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# Extraction and Analytical Method for N-Nitrosoatrazine Using Liquid Chromatography Paired with Tandem Mass Spectrometry

Carolyn Billings
*University of Nebraska - Lincoln*

# EXTRACTION AND ANALYTICAL METHOD FOR *N*-NITROSOATRAZINE USING LIQUID CHROMATOGRAPHY PAIRED WITH TANDEM MASS SPECTROMETRY

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Carolyn Billings

Forensic Science

College of Agricultural Sciences and Natural Resources

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Thesis Project Mentors:

Martha G. Rhoades, PhD., School of Natural Resources

Daniel D. Snow, PhD., School of Natural Resources

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## ABSTRACT

Atrazine is an herbicide used in the United States to control weeds. When combined with nitrite, atrazine can be nitrosated to form *N*-nitrosoatrazine (NNAT) *in vivo* and *in vitro* under acidic conditions. Atrazine can also be metabolized to atrazine mercapturate, deethylatrazine (DEA), didealkylatrazine (DDA), and deisopropylatrazine (DIA). Atrazine is monitored in drinking water due to its possible carcinogenicity. The objective of this study was to develop extraction and analytical methods for NNAT, atrazine, and atrazine metabolites. An instrument detection limit, extraction methods comparison, and extraction method detection limit were determined. To compare methods, Fischer 344 rat tissue samples were spiked with analytes and internal standard ($^{13}C_3$ atrazine). Method 1 consisted of acetonitrile solvent extraction, mixing by centrifuge, and filtering. Method 2 consisted of acetonitrile solvent extraction, mixing by shaker followed by centrifuge, and filtering. Method 1 had a higher NNAT recovery (26.8%) compared to method 2, so was used for all extraction method detection limit tests. Several extraction method adaptations were performed to improve NNAT recovery and determine the method detection limit. Sand samples were spiked with analytes, surrogate (terbuthylazine), and internal standard ($^{13}C_3$ atrazine). The samples were subjected to sequential solvent extraction using acetonitrile and were filtered and analyzed using liquid-chromatography paired with tandem mass spectrometry (LC-MS-MS). The average recoveries in sand spikes were 149, 140, 153, 124, 135, and 75.4 percent for atrazine, atrazine mercapturate, DDA, DEA, DIA, and NNAT, respectively. After further evaluations with spiked tissues, this method will be used to quantify placental transfer of these compounds in animal models.



Keywords: *N*-Nitrosoatrazine, tissue extraction, LC-MS-MS quantification

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# INTRODUCTION AND LITERATURE REVIEW

## *N*-Nitrosoatrazine

*N*-nitrosoatrazine (NNAT) is a nitrosamine formed by the nitrosation of atrazine. This reaction can occur in the environment and *in vivo.* In an acidic environment, nitrite is converted to nitrous acid and then transformed to a nitrous acidium ion. The nitrosating agent, the nitrosonium ion, is formed from the nitrous acidium ion (1). Secondary amines, such as those found in the structure of atrazine, can react with the nitrosonium ion, and form a nitrosamine, such as NNAT (2). The major sources of exposure to nitrosamines include tobacco smoke, food, drinking water, and personal care products (3). A 1975 review (1) reported that nitrite and pesticides such as atrazine can form carcinogenic nitrosamines *in vitro*. The authors predicted that this reaction could also take place *in vivo* and concluded that the amount of carcinogenic product depends on how readily the amine is nitrosated. As these reactions occur at acidic pH, the stomach is a suitable environment (4). Krull et al. (5) found that atrazine can be converted to NNAT in mice although *in vivo* nitrosation varied at different concentrations. The authors performed an *in vitro* test and determined that the highest doses of atrazine (1000 μg) and nitrite (500 μg) resulted in about 0.4% conversion to NNAT (5).

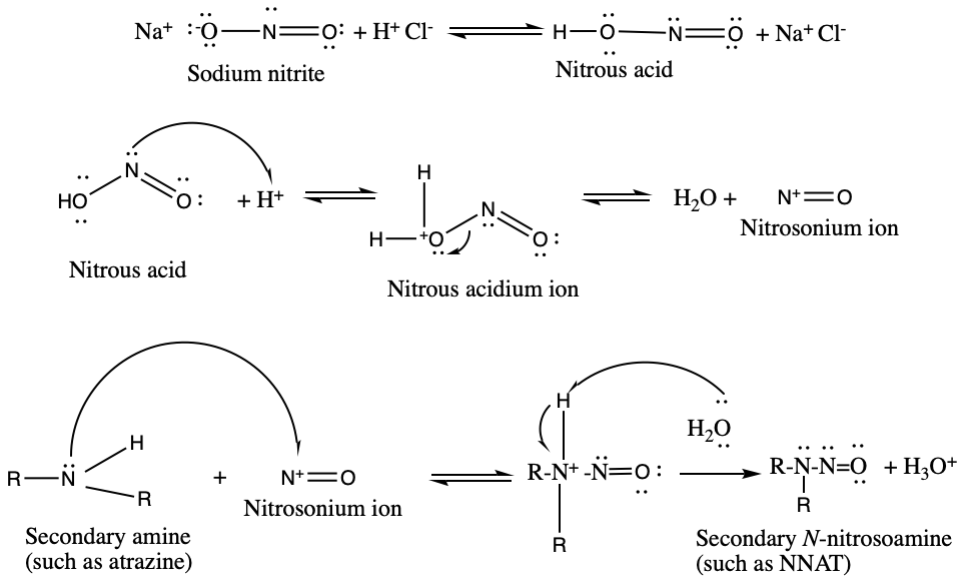
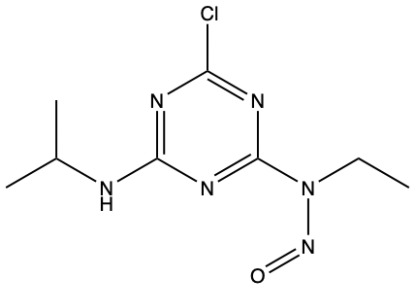



**Figure 1.** Formation of a secondary *N*-nitrosamine (such as NNAT) from a secondary amine (such as atrazine) and nitrite.



*N*-nitrosoatrazine

**Figure 2.** Structure of *N*-nitrosoatrazine, the main compound of interest.

## Atrazine

Atrazine is a chlorinated triazine herbicide used to control broadleaf and grass weeds. It is widely used in agriculture, mostly in field corn. It is also permitted on roadsides and in warm season turfgrasses, such as golf courses or residential areas. In the United States, through the Safe Drinking Water Act, atrazine is monitored in drinking water and ecosystems to determine exposure. The current maximum contaminant level (MCL) in drinking water for atrazine is 0.003 µg/mL (6). According to the U.S. Environmental Protection Agency (EPA), the main toxicological impacts are neuroendocrine effects (7). According to Rickard et al., the main

concern is the parent compound (atrazine) and three of its metabolites: deethylatrazine (DEA), deisopropylatrazine (DIA), and didealkylatrazine (DDA) (8). Joo et al. (9) determined that humans can metabolize atrazine in the liver. The major metabolites present in human liver microsomes included DEA and DIA, but NNAT was not assessed. Humans can be exposed to atrazine through some foods and drinking water, along with occupational exposures. A 2018 EPA report (8) concluded that occupational exposure to atrazine often results in dermal and inhalation risk estimates that are of concern, even when the handler is wearing long sleeves, long pants, shoes, socks, and chemical gloves. Other herbicides similar to atrazine in structure and reactivity include propazine and simazine.

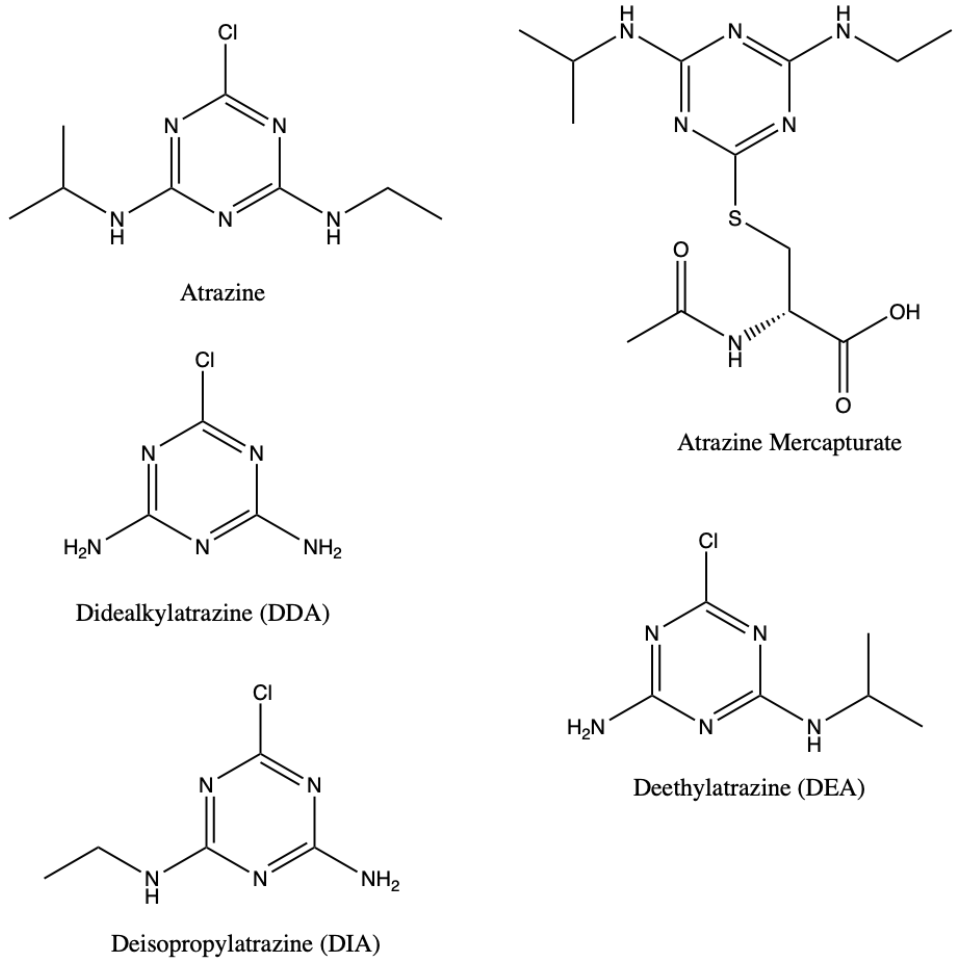



**Figure 3.** The structure of atrazine and its metabolites included in this study.

## Teratogenicity/Toxicity

### Nitrosamines

The Centers for Disease Control and Prevention (CDC) reports that defects occur in about one in every 33 U.S. births (10). A 2017 study (11) determined that NNAT delayed chicken embryo development and increased the occurrence of defects. The author concluded that NNAT (0.26, 0.58, and 0.70 μmol) in the developing embryos was associated with smaller gestational stages (weight less than 0.17 g), and embryos with smaller gestational stages were likely to have malformations (11). A 2018 paper (12) reviewed the impacts of nitrate on human health. The authors found that most studies since 1980 concluded that higher exposure to nitrate during pregnancy led to birth defects. A 2009 study on human placentas reported that when the mother is exposed to nitrosodimethylamine, the fetus is likely exposed and endangered (13). Blaisdell et al. (14) connected nitrate in drinking water to limb deficiencies, congenital heart defects, and neural tube defects. However, Brender et al. (15) found no association between nitrate in drinking water and birth defects. Some nitrosamines are also considered carcinogenic. In a 1977 study, Mirvish (16) recommended nitrosamine monitoring due to the potential for nitrosamines to cause cancer in humans. The study found that nitrosamines induced pancreatic tumors in hamsters. This caused the author to look further into the possibility that nitrosamines are a cause of pancreatic cancer in humans in the U.S. Another 1978 research paper determined that nitrosamines, such as *N*-nitrosodiethylamine, had carcinogenic effects in cats and chickens. The liver was the organ where these effects occurred most often (17). Magee and Barnes (18) found that *N*-nitrosodimethylamine caused liver tumors in 19 of 20 rats.

### Pesticides

Lin et al. (19) developed a model to predict transfer of atrazine and its metabolites in rats and mice. They determined there was potential placental transfer of atrazine and its metabolites, and that the fetus would be exposed to about the same levels as the mother. Ibrahim et al. (20) reported that atrazine (as low as 1 μmol/mL) caused apoptosis and necrosis in placental cells. Atrazine also inhibited and changed the growth of the cells, including a decreased P53 (tumor suppressor) expression. A 2017 California study reported that the population falling within the high exposure range of pesticides applied (about 4200 kg) for agricultural use during gestation led to a 9% increase in birth defects in the San Joaquin Valley of California (21). The authors stated that the average annual pesticide use between 1997 and 2011 was about 975 kg per 2.6 $km^2$ area. In the high exposure group, there was a decrease in birth weight (about 0.4%), decrease in gestational length (about 0.1%), and an increase in the probability of premature birth (about 8%). Roustan et al. (22) found that atrazine and pesticide mixtures of pesticides damaged chromosomes in Chinese hamster ovary cells and in human lymphocytes. They concluded that exposure to pesticide mixtures containing atrazine, glyphosate, aminomethyl phosphoric acid, and deethylatrazine cause damage to DNA at lower concentrations than a single pesticide. Rhoades et al. (23) reported an increased risk of developing non-Hodgkin lymphoma when both nitrate and atrazine are present in drinking water. They hypothesized that the formation of NNAT was the cause of the carcinogenesis. A 2021 study (24) linked pediatric cancer in Nebraska to atrazine surface and groundwater concentrations. The authors found 4.6% of groundwater samples taken between April and October in 1987 through 2016 exceeded the EPA MCL (0.003 μg/mL) for atrazine.

## Pesticide Use/Problem

The EPA released a pesticide report in 2017, based on 2008-2012 usage (25). In 2012, approximately 387 million kilograms of pesticide active ingredients were applied in the U.S. and about 90% were used in agriculture. Atrazine was the second most used pesticide in 2012. A United States Geological Survey preliminary report (26), estimated 32 to 33 million kilograms of atrazine were used in 2019. Buckley et al. (27) found high exposure to herbicides, fungicides, and insecticides among pregnant women in the U.S. Seventy-three of 89 analytes, including atrazine, were detected in the urine of at least one expectant mother participating in the study. Ninety percent of urine samples contained analytes not currently included in the National Health and Nutrition Examination Survey, which monitors chemicals in the U.S. Their study showed that the population is exposed to pesticides and other chemicals by agriculture and home use.

Atrazine is a pesticide frequently detected in American water sources. A 2020 Nebraska Groundwater Quality Monitoring Report (28) stated that atrazine and DEA exceeding the reporting limit were found in about 21% and 26% of monitoring and irrigation wells sampled, respectively. Nitrate exceeded the reporting limit in nearly 92% of wells monitored. Atrazine has been banned in the European Union but is still used in the U.S. However, the EPA proposed more regulations for atrazine in June 2022 (29). If issued, application of atrazine will be restricted to specific times depending on environmental conditions. Jablonowski et al. (30) found metabolites of atrazine in water in some countries years after it was banned. They concluded that continuous use created large accumulations of atrazine in the soil. They also stated a need for continuous monitoring and more research on the effects of atrazine and its metabolites on humans.

## Placental Transfer

Some pesticides have been found to transfer from mother to fetus. This occurs when substances are exchanged between the mother's and fetus's blood circulation. The rate of transport depends on the properties of the substance, such as lipid solubility and molecular weight. Low molecular weight substances tend to cross the placental barrier faster; however, larger molecules can also be transferred. A 2020 study in Denmark (31) studied placental transfer of two fungicides (propiconazole and bitertanol) and one insecticide (cypermethrin) in human placental perfusions. The authors concluded that the two fungicides were transferred across the placenta at a faster rate than the insecticide because of passive diffusion of the fungicides. Metabolites of these pesticides were also detected in the placenta, and concentrations increased over time. Fraites et al. (32) studied the effects and transfer of atrazine after exposure *in utero*. They detected DIA, DEA, and DDA in the fetus at concentrations similar to those found in the mother. They concluded that the placenta did not hinder the transfer of compounds to the fetus.

## *N*-Nitrosoatrazine Extraction and Analysis Methods

Extraction and analysis methods for many nitrosamines have been used previously. Gas chromatography (GC), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) are used to quantify nitrosamines and pesticides. Beeson et al. (33) developed an HPLC-MS method for detecting levels of atrazine in urine. The method has sufficiently high specificity and low limits of detection (approximately 0.0005 µg atrazine/mL of urine) for quantifying pesticide exposure. The method can also be used for atrazine mercapturate, an important glutathione-derived metabolite of atrazine. A 2020 study (34) used HPLC paired with photochemical reaction and chemiluminescence detection to analyze *N*-nitrosamines in water

samples. The method reporting limits for the *N*-nitrosamines ranged from $1.2 \times 10^{-6}$ to $5.0 \times 10^{-6}$ µg/mL, which were similar or slightly better than published methods using mass spectrometry. Sepehr et al. (35) developed an extraction and analytical method for quantifying tobacco-specific *N*-nitrosamines in urine using liquid chromatography tandem mass spectrometry (LC-MS-MS). This method had greater selectivity and sensitivity than previous methods. The extraction method used by Hu et al. (36) is one of the few available for *N*-nitrosamines in a blood or tissue sample. They successfully extracted and quantified the analyte using HPLC-MS. The limits of detection were 0.0003 µg/mL for NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) and 0.0002 µg/mL for NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol). Wei et al. (2) successfully quantified NNAT in soil using HPLC.

## PROJECT OBJECTIVES

To my knowledge, few methods for measuring NNAT in tissue samples have been published. The first paper (5) was published in 1980 and determined the *in vivo* and *in vitro* conversion of atrazine to NNAT. The recovery of NNAT in that method was only 48 ± 13%. The second paper (37) was a study on NNAT and atrazine in the urine and liver of rats. The detection limit was 2.0 ng, and the recovery was 69 ± 7.0%. The goal of the present study was to develop a method for extraction and analysis of *N*-nitrosoatrazine, atrazine, and atrazine metabolites in blood and tissue samples from F344 rodents. Atrazine metabolites are present in the environment, and atrazine mercapturate can be produced *in vivo,* therefore it is important to quantify those compounds as well. The method will have potential use in future research on NNAT and other nitrosatable compounds. It is envisioned that the method developed herein could be used to determine NNAT transfer across the placental barrier in F344 rodents and to further investigate the potential cause of fetal malformations reported by other researchers. It is envisioned that the

method developed herein could be used to determine NNAT transfer across the placental barrier in F344 rodents and to further investigate the potential cause of fetal malformations reported by other researchers.

## MATERIALS AND METHODS

Due to an instrument failure during this study, two LC-MS-MS instruments were used. This section is divided into the analyses performed on a Waters Quattro LC-MS-MS and those performed later on a Xevo TQS micro LC-MS-MS both manufactured by Waters Corporation and housed at the University of Nebraska Water Sciences Laboratory.

### Chemical Reagents and Animals

The following procedures were carried out under a fume hood, due to the carcinogenic nature of the compounds. Protective equipment, including safety gloves, glasses, and a lab coat, were used. NNAT was purchased from Toronto Research Chemicals, Inc. (Toronto, ON, Canada); DIA, DEA, terbuthylazine and sodium chloride from Sigma-Aldrich (St. Louis, MO, USA); DDA and atrazine from Chem Service (West Chester, PA, USA); atrazine mercapturate from Cambridge Isotope Laboratories (Tewksbury, MA, USA); ammonium formate from Honeywell (St. Muskegon, MI); and $^{13}C_3$ atrazine (stable isotope label at C-3) from MSD Isotopes (Rahway, NJ, USA). Acetonitrile was purchased from Fischer Chemical (Fair Lawn, NJ, USA). Rodent tissue and blood samples were provided by Dr. Eric Peeples and Dr. Katie Kim at the University of Nebraska Medical Center (stored in a -80 °C freezer until used).

## Waters Quattro LC-MS-MS

### Instrument Conditions:

The samples analyzed on the Waters Quattro LC-MS-MS were separated using a HyPurity C-18 column (250 mm × 2.1 mm, 5 µm film) at 50 °C with a 25 µL injection at a flow rate of 0.2 mL/min. The mobile phases for the HPLC were (A) 0.1% formic acid in water and (B) methanol. The gradient was: initial concentration 95:5 (A:B) for 2 min, linearly changed to 60:40 (A:B) at 9 min and 20:80 (A:B) at 15 min, 100% B for 8.5 min, and then returned to initial concentration (95:5) for 6.5 min. The total run time was 30 min. The tandem quadrupole mass spectrometer was in positive electrospray (ES+) mode. Table A-3 shows the parameters of each analyte, internal standard, and surrogate.

### Solution Preparation:

Stock analyte solutions were prepared from solids as received, except for atrazine mercapturate, DIA, DEA, atrazine, terbuthylazine, and $^{13}C_3$ atrazine, which had already been prepared in solution. Analyte and internal standard mixtures were prepared from the stock solutions to determine an LC-MS instrument detection limit (IDL) on the Quattro LC-MS. Analyte and internal standard mixtures were prepared to spike samples and for instrument calibrations. Calibration solutions were used to produce a calibration curve and determine the instrument detection limit.

*Stock solutions*: A DDA stock solution was prepared by weighing out 0.00190 g DDA and mixing with about 3.80 mL of methanol (3.00732 g) to obtain a final concentration of 500.0 ng/µL. This was done using an analytical balance that went to five decimal places. The solid was placed on the balance in a vial, the balance was zeroed, methanol was added to the solid, and

allowed to evaporate to the exact mass. An NNAT stock solution was prepared by weighing out 0.01912 grams of NNAT and mixing with about 3.82 mL of methanol (3.02631 g) to obtain a final concentration of 5000.0 ng/µL. DIA, DEA, atrazine, terbuthylazine, and $^{13}C_3$ atrazine were already in solution at a concentration of 5000.0 ng/µL. Atrazine mercapturate was also in solution at a concentration of 100.0 ng/µL.

*Analyte mixtures*: Two analyte mixtures (A and B) were prepared containing DDA, DIA, DEA, NNAT, atrazine, and atrazine mercapturate. Both mixtures were subjected to a series of dilutions to prepare a range of concentrations from 1.0 to 100.0 ng/µL. Analyte mixture A (100.0 ng/µL) was prepared by mixing 1000 µL of DDA and 100 µL of DIA, DEA, NNAT, atrazine, and terbuthylazine with methanol to volume in a 5 mL volumetric flask. It was then diluted to 1.00 ng/µL by mixing 100 µL of the original mixture A and 100 µL of atrazine mercapturate with methanol to volume in a 10.0 mL volumetric flask. Analyte mixture B (100.0 ng/µL) was prepared by mixing 1000 µL of DDA and 100 µL of DIA, DEA, NNAT, and atrazine with methanol to volume in a 5.00 mL volumetric flask. It was then diluted to 1.00 ng/µL by mixing 100 µL of the original mixture B and 100 µL of atrazine mercapturate with methanol to volume in a 10.00 mL volumetric flask. The final analyte concentration in both mixtures was 1.00 µg/µL. This analyte mixture was used to prepare IDL calibration solutions. It was diluted once more for the correct spike concentration. The analyte spike was prepared by mixing 200 µL of analyte mixture A with acetonitrile to volume in a 10.00 mL volumetric flask. The final concentration of the spike solution was 0.02 ng/µL.

*Internal standard mixture*: An internal standard mixture (100.00 ng/µL) was prepared by mixing 100 µL of $^{13}C_3$ atrazine with methanol to volume in a 5.00 mL volumetric flask. It was then diluted by mixing 500 µL of the original mixture with methanol to volume in a 5.00 mL

volumetric flask. The final internal standard mixture concentration was 10.00 ng/µL. This internal standard mixture was used for the IDL calibration solutions. It was diluted once more to the correct spiking concentration. The internal standard spike was prepared by mixing 400 µL of internal standard mixture with acetonitrile to volume in a 10.00 mL volumetric flask for a final concentration of 0.40 ng $^{13}C_3$ atrazine/µL.

*Calibration solutions*: Five calibration solutions ranging from 0.01 to 0.10 ng/µL were prepared using analyte mixture A, internal standard mixture, distilled deionized water, and methanol (Table 1). The final internal standard concentration in the IDL calibration solutions was 0.10 ng/µL.

**Table 1.** Amounts of analyte, internal standard, water, and methanol added to prepare calibration solutions and blanks for the Quattro LC-MS.

| ***Calibration Solution*** | ***Analyte Mix A (µL)*** | ***Internal Standard Mix (µL)*** | ***Water (µL)*** | ***Methanol (µL)*** |
|---|---|---|---|---|
| *Blank* | 0 | 0 | 7500 | 2500 |
| *1 (0.10 ng/µL)* | 1000 | 100 | 7500 | 1400 |
| *2 (0.075 ng/µL)* | 750 | 100 | 7500 | 1650 |
| *3 (0.05 ng/µL)* | 500 | 100 | 7500 | 1900 |
| *4 (0.02 ng/µL)* | 200 | 100 | 7500 | 2200 |
| *5 (0.01 ng/µL)* | 100 | 100 | 7500 | 2300 |

*Extraction buffer*: A 500 mM ammonium formate buffer was prepared by dissolving 3.15 g of ammonium formate in 100.0 mL of distilled deionized water.

**Extraction and Recovery Comparison:**

To develop an extraction method with optimal recovery of the analytes of interest, the methods of Hu et al. (36) and Wei et al. (2) were compared. The sample weights used for each type of tissue and method are reported in Table A-1. All samples were homogenized in 250 µL of distilled water and stored at -80 °C. The samples were then placed in a fume hood for 15 min to

thaw. Samples for both methods were spiked with 150 µL of analyte mix A (NNAT, DDA, DIA, DEA, ATZ, atrazine mercapturate, and terbuthylazine). After 1 hour, acetonitrile (460 µL) was added to each sample and mixed by vortexing.

**Extraction Method 1:**

As described by Hu et al., 20 µL of the ammonium formate buffer was added to each sample after the acetonitrile addition. The samples were vortexed and centrifuged for 20 min. at 6000 rpm. The supernatant was transferred to a clean 1.5-mL centrifuge tube and the 20-min centrifuging was repeated. The extract was then filtered using a 0.2- µm PVDF syringe filter into a clean 1.5-mL centrifuge tube and 115 µL of internal standard mixture was added. The extracts were transferred to LC-MS-MS vials for analysis.

**Extraction Method 2:**

As described by Wei et al., the samples processed using this procedure were placed in the Barnstead Max$^{Q}$ 4000 shaker at 200 rpm for 1 h after the addition of acetonitrile. The samples were then centrifuged at 5000 rpm for 15 min. The supernatant was transferred to a clean 1.5-mL centrifuge tube and centrifuged again at 5000 rpm for 15 min. There was no pellet remaining and the extracts were filtered through a 0.2- µm PVDF syringe filter, collected in a clean centrifuge tube, and 115 µL of internal standard mixture was added. Extracts containing internal standard were transferred to LC-MS-MS vials for analysis.

For the extraction and recovery comparison, method blanks were prepared following the same steps, but without the addition of analyte.

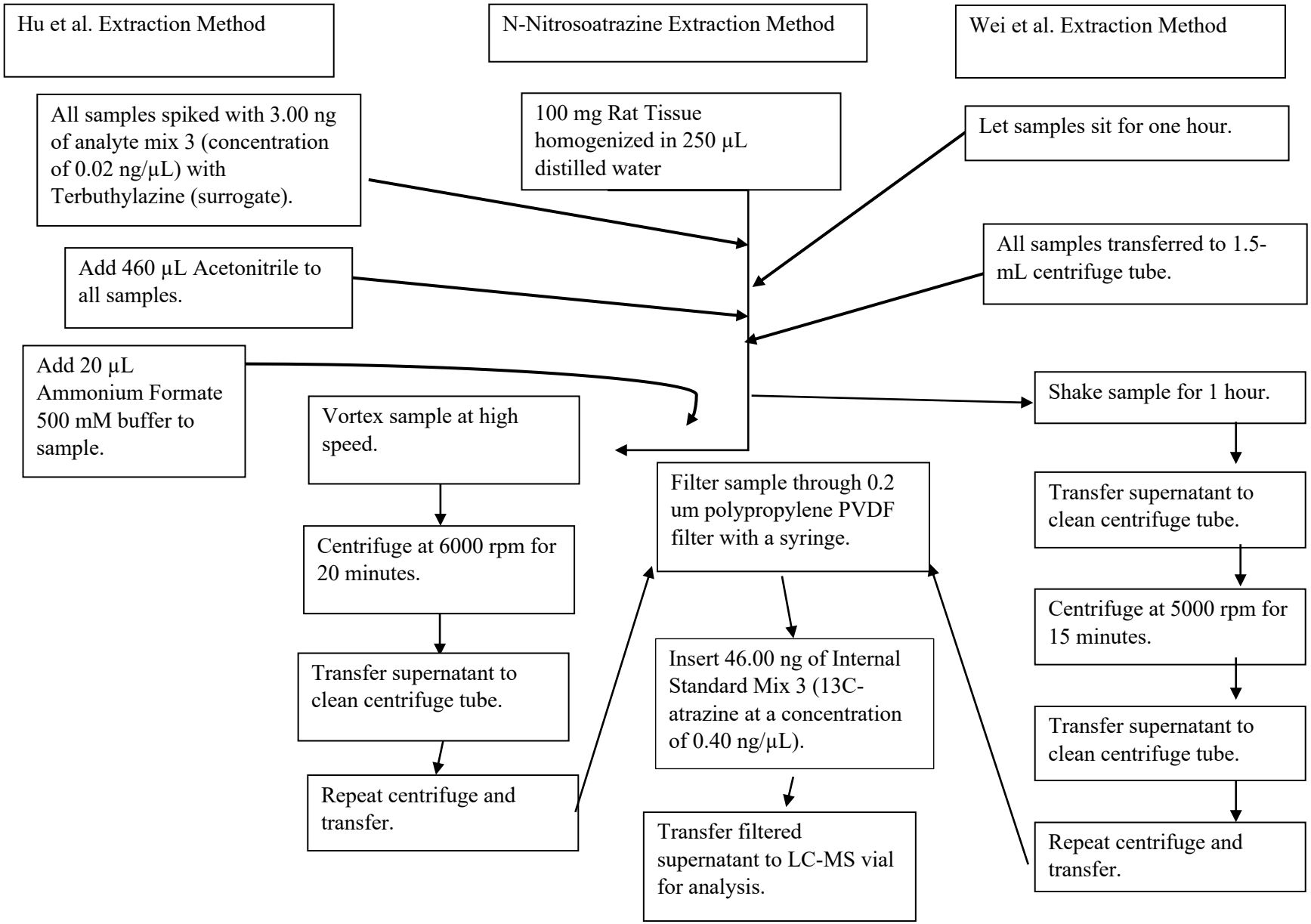



**Figure 4.** Flow chart of procedure for method comparison test.

All samples and method blanks were placed on the Waters Quattro LC-MS-MS for analysis. Blanks of acetonitrile, spiking solution, and internal standard were also included. Calibration standards were included in the sample run for quality control.

## Xevo TQS micro LC-MS-MS

Due to instrument failure after the recovery test was performed, a new LC-MS-MS Xevo TQS micro was used for further analysis. Since a new instrument was being used, an instrument detection limit had to be determined. DDA was also included in this analysis because the new instrument was more sensitive than the Waters Quattro LC-MS-MS and was better able to detect this compound. The following sections outline the methods used for this new instrument.

**Instrument Conditions:**

The samples analyzed on the Xevo TQS micro LC-MS-MS were separated using an Acquity UPLC BEG C-18 reverse phase HPLC column (50 mm × 2.1 mm, 1.7 µm film) at 40 °C with a 2 µL injection volume at a flow rate of 0.6 mL/min. The mobile phases for the HPLC were (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The gradient was: initial concentration of 95:5 (A:B), gradually increased to 5:95 (A:B) until 3 min, hold for 0.5 min, and return to initial concentration (95:5). The total run time was 5 min. The mass spectrometer was in UniSpray in positive ion (+) mode. Table A-4 shows the parameters of each analyte, internal standard, and surrogate. Example chromatograms are reported in Figures A-1 and A-2.

**Solution Preparation:**

*Stock solutions*: $^{13}C_3$ atrazine solution was prepared by dissolving 0.00445 g in about 4.49 mL of methanol (3.55703 g) for a final concentration of 1.00 µg/µL. Terbuthylazine solution was

prepared by dissolving 0.00196 g in about 1.96 mL of methanol (1.54817 g) for a final concentration of 1.00 µg/µL. Atrazine solution was prepared by dissolving 0.00208 g in about 2.08 mL of methanol (1.64354 g) for a final concentration of 1.00 µg/µL. DEA solution was prepared by dissolving 0.00291 g in about 2.91 mL of methanol (2.30297 g) for a final concentration of 1.00 µg/µL. DDA solution was prepared by dissolving 0.00101 g in about 1.01 mL of methanol (0.79836 g) for a final concentration of 1.00 µg/µL. DIA solution was prepared by dissolving 0.00111 g in about 1.11 mL of methanol (0.87908 g) for a final concentration of 1.00 µg/µL. NNAT solution was prepared by dissolving 0.00263 g in about 2.63 mL of methanol (2.08138 g) for a final concentration of 1.00 µg/µL. Atrazine mercapturate was already in solution at a concentration of 0.100 µg/µL.

*Internal Standard Mixture and Spike*: For the internal standard mixture, 10 µL of $^{13}C_3$ atrazine stock solution was added to a 10.00 mL volumetric flask and it was filled to volume with methanol for a final concentration of 1.00 ng/µL. For the internal standard spike, 5 µL of $^{13}C_3$ atrazine stock solution was added to a 50.00 mL volumetric flask and it was filled to volume with methanol for a final concentration of 0.10 ng/µL. An internal standard spike was prepared for the second method detection limit test by diluting the original with methanol to 0.80 ng/µL.

*Surrogate Spike*: Terbuthylazine stock solution (5 µL) was added to a 50.00 mL volumetric flask and filled to volume with methanol for a final concentration of 0.10 ng/µL.

*Analyte Mixture and Spike*: For the analyte mixture, 10 µL of terbuthylazine, atrazine, DEA, DDA, DIA, and NNAT stock solutions were added to a 10.00 mL volumetric flask, along with 100 µL of atrazine mercapturate stock solution, and diluted to 10.00 mL with methanol. The final concentration of each analyte was 1.00 ng/µL. For the spike, 5 µL of atrazine, DEA, DDA, DIA, and NNAT stock solutions were added to a volumetric flask, along with 50 µL of atrazine

mercapturate stock solution, and diluted to 50.00 mL with methanol. The final concentration of the analyte spike was 0.10 ng/µL. The analyte spike was further diluted with methanol to 0.001 ng/µL to use in the method detection limit test. A second and third method detection limit tests were performed for which the analyte spike was prepared by diluting the original analyte spike with methanol to 0.005 ng/µL.

*Calibration solutions*: Six calibration solutions ranging from 0.10 to 50.00 pg/µL and a blank were prepared using the internal standard mixture, analyte mixture, deionized-distilled water, and methanol (Table 2). The final internal standard concentration in all calibration solutions was 20.00 pg/µL.

**Table 2.** Amounts of analyte, internal standard, water, and methanol added to prepare calibration solutions and blank for the Xevo TQS micro LC-MS-MS.

| ***Calibration Solution*** | ***Analyte Mix (µL)*** | ***Internal Standard Mix (µL)*** | ***Water (µL)*** | ***Methanol (µL)*** |
|---|---|---|---|---|
| *Blank* | 0 | 200 | 8000 | 1800 |
| *1 (50.00 pg/µL)* | 500 | 200 | 8000 | 1300 |
| *2 (20.00 pg/µL)* | 200 | 200 | 8000 | 1600 |
| *3 (10.00 pg/µL)* | 100 | 200 | 8000 | 1700 |
| *4 (5.00 pg/µL)* | 50 | 200 | 8000 | 1750 |
| *5 (1.00 pg/µL)* | 10 | 200 | 8000 | 1790 |
| *6 (0.10 pg/µL)* | 1 | 200 | 8000 | 1799 |

**Extraction Method Detection Limit Test:**

After determining that extraction method 1 (from Hu et al.) had better recovery of NNAT (27%, Table 4), initial method detection limit tests were performed using sand. Additional tests with blood and tissue samples will be needed to determine the acceptability of the method for rat blood and tissue. Eight replicates were prepared by weighing out about 100 mg of sand and adding 250 µL of deionized distilled water. All samples were spiked with 0.20 ng of analyte

spike (at a concentration of 0.001 ng/µL) and 10.00 ng of surrogate spike. Acetonitrile (460 µL) and 20 µL of 500 mM ammonium formate buffer were added to all samples. They were vortexed and centrifuged at 5000 rpm for 20 min. Supernatants were transferred to clean centrifuge tubes, again centrifuged at 5000 rpm for 20 min, filtered through a 0.2-um PVDF syringe filter into clean centrifuge tubes, and spiked with 10.00 ng of internal standard spike. All samples were transferred to LC-MS vials for analysis. An unspiked ninth replicate of sand and deionized distilled water was prepared as a blank, following the same procedure.

A second method detection limit determination was performed due to low recovery of NNAT (37%, Table 6) in the first and no detection of DDA or DIA. Samples were prepared in the same way as the first, except they were spiked with 0.50 ng of analyte and 80.00 ng of internal standard.

A third method detection limit test was performed due to even lower recovery of NNAT (11%, Table 7). A new flow chart was created in which water and acetonitrile volumes were increased, and sodium chloride was added. The exact procedure is reported in Figure 5.

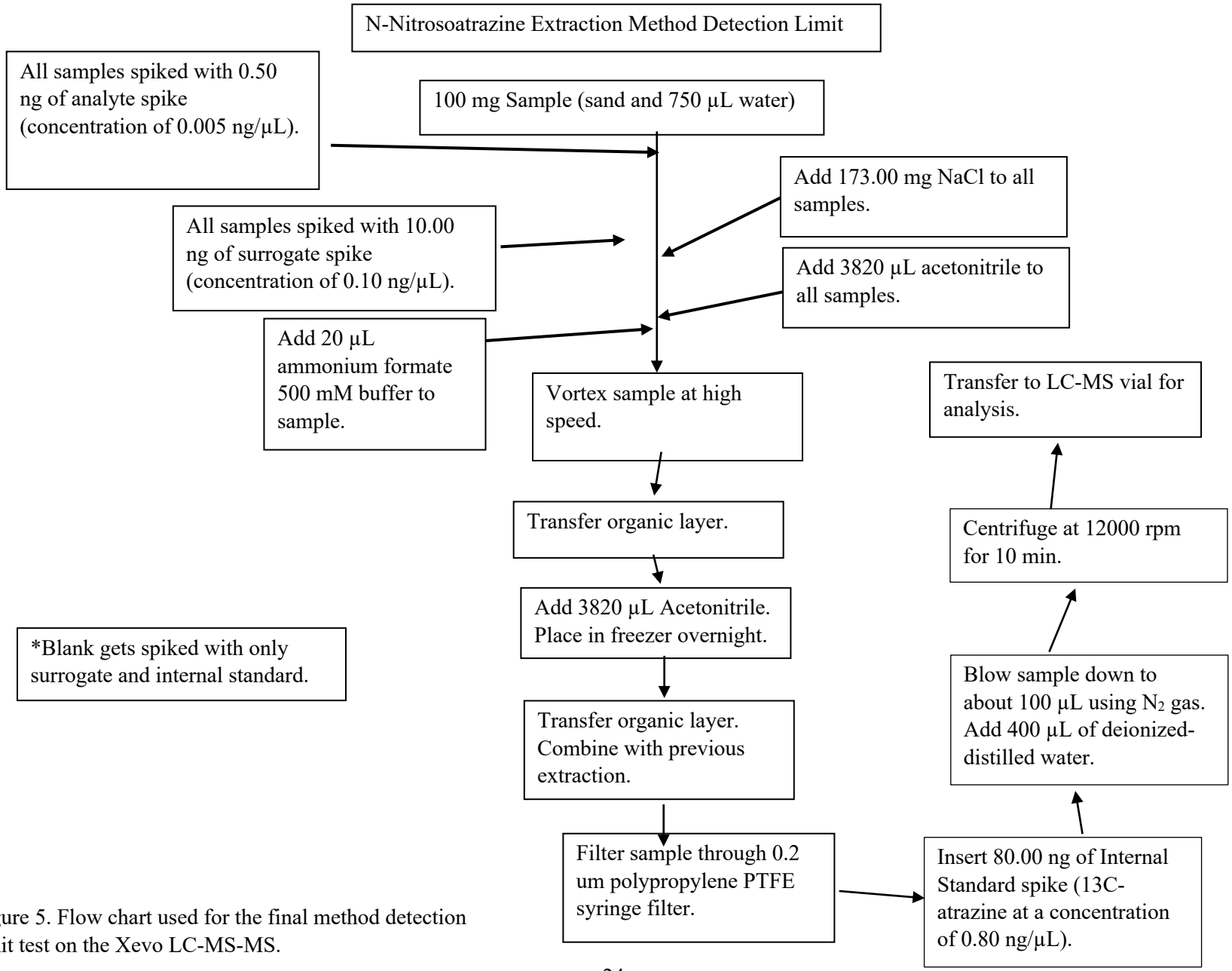



Figure 5. Flow chart used for the final method detection limit test on the Xevo LC-MS-MS.

## Statistical Analysis

Instrument Detection Limit: To determine the Waters Quattro LC-MS-MS instrument detection limit (IDL), calibration solution five (0.01 ng/µL) was analyzed eight times. To determine the Xevo TQS micro LC-MS-MS IDL, calibration solution six (0.10 pg/µL) was analyzed eight times. The mean and standard deviation for analyte measured by the instrument were determined. The IDLs were calculated as follows (38):

$$DL = s \cdot t\,value \quad \textbf{[1]}$$

Equation [1] is used to determine the IDL, where s is the standard deviation and DL is the detection limit in picograms. The t value is based on the number of samples, and this t value is for a 99% confidence interval. The t values are listed in Table A-2. Equation [1] can also be used to determine the method detection limit (MDL) in ng/g by using the original weight of the sample and finding the concentration:

$$concentration = measured \cdot m \quad \textbf{[2]}$$

Equation [2] is used to determine the concentration of the measured analyte, where concentration is in ng/g, measured is the value obtained from the instrument, and m is the mass of the sample in g. After finding the concentration of all measurements, the standard deviation is calculated and used in equation [1] to determine a detection limit in ng/g.

Extraction and Recovery Comparison: To determine the appropriate amount of analyte for spiking the samples, the detection limit was converted to a concentration (equation [2]) and multiplied by the amount of extract solvent (460 µL) used. This amount was multiplied by three (by convention) to be certain the instrument would detect analyte. After conducting both

extraction methods and analyzing the samples using the Waters Quattro LC-MS-MS, percent recovery was determined as follows:

$$\%\, R \;=\; \left(\frac{measured}{actual}\right) \cdot 100 \qquad \textbf{[3]}$$

Equation [3] is used to determine percent recovery, where % R is the recovery, measured is the mass of the analyte in nanograms as detected by the instrument, and actual is the mass of analyte (nanograms) used to spike the samples. This equation was also used to determine the percent recovery for the detection limit determinations. After percent recovery was found for each sample, the average percent recovery was determined.

# RESULTS

## Waters Quattro LC-MS-MS IDL

The average detection of NNAT in the calibration samples was 261 ± 16.0 pg. The average detection of DEA was 259 ± 8.00 pg. The average detection of DIA was 235 ± 10.0 pg. The average detection of atrazine mercapturate was 232 ± 13.0 pg. The average detection of atrazine was 251 ± 4.00 pg. Table 3 reports the instrument detection limit and average percent recovery for each analyte using equations [1] and [3].

**Table 3.** Waters Quattro LC-MS-MS instrument detection limit and average percent recovery for each analyte.

| ***Analyte*** | ***IDL (pg)*** | ***Average Recovery (%)*** |
|---|---|---|
| *Atrazine* | 10.8 | 100 ± 1.45 |
| *Atrazine mercapturate* | 40.3 | 93.0 ± 5.38 |
| *DEA* | 25.1 | 103 ± 3.35 |
| *DIA* | 31.4 | 94.0 ± 4.19 |
| *NNAT* | 48.4 | 105 ± 6.46 |

## Extraction and Recovery Comparison

Using the IDL for NNAT (48.4 pg), the amount of analyte to spike into the rat tissue was determined using the calculations from the statistical analysis section. The amount spiked was calculated to be 3.00 ng of a 0.02 ng/µL analyte mixture (150 µL). The recovery of each analyte in both methods was calculated using equation [3]. The average recovery of NNAT in method 1 (from Hu et al.) is 26.8%, with a standard deviation of 14.5. The average recovery of NNAT in method 2 (from Wei et al.) is 23.9%, with a standard deviation of 21.6. Table 4 shows the average detection and average percent recovery of each analyte. The average detection and percent recovery for each analyte and tissue sample is reported in Table A-5.

**Table 4.** Average detection and recovery for each analyte and method using the Waters Quattro LC-MS-MS instrument.

| *Analyte* | *Method* | *Average Detection (ng)* | *Average Recovery (%)* |
|---|---|---|---|
| *Atrazine* | 1 | 2.85 | 95.0 ± 12.6 |
| | 2 | 2.67 | 89.1 ± 17.0 |
| *Atrazine mercapturate* | 1 | 1.83 | 61.0 ± 13.0 |
| | 2 | 1.77 | 58.9 ± 15.5 |
| *DEA* | 1 | ND* | ND* |
| | 2 | ND* | ND* |
| *DIA* | 1 | ND* | ND* |
| | 2 | ND* | ND* |
| *NNAT* | 1 | 0.804 | 26.8 ± 14.5 |
| | 2 | 0.718 | 23.9 ± 21.6 |

*ND: no detection

## Xevo TQS micro LC-MS-MS IDL

The average detection of NNAT in calibration solution six (0.10 pg/µL) was 0.145 ± 0.005 pg. The average detection of atrazine was 0.222 ± 0.0200 pg. The average detection of atrazine

mercapturate was 0.675 ± 0.0300 pg. The average detection of DDA was 0.222 ± 0.100 pg. The average detection of DEA was 0.160 ± 0.0100 pg. The average detection of DIA was 0.157 ± 0.0200 pg. Table 5 shows the instrument detection limit and average percent recovery for each analyte calculated using equations [1] and [3].

**Table 5.** Xevo TQS micro LC-MS-MS instrument detection limit and average percent recovery for each analyte.

| *Analyte* | *IDL (pg)* | *Average Recovery (%)* |
|---|---|---|
| *Atrazine* | 0.0725 | 111 ± 12.1 |
| *Atrazine mercapturate* | 0.0767 | 338 ± 12.8 |
| *DDA* | 0.285 | 111 ± 47.5 |
| *DEA* | 0.0259 | 80.0 ± 4.31 |
| *DIA* | 0.0644 | 78.6 ± 10.2 |
| *NNAT* | 0.0157 | 72.4 ± 2.50 |

## Xevo TQS micro LC-MS-MS Extraction MDL

MDL 1: Using method 1 and the IDL for NNAT on the Xevo TQS micro LC-MS-MS (0.02 pg), the amount of analyte spike was determined from the calculations explained in the statistical analysis section. The amount spiked was calculated to be 0.20 ng of a 0.001 ng/µL analyte spike (200 µL). The method detection limit (MDL) was calculated using equations [1] and [2]. The recovery of each analyte was calculated using equation [3]. Table 6 reports the method detection limit and average percent recovery of each analyte.

**Table 6.** Extraction method detection limit and average percent recovery for each analyte (0.2 ng of 0.001 ng analyte/µL spike) using the Xevo TQS micro LC-MS-MS instrument.

| *Analyte* | *MDL (ng/g)* | *Average Recovery (%)* |
|---|---|---|
| *Atrazine* | 1.22 | 302 ± 20.3 |
| *Atrazine mercapturate* | 1.03 | 249 ± 17.2 |
| *DDA* | ND* | ND* |
| *DEA* | 1.00 | 234 ± 16.7 |
| *DIA* | ND* | ND* |
| *NNAT* | 0.327 | 36.7 ± 5.20 |

*ND: no detection

MDL 2: Using method 1 and increasing the analyte spike amount to 0.50 ng and the internal standard to 80.00 ng, the method detection limit test was repeated to improve recovery of DDA, DIA, and NNAT. The MDL was calculated using equations [1] and [2], and the percent recovery was calculated using equation [3]. Table 7 reports the MDL and average percent recovery of each analyte.

**Table 7.** The extraction method detection limit and average percent recovery for each analyte (0.5 ng of 0.001 ng analyte/µL spike) as analyzed on the Xevo TQS micro LC-MS-MS.

| *Analyte* | *MDL (ng/g)* | *Average Recovery (%)* |
|---|---|---|
| *Atrazine* | 0.681 | 116 ± 4.05 |
| *Atrazine mercapturate* | 2.16 | 116 ± 13.7 |
| *DDA* | 3.50 | 93.4 ± 23.3 |
| *DEA* | 0.556 | 72.1 ± 3.30 |
| *DIA* | 3.18 | 126 ± 21.2 |
| *NNAT* | 0.0313 | 10.8 ± 0.167 |

MDL 3: Repeating the procedure and adding 173.00 mg sodium chloride and increasing the volume of water (750 µL) and acetonitrile (3820 µL), the method detection limit was determined. The MDL was calculated using equations [1] and [2], and the percent recovery was

calculated using equation [3]. Table 8 reports the MDL and average percent recovery of each analyte.

**Table 8.** Xevo TQS micro LC-MS-MS extraction method detection limit and average percent recovery for each analyte with the addition of sodium chloride and larger volumes of water and acetonitrile.

| *Analyte* | *MDL (ng/g)* | *Average Recovery (%)* |
|---|---|---|
| *Atrazine* | 0.253 | 149 ± 29.4 |
| *Atrazine mercapturate* | 4.15 | 140 ± 28.6 |
| *DDA* | 6.31 | 153 ± 43.6 |
| *DEA* | 4.08 | 124 ± 28.2 |
| *DIA* | 2.05 | 135 ± 13.0 |
| *NNAT* | 1.46 | 75.4 ± 10.0 |

# DISCUSSION

The objective of this study was to develop extraction and analytical methods to quantify NNAT. Upon additional experiment with blood and tissue samples, this method could be used in rodent tissue and blood. This method could be used in further research on NNAT and other nitrosamines, and it will also be used in the next stage of this research to investigate the placental transfer of NNAT and atrazine. The instrument detection limit test performed on the Waters Quattro LC-MS-MS yielded recovery that was within the acceptable range of 80-120%. Throughout this study, there were average percent recoveries from the Xevo TQS micro LC-MS-MS higher than 120% and there were some around 300%, which is impossible. Those results could have been due to pipetting or matrix error. If there was less internal standard than what was assumed, the results would be falsely high. Based on the Waters Quattro LC-MS-MS IDL results, the extraction and recovery comparison was performed. DIA and DEA were not detected during this test. However, since they are not the main compounds of interest, the results did not

impact the decision on which method to use. This is a limitation as DIA and DEA are atrazine metabolites. NNAT can denitrosate to atrazine and then be further metabolized to DIA and DEA. Recovery of NNAT was not very high but other studies (5) also had recoveries below 80%. Therefore, it was determined that method 1 would be used since the average percent recovery of NNAT (26.8%) was higher than that in method 2 (23.9%).

The instrument detection limit test performed on the Xevo TQS micro LC-MS-MS also yielded recovery that was within the acceptable range of 80-120%. Atrazine mercapturate had a recovery exceeding 120%, but it is not one of the main compounds of interest, so it was determined that the result did not impact the instrument detection limit test. As previously mentioned, the high recovery could have been due to pipetting or matrix error. However, this would have affected all analytes, so it is possible that it is due to another error.

The first extraction method detection limit test performed yielded substantial recovery for atrazine, atrazine mercapturate, and DEA. Some of the recoveries were higher than 100%, again due to either pipetting or matrix error. DDA and DIA were not detected, and NNAT had a low recovery. Therefore, it was determined that a second extraction method detection limit test should be performed at a higher concentration of analyte and internal standard to improve recovery. The second MDL test yielded acceptable recovery for all analytes except NNAT (lower recovery). Therefore, a third extraction method detection limit test was performed to improve recovery. The third method increased the amount of water and acetonitrile added to better see the separation between the aqueous and organic layers. “Salting out” was added to the method to increase the recovery of analytes. The addition of a salt can be used to create better separation between acetonitrile and water during solvent extraction. Valente et al. found that sodium chloride was the optimum salt when separating acetonitrile and water (39). After adding

increased acetonitrile and water, along with salt, the separation between the two layers was easier to see. A second solvent extraction using acetonitrile was performed during this MDL test to ensure that all the analyte had been extracted. This time the solution had to be chilled in order to see the separation better. Since the volume of sample had been increased due to more water and acetonitrile, blow-down was required to get it to a volume that would fit inside an LC-MS-MS vial. After making all the changes, the recovery of NNAT from spiked sand improved to 75.4%. A comparison of recoveries between each method is reported in Table A-6. The other analytes in the final extraction MDL determination were slightly over 120% recovery, again due to either pipetting or matrix error. This could also have caused an inflation in the recovery of NNAT. In order to decrease this error, spike checks will be used in further research.

## CONCLUSION

The final method detection limit test resulted in acceptable recovery of NNAT spiked sand (Xevo TQS micro LC-MS-MS). Upon additional experiments with blood and tissue samples, this method could be used for the extraction and analysis of NNAT in rodent blood and tissue samples. The method could potentially be useful for further research and to investigate the placental transfer of NNAT and atrazine in rodent blood and tissue. The method had a detection limit of 1.46 ng/g and recovery of 75.4% for NNAT from sand. The detection limit for atrazine was 0.253 ng/g with a recovery of 149%.

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# APPENDIX

**Table A-1.** Tissue sample types, weights, and extraction methods used.

| *Sample Type* | *Sample Weight (mg)* | *Extraction Method* |
|---|---|---|
| *Rat Brain* | 100 | 1 |
| *Rat Brain* | 100 | 2 |
| *Rat Liver* | 100 | 1 |
| *Rat Liver* | 100 | 2 |
| *Rat Heart* | 100 | 1 |
| *Rat Heart* | 100 | 2 |
| *Rat Kidney* | 100 | 1 |
| *Rat Kidney* | 100 | 2 |
| *Rat Lungs (Sonicated)* | 100 | 1 |
| *Rat Lungs (Sonicated)* | 100 | 2 |
| *Rat Lungs (Homogenized)* | 100 | 1 |
| *Rat Lungs (Homogenized)* | 100 | 2 |
| *Mouse Placenta* | 132 | 1 |
| *Mouse Placenta* | 107 | 2 |
| *Mouse Fetal Tissue* | 51 | 1 |
| *Mouse Fetal Tissue* | 39 | 2 |

**Table A-2.** The t-values used for the number of samples analyzed on both the Quattro micro LC-MS-MS and Xevo LC-MS-MS.

| *Number of Samples* | *T-value (99% Confidence)* |
|---|---|
| *2* | 31.821 |
| *3* | 6.965 |
| *4* | 4.541 |
| *5* | 3.747 |
| *6* | 3.365 |
| *7* | 3.143 |
| *8* | 2.998 |
| *9* | 2.896 |
| *10* | 2.821 |
| *11* | 2.764 |

**Table A-3.** Parameters for each analyte, internal standard, and surrogate on the Waters Quattro LC-MS-MS.

| *Compound* | *MRM* | *Cone Energy (V)* | *Collision Energy (eV)* | *Retention Time (min)* |
|---|---|---|---|---|
| *$^{13}C_3$ Atrazine* | 219.1>177.05 | 33 | 17 | 20.8 |
| *Terbuthylazine* | 230.1>174.05 | 33 | 17 | 22.0 |
| *NNAT* | 245.05>215.05 | 20 | 10 | 22.0 |
| *Atrazine* | 216.05>174.05 | 33 | 17 | 20.8 |
| *DEA* | 188.05>145.95 | 33 | 17 | 17.3 |
| *DIA* | 174.05>95.90 | 32 | 17 | 14.6 |
| *Atrazine mercapturate* | 343.00>214.00 | 28 | 22 | 18.8 |

**Table A-4.** Parameters for each analyte, internal standard, and surrogate on the Xevo TQS micro LC-MS-MS.

| *Compound* | *MRM* | *Cone Energy (V)* | *Collision Energy (eV)* | *Retention Time (min)* |
|---|---|---|---|---|
| *$^{13}C_3$ Atrazine* | 219.117>97.966 | 46 | 32 | 2.75 |
| *Terbuthylazine* | 230.096>173.948 | 38 | 14 | 3.02 |
| *NNAT* | 244.984>215.043 | 24 | 8 | 3.01 |
| *Atrazine* | 215.994>96.085 | 16 | 24 | 2.75 |
| *DEA* | 187.964>145.95 | 20 | 22 | 2.09 |
| *DIA* | 173.88>96.028 | 24 | 18 | 1.71 |
| *Atrazine mercapturate* | 343.236>214.07 | 28 | 16 | 2.33 |
| *DDA* | 145.914>78.981 | 24 | 16 | 0.63 |

**Table A-5.** Results for each tissue analyzed on the Waters Quattro LC-MS-MS in the extraction and recovery comparison.

| ***Sample*** | ***Method*** | ***Atrazine*** | | ***Atrazine mercapturate*** | | ***DEA*** | | ***DIA*** | | ***NNAT*** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mass (ng) | Recovery (%) | Mass (ng) | Recovery (%) | Mass (ng) | Recovery (%) | Mass (ng) | Recovery (%) | Mass (ng) | Recovery (%) |
| *Brain* | 1 | 2.85 | 94.8 | 1.93 | 64.2 | 0 | 0 | 0 | 0 | 1.33 | 44.2 |
| | 2 | 3.08 | 103 | 2.17 | 72.4 | 0 | 0 | 0 | 0 | 0.449 | 15.0 |
| *Liver* | 1 | 2.13 | 70.9 | 2.34 | 77.8 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 1.83 | 70.0 | 1.39 | 46.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Heart* | 1 | 2.97 | 98.9 | 1.64 | 54.5 | 0 | 0 | 0 | 0 | 0.878 | 29.3 |
| | 2 | 2.13 | 71.1 | 2.00 | 66.6 | 0 | 0 | 0 | 0 | 0.39 | 58.6 |
| *Kidney* | 1 | 3.29 | 110 | 1.80 | 60.1 | 0 | 0 | 0 | 0 | 1.27 | 42.2 |
| | 2 | 2.46 | 82.0 | 2.53 | 84.5 | 0 | 0 | 0 | 0 | 1.76 | 9.74 |
| *Sonicated Lungs* | 1 | 2.85 | 95.0 | 1.19 | 39.8 | 0 | 0 | 0 | 0 | 0.585 | 19.5 |
| | 2 | 2.62 | 87.3 | 1.14 | 38.0 | 0 | 0 | 0 | 0 | 0.292 | 7.42 |
| *Homogenized Lungs* | 1 | 3.29 | 109.8 | 2.16 | 72.0 | 0 | 0 | 0 | 0 | 0.499 | 16.6 |
| | 2 | 2.98 | 99.3 | 1.37 | 45.8 | 0 | 0 | 0 | 0 | 0.223 | 39.5 |
| *Placenta* | 1 | 2.88 | 95.9 | 1.45 | 48.3 | 0 | 0 | 0 | 0 | 0.963 | 32.1 |
| | 2 | 2.95 | 98.4 | 1.85 | 61.5 | 0 | 0 | 0 | 0 | 1.19 | 48.2 |
| *Fetal Tissue* | 1 | 2.56 | 85.4 | 2.15 | 71.5 | 0 | 0 | 0 | 0 | 0.918 | 30.6 |
| | 2 | 3.33 | 111 | 1.70 | 56.6 | 0 | 0 | 0 | 0 | 1.45 | 23.9 |

**Table A-6.** Comparison of NNAT average percent recovery results between extraction method detection limit determinations.

| ***Procedure*** | ***NNAT Average % Recovery*** |
|---|---|
| *Extraction and Recovery Comparison* | 26.8 ± 14.5 |
| *MDL 1* | 36.7 ± 5.20 |
| *MDL 2* | 10.8 ± 0.167 |
| *MDL 3* | 75.4 ± 10.0 |

**Figure A-1.** Example chromatograms of each analyte when using the Xevo TQS micro LC-MS-MS instrument.

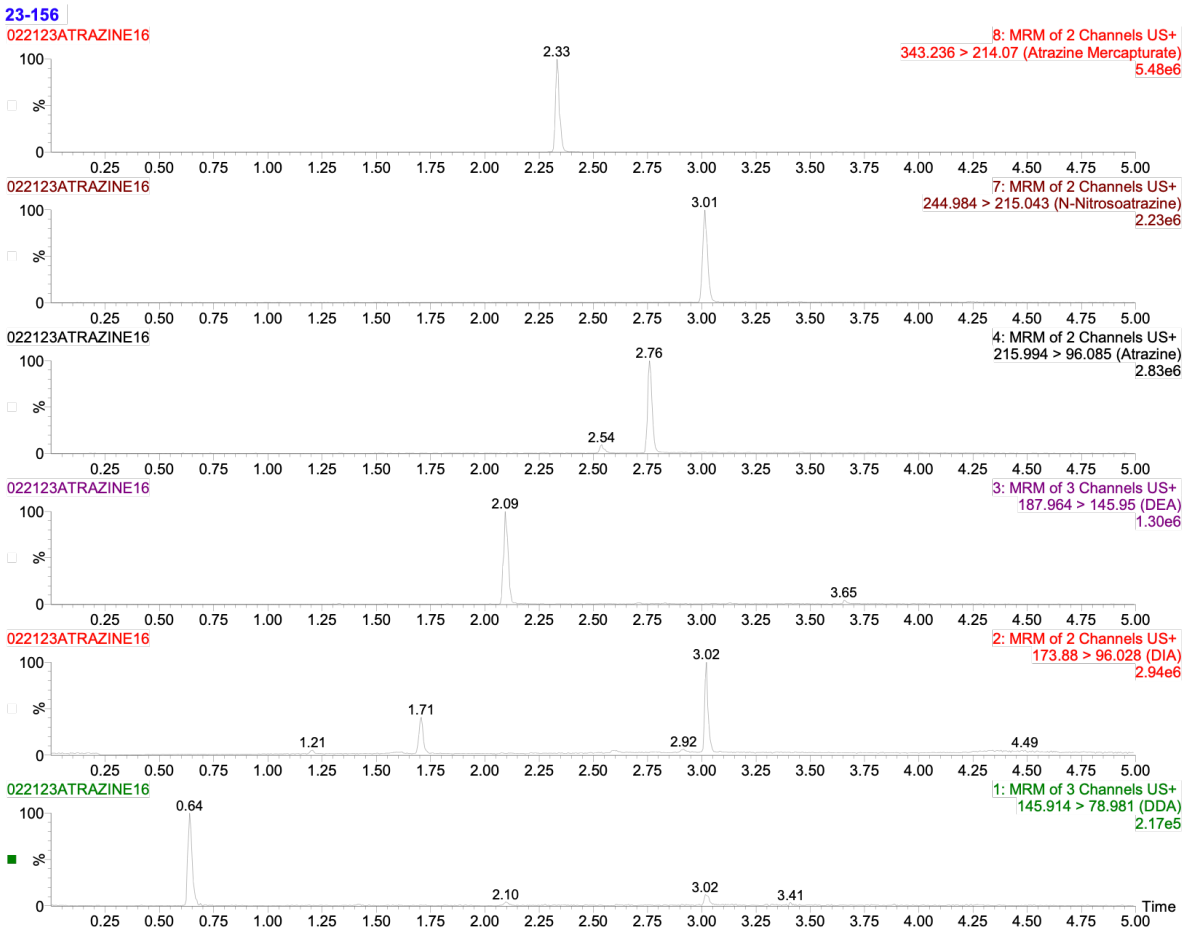

**Figure A-2.** Example chromatograms of the internal standard and surrogate when using the Xevo TQS micro LC-MS-MS instrument.