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Analysis of ketamine and xylazine in fur and bones using multidimensional liquid chromatography tandem mass spectrometry

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ANALYSIS OF KETAMINE AND XYLAZINE IN FUR AND BONES USING MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

by

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Dedicated in loving memory to S. Holla and S.L. Karanth

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ABSTRACT

While ketamine is traditionally administered for anesthesia or pain management, illicit usage is often seen in forensic cases either as a recreational drug or as a tool in drug-facilitated sexual assault. Xylazine is an anesthetic agent used in veterinary medicine and does not have FDA approval for use in humans. However, it has recently been observed as a cutting agent in heroin. Post-mortem specimens present many challenges when it comes to toxicological analysis. Due to compound degradation and decomposition factors, analytes present at trace levels may be missed in blood and urine. Hair, bone, and insects have recently been investigated as alternative matrices for postmortem analysis due to their increased durability compared to more traditional matrices. However, this durability increases the difficulties in extracting and isolating compounds of interest from these matrices via traditional extraction and chromatography methods. These methods require lengthy extraction times and extensive cleanup steps in order to obtain samples suitable for analysis. Utilizing multiple instrumentation combinations, analysts are able to detect compounds at trace levels. Through the use of multidimensional chromatography, several time-

consuming extraction steps can be eliminated while still retaining the ability of trace level detection and quantitation. Using Waters Oasis® HLB PRiME solid phase extraction cartridges using a methanol pH10 loading and an acetonitrile pH3 elution, a solvent extraction yielded linear dynamic ranges of 2pg/mL-1ng/mL and 5pg/mL-1ng/mL for xylazine and ketamine respectively.

Rat specimens utilized in this project were treated as per an Institutional Animal Care and Use Committee (IACUC) protocol. The test rodents received an acute dosage of 2mg/mL of xylazine and 24mg/mL of ketamine approximately half an hour prior to death. The 14 test samples were placed outside directly on the ground at the Boston University Forensic Anthropology Outdoor Research Facility (Holliston, MA, U.S.A.) for a period of 6 months. A 15^{th} rat was kept in -20°C until analysis to serve as a Time=0 sample. The outdoor samples were recovered and de-fleshed along with the Time=0 sample manually.

Drug-free hair samples were donated anonymously as per Internal Review Board (IRB) protocols.

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LIST OF ABBREVIATIONS

cc	Cubic centimeter
DEA	Drug Enforcement Administration
DFSA	Drug-facilitated Sexual Assault
FDA	Food and Drug Administration
HLB	Hydrophilic-Lipophilic Balance
HPLC	High Performance Liquid Chromatography
IACUC	Institutional Animal Care and Use Committee
IRB	Internal Review Board
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
M	Molar
MCX	Mixed mode, strong Cation Exchange
mg	Milligram
mL	Milliliter
mm	Millimeter
MS	Mass Spectrometry
µg	Microgram
N	Normal
NFLIS	National Forensic Library Information System
ng	Nanogram

pg	Picogram
ppt	Points per trillion
UPLC	Ultra Performance Liquid Chromatography

1. INTRODUCTION

1.1 Postmortem Toxicology

1.1.1 Current sample matrices

Toxicology involves the detection and/or quantification of drugs or poisons within a biological matrix. Postmortem toxicology focuses on the testing of samples from deceased individuals. Certain matrices such as blood, urine, soft tissue, vitreous humor, and oral fluid are most commonly used due to accessibility and ease of analysis. This is due to the fact that, with the exception of soft tissue, these samples are liquids, requiring significantly less sample preparation prior to analysis. In an ideal scenario, these samples are collected at autopsy soon after death with minimal exposure to external contaminants. In addition, there is a sufficient quantity of each matrix from which samples can be acquired from multiple locations with clean collection methods and containers[1]. In order to isolate substances that are inconsistent with that individual's typical medications and environmental exposure, forensic toxicologists use the deceased individual's medical history, collected by medico-legal death investigators. This information, along with case findings, is influential in determining screening methods and identifying which analytes are important for subsequent analysis.

1.1.2 Complications with traditional matrices

Death brings complications of decomposition factors and drug degradation. Unlike with clinical toxicology, there is a finite supply of different

matrices in the deceased, depending on the many factors influencing decomposition. Care must be taken to reduce contamination and ensure an adequate supply for immediate and future testing. Autolysis and bacterial decomposition begin almost immediately after death, compromising the integrity of the biological fluids and potentially the drugs within these biological matrices. Postmortem redistribution, for example, is the result of observing inconsistent concentrations of a xenobiotic substance from samples taken from different locations. Depending on the drug and body composition, there can be significant differences in concentrations from blood taken from the heart versus blood taken from peripheral extremities[2]. The degree of drug degradation is dependent on a number of factors: the type of drug, the drug's pharmacokinetic and pharmacodynamic properties, exposure to additional chemicals, environmental conditions, and postmortem interval[1]. For example, drugs or toxins in a corpse exposed to high temperatures in a dry environment can have different concentrations than those in a corpse exposed to low temperatures in a wet environment, even if they started with identical perimortem drug concentrations.

Embalmed and interred remains pose a problem in that traditional matrices are either removed or too degraded for use in typical analytical methods. Some examples include "cold" cases, or cases in which the original cause and manner of death are called into question. Alternative matrices are considered more stable, so they are still suitable for analysis once an optimal analytical method has been developed.

1.2 Alternative Matrices

1.2.1 Hair

Hair is a collection of keratinized cells that are fused together to form strands. Those strands are formed in hair follicles found over the skin's surface. Hair structure contains three layers: the cortex, the medulla, and the cuticle.

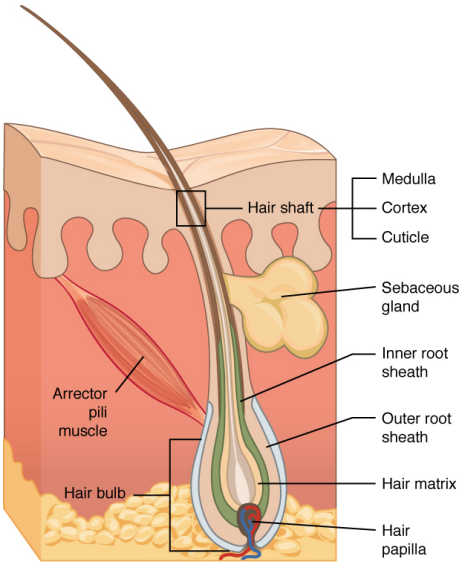

Figure 1: Hair follicle cross-section[3]

Xenobiotics are incorporated into the hair through two processes: the primary mechanism during root formation through attachment to the medulla and the secondary mechanism when the drugs enter the cuticle and disperse through the hair[4]. The primary mechanism occurs at the hair papilla inside the hair bulb. The hair papilla is vascularized, as seen in Figure 1, so drugs inside the blood are integrated within the hair matrix. The secondary mechanism occurs during external deposition of drugs onto the cuticle and their subsequent diffusion into

the hair. Factors that influence the amount and rate of disposition include surrounding enzyme activity, rate of hair growth, and drug type and dosage.

There are many factors that affect the long-term stability of hair for use as a toxicological matrix. Ultra violet radiation, heat, and cosmetic chemical treatments are the major contributors to reduction in drug concentrations in hair[5–7]; exposure to any of these conditions should be recorded by law enforcement or medicolegal death investigators, if possible. Storage conditions should also ensure protection from those factors. The Society of Hair Testing's Consensus on Hair Analysis states that hair samples should be stored dry, in the dark, and at room temperature[8]. These conditions do not require additional equipment or resources, so this is easier for laboratories with limited space or budgets, both problems commonly faced by public testing labs.

Aside from post-mortem examinations, hair analysis can also be utilized in cases such as workplace drug testing, child custody disputes involving parental drug abuse, and drug-facilitated sexual assaults (DFSA). Due to delayed reporting of DFSAs, incapacitating drugs may not be present in blood or urine during examination, which are the typical toxicological specimens taken in a sexual assault kit. However, the Sexual Assault Nurse Examiner also collects hair samples; these are typically used for trace analysis and fiber comparisons. As a toxicological matrix, hair has a longer window of detection as opposed to blood and urine. Using hair as an alternative testing material in sexual assault cases can provide evidence of drug administration for months after the fact.

1.2.2 Bones

The skeletal system is comprised of cartilage and rigid calcified organs known as bones. The skeletal system “supports the body, facilitates movement, protects internal organs, produces blood cells, and stores and releases minerals and fat.”[3] Bones are classified as “a hard, dense connective tissue”[3] comprising of cortical bone, cancellous bone, and bone marrow. Cortical, or compact bone is the solid outer layer of the bone, providing shape and structural support. Cancellous, or spongy bone is similar to honeycomb in appearance, and is typically located at the ends of long bones, inside vertebrae, and at load-bearing joints[3]. Figure 2 illustrates typical bone structure. These characteristics are harder to view in other types of bone due to their different shapes and functions.

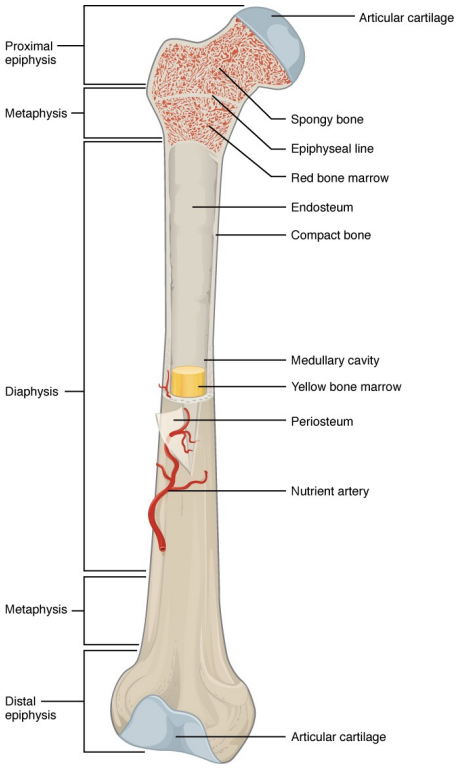

Figure 2: Gross bone anatomy and cross-section[3]

There are four different types of cells within bone: osteogenic cells, osteoblasts, osteocytes, and osteoclasts[3]. All of these cells have access to blood vessels that enter the bone through nutrient foramina, small holes in the bone's surface, into the osteon system (Figure 3). These vessels run through the bone shaft via Haversian, or central canals located in the center of each osteon, and nutrients are distributed to the osteoblasts and osteocytes through Volkmann's, or perforating canals[3].

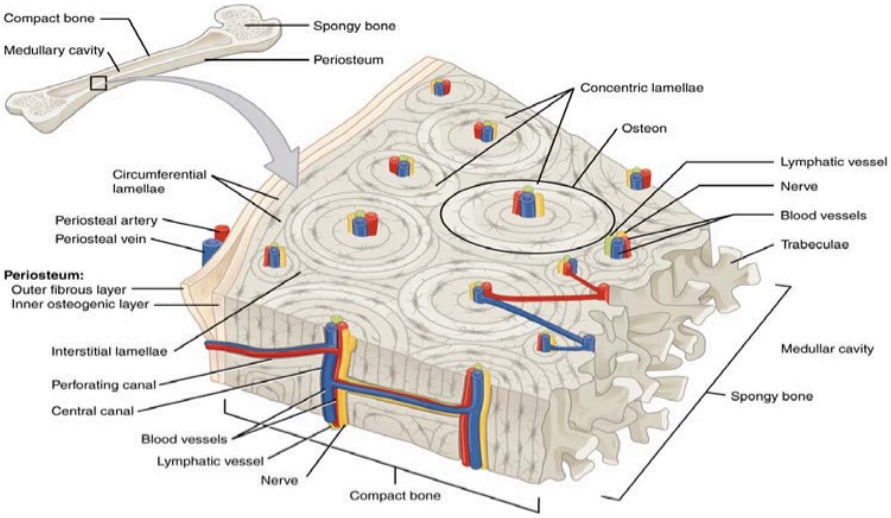

Figure 3: Osteon system cross-section[3]

There is constant activity by all the bone cells, so substances circulating in the blood are incorporated into the bone tissue by osteocytes and osteoblasts. In addition, if the bones have maintained their structural integrity, water-soluble compounds would most likely be found within red marrow, and fat-soluble compounds would be stored within yellow marrow.

As a toxicological matrix, bone is effective because it can be stored for long term without requiring refrigeration or freezing to maintain viability. In cases where the body is significantly decomposed, bone may be the only toxicological sample available.

1.2.3 Complications with alternative matrices

The attributes that help hair and bone samples retain their stability also pose issues for extraction and analysis. With both matrices, the extraction process begins with either the sample being mechanically broken down into smaller pieces. This increases the surface area of sample exposed to future

extraction steps, allowing access to internal components such as marrow or the medulla where drugs are also incorporated. Examples in the literature include scissors[9], pliers[10], domestic grinders[11], and a mortar and pestle[11], which can be effective, but bead mills are preferred due to their ability to rapidly breakdown solid material into minute particles [9] rather than the larger fragments produced by the previous methods. Particular care must be taken with hair because the strands are susceptible to static charge and air movement, especially in cases with small sample sizes.

After the sample has been separated into smaller pieces, the analyte of interest will direct the reagents used for extraction; in cases where the history is unknown, these must be carefully considered. Common extraction techniques involve acidic or basic digestion, solvent extraction, heated incubation, or enzymatic hydrolysis[9]; if the analyte of interest is unstable in any of those conditions, any trace of it will be lost or rendered undetectable.

In addition, the matrix itself can have inconsistencies that can complicate analysis. For instance, hair pigmentation levels play a role in the concentration of drugs incorporated into hair; individuals with increased melanin production will have an increased drug concentration than individuals with less melanin, even if they are taking the same dosages[12]. With bone, surface contamination from surface decomposition can enhance or reduce drug concentrations[10].

1.3 Ketamine and Analysis in Toxicology Labs

1.3.1 Mechanism of action and appearance in casework

Ketamine is an arylcyclohexamine used in both human and veterinary medicine for its sedative and analgesic effects. Arylcyclohexamines act as N-methyl-D-aspartate (NMDA) antagonists and as dopamine D2 receptors agonists in the brain[13].

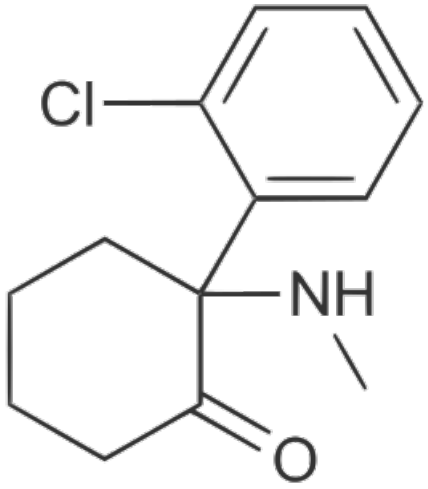

Figure 4: Ketamine Molecular Structure[14]

Arylcyclohexamines have anticonvulsant, dissociative, and anesthetic properties, which lend themselves to a variety of pharmacological applications[13]. Due to the variety of functional groups present, those effects can vary in type and severity from compound to compound; for example, ketamine and phencyclidine have similar dissociative effects, but ketamine's are shorter in duration and less intense than phencyclidine. Ketamine induces tranquilizing and dissociative effects upon administration, and in addition to sedation and pain relief, individuals will total amnesia [15].

The Food and Drug Administration (FDA) has approved Ketamine for both human and veterinary uses. Clinical uses for ketamine include inducing dissociative sedation and analgesia for shortened procedures or to reduce stress to a patient during a potentially traumatizing procedure or exam.

The Controlled Substances Act classifies ketamine as a Schedule III drug for due to its medical uses with some potential for abuse[16]. As such, the Drug Enforcement Administration (DEA) compiles reported cases in the National Forensic Library Information System (NFLIS) under the tranquilizers and depressants category[17]. The 2017 NFLIS-Drug 2017 Midyear Report has 731 reported cases involving ketamine nationwide, an increase of 3.4% from the 2016 NFLIS-Drug Midyear report, and 58.6% of the total ketamine cases reported in 2016[17–19]. Ketamine is most often seen in club settings, going by the street names of “Special K”, “K”, and “Kit Kat”, and the dissociative effects are referred to as a “K-Hole”[16].

Due to its ability to induce a catatonic state in conjunction with total amnesia, ketamine is frequently seen in DFSA cases. This can be through consensual or nonconsensual administration. In 2015, The Washington Post and the Kaiser Family Foundation conducted a survey of current college students and recent graduates; 14% of women and 4% of men surveyed reported DFSA when answering the questions posed[20] . While there is little information on the breakdown of which drugs are present in DFSA case, it is important when

interviewing victims to ask if they can recall any symptoms and if so, what they were. This can provide guidelines for screening tests.

1.3.2 Current analytical techniques

There is currently a multidimensional liquid chromatography tandem mass spectrometry method for ketamine in rat tissue yielding limits of detection of 0.5pg/mL [21], utilizing mixed-mode cation exchange(MCX) solid phase extraction(SPE) for sample preparation. The most commonly used methods are High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) or Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS/MS)[11,22–24]. Limits of detections for those studies range from 0.02ng/mL[23] to 5ng/mL[11], using SPE for sample preparation of the hair and bone samples. In the Watterson study, further research into whether the body incorporated more ketamine in cancellous or cortical bone, and if marrow residue introduced a potential contamination issue. Baretto et al's method involved an extensive washing process prior to sample preparation and extraction including an hour long drying cycle.

1.4 Xylazine and Analysis in Toxicology Labs

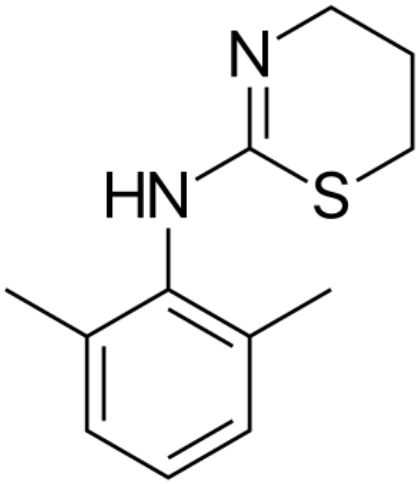

Figure 5: Xylazine Molecular Structure[25]

1.4.1 Mechanism of action and appearance in casework

Xylazine is a veterinary anesthetic agent often administered in conjunction with ketamine. It acts as an α-2 andrenergenic agonist to produce sedation and analgesia during veterinary procedures[26,27]. In addition, side effects include bradycardia, respiratory depression, muscle relaxation, and central nervous system depression. However, it is unclear how xylazine interacts with the human body to create these symptoms because there is little research on its mechanism of action. Clinical trials for use in humans were halted due to severe hypotension that occurred[28], so the FDA has only approved it for veterinary use.

Because xylazine is not approved for human use, it is not a controlled substance. This means there is no case data reported to NFLIS; however, there have been case reports in the literature involving xylazine overdoses. For example, in Puerto Rico, xylazine was observed to be an adulterant in both

heroin and cocaine samples, which resulted in fatal and non-fatal overdoses[29,30]. This is most likely the result of dealers increasing the amount of product they can sell by supplementing their product with a drug that induces similar effects.

1.4.2 Current analytical techniques

Because it is not a controlled substance, it is unlikely that many forensic toxicology departments have pre-established methods for detecting xylazine in case samples. However, veterinary pharmacological research has resulted in quantitative methods in whole blood samples using liquid-liquid extraction (LLE) and either HPLC-MS or UPLC-MS-MS[26–28]. Testing on tissue and postmortem blood have yielded limits of detection of 1ng/mL, 2.81ng/mL, and 5.1[28,31,32] in humans, which is well above the trace levels necessary for bone and hair analysis. However, there is limited case history on the dosage consumed or decomposition period, so those factors may have influenced how much entered those matrices. It is recommended that multiple screening methods should be employed in clinical settings due to the rarity of its appearance.

1.5 Instrumentation Theory

1.5.1 Single-dimension liquid chromatography-tandem mass spectrometry

Liquid chromatography is the separation of compounds by dissolving them into a liquid mobile phase and interacting with a solid stationary phase based on their chemical properties in order to reach a detector at different retention times.

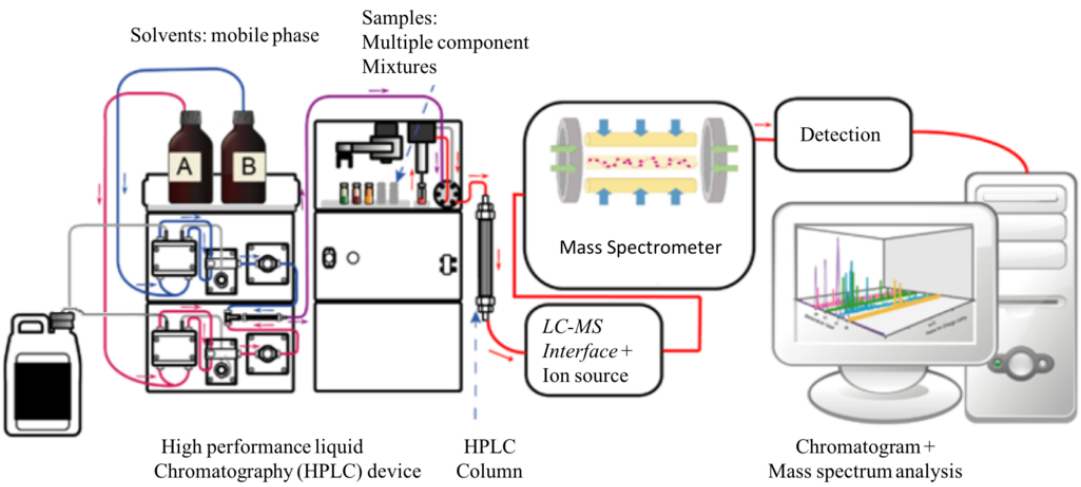

Figure 6: LC-MS schematic

Liquid chromatography uses a liquid mobile phase facilitate the movement of compounds through the column into the detector. Water and an organic solvent such as methanol or acetonitrile are the most commonly used mobile phase combinations. These mobile phases can be pH-adjusted to improve the separation of compounds and reduce the effects of ion suppression or enhancement once the compound reaches the detector[33].

Single-dimensional liquid chromatography-tandem mass spectrometry, or HPLC-MS and UPLC-MS/MS, refers to the utilization of a single analytical column for the separation of compounds. Two common chromatographic

techniques include hydrophilic interaction liquid chromatography (HILIC), a type of normal phase chromatography, or reverse phase chromatography. With HILIC methods, the analytical column contains a stationary phase that is more polar than the mobile phase, which results in a stronger retention of hydrophilic compounds in the sample[34]. The stationary phase will consist of either silica particles with no attached functional groups or with polar molecules such as amino, cyano, or amide groups attached to form a hybrid stationary phase. Reverse-phase chromatography utilizes a non-polar stationary phase in conjunction with a polar solvent. Typical results include shorter retention times for polar compounds than non-polar compounds due to their different affinity towards the mobile phase[35]. The reverse-phase columns are packed with silica particles with a carbon-based ligand that varies based on the compounds of interest. An example of a common ligand is C_{18}, which is an eighteen carbon chain, which is bonded to the silica particle[36]. Because C_{18} interacts with a large variety of compounds, it is typically the first column chemistry of choice when developing a method. Other ligands include C_4, C_8, phenyl, and fluoro, which have different degrees of selectivity for compounds. During method validation, multiple different columns are evaluated to discover optimal separation conditions for the analytes of interest.

Columns are selected based on the following criteria: length, internal diameter, particle size, and chemistry. The internal diameter of the column determines the pressure within the column as the mobile phase travels through it.

Particle size refers to the size of the materials that make up the stationary phase. The Van Deemter equation represents the relationship between eddy diffusion, longitudinal diffusion, and interphase mass transfer, which are impacted by both particle size and internal diameter[37]. Eddy diffusion describes the pathways an analyte can travel through the analytical column; the longer the analyte travels, the longer the retention time. Eddy diffusion is controlled by stationary phase particle size and the how compact those particles are pressed within the column. Longitudinal diffusion refers to the spread of the compound of interest from high to low areas of concentration, and is controlled by the mobile phase's type, flow rate, and viscosity as it moves through the circuit. Interphase mass transfer is related to the equilibration time required for a particle to stabilize between the stationary and mobile phases. Smaller particle sizes reduce the peak broadening caused by increased eddy diffusion, increasing resolution and sensitivity. However, smaller particle sizes also result in increased pressure, which can negatively affect interphase mass transfer, which must be considered when choosing a technique[35]. For example, HPLC typically uses particle sizes of 3.5µm to 5µm, which can handle up to 6000 pounds per square inch, or psi, while UPLC will use particle sizes of 1.75 μm to 2.5 μm and can take up to 18,000psi. If sensitivity is not a priority, then a larger particle size is suitable, whereas in samples with trace amounts, a smaller particle size is required. Column chemistry is determined by the type of stationary phase packed within the column. Methods for detecting drugs of abuse most commonly use reverse-

phase chromatography due to their lower molecular weight, and polarity[36]. Typical drugs of interest such as cocaine and ketamine are water soluble and more polar, which results in shorter retention times in reverse phase chromatography.

1.5.2 Multidimensional chromatography

While multidimensional chromatography utilizes the general concepts behind traditional liquid chromatography, it incorporates two additional elements to improve separation, resolution, and sensitivity. First, it adds an additional column chemistry contained in a trap column, which is located after the sample manager but before the analytical column. Secondly, multiple pumps are added to the instrumentation configuration to increase sensitivity and improve resolution. This experiment utilized a trap-and-elute two-dimensional set-up as shown in Figure 7.

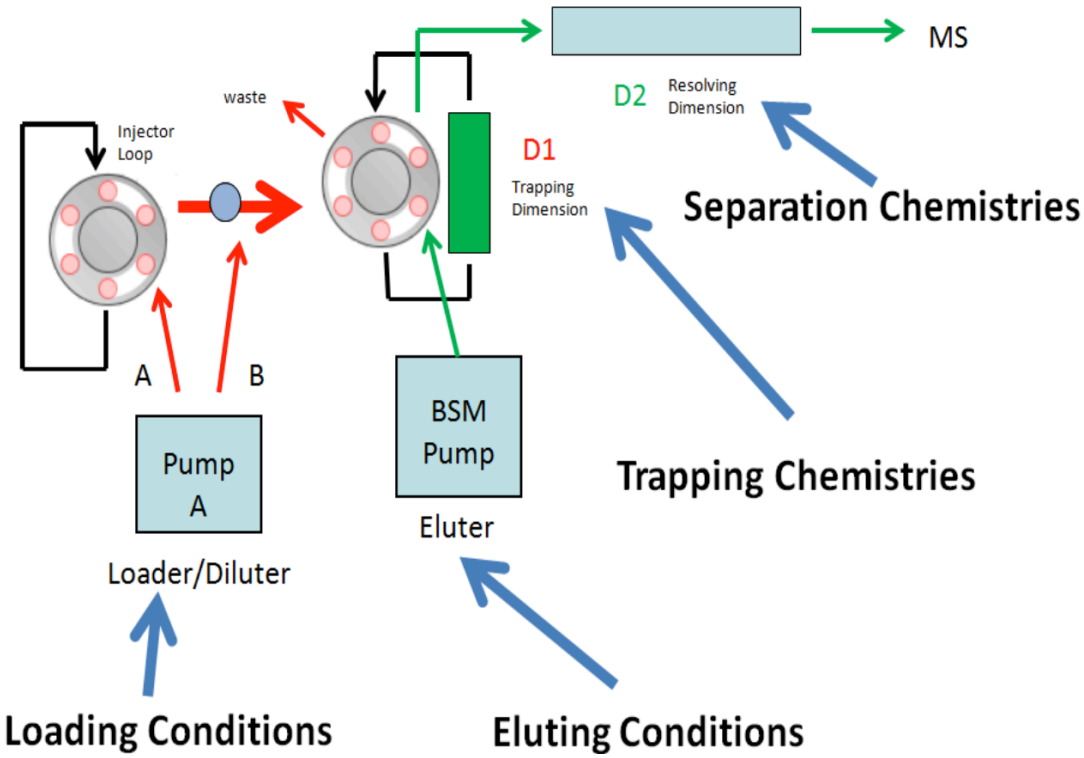

Figure 7: 2D-LC system configuration[38]

In a trap-and-elute scenario, there is a valve in the column manager separating the trap column and the analytical column. When the system is in trap mode, the valve closes to prevent mobile phase from reaching the analytical column. The sample, made up entirely of organic solvent enters the fluid circuit at this point and is loaded onto the trap column. There, additional aqueous mobile phase flows from the dilutor line to mix with the sample at the mixer in a process known as at-column dilution. This increases the sample volume and creates an organic/aqueous sample mixture to prevent breakthrough on the analytical column. Once the sample has been sufficiently diluted, the column manager valve opens, allowing the sample to enter the analytical column. By increasing the sample volume, this increases the amount of analyte being injected into the

system, allowing for lower limits of detection and quantitation. The at-column dilution process improves sensitivity because it allows for the injection of a larger organic sample volume, which increases sensitivity while also reducing sample preparation time. This is achieved by eliminating the reconstitution step of sample preparation, which ranges from hours to overnight evaporation.

1.5.3 Comparison of sample preparation methods for single dimensional and multidimensional chromatography

While there are some similarities between sample preparation methods for traditional single dimensional and multidimensional chromatography, there is a key difference, which allows for the increased sensitivity. That difference is the sample composition when it is injected into the chromatographic system. In order for effective retention on the analytical column, a sample must be a mixture of aqueous and organic solvents. When a 100% organic sample is injected onto an analytical column, it does not interact with the analytical column's stationary phase. This lack of interaction causes the sample to be eluted at the initial stages of the chromatographic run, rather than during the gradient. This phenomenon, known as breakthrough, drastically reduces the method's specificity due to insufficient time for multiple analytes within the sample to interact with the stationary phase. Each analyte will pass through an analytical column at different rates due to their increased or decreased retention on the stationary phase, which is exhibited by their different retention times. To prevent breakthrough, traditional HPLC or UPLC sample preparation methods require that an organic

eluent from a sample preparation scenario be evaporated to dryness to remove all of the organic solvent and subsequently reconstituted with a 95:5 mixture of the aqueous and organic mobile phased used in the analytical method. This process can be time-consuming, either in the form of allowing the solvent to passively evaporated, or accelerating the process with an evaporator or heating block, which can still take hours. This reduces the time available for sample analysis and preparation of additional samples. Once a sample is reconstituted, only a small amount can be injected into the system, to prevent any possibilities of breakthrough. The small injection volume reduces the likelihood that an accurate representation of the sample has made it into the system, diminishing the absolute confidence in one's results.

Conversely, the at-column dilution method utilized in multidimensional chromatography eliminates the reconstitution stage of sample preparation entirely. This is because the organic eluent is mixed with water at the mixer rather than after the sample has been dried and reconstituted with an organic and aqueous mixture prior to injection. This allows for an increased injection volume, which improves the likelihood that all analytes present in the sample are represented in the injection. In addition, the increased injection volume increases sensitivity by allowing more sample to reach the detector.

A method's analytical parameters can be created from hundreds of combinations, including mobile phases, pH, and column chemistries. By using a 6x6 grid to test 36 combinations of those characteristics, a wide range of possible

methods can be tested to inform future steps. When combined with automation, those can be evaluated overnight to increase productivity during working hours. An analyte's pK_a will affect its ionization state at different pHs, which in turn effects how it interacts with both the stationary and mobile phases. For example, ketamine has a pK_a of 7.5, which means that it would be ionized at low pH values and neutral at high pH values. By testing a range loading pHs, the analyte's interaction with the trap column's stationary phase can be enhanced or hindered by whether the compound of interest is in an ionized or neutral state. This testing scheme can also be applied to the elution solvent to determine the optimal pH for analysis. The trap column sorbents range from C_8, which has the lowest retention strength, to HLB, which has the highest retention strength. The retention strength can increase or decrease the retention time in order to keep it within the gradient. By observing the chromatograms from the 36 methods, a choice can be made for method development going forward.

1.6 Research Objective

This research hopes to determine whether or not an acute dosage of both ketamine and xylazine can be detected in bone and fur samples following six months of decomposition using 2D-LC/MS/MS technologies, which will be coupled with accelerated extraction methods to facilitate trace-level detection at points per trillion (ppt)

2. MATERIALS AND METHODS

2.1 Instrumentation

Compound optimization and chromatography method development were performed using Waters ACQUITY UPLC® QSM, BSM, FTN Sample Manager, Column Manager, and Xevo TQ-S tandem mass spectrometer (Waters Corporation, Milford, MA, USA). MassLynx© version 4.1 (Waters Corporation, Milford, MA, U.S.A.) software was used to control instrumentation, view chromatograms and spectra, and monitor instrumentation conditions.

2.2 Standards and Reagents

Analytical reference standards for ketamine and ketamine-D4, were obtained from Cerilliant© (Round Rock, TX, U.S.A.) in the form of 1mg/mL solution dissolved in methanol for ketamine and 100µg/mL solution dissolved in methanol for ketamine-D4.

Xylazine was obtained from Cayman Chemicals© (Ann Arbor, MI, U.S.A.) in powder form and mixed into solution using Optima™ grade methanol.

Xylazine-D6 was obtained from Frontier Biopharm© (Richmond, KY, U.S.A.) in powder form and mixed into solution using Optima™ grade Methanol.

Optima™ grade methanol, acetonitrile, hexane, acetone, water, and isopropanol were obtained from Fisher Scientific™ (Waltham, MA, U.S.A.) Formic acid of 99% purity, ammonium hydroxide, and hydrochloric acid were obtained from Millipore Sigma© (St. Louis, MO, U.S.A.).

AQCUITY UPLC BEH C18 analytical column, 130Å, 1.7µm 2.1mm X 50mm and AQCUITY UPLC BEH C18 VanGuard Pre-column, 130Å, 1.7µm 2.1mm X 30mm guard columns were obtained from Waters Corporation (Milford, MA, U.S.A.). XBridge BEH C18 Direct Connect HP Column, 130Å, 10 µm, 2.1 mm X 30 mm, XBridge BEH C8 Direct Connect HP Column, 130Å, 10 µm, 2.1 mm X 30 mm, and XBridge BEH HLB Direct Connect HP Column, 130Å, 10 µm, 2.1 mm X 30 mm were acquired from Waters Corporation (Milford, MA, U.S.A.).

Oasis® HLB, HLB PRiME, and MCX solid phase extraction cartridges were obtained from Waters Corporation in 3cc/60mg, 6cc/150mg, and 6cc/200mg sizes (Milford, MA, U.S.A.). Chrom Tech® centrifuge filter tubes, MIDI PFTE 0.45µm, were obtained from Chrom Tech®(Apple Valley, MN, U.S.A). Vivaspin® 6 centrifuge filter tubes were acquired from Sartorius (Goettingen, Germany).

Ultrapure water was obtained using a Milli-Q® IQ 7000 water purification system with a LC-Pak® Polisher filter attachment purchased from rom Millipore Sigma© (St. Louis, MO, U.S.A.).

A Precellys Evolution homogenizer equipped with a 15mL holder pack was used to homogenize the bone and hair samples in 15mL extraction tubes with 4 ceramic ball bearings from Bertin Corporation (Rockville, MD, U.S.A).

2.3 Rats and Hair

The hair used for method development was donated by an anonymous donor as per IRB protocol.

The rats were obtained from the Boston University Animal Science Center (Boston, MA, U.S.A.). They were dosed with 2mg/mL of xylazine and 24mg/mL of ketamine prior to euthanasia. Fourteen of the fifteen rats procured were placed outside at the Boston University Forensic Anthropology Outdoor Research Facility (Holliston, MA, U.S.A.) for a period of six months. They were placed directly on the ground and secured using a wire mesh screen (Figure 8).

Figure 8: Rat specimens at (L-R): initial deposition, 3 months, and 6 months

After six months, the rats were removed from the site, and any remaining fur or bones in addition to the skeletal remains were collected and placed into -20°C storage. The fifteenth rat was kept at -20°C to act as a Time:0 sample. Flesh, grass, insects, and fur were then removed manually from each skeleton.

2.4 Method Development

2.4.1 Compound optimization

Compound optimization was conducted by performing multiple reaction monitoring (MRM) for both ketamine and xylazine and evaluating the effects of different pH, electrospray charge, collision energy, cone voltage, and solvents had on signal strength. Each compound was directly infused into the mass

spectrometer; the infusion solution consisted of a 10ppb standard concentration in a 50:50 methanol:water solution. The different pH evaluations were done by adding formic acid, ammonium hydroxide, or neither additive. The infusion samples were run using MS1 mode to determine the precursor ion; the cone voltage and probe position were varied in order to produce the most intense signal. The product ions were determined using MS/MS mode with multiple collision energies evaluated. This determines the different product ions created at the different collision energies; there is increased molecule fragmentation when the collision energy becomes higher. The quantitative and qualitative ions were chosen based on the 2 fragments with the highest signal intensities. For MRM conditions, the collision energies that produced the highest signal were selected for that specific ion. The experimental values are listed in Table 1.

Table 1: Compound MRM Values acquired on Xevo TQ-S

Compound	Ion Mode	Precursor Ion	Cone Voltage	Product Ions	Collision Energy
Ketamine	ESI+	238.2	30	125.0	25
				222.2	15
Ketamine-D4	ESI+	242.2	30	129.1	25
				224.2	10
Xylazine	ESI+	221.2	30	90.1	20
				164.0	20
Xylazine-D6	ESI+	227.2	30	90.2	20
				170.1	25

2.4.2 Chromatography optimization

36 LC methods were evaluated to determine the optimal conditions for the analysis of both ketamine and xylazine. This was done by testing different

combinations of solvent types, pH, and trap column sorbent. The grid with the 36 permutations appears in two parts in Tables 2 and 3.

Table 2: Acetonitrile Chromatography Evaluation Parameters

	Water/Solvent	**Water**	**Trap Column Chemistry**		
Solvent Type	Elution pH	Loader/Diluter pH	C8	C18	HLB
Acetonitrile	pH10	pH 10	16	17	18
		pH 7	13	14	15
		pH 3	10	11	12
	pH 3	pH 10	7	8	9
		pH 7	4	5	6
		pH 3	1	2	3

Table 3: Methanol Chromatography Evaluation Parameters

	Water/Solvent	**Water**	**Trap Column Chemistry**		
Solvent Type	Elution pH	Loader/Diluter pH	C8	C18	HLB
Methanol	pH10	pH 10	34	35	36
		pH 7	31	32	33
		pH 3	28	29	30
	pH 3	pH 10	25	26	27
		pH 7	22	23	24
		pH 3	19	20	21

The flow rates, column manager temperature, sample manager temperature, and MRMs were kept the same for each method. Each method was tested using 10ng/mL concentrations of ketamine and xylazine in water, methanol, and acetonitrile; injections were performed in triplicate for each solvent. Chromatograms for each method were evaluated based on peak shape, signal strength, and retention time; each method was assigned a color designation of red, yellow, or green, from green representing optimal signal recordings – defined here as Gaussian peak shapes with at least an e6 signal intensity - to red

representing no signal present. Methods 1-9 involved eluting with a 5-95% acetonitrile ph3 gradient, while Figure 9 provides representative peaks from each method.

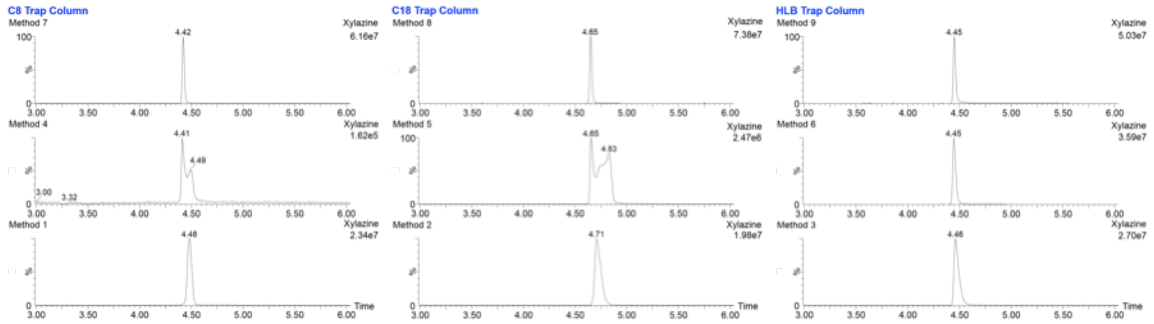

Figure 9: Xylazine Chromatograms, Methods 1-9

Table 4: 3X9 Chromatography Evaluation for each compound, Eluting with Acetonitrile, pH3

	Method	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	Column	C8	C18	HLB	C8	C18	HLB	C8	C18	HLB
	Loading pH	pH3	pH3	pH3	pH7	pH7	pH7	pH10	pH10	pH10
Solvent	**Eluting pH**	pH3	pH3	pH3	pH3	pH3	pH3	pH3	pH3	pH3
Water	Ketamine	e7 4.35	e7 4.55	e7 4.34		e6 4.65 lead	e7 4.33	e7 4.30	e7 4.50	e7 4.33
	Xylazine	e7 4.48	e6 4.71	e7 4.46		e6 4.65 split	e7 4.45	e7 4.42	e7 4.65	e7 4.45
MeOH	Ketamine	e7 4.36	e7 4.55	e7 4.35	e4 4.28 split	e6 4.65 lead	e7 4.33	e7 4.31	e7 4.50	e7 4.33
	Xylazine	e7 4.48	e7 4.71	e7 4.46	e5 4.41 split	e6 4.84 split	e7 4.45	e7 4.42	e7 4.65	e7 4.45
ACN	Ketamine	e7 4.35	e7 4.55 tail	e7 4.35	e4 4.30 split	e6 4.62 lead	e7 4.33	e7 4.31	e7 4.50	e7 4.33
	Xylazine	e7 4.48	e7 4.71 tail	e7 4.46	e5 4.41 split	e6 4.79 split	e7 4.45	e7 4.43	e7 4.65	e7 4.45

After evaluating the peak shapes and signals, Method 6 was determined to be the final method.

During extraction evaluation, it became apparent that there was a carryover issue involving both compounds, even with running solvent blanks between each sample set (Figure 10).

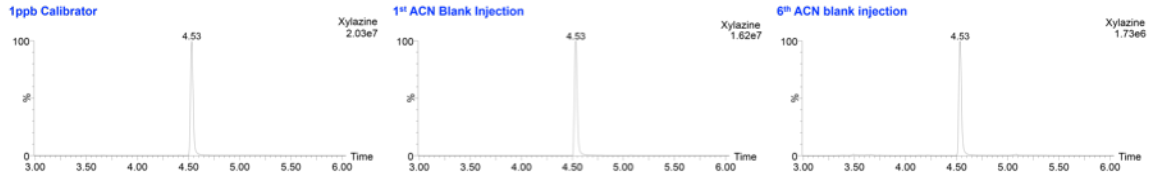

Figure 10: Old method carryover study

The cause of these issues was determined to be insufficient cleaning of the mixer connecting the loader and diluter lines to the column manager. To resolve this, a reconditioning method resulting in changes to loader flow rate, solvent type, and gradient duration was added. In addition, 6 injections from a hexane blank were added between each sample set to flush out the system and remove any potential contamination. This was done by priming the loader line first with a 1:1:1 Isopropanol:Acetonitrile:Acetone mixture with 2% formic acid for 10 minutes at 2ml/min for three blank hexane injections, and then followed by two blank hexane injections during which the line was primed with Milli-Q® water for 10 minutes. The same was repeated with the dilutor pump, but instead of 1:1:1 Isopropanol:Acetonitrile:Acetone mixture with 2% formic acid, it was flushed with a 1:1:1 Isopropanol:Acetonitrile:Acetone mixture with no addition. Finally, the final elution method was run to determine the presence of carryover.

A reconditioning step was also added within the run itself to further reduce carryover of analytes from one sample to the next. Using the same 1:1:1 Isopropanol:Acetonitrile:Acetone mixture with 2% formic acid, the loader line was flushed out for 2 minutes at 2mL/min after the gradient was completed.

The gradient was reduced from five minutes to three minutes to accommodate the additional step without increasing the overall run time. This successfully removed the carryover, even when following the highest concentration calibrator, as seen in Figure 11:

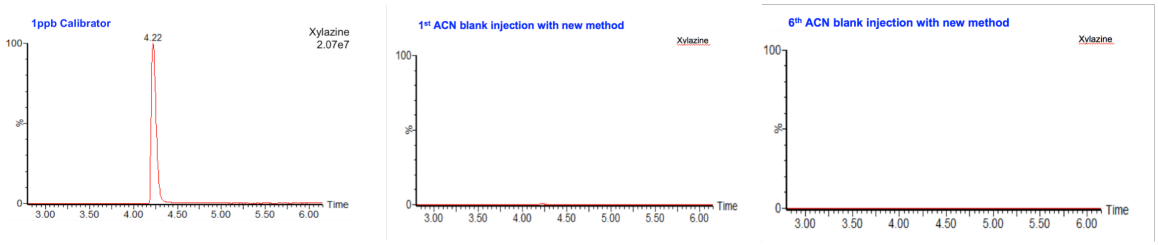

Figure 11: New method carryover study

The final run conditions are listed below, compared to a typical run scenario:

Table 5: Run condition comparison

Carryover Reduction Run Conditions	Typical Run Conditions
1) Loading onto trap column: 3 minutes	1) Loading onto trap column: 3 minutes
2) 5%-95% ACN gradient: 3 minutes	2) 5%-95% ACN gradient: 5 minutes
3) Hold at 95%ACN: 1 minute	
4) Flush out loader circuit with 1:1:1 Acetone:Acetonitrile:Isopropanol with 2% Formic Acid: 2 minutes	3) Hold at 95%ACN: 1 ½ minutes
5) Flush out loader circuit with water, no addition: 1 ½ minutes	
6) Return to initial conditions: 30 seconds	4) Return to initial conditions: 30 seconds
Total Run Time: 10 minutes	**Total Run Time: 10 minutes**

2.4.3 Extraction evaluation

Three different solid phase extraction cartridges were evaluated for extraction. Waters Oasis® HLB, Oasis® PRiME HLB, and Oasis® MCX extraction cartridges were used. Each cartridge type was 3cc capacity with a 60mg sized sorbent bed, through which 2mL water samples were passed at a concentration of either 1ppb or 10ppb, depending on the cartridge. HLB and MCX contain reversed-phase extraction sorbents that perform captive extraction. Captive extraction is where analytes of interest are trapped onto the sorbent bed, while interferences are removed from the sorbent bed through wash steps. The analytes of interests are then released from the sorbent bed with a final elution solvent. To reduce the extraction time, the samples are placed on a vacuum pressure manifold, using negative pressure to move each step

The HLB extraction protocols consist of the following steps:

1. Condition columns first with MeOH, then water
2. Load 2mL of organic sample in 100mL water with a pH of 3, 7, or 10
3. Wash sorbent bed with 2mL of a percentage of organic solvent in water at either pH 3 or pH 10
4. Elute analyte from sorbent bed with 2mL of a percentage of organic solvent in water at either pH3 or pH 10

The MCX extraction protocols consist of the following steps:

1. Condition columns first with MeOH, then water
2. Load 2mL of organic sample at pH7

3. Wash sorbent bed with 2mL of 0.1N HCl in water
4. Wash sorbent bed with 2mL of organic solvent with 2% formic acid (pH3)
5. Elute sample with 2mL of organic solvent with 2% ammonium hydroxide

HLB PRiME cartridges also contain a reversed-phase extraction sorbent, but it utilizes passive extraction so that the compound of interest is eluted when the sample is loaded onto the cartridge. To evaluate this, samples were created in a percentage of organic in water, ranging from 50% to 90% organic at pH 3, 7, or 10.

For the SPE cartridge recovery evaluation, the loading, wash steps, and elution eluents for each sample were retained and analyzed using the final chromatographic method. The HLB, MCX, and HLB PRiME recovery percentages were calculated by dividing the average area count of each loading, wash, or elution eluents by the total area count of the sample peaks. TargetLynx was used to obtain the area counts of any peaks that were present.

2.4.4 Bone and hair sample preparation prior to SPE

The bone preparation protocol was taken from Mella et al with no alterations [38]. The hair preparation protocol was adapted from Mella et al's procedure to accommodate the smaller hair sample amounts collected in a forensic setting. In previous toxicological research with hair, the largest sample mass used was 100mg [9], but in the interest of ensuring the supply of drug-free

hair would not run out, 50mg was decided as the sample mass. The protocols are as featured in Table 6.

Table 6: Bone and Hair Extraction Protocols

Bone Extraction Protocol	Hair Extraction Protocol
1. 1g bone material and 4mL of solvent added to 15mL bead mill tube with 4 4mm ceramic ball bearings and sealed	1. 50mg hair material and 4mL of solvent added to 15mL bead mill tube with 4 4mm ceramic ball bearings and sealed
2. Bead mill tube inserted into Precellys bead mill and shaken for three 90 second cycles at 5000rpm	2. Bead mill tube inserted into Precellys bead mill and shaken for three 90 second cycles at 5000rpm
3. Bead mill tube placed into centrifuge for 5 minutes at 3500rpm	3. Bead mill tube placed into centrifuge for 5 minutes at 3500rpm
4. Sample decanted from bead mill tube into centrifuge filter tube and placed back into centrifuge for 5 minutes at 3500rpm	4. Sample decanted from bead mill tube into centrifuge filter tube and placed back into centrifuge for 5 minutes at 3500rpm
5. SPE is performed	5. SPE is performed

Two types of centrifuge filter tubes were used in this experiment due to their availability: Chrom Tech® PFTE 0.45µm, Midi tubes and Sartorius Vivaspin® 6 tubes with a PES filter.

2.4.5 Data processing

TargetLynx was used to acquire limit of detection, limit of quantitation, linear dynamic range, recovery, calibration curves, bias, and precision data. A ten-point calibration curve was run in triplicate. Samples ranged in concentration

from 1pg/mL to 1ng/mL, and 15pg/mL and 300pg/mL samples acted as low and high quality controls (QC).

A linear calibration model with a 1/x weighting factor was used to create a curve to which samples could be compared. This curve also determines limit of detection (LOD), limit of quantitation (LOQ), percent bias, percent precision, and linear dynamic range (LDR). According to the Scientific Working Group for Forensic Toxicology, LOD is defined as "an estimate of the lowest concentration of an analyte in a sample that can be reliably differentiated from blank matrix and identified by the analytical method"[39]; from a chromatographic stand point, LOD is represented by lowest concentration where the signal height of the peak of interest is greater than $1/10^{th}$ the signal height of the baseline noise. However, LOQ is defined as an estimate of the lowest concentration of an analyte in a sample that can be reliably measured with acceptable bias and precision"[39]; on a chromatogram, this is the lowest concentration at which the signal height of the peak of interest is greater than $1/3^{rd}$ the height of the baseline noise. Bias refers to how close your data points are to the expected concentration, and precision refers to how close your data points are to each other for a given concentration. To provide a reference point, all samples were spiked with an internal standard mixture of Ketamin-D4 and Xylazine-D6 post extraction at a concentration of 2ng/mL. This provides a known concentration for the calibration model to compare to the calibration curve samples and acts as an indicator that the instrumentation was functioning correctly for the duration of the run.

3. RESULTS AND DISCUSSION

3.1 SPE Cartridge Evaluation

Out of the three SPE cartridge sorbents evaluated, HLB Prime showed the highest sample recovery percentage.

Table 7: Percent Recovery for HLB 3cc, 60mg extraction

	0	10	20	30	40	50	60	70	80	90	100
Ketamine ACN pH3	0.06	85.22	13.15	1.19	0.20	0.06	0.04	0.04	0.03	0.00	0.01
Ketamine ACN pH10	0.12	0.12	12.54	81.45	3.90	0.68	0.35	0.28	0.17	0.18	0.21
Ketamine MeOH pH3	0.16	4.61	83.98	7.70	1.58	0.82	0.44	0.29	0.19	0.22	0.00
Ketamine MeOH pH10	0.05	0.12	2.76	50.84	15.74	20.33	5.17	2.74	1.05	0.80	0.41
Xylazine ACN pH3	0.03	52.57	34.09	7.69	2.47	1.29	0.56	0.52	0.42	0.24	0.11
Xylazine ACN pH10	0.16	0.93	1.08	60.31	25.50	5.62	2.20	1.51	0.85	0.58	1.26
Xylazine MeOH pH3	0.19	1.47	78.15	10.28	3.15	2.06	1.60	0.95	1.18	0.97	0.00
Xylazine MeOH pH10	0.17	0.46	0.77	26.00	12.12	24.71	12.79	12.07	5.77	3.14	1.99

Maximum Recovery

Table 8: Percent Recovery for HLB PRiME 3cc, 60mg

	50	55	60	65	70	75	80	85	90
Ketamine ACN pH3	95.29	93.41	94.26	92.68	90.38	92.42	92.62	90.89	88.42
Ketamine ACN pH7	50.73	61.55	62.77	65.90	71.64	74.51	79.49	80.48	81.49
Ketamine ACN pH10	67.87	68.29	70.48	78.37	79.14	81.96	82.12	82.84	85.07
Ketamine MeOH pH3	86.84	90.71	88.44	89.45	89.09	89.09	89.68	89.97	96.53
Ketamine MeOH pH7	2.98	17.78	30.98	45.67	54.08	63.25	67.93	72.62	91.80
Ketamine MeOH pH10	4.15	20.25	35.98	55.01	64.81	75.92	77.60	81.92	94.14

	50	55	60	65	70	75	80	85	90
Xylazine ACN pH3	92.11	87.99	90.60	89.51	85.63	89.55	89.31	88.45	87.45
Xylazine ACN pH7	15.62	25.26	29.19	33.61	42.51	39.75	48.72	49.76	53.66
Xylazine ACN pH10	28.83	31.91	37.83	53.28	51.13	56.70	57.25	55.86	56.12
Xylazine MeOH pH3	79.55	85.21	82.25	85.30	85.82	84.95	86.18	87.20	95.20
Xylazine MeOH pH7	0.56	12.48	13.44	23.98	28.08	35.34	32.90	36.20	68.41
Xylazine MeOH pH10	1.68	12.92	23.56	38.06	56.38	63.00	72.29	72.54	92.07

Maximum Recovery

Table 9: Percent Recovery for MCX 3cc, 60mg extraction

	Loading	Wash 1 pH3	Wash 2 pH3	Elution pH10
Ketamine LpH3 ACN	0.00	0.00	85.84	14.15
Ketamine LpH7 ACN	0.04	0.02	86.03	13.91
Ketamine LpH10 ACN	0.28	0.02	99.70	0.00
Ketamine LpH3 MeOH	0.03	0.01	89.93	10.03
Ketamine LpH7 MeOH	0.04	0.05	86.98	12.93
Ketamine LpH10 MeOH	0.32	0.03	99.65	0.00
Xylazine LpH3 ACN	0.14	0.08	88.94	10.84
Xylazine LpH7 ACN	0.07	0.06	89.47	10.39
Xylazine LpH10 ACN	0.25	0.02	99.73	0.00
Xylazine LpH3 MeOH	0.17	0.08	88.30	11.44
Xylazine LpH7 MeOH	0.19	0.22	84.97	14.61
Xylazine LpH10 MeOH	0.58	0.04	99.38	0.00

Maximum Recovery

In the initial MCX cartridge experiment, approximately 83.26% of the target analytes were being eluted in the second wash step (Table 9). To determine the cause, increasing percentages from 10% to 100% of organic solvent in water at 5% formic acid were used to wash the cartridge, and the area counts of each wash sample were evaluated (Table 10).

Table 10: Percent Recovery for MCX 3cc, 60mg cartridges, Wash 2 10-100% Organic Percentages

Sample	Ketamine % Recovery	Xylazine % Recovery
Loading	0.07	0.13
W1 pH3	0.08	0.10
W2 10% MeOH pH3	0.15	0.04
W2 20% MeOH pH3	0.31	0.16
W2 30% MeOH pH3	0.17	0.08
W2 40% MeOH pH3	0.23	0.04
W2 50% MeOH pH3	0.11	0.02
W2 60% MeOH pH3	0.22	0.03
W2 70% MeOH pH3	0.24	0.07
W2 80% MeOH pH3	0.14	0.05
W2 90% MeOH pH3	0.06	0.03
W2 100% MeOH pH3	0.10	0.07
Elute 100% MeOH pH10	98.13	99.20

Maximum Recovery

In addition, a larger MCX sorbent bed size of 6cc, 150mg was evaluated (Table 11).

Table 11: Percent Recovery for MCX, 6cc, 150mg solvent extraction

	Loading	Wash 1	Wash 2	Elution
Ketamine in ACN	1.69	0.19	1.15	96.97
Ketamine in MeOH	1.76	0.08	1.54	96.63
Xylazine in ACN	0.22	0.15	0.46	99.18
Xylazine in MeOH	1.90	0.45	0.39	97.25

Maximum Recovery

While the increasing organic percentages and larger sorbent sizes for the MCX cartridges did produce chromatograms replicating similar results from previous studies, the HLB PRiME 6cc, 200mg SPE cartridges showed less extraneous peaks in comparison, as seen in Figure 12.

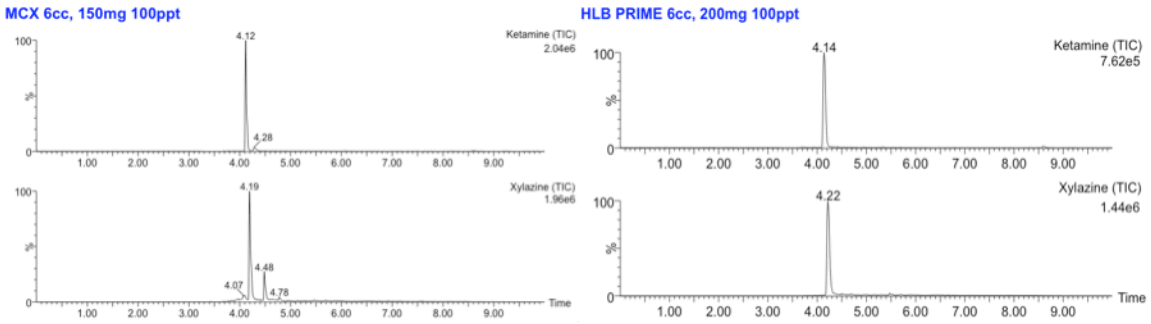

Figure 12: MCX versus HLB PRiME comparison

Table 12: Percent Recovery for HLB PRiME 6cc, 200mg extraction evaluation, Loading 50%MeOH pH10, Eluting 50%ACN pH3

	Load 50%MeOH pH10	Elute 50%ACN pH3	Wash 100% ACN pH3
Ketamine Solvent A	-	94.82	5.18
Ketamine Solvent B	-	95.64	4.36
Ketamine Solvent C	0.68	**97.71**	1.61
Ketamine Solvent D	0.60	**98.16**	1.24
Xylazine Solvent A	-	92.82	7.18
Xylazine Solvent B	-	93.89	6.11
Xylazine Solvent C	0.35	**96.85**	2.80
Xylazine Solvent D	0.25	**97.41**	2.34

Maximum Recovery

After determining the SPE cartridge, sample preparation commenced. While running the hair extraction calibration curves, an abnormally large concentration of ketamine was observed in even the lowest calibrators that had not been observed in the bone extractions. Two types of centrifuge filter tubes were used in this experiment: Chrom Tech® PFTE 0.45 μm, Midi tubes and Sartorius Vivaspin® 6 tubes with a PES filter. The bone extractions were performed with the Chrom Tech® tubes, and the hair extractions were performed with the Vivaspin® tubes. Blank hair extracted with the Vivaspin® tubes showed

an approximate e7 signal for ketamine's 125.1 quantitation ion, as shown in Figure 13.

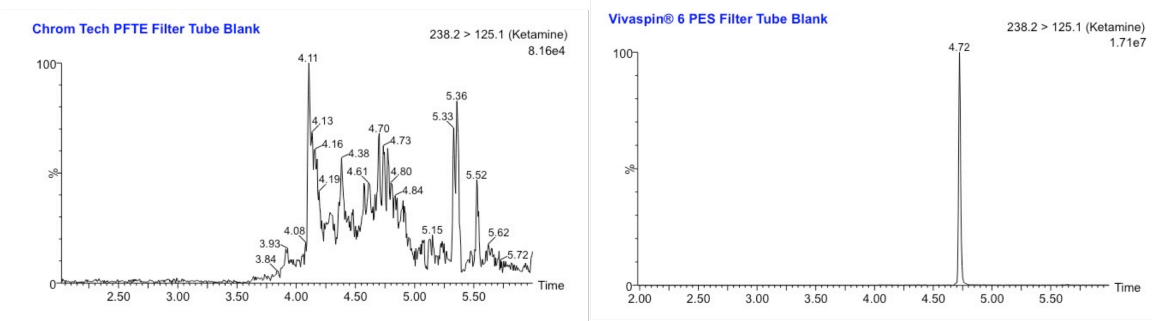

Figure 13: Chrom Tech® and Vivaspin® Contamination Comparison

This may be due to the PES filter or the plastic material of the tube itself. Thus solvent extractions were performed to evaluate LOD, LOQ, accuracy, bias and precision.

3.2 Solvent Extraction Calibration

The linear dynamic range is determined to be 2pg/mL-1ng/mL for xylazine and 5pg/mL-1ng/mL for ketamine. The LOD and LOQ values are 2pg/mL for xylazine and 5pg/mL for ketamine. These numbers were determined by evaluating the three calibration curves and determining the ranges that achieved r^2 values of 0.98 or better, as per SWGTOX guidelines and can be seen in Figures 14 and 15.

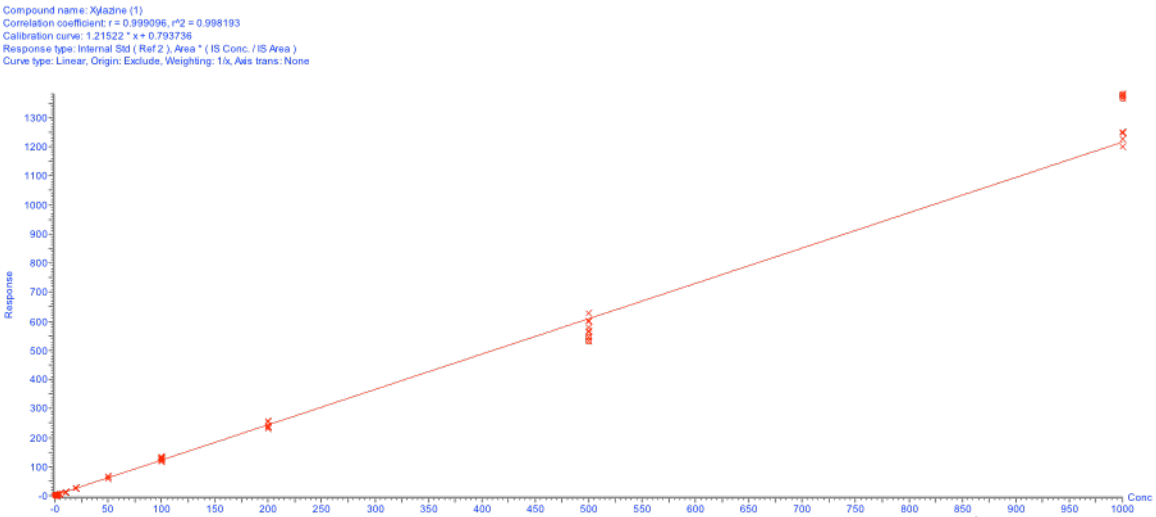

Figure 14: Xylazine Solvent Extraction, Calibration Curves 1-3, r^2=0.998

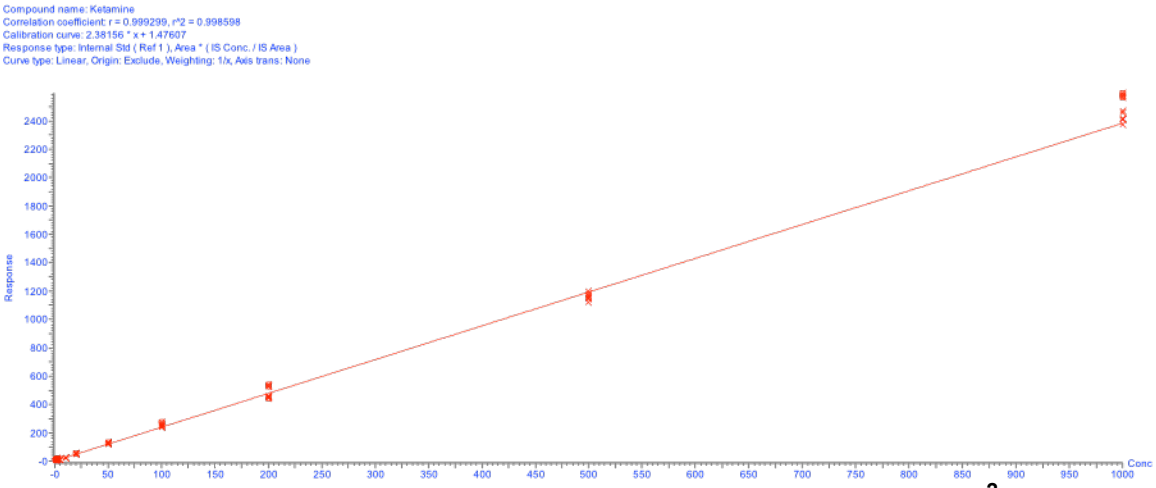

Figure 15: Ketamine Solvent Extraction, Calibration Curves 1-3, r^2=0.998

Both the ketamine and xylazine curves have r^2 values of 0.998. Those values fit the curve by maintaining a line of best fit within ±20% percent for accuracy and precision.

4. CONCLUSIONS

This research focused on developing an efficient method for detection of ketamine and xylazine in rat fur and bone samples following a six-month outdoor exposure period, in order to mimic forensic casework involving bones and hair. Current methods are time-consuming, so reducing extraction and evaluation times would increase case outputs in forensic labs while reducing the amount of sample and consumables required. Multidimensional chromatography reduces preparation times by eliminating the drying down and reconstitution of samples, which can take hours as opposed to half an hour total extraction time shown in this study. The opioid epidemic has increased the amount of forensic casework, including toxicological samples. With these reduced preparation times, forensic toxicology labs will be able to efficiently process their samples without sacrificing sensitivity.

In developing the extraction method, HLB PRiME, a different SPE sorbent bed chemistry, produced equivalent recovery percentages and signal strengths with improved peak shape when compared to the MCX cartridges used in previous studies. This opens up additional avenues of exploration for different analytes of interest that may respond better to the HLB PRiME sorbent

Using a solvent extraction, the linear dynamic ranges of both ketamine and xylazine were established to be 5pg/mL-1ng/mL and 2pg/mL-1ng/mL in both LOD and LOQ, which indicates a sensitivity suitable for quantitation down to trace levels of detection.

5. FUTURE DIRECTIONS

This study has opened up new avenues of exploration for forensic toxicology. Now that HLB PRiME has proven to be a suitable SPE sorbent for multidimensional chromatography, this increases the range of drugs that can be analyzed using this method. Because designer drugs are appearing in an increasing number of forensic cases, having multiple SPE options increases the likelihood that they will be detectable using multidimensional chromatography.

In addition, future analysis of unknown samples will assist in the development of forensic methods. The Chrom Tech® tubes were effective in removing physical contaminants without introducing new ones, so they should be used going forward. The bone and fur samples utilized here will provide case analogs with known dosages for comparison. In addition to postmortem toxicology, DFSA cases would be another case type where multidimensional chromatography would prove useful. The process of incorporating drugs into hair results in detection at least three weeks post-exposure, which means victims who report after the window for analysis via blood and urine has closed may still have toxicological evidence that can be non-invasively collected.

The use of nontraditional matrices can also be incorporated into drug chemistry cases. More states are legalizing the use of marijuana for both medical and recreational uses, leading to an increased presence of tetrahydrocannabinol, its metabolites, and its analogs in casework, not just in plant form, but in oil and

edible formulations. The efficient preparation and extraction processes utilized in this study can be applied to analyzing those samples with the same sensitivity.

BIBLIOGRAPHY

1. Dinis-Oliveira RJ, Carvalho F, Duarte JA, Remião F, Marques A, Santos A, et al. Collection of biological samples in forensic toxicology. Toxicology Mechchanisms and Methods 2010;20(7):363–414.

2. Skopp G. Postmortem toxicology. Forensic Science, Medicine and Pathology [Internet] 2010;6(4):314–25. Available from: http://link.springer.com/10.1007/s12024-010-9150-4

3. Openstax. Muscle Tissue [Internet]. 2013; Available from: https://openstax.org/details/books/anatomy-and-physiology

4. White RM. Drugs in hair. Part I. Metabolisms of major drug classes. Forensic Science Review 2017;29(1):23–55.

5. Grass J, Miller R, Carlitz EHD, Patrovsky F, Gao W, Kirschbaum C, et al. In vitro influence of light radiation on hair steroid concentrations. Psychoneuroendocrinology [Internet] 2016;73:109–16. Available from: http://dx.doi.org/10.1016/j.psyneuen.2016.07.221

6. Ettlinger J, Yegles M. Influence of thermal hair straightening on cannabis and cocaine content in hair. Forensic Science International [Internet] 2016;265:13–6. Available from: http://dx.doi.org/10.1016/j.forsciint.2016.01.002

7. Crunelle CL, Yegles M, De Doncker M, Dom G, Cappelle D, Maudens KE, et al. Influence of repeated permanent coloring and bleaching on ethyl glucuronide concentrations in hair from alcohol-dependent patients. Forensic Science International [Internet] 2015;247:18–22. Available from: http://dx.doi.org/10.1016/j.forsciint.2014.11.023

8. Society of Hair Testing. Recommendations for Hair Testing in Forensic Cases Society of Hair Testing Criteria for mass spectrometric analysis. Forensic Science International [Internet] 2004;145:83–4. Available from: http://www.soht.org/pdf/Consensus_on_Hair_Analysis.pdf

9. Baciu T, Borrull F, Aguilar C, Calull M. Recent trends in analytical methods and separation techniques for drugs of abuse in hair. Analytica Chimica Acta 2015;856:1–26.

10. Desrosiers NA, Watterson JH. The effects of burial on drug detection in skeletal tissues. Drug Testing and Analysis 2010;2(7):346–56.

11. Watterson JH, VandenBoer TC. Effects of tissue type and the dose-death interval on the detection of acute ketamine exposure in bone and marrow with solid-phase extraction and ELISA with liquid chromatography-tandem mass spectrometry confirmation. Journal of Analytical Toxicology 2008;32(8):631–8.

12. Rollins DE, Wilkins DG, Krueger GG, Augsburger MP, Mizuno A, O'Neal C, et al. The Effect of Hair Color on the Incorporation of Codeine into Human Hair. Journal of Analytical Toxicology 2003;27(8):545–51.

13. Seeman P, Ko F, Tallerico T. Dopamine receptor contribution to the action of PCP, LSD and ketamine psychotomimetics. Molecular Psychiatry 2005;10(9):877–83.

14. Mrgreen71. Ketamine [Internet]. 2009;Available from: https://commons.wikimedia.org/wiki/File:Ketamine.svg

15. Green SM, Roback MG, Kennedy RM, Krauss B. Clinical practice guideline for emergency department ketamine dissociative sedation: 2011 update. Annals of Emergency Medicine [Internet] 2011;57(5):449–61. Available from: http://dx.doi.org/10.1016/j.annemergmed.2010.11.030

16. Drug Enforcement Administration. Ketamine. Office of Diversion Control 2013;1.

17. U.S. Drug Enforcement Administration: Office of Diversion Control. NFLIS-Drug 2017 Midyear Report. 2017;

18. U.S. Drug Enforcement Administration: Office of Diversion Control. NFLIS-Drug 2016 Midyear Report. 2016;

19. U.S. Drug Enforcement Administration: Office of Diversion. 2016 ANNUAL REPORT Special NFLIS Announcement. 2018;(April).

20. The Washington Post/Kaiser Family Foundation Survey Project. Poll: One in Five women say they have been sexually assaulted in college [Internet]. Washington Post. 2015 [cited 2018 Jul 1];Available from: http://www.washingtonpost.com/graphics/local/sexual-assault-poll/

21. Mella M, Schweitzer B, Botch-Jones SR, Mallet CR, Ph D. [APPLICATION NOTE] Analysis of Ketamine and Xylazine in Rat Tissues Using the ACQUITY UPLC with 2D Technology [APPLICATION NOTE]. Waters Corporation 2016;1–11.

22. Favretto D, Vogliardi S, Tucci M, Simoncello I, El Mazloum R, Snenghi R. Occupational exposure to ketamine detected by hair analysis: A retrospective and prospective toxicological study. Forensic Science International [Internet] 2016;265:193–9. Available from: http://dx.doi.org/10.1016/j.forsciint.2016.03.010

23. Barreto AS, Brant VF, Spinelli E, Rodrigues S V. Validation of a SPE-LC–MS/MS method for the determination of ketamine and norketamine in micropulverized hair after a single IV dose. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences [Internet] 2016;1033–1034:200–9. Available from: http://dx.doi.org/10.1016/j.jchromb.2016.08.027

24. Tabernero MJ, Felli ML, Bermejo AM, Chiarotti M. Determination of ketamine and amphetamines in hair by LC/MS/MS. Analytical and Bioanalytical Chemistry 2009;395(8):2547–57.

25. Vaccinationist. Xylazine [Internet]. 2015;Available from: https://commons.wikimedia.org/wiki/File:Xylazine.svg

26. Gehring R, Coetzee JF, Tarus-Sang J, Apley MD. Pharmacokinetics of ketamine and its metabolite norketamine administered at a sub-anesthetic dose together with xylazine to calves prior to castration. Journal of Veterinary Pharmacology and Therapeutics 2009;32(2):124–8.

27. Santonastaso A, Hardy J, Cohen N, Fajt V. Pharmacokinetics and pharmacodynamics of xylazine administered by the intravenous or intra-osseous route in adult horses. Journal of Veterinary Pharmacology and Therapeutics 2014;37(6):565–70.

28. Ruiz-Colón K, Martínez MA, Silva-Torres LA, Chavez-Arias C, Meléndez-Negrón M, Conte-Miller MS, et al. Simultaneous determination of xylazine, free morphine, codeine, 6-acetylmorphine, cocaine and benzoylecgonine in postmortem blood by UPLC-MS-MS. Journal of Analytical Toxicology 2012;36(5):319–26.

29. Torruella RA. Xylazine (veterinary sedative) use in Puerto Rico. Substance Abuse: Treatment, Prevention, and Policy [Internet] 2011;6(1):7. Available from: http://www.substanceabusepolicy.com/content/6/1/7

30. Reyes JC, Negrón JL, Colón HM, Padilla AM, Millán MY, Matos TD, et al. The emerging of xylazine as a new drug of abuse and its health consequences among drug users in Puerto Rico. Journal of Urban Health 2012;89(3):519–26.

31. Capraro AJ, Wiley JF, Tucker JR. Severe intoxication from xylazine inhalation. Pediatric Emergency Care 2001;17(6):447–8.

32. Silva-Torres L, Velez C, Lyvia Alvarez J, Ortiz JG, Zayaz B. Toxic Effects of Xylazine on Endothelial Cells in COmbination with Cocaine and 6-monoacetylmorphine. Toxicology In Vitro 2014;28(7):1312–9.

33. Mallet CR, Lu Z, Mazzeo JR. A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. Rapid Communications in Mass Spectrometry 2004;18(1):49–58.

34. Buszewski B, Noga S. Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. Analytical and Bioanalytical Chemistry 2012;402(1):231–47.

35. Meyer V. Practical high-performance liquid chromatography. 4th ed. Chichester: John Wiley, 2004;

36. Neue U. HPLC columns: theory, technology, and practice. New York: Wiley-VCH, 1997;

37. Lenehan C. Chromatography: Basic Principles. In: Houck, PhD, FRSC MM, editor. Forensic Chemistry. San Diego: Elsevier Inc., 2015; 62–71.

38. Mella M. Detection of cocaine and its major metabolites in bone following outdoor decomposition after chronic cocaine administration using 2D-LC/MS/MS. 2017;

39. Scientific Working Group for Forensic Toxicology. Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology Report from the Scientific Working Group for Forensic Toxicology. Journal of Analytical Toxicology [Internet] 2013;37(7):452–74. Available from: https://vula.uct.ac.za/access/lessonbuilder/item/177330/group/57183a37-6c5d-4efd-ad65-310b5f4140a1/Required Reading/Method Validation_SWGTOX.pdf

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