-SIM-MS.

Non-hydrolyzed and Reagent Blank Experiments

To investigate whether any unbound 4-AMX metabolite was present in the carp Hb, a control experiment without hydrolysis by base was carried out. In this experiment, except for the NaOH, all chemicals and solvents were added to the Hb, and the same extraction and proconcentration procedures were followed as described in the hydrolysis work. A laboratory or reagent blank control experiment was also performed by taking the same amounts of solvents, chemicals, and reagents used for the hydrolysis experiment, but without the Hb.

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Gas Chromatography and Mass Spectrometry

An Agilent Technologies HP 6890 series GC system equipped with a HP 5973 mass selective detector (MSD) connected to an Agilent 7683 auto sampler and an Agilent 6890 GC were used. The helium carrier gas was passed through a DB-5 (J&W Scientific, Agilent Technologies, CA) capillary column (40 m long, 0.180 mm i.d., and 0.18 μm film thickness) at a constant flow rate (for EIMS) of 0.5 mL/min (average linear velocity 22 cm/sec) using the pulsed splitless mode. The auto sampler injected a 2-μL volume of sample or standard solution into the GC with oven temperature gradients starting at 60°C for 1 min, 150°C at 10°C min^{-1}, 250°C at 8°C min^{-1}, and 300°C at 10°C min^{-1}, holding the final temperature for 6 min. The injector and transfer line temperatures were 250 and 280°C, respectively. The ion source temperature was 230°C and the source operated in the 70 eV EI mode. By selecting the base peak and confirming ions of the IS and the target compounds, the mass spectral acquisitions were performed with dwell times of 25 msec/ion using the GC/MSD Agilent ChemStation software, version B.02.05.

In the EI mode the following sets of ions were monitored: m/z 136 for d_8-naphthalene (IS); m/z 294, 279, 128 for MK; m/z 268, 253, and 223 for musk ambrette (MA); m/z 297, 282, and 115 for MX; m/z 267, 252, and 218 for 4-AMX; m/z 264, 249, 215, and 191 for 2-AMK. For EC NICIMS work the following parameters were used: a setting of 38 for the methane flow (38% of 2 mL/min), source temperature 150°C, quadrupole analyzer 106°C, filament emission 49.4 mA, and 2000 V on the electron multiplier. A constant pressure method was used for the GC flow rate with the same temperature program as above, which resulted in longer retention times for the analytes (Table I). Ions monitored included an IS2 (pentafluorobenzophenone), m/z 272; m/z 267, 268 for 4-AMX; m/z 264, 265 for 2-AMK; m/z 294 for MK (m/z 264 already included); m/z 297 for MX (267 already included); m/z 268, 253, 238 for MA. In both the EIMS and EC NICIMS, musk ketone, musk xylene, and musk ambrette were monitored qualitatively since their responses would also have some ions in common with the metabolites, although with different retention times.

TABLE I Retention times of parent nitro musk compounds and aminomusk metabolites

Compound (MW)	*EIMS, m/z*		*EC NICIMS, m/z*	
	Quant. ion[a]	*RT (min)*	*Quant. ion*[a]	*RT (min)*
Musk ketone (294)	279*	22.20	264*	23.43
Musk Ambrette (268)	268*	20.34	268*	21.26
Musk Xylene (297)	282*	20.70	267*	21.83
4-AMX (267)	252	24.43	267	34.18
2-AMK (264)	264	23.88	264	33.84
IS[b] (136), IS2[b] (272)	136	11.23	272	16.55

[a]Ions indicated * are potential quantitation ions but these compounds were not quantitated in this study.
[b]IS and IS2 represent naphthalene-d_8 and pentafluorobenzophenone, respectively.

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Calibration Curve

A regression analysis was carried out on the ratio of areas (analyte area divided by internal standard area) versus the ratio of 4-AMX and 2-AMK concentration to internal standard concentration, resulting in a six-point calibration curve. Unweighted regression was considered and resulted in an R^2 value of 0.998 for EI and 0.995 for EC by forcing the equation through zero signal. The calibration curve resulting from forcing the equation through the point 0,0 was used to determine the 4-AMX and 2-AMK in Hb samples because the concentration of 4-AMX and 2-AMK were expected to be near the low end of the calibration line. The resulting equation of the line was used to calculate the concentrations of 4-AMX and 2-AMK in the samples and check standards run during the course of analysis. The concentration of 4-AMX and 2-AMK was calculable based on the known volume and concentration of internal standard added to the sample and the known mass of the sample being analyzed.

Quality Assurance/Quality Control (QA/QC)

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 of the standard. Deviations from the calibration curve greater than ±10% would cause rerunning of standards, construction of a new calibration curve, or replacement of the capillary GC injector as a result of poor peak shape (tailing), which affected quantitation. Retention time variations were generally less than 0.08%, and peak widths at half-height were about 3 s.

A laboratory reagent blank was analyzed using the same procedure as was used for the Hb samples, except that no Hb was extracted. No 4-AMX or 2-AMK were detected as background contaminants. Standards run on the day of analysis were followed by a solvent blank run, followed by the extract. No carryover of 4-AMX or 2-AMK was observed. Confirmation of identity was based on the presence of two to six ions whose relative abundances agreed to within ± 20% of the relative abundances of the standard, and whose retention time was within 2 s of that of the standard. Fewer fragment ions are available in the EC mode relative to EIMS and specificity needs to be carefully considered.

RESULTS AND DISCUSSION

Table I presents the various compounds, ions, and retention times used in the study. The parent compounds were monitored during the study but were not quantitated. Accurate assays of parent compounds require strict access limitations to the laboratory and near clean-room conditions in order to avoid inadvertent background contributions due to the nearly ubiquitous distribution of the musks (but not the metabolites). However, because some ions are common to parents and metabolites, especially in EC NICIMS, parent compound responses were recognized at their unique retention times. A change in choice of internal standard between EIMS and EC NICIMS was necessitated by the need for an EC response in the NI mode. The retention times also reflected different flow programs (one constant flow, one constant pressure) but would have differed in any case because of periodic column removal from the injection end each time the injection liner was replaced in response to degradation of the chromatography. The relative order of elution of all the compounds was the same in either flow program with 2-AMK eluting before 4-AMX. The metabolites were well separated from the parent compounds.

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Validation of the Presumption of Bound Residues

In addition to alkaline hydrolysis of Hb, non-hydrolyzed and reagent blank experiments were performed to support the premise that 4-AMX and 2-AMK metabolites originated from the carp Hb as bound residues. In the alkaline hydrolysis, the musks bound to carp Hb were released as the aminomusk metabolites that were extracted into *n*-hexane. Therefore, it was necessary to demonstrate that no unbound 4-AMX or 2-AMK compounds were present in the samples. To investigate the possible presence of unbound metabolites, the non-hydrolyzed and reagent blank experiments were also performed. The preconcentrated extracts obtained from the non-hydrolyzed and reagent blank experiments were spiked with a known concentration of IS solution. A 2-µL volume of each of the standard, solvent blank, non-hydrolyzed Hb extract, solvent blank, and reagent blank extract was injected in that order into the GC-MS, by the autosampler. In the case of the standard solution, it was observed that the IS, 2-AMK, and 4-AMX metabolite eluted from the GC capillary column at 11.19, 23.88, and 24.43 min, respectively, and the appropriate base peak and confirming ions, in the correct relative intensity ratios, were also observed at these times. When non-hydrolyzed and reagent blank extracts were considered, the GC-MS chromatograms did not show any response for 4-AMX or 2-AMK at the respective retention times. This indicated that no unbound 4-AMX or 2-AMK metabolites were present in the carp Hb investigated in this study. This further supports the mechanism that 4-AMX and 2-AMK result from liberation, by hydrolysis, from the Hb-bound metabolites [21].

Detection and Quantitation of Bound 4-AMX and 2-AMK Metabolites in the Carp Hb

To determine the 4-AMX and 2-AMK metabolites in the carp Hb, the preconcentrated extracts obtained from the hydrolyzed experiments were spiked with a known concentration of the IS solution. A standard solution (50 pg/mL) of 4-AMX and 2-AMK metabolites containing 200 pg/mL IS was also prepared. A 2-µL volume each of standard, solvent blank, and hydrolyzed Hb extract was injected in this order into

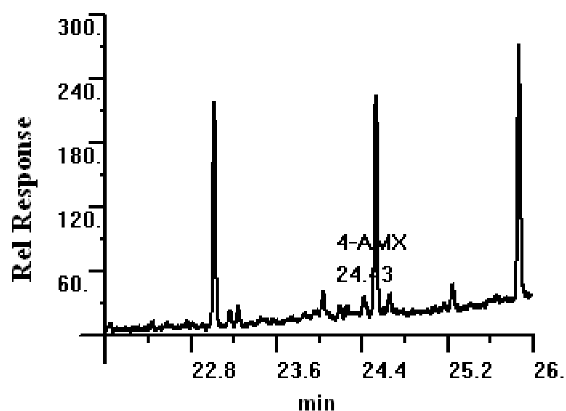

FIGURE 1 Ion chromatogram for *m/z* 267 (molecular ion of 4-AMX) under EIMS with the retention time of 24.43 min for the metabolite. Conditions in Experimental section.

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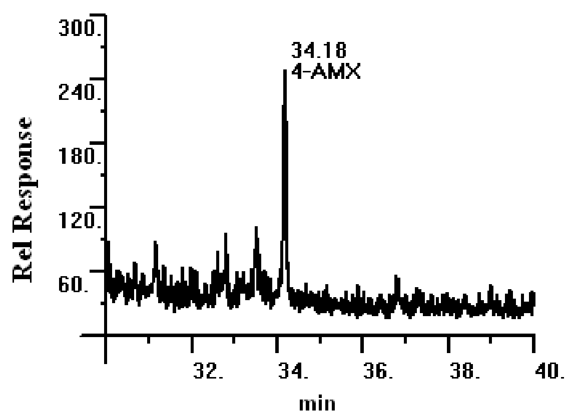

FIGURE 2 Ion chromatogram for *m/z* 267 of 4-AMX under EC NICIMS with retention time 34.18 min. Conditions in Experimental section.

the GC-MS by the autosampler. The 4-AMX and 2-AMK metabolites were found only in the hydrolyzed extract solution. Figure 1 shows the *m/z* 267 trace of 4-AMX by EIMS and Fig. 2 shows the same ion chromatogram under EC NICIMS. The trace by EC NICIMS reveals a clear response at 34.18 min and relatively small background responses. In contrast, the EI trace of *m/z* 267 exhibits several relatively large responses, and the target response is a relatively small response at 24.43 min. The EC NICIMS background-subtracted mass spectrum is shown in Fig. 3. The reconstructed mass spectrum from SIM data provides confirmation of identity along with the retention time. A similar result for the *m/z* 264 ion of 2-AMK is shown in Figs. 4–6. Here again, the EC NICIMS trace more clearly reveals the analyte relative to background responses. The resulting EC NICIMS background-subtracted reconstructed mass spectrum, shown in Fig. 6, reveals an additional ion present at *m/z* 268, but this ion cannot logically be related to *m/z* 264 under these ionization conditions. The *m/z* 268 ion also exhibits a slightly different retention time from that of *m/z* 264. Examples of EI background-subtracted spectra have been illustrated previously, and EI quantitations were unable to

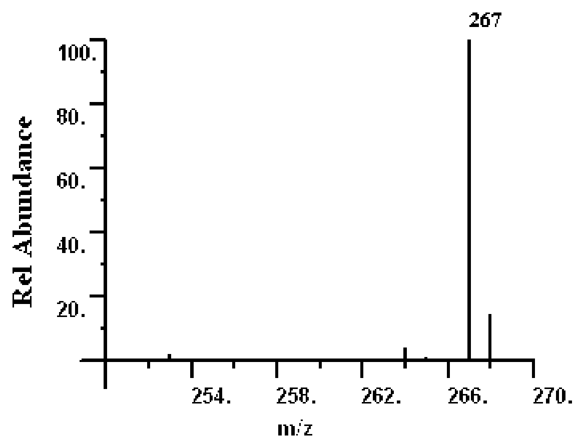

FIGURE 3 Background-subtracted spectrum of 4-AMX under EC NICIMS conditions.

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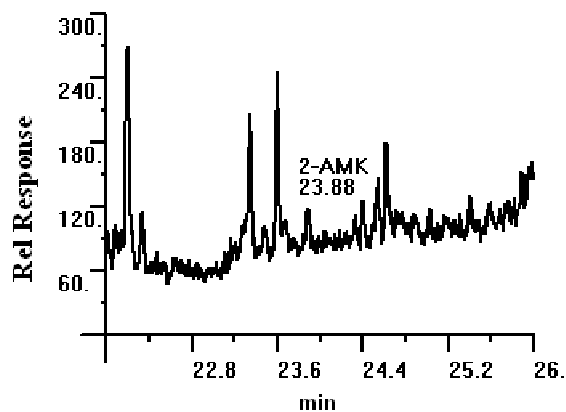

FIGURE 4 Ion chromatogram of m/z 264 (molecular ion of 2-AMK) under EIMS with retention time of 23.88 min. Conditions in Experimental section.

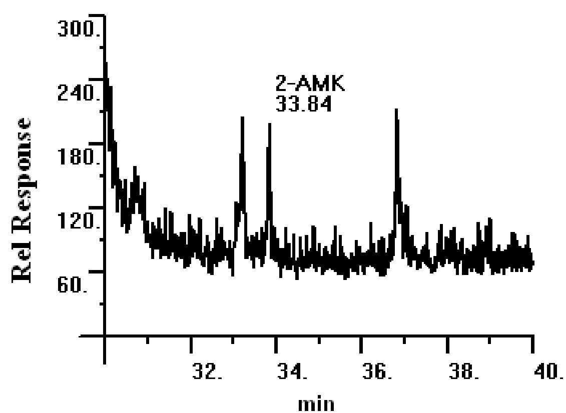

FIGURE 5 Ion chromatogram of m/z 264 of 2-AMK under EC NICIMS with retention time of 33.84 min. Conditions in Experimental section.

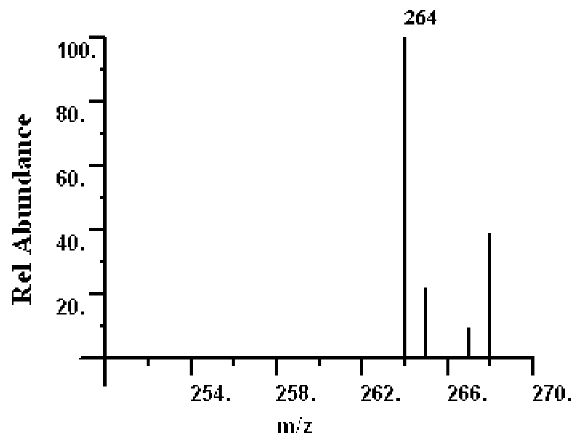

FIGURE 6 Background-subtracted spectrum of 2-AMK under EC NICIMS conditions.

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detect the 2-AMK [22]. In the EC NICIMS mode, both 4-AMX and 2-AMK metabolites were detected (Figs. 2,3,5,6) and the sensitivity of EC NICIMS was found to be a factor of five greater than EIMS. The 4-AMX was quantified for a signal-to-noise ratio of 3 to 1 (3:1) with a limit of detection of 3 to 4 ng/g, based on the matrices.

Biomarker analyses are characterized by a relatively small available sample. Therefore, highly sensitive and specific techniques are sought that minimize sample handling and are able to detect the relative levels needed. In the case of the musks, the EC NICI response is sensitive and selective enough to be of significant value for these kinds of analyses. EIMS is also applicable to these analyses but is characterized by more matrix responses and sometimes less sensitivity depending on compound structure and the extent of mass spectral fragmentation of the target compound (i.e., greater fragmentation may improve specificity at the expense of sample ion current divided among several ions and therefore decreasing the signal-to-noise ratio). The EC NICIMS detection limit was approximately 0.8 ng/g, whereas the EIMS limit was about 4 ng/g, depending on the matrices, with both values based on the freeze dried weight of hemoglobin. The 4-AMX and 2-AMK metabolites provided reproducible and robust calibration plots for quantitation by EIMS or EC NICIMS. Provided that the chromatography did not degrade during the performance period, the plots were useful for several weeks using continuing calibration checks on a daily basis.

CONCLUSION

The binding of 4-AMX and 2-AMK metabolites, presumably with cysteine, has been detected in carp hemoglobin (Hb) by both EIMS and EC NICIMS, and the approaches compared. Monitoring and assessment of MX and MK exposure via analysis of the reduced Hb adducts is therefore a useful approach. Previous studies have suggested that the reduction of a nitro group in MX and MK would yield intermediate nitroso compounds capable of forming adducts of Hb that could, on alkaline hydrolysis, yield amines that would be suitable as analytical endpoints. The concentrations of bound 4-AMX and 2-AMK metabolites were found in the carp Hb in the low nanogram per gram range. The use of Hb adducts as biomarkers of exposure to nitro

musks in fish populations appears to be well served by either the EIMS or the EC NICIMS approach, with the latter exhibiting more sensitivity and selectivity. In the EC NICIMS mode, both metabolites were detected, whereas only 4-AMX was detected by EIMS. The EC NICIMS approach exhibited fewer matrix responses.

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