## Contents

## Abstract

The synthetic cathinones are powerful psychostimulants that have been associated with impairment, intoxication, and fatal overdose. Forensic laboratories must be able to identify these new drugs as part of antemortem and postmortem toxicology investigations. Anecdotally, and in a small number of preliminary reports, some of the cathinones are reported to be unstable. It is important to understand drug stability in biological evidence in order to interpret analytical findings in criminal and death investigations. The purpose of this study was to systematically evaluate the stability of synthetic cathinones in urine and blood using liquid-chromatography/quadrupole-time of flight-mass spectrometry (LC-Q/TOF-MS). The stability of twenty-two synthetic cathinones were investigated in terms of pH, temperature, and structural characteristics.

Solid phase extraction (SPE) and LC-Q/TOF-MS was used to evaluate the stability of synthetic cathinones in blood and urine over a period of six months. Cathinone stability was systematically evaluated in urine at pH 4 and 8 and at physiological pH in blood to determine analyte, pH, concentration, and temperature (-20°C, 4°C, 20°C, and 32°C) dependence. A total of twenty-two cathinones were selected based on their structural features and functional substituents. Cathinones bearing secondary and tertiary (pyrrolidinyl) amines, ring substituents, and methylenedioxy substituents were included.

Cathinone stability was highly temperature dependent, pH dependent, and analyte dependent. No concentration dependence was observed. Cathinones were most stable when frozen in acidic urine and least stable under alkaline conditions at elevated temperatures. Moreover, the chemical structure of these polyfunctional aminoketones had a profound influence on stability. Under some conditions, drugs were completely undetectable within twenty-four hours of storage. Under all conditions tested, the pyrrolidine-type cathinones were the most stable, followed by the methylenedioxy-type, ring substituted, and unsubstituted cathinones. Both the methylenedioxy and pyrrolidinyl substituents exerted a significant stabilizing effect.

Finally, authentic urine samples from cathinone users were reanalyzed following specified periods of storage. A total of 188 specimens were investigated, yielding a total of 197 cathinone positive findings. Analyte-dependent differences in stability were consistent with experimentally determined data using fortified samples. These findings also confirmed the critical importance of specimen pH on cathinone stability.

Biological evidence may be subjected to a variety of environmental conditions prior to, and during transport to the forensic laboratory. These findings demonstrate the inherent instability of certain cathinone species in biological evidence under some conditions. Moreover, this study highlights the need for quantitative drug findings in toxicological investigations to be interpreted cautiously, and within the context of specimen storage and integrity.

## Executive Summary

Synthetic cathinones are a class of designer drug derived from cathinone, the principal psychoactive component of "khat" (*Catha edulis)*. Due to their amphetamine-like structure, the drugs can be classified as sympathomimetic amines. However, synthetic cathinones can produce a complex array of adrenergic and serotonergic effects, and the combination of stimulant and mood-altering sensations have contributed to the popularity of these substances among recreational drug users.

The federal government exercised its emergency scheduling authority to address cathinone abuse in late 2011. Since then, many of the synthetic cathinones have been permanently scheduled, principally as Schedule I drugs, in the Federal Controlled Substances Act. Despite regulation and enforcement efforts, new cathinones continue to emerge. Detection of these substances in toxicological investigations is of paramount importance because of their potential impact on public health and safety. There have been numerous reports of impaired driving, fatal intoxications, and adverse consequences following cathinone use. Quantitative drug toxicology, most frequently using blood, may be used interpretively in forensic investigations. However, inherent instability, or changes that take place during storage, transportation, or during human decomposition, may change the concentration of the drug. Potential differences in concentration between the time of interest (*i.e.* death, driving, or other activity where drug use is of interest) and the time of testing, must be carefully considered. Currently the literature pertaining to cathinone stability in biological samples is relatively limited. In this report we describe a systematic and comprehensive approach to evaluate cathinone stability in terms of matrix, pH, temperature, concentration, and analyte dependence. This approach will not only aid in the interpretation of toxicological findings, but will also improve our understanding of future designer drugs within the class.

Twenty-two synthetic cathinones were included in the study, reflecting common cathinones of abuse and compounds with a variety of benzylic and amine substituents. These included methcathinone, ethcathinone, pentedrone, buphedrone, 3-fluoromethcathinone (3-FMC), 4-

fluoromethcathinone (4-FMC), 4-methylethcathinone (4-MEC), 4-ethylmethcathinone (4-EMC), mephedrone, methedrone, 3,4-dimethylmethcathinone (3,4-DMMC), ethylone, butylone, pentylone, eutylone, methylone, methylenedioxypyrovalerone (MDPV), 4-methylpyrrolidinobutiophenone (MPBP), 3,4-methylenedioxypyrrolidinobutiophenone (MDPBP), α-pyrrolidinopentiphenone (α-PVP), pyrovalerone, and naphyrone. Of the twenty-two drugs selected, sixteen were secondary amines. Of these, four were not substituted at the benzene ring (methcathinone, ethcathinone, buphedrone, and pentedrone), seven were ring substituted (mephedrone, 4-MEC, 4-EMC, methedrone, 3,4-DMMC, 3-FMC, and 4-FMC) and five were methylenedioxy substituted (ethylone, methylone, butylone, pentylone, and eutylone). Six tertiary amines (pyrrolidines) were also included and of these, two were methylenedioxy substituted.

Cathinone stability was evaluated in preserved blood (pH 7) and urine (pH 4 and 8) at two concentrations (100 and 1,000 ng/mL) at four storage temperatures. These were chosen to reflect frozen (-20°C) and refrigerated (4°C) long- and short-term storage temperatures at the laboratory; exposure to ambient (20°C) or room temperature during routine processing and handling; and finally, potential exposure to elevated temperatures during shipping and transport to the laboratory (32°C). A total of nine deuterated internal standards were utilized. All quantitative measurements were performed using LC-Q/TOF-MS following isolation of the drugs by SPE. The analytical procedure was fully validated in accordance with recognized guidelines (SWGTOX, 2013). Extraction efficiencies were 84-104% and 81-93% in urine and blood, respectively. Limits of quantitation in both matrices were 0.25 – 5 ng/mL. Precision, bias, and matrix effect were all within acceptable thresholds and the assay was free from more than fifty interferences.

Drug stability was evaluated to determine analyte, concentration, pH, matrix, and temperature dependence. Although no concentration dependence was observed, cathinone stability was highly analyte dependent. Structural features and substituents within these arylaminoketones exerted significant stabilizing and destabilizing effects. Notably, 3-FMC was the least stable of all

of the drugs tested. Significant differences were observed between secondary and tertiary amines. Pyrrolidinyl analogs were inherently more stable, demonstrating far greater resilience than their secondary amine counterparts. With the exception of fluorine substitution, stability within the ring substituted cathinones was not significantly different. Both the unsubstituted and ring substituted cathinones were equally unstable under most conditions. In contrast, however, the methylenedioxy substituted cathinones were significantly more stable. The stabilizing effect of the methylenedioxy group was observed for both the secondary amines and the pyrrolidines. As a result, cathinone species that contained both a methylenedioxy and a pyrrolidine were the most stable drugs tested. Stability was also highly pH dependent. Cathinones were considerably more stable under acidic conditions. Degradation of the drug was accelerated dramatically under alkaline conditions, for even the most stable drugs. Significant temperature dependent stability was observed for all cathinones. Exposure to elevated temperature decreased estimated half-lives by several orders of magnitude for some drugs. With the exception of the methylenedioxy substituted pyrrolidines, significant degradation was observed for all drugs within hours following exposure to elevated temperatures (32$^{o}$C). With the exception of 3-FMC, cathinones were stable, or underwent only moderate degradation in blood when frozen. At refrigerated temperatures in blood, all drugs except 3-FMC were stable or underwent moderate losses (<40%) during the first 30 days of storage.

At elevated temperatures in blood, all of the cathinones demonstrated significant (>20%) loss within 5.5 hours (3-FMC) to 7 days (for the most stable methylenedioxy substituted pyrrolidines). At refrigerated temperature, significant losses were seen within 7 days to more than five months in blood, and 1 day to more than six months in pH 8 urine. These results highlight the critical role of chemical structure among these complex arylaminoketones. Although frozen temperatures provided the greatest protection from loss, this is not necessarily feasible in many laboratories, except for long-term storage. Studies using fortified matrix show that exposure of biological evidence to elevated or ambient temperatures can significantly decrease concentrations over time. However, given the analyte dependent differences in stability, cathinones bearing secondary amines are the most susceptible to loss.

Urine specimens (n=188) obtained from cathinone users were also investigated following specified periods of refrigerated storage. The 188 samples yielded a total of 197 cathinone positive findings for nine cathinones. Of these, quantitative comparisons were made in 162 instances. Quantitative comparisons using authentic urine samples from cathinone users were in good agreement with experimentally determined stability data using fortified matrix. This data also underscored the critical importance of specimen pH on overall drug stability. Moreover, the limited degradation of some drugs following extended periods of storage suggest that pH dependent variables were equally as important as conventional time dependent interpretation of drug stability.

Upon receipt in the laboratory, forensic toxicology specimens are typically stored at refrigerated or frozen temperatures for short- or long-term storage. During specimen transport and routine handling, these items may be subjected to ambient or elevated temperatures. For unstable drugs, the concentration at the time of testing may be significantly different from the time of interest. Even in the presence of commonly used preservatives, degradation may be inevitable. As a result, evidence disposition and conditions of storage must be considered when interpreting toxicological findings related to the synthetic cathinones.

## I. Introduction

### Statement of the Problem

The proliferation of new psychoactive substances (NPS) and designer drugs has received widespread attention, both nationally and globally. Designer drugs are often perceived by drug users to be advantageous from both a pharmacological and legal standpoint. Small alterations in structure may produce considerable changes in terms of the perceived effects by the drug user, but may also circumvent existing drug legislation. Demand from recreational drug users, and the clandestine supply and effective "marketing" of designer drugs via the Internet, has significantly outpaced the ability of government to regulate, legislate, and enforce those actions. Although legislative actions typically reduce the use of a particular drug, they are quickly replaced by new analogs.

Cathinones are one of the many classes of designer drug. Abused principally for their psychostimulant or amphetamine-like effects, they have been associated with a wide array of toxicological investigations, including impaired driving, overdose, and fatal intoxications.

Since the federal government first exercised emergency scheduling authority to control a small number of synthetic cathinones in 2011, at least 43 states and Puerto Rico have enacted legislation to control their abuse (National Conference of State Legislatures, 2015). Synthetic cathinones are derivatives of cathinone, a psychoactive substance of natural origin (*Catha edulis*, or "khat"). They were initially marketed as bath salts, plant food, insect repellant, pond cleaner, vacuum freshener, or research chemicals. Although clearly intended for recreational purposes, most are labeled "not for human consumption" in an effort to avoid criminal prosecution. Marketed under a wide variety of street names (Ivory Wave, Vanilla Sky, Cotton Cloud, Snow Day, and Ocean Snow and more), these synthetic derivatives can produce a complex array of stimulant and psychedelic effects.

Liquid chromatography-mass spectrometry has been used to identify several synthetic cathinones in biological samples. Gas chromatography-mass spectrometry (GC/MS) is also used,

but is somewhat limited by the relatively poor mass spectral qualities of some of the synthetic cathinones. Nevertheless, concentrations of drugs have been reported over a wide range in both criminal and death investigations. Forensic toxicology laboratories responsible for analyzing antemortem and postmortem specimens go to considerable lengths to ensure that the analytical methods that are used are of sufficient quality to produce reliable quantitative results. However, questions concerning the stability of cathinones have arisen, but have not yet been fully or systematically investigated.

Forensic toxicology specimens may be subjected to a variety of conditions during sample transport, shipping, storage, and analysis that may cause drug concentrations to change considerably between the time of collection and the time of analysis. Furthermore, information is limited because not all forensic toxicology laboratories routinely screen for these drugs. Significant knowledge gaps exist with respect to their stability. In order for toxicological results to be reliably interpreted in forensic investigations, factors that influence drug stability must be considered. In this report, we describe a systematic approach to evaluate cathinone stability in blood and urine to aid in the investigation and interpretation of forensic toxicology findings.

**Literature Citations and Review**

Use and Abuse

Synthetic cathinones are a rapidly evolving class of designer drug that are structurally related to cathinone, the principal psychoactive component of khat. These sympathomimetic amines are potent modulators of the monoamine transporters dopamine, noradrenaline, and serotonin, but their selectivity for the transporter varies significantly, producing a complex array of adrenergic and serotonergic effects. The combination of stimulant and mood-altering sensations has contributed to their popularity among recreational drug users. They are most frequently encountered as pills, powders, or capsules. Drug users typically ingest, inhale, inject, smoke, or insufflate these drugs, which sell for approximately $20-35 per gram (Prosser, 2012). Desired effects of the drug include increased energy, empathy, openness, and increased libido. However,

cardiac, psychiatric, and neurological effects are common among users that require medical treatment.

The synthetic cathinones of forensic importance to date consist of arylamino ketones that can be categorized into alkylamines (principally secondary amines) and pyrrolidines (tertiary amines). Their chemical behavior is dominated by two functional groups: the ketone and the amine. The cathinones are either ring substituted ($R_1$ and $R_2$), formed by the variation of the alpha-carbon substituent ($R_3$), or *N*-alkylated ($R_4$ and $R_5$) (**Figure 1**).

Although gas chromatography-mass spectrometry (GC/MS) is a widely used technique in forensic toxicology laboratories, several of the synthetic cathinones produce electron ionization (EI) mass spectra of relatively poor specificity. Additionally, cathinones may undergo thermal degradation to oxidative breakdown products in situ (Noggle, 1994; DeRuiter, 1994; Archer, 2009; Tsujikawa, 2013; Kerrigan, 2016). For these reasons, liquid chromatography-mass spectrometry (LC/MS) and LC/MS/MS have been the most widely used techniques for the identification of cathinones in biofluids to date. Ammann was the first to report the use of LC/MS/MS to simultaneously identify a large number of cathinones in blood (Ammann, 2012). At the inception of this study, a total of twenty-two synthetic cathinones were commercially available and the stability of these compounds in biological fluids was investigated (**Table 1**).

**Figure 1.** General cathinone structure.

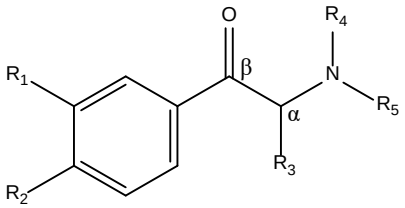


**Table 1.** Synthetic cathinones included in the study.

| Common Name | IUPAC Name | Other Names |
|---|---|---|
| 3,4-DMMC | 1-(3,4-dimethylphyenyl)-2-(methylamino)-propan-1-one | 3,4-dimethylmethcathinone |
| 4-EMC | 1-(4-ethylphenyl)-2-methylaminopropan-1-one | 4-ethylmethcathinone |
| 3-FMC | 1-(3-fluorophenyl)-2-(methylamino)-propan-1-one | 3-fluoromethcathinone |
| 4-FMC | 1-(4-fluorophenyl)-2-(methylamino)-propan-1-one | 4-fluoromethcathinone, flephedrone |
| 4-MEC | 1-(4-methylphenyl)-2-ethylaminopropan-1-one | 4-methylethcathinone |
| Buphedrone | 2-methylamino-1-phenyl-butan-1-one | alpha-methylamino-butyrophenone |
| Butylone, bk-MBDB | 2-methylamino-1-(3,4-methylenedioxy-phenyl)-butan-1-one | beta-keto-*N*-methylbenzodioxoyl-butanamine |
| Ethcathinone | 2-ethylamino-1-phenyl-propan-1-one | *N*-ethylcathinone |
| Ethylone, bk-MDEA | 2-ethylamino-1-(3,4-methylenedioxy-phenyl)-propan-1-one | 3,4-methylenedioxy-*N*-ethylcathinone |
| Eutylone, bk-EBDB | 1-(1,3-benzodioxol-5-yl)-2-(ethylamino)-butan-1-one | beta-keto-ethylbenzodioxolylbutanamine |
| MDPBP | 1-(1,3-benzodioxol-5-yl)-2-(1-pyrrolidinyl)-1-butan-1-one | 3,4-methylenedioxy-alpha-pyrrolidinobutyrophenone |
| MDPV | 1-(1,3-benzodioxol-5-yl)-2-(pyrrolidinyl)-pentan-1-one | 3,4-methylenedioxypyrovalerone |
| Mephedrone, 4-MMC | 2-methylamino-1-(4-methylphenyl)-propan-1-one | 4-methylmethcathinone |
| Methcathinone | 2-methylamino-1-phenyl-propan-1-one | ephedrone |
| Methedrone, bk-PMMA | 1-(4-methoxyphenyl)-2-(methylamino)-propan-1-one | 4-methyoxy-methcathinone |
| Methylone, bk-MDMA | 2-methylamino-1-(3,4-methylenedioxyphenyl)-propan-1-one | 3,4-methylenedioxy-*N*-methylcathinone |
| MPBP | 1-(4-methylphenyl)-2-(1-pyrrolidinyl)-butan-1-one | 4-methyl-alpha-pyrrolidinobutiophenone |
| Naphyrone | 1-(2-naphthyl)-2-(1-pyrrolidinyl)-pentan-1-one | naphthylpyrovalerone |

| | | |
|---|---|---|
| Pentedrone | 2-methylamino-1-phenyl-pentan-1-one | alpha-methyalmino-valerophenone |
| Pentylone | 1-(1,3-benzodioxol-5-yl)-2-(methylamino)-pentan-1-one | beta-keto-methylbenzodioxolylpentanamine |
| α-PVP | 1-phenyl-2-(1-pyrrolidinyl)-pentan-1-one | alpha-pyrrolidinovalerophenone |
| Pyrovalerone | 1-(4-methylphenyl)-2-(1-pyrrolidinyl)-pentan-1-one | - |

Cathinones in Forensic Toxicology Investigations

Due to their central nervous system (CNS) stimulant-like effects, cathinones pose a significant risk of abuse and addiction. Reported effects include elevated blood pressure, increased heart rate, agitation, hallucinations, panic attacks, paranoia, delusions, and psychosis. MDPV, 4-MEC, α-PVP, mephedrone, methcathinone, and methylone have been reported in impaired driving investigations and following recreational use (**Table 2**). An even larger number of cathinones have been reported in death investigations (**Table 3**). Like many of their counterparts, drug concentrations in fatal and non-fatal investigations overlap, making interpretation of the results more complex (**Tables 2 and 3**). Cathinone concentrations in blood have been reported from the low ng/mL to several thousand ng/mL in both impaired driving and death investigation casework. Although numerous published case reports exist, their prevalence is likely underestimated because not all laboratories routinely screen for cathinones, or have the ability to detect only a limited number of analogs within the class.

**Table 2**. Published case reports in living subjects (antemortem toxicology).

| Drug | Concentration | Investigation | Reference(s) |
|---|---|---|---|
| 4-MEC | 46 ng/mL (blood) | Recreational Drug Use | Gil, 2013 |
| MDPV | 306 ng/mL (blood) | Recreational Drug Use | Adamowicz, 2013a |
| | 124 ng/mL (blood) | Driving Under the Influence | Adamowicz, 2013a |
| | 75 µg/L (serum) | Recreational Drug Use | Truscott, 2013 |
| | <10 – 530 ng/mL (serum) | Recreational Drug Use (n=20) | Grapp, 2017 |
| | 200-8,400 ng/mL (blood) | Driving Under the Influence (n=25) | Kriikku, 2011 |
| | <10-368 ng/mL (blood) | Driving Under the Influence/Recreational Drug Use (n=9) | Marinetti, 2013 |
| Mephedrone | 0.08-0.66 mg/L (blood) | Driving Under the Influence (n=9) | Cosbey, 2013 |
| | 150 ng/mL (serum) | Recreational Drug Use | Wood, 2010 |
| Methcathinone | 500 ng/mL (serum), 17,420 ng/mL (urine) | Recreational Drug Use | Belhadj-Tahar, 2005 |
| Methylone | 6.1 ng/mL (blood) | Driving Under the Influence | Knoy, 2014 |
| | 7 ng/mL (blood) | Driving Under the Influence | Marinetti, 2013 |
| α-PVP | 6.4-99 ng/mL (blood) | Driving Under the Influence (n=24) | Adamowicz, 2016 |
| | 1.2-56 ng/mL (blood) | Recreational Drug Use (n=4) | Adamowicz, 2016 |
| | 63 ng/mL (blood) | Driving Under the Influence | Knoy, 2014 |
| | 20-360 ng/mL (blood) | Driving Under the Influence (n=2) | Rojek, 2016 |
| | 70-100 ng/mL (blood) | Recreational Drug Use (n=2) | Dumestre-Toulet, 2017 |

**Table 3**. Published case reports involving fatalities (postmortem toxicology).

| Drug | Concentration | Reference(s) |
|---|---|---|
| 3,4-DMMC | 3.31 µg/mL (blood) | Sykutera, 2015 |
| 4-MEC | 152 ng/mL (blood), 122 ng/mL (urine) | Gil, 2013 |
| | 56 ng/mL (blood), 14.3 µg/mL (urine) | Gil, 2013 |
| | 0.170-1.73 mg/L (PM femoral blood) (n=2) | Smith, 2016 |
| Buphedrone | 127 ng/mL (blood) | Adamowicz, 2013a |
| | 3-127 ng/mL (blood) (n=2) | Zuba, 2013 |
| Ethylone | 0.39 mg/L (peripheral blood), 0.38 mg/L (central blood), 20 mg/L (urine) | McIntyre, 2014 |
| MDPV | 17-38 ng/mL (blood) (n=2) | Adamowicz, 2013a |
| | 670 ng/mL (urine), 82 ng/mL (serum) | Murray, 2012 |
| | 1,200 ng/mL (cardiac blood) | Namera, 2013 |
| | 39-130 ng/mL (femoral blood), 760-3,800 ng/mL (urine) (n=2) | Wright, 2013 |
| | 38 ng/mL (blood) | Zuba, 2013 |
| | 32-576 ng/mL (serum) (n=3) | Grapp, 2017 |
| | 470 ng/mL (heart blood) | Cawrse, 2012 |
| | 10-640 ng/mL (blood) (n=18) | Marinetti, 2013 |
| Mephedrone | 5.5 µg/mL (blood), 7.1 µg/mL (vitreous) | Adamowicz, 2013b |
| | 0.06 mg/L-2.10 mg/L (blood) (n=12) | Cosbey, 2013 |
| | 0.50 mg/L (blood), 198 mg/L (urine) | Dickson, 2010 |
| | 1.33 mg/L (heart blood), 144 mg/L (urine) | Gerace, 2014 |
| | 5.1 mg/L (femoral blood), 186 mg/L (urine), 1.04 g/L (stomach contents) | Lusthof, 2011 |
| | 1,200-22,000 ng/mL (blood) (n=4) | Torrance, 2010 |
| | 130-2,240 ng/mL (femoral blood) (n=4) | Maskell, 2011 |
| Methylone | 3.4 mg/L (iliac blood), 3.4 mg/L (central blood) | McIntyre, 2013 |
| | 0.56-3.3 mg/L (peripheral blood) (n=3) | Pearson, 2012 |
| | 500 ng/mL (peripheral blood), 39,770 ng/mL (urine) | Shimomura, 2016 |
| | 729 ng/mL (heart blood) | Marinetti, 2013 |
| | 60-1,100 ng/mL (heart blood), 220-38,000 ng/mL (urine) | Cawrse, 2012 |
| Pentedrone | 8,794 ng/mL (femoral blood) | Sykutera, 2015 |
| α-PVP | 1.1-6,200 ng/mL (blood) (n=12) | Adamowicz, 2016 |

| | | |
|---|---|---|
| | 174 ng/mL (peripheral blood), 401 ng/mL (urine) | Potocka-Banaś, 2017 |
| | 901 ng/mL (femoral blood) | Sykutera, 2015 |
| Pyrovalerone | 42 ng/mL (femoral blood), 59 ng/mL (heart blood) | Marinetti, 2013 |

Although forensic toxicologists must also rely on other factors when forming scientific opinions, interpretation of quantitative toxicology results is influenced by the relative stability (or instability) of the drug. As the data in **Tables 2 & 3** suggests, synthetic cathinones have been reported in blood over a very wide range of concentrations, spanning several orders of magnitude. If drugs are unstable, single doses of a drug that may impair, could be undetectable at the time of testing if the analysis is not performed expeditiously, or if exposed to unfavorable conditions. Many toxicology laboratories suffer from backlogged cases and in some instances, it can be days, weeks, or months before testing is complete. During this time, specimens are refrigerated or, less frequently, frozen for longer-term storage. Additionally, specimens may be subjected to elevated temperatures during routine shipping and transport to the laboratory. In postmortem investigations, forensic pathologists rely heavily on forensic toxicologists to assist with the interpretation of quantitative drug toxicology, particularly concerning new and emerging drugs, with which they may be less familiar.

Cathinone Stability

Drug stability is an important consideration in forensic toxicology, particularly if quantitative drug results are to be heavily relied upon. Stability is influenced by the physicochemical properties of the drug, characteristics of the specimen, container selection, storage temperature, and use of preservatives and other additives (Kerrigan, 2012). Aside from the external or environmental conditions, stability in forensic toxicology samples can be complicated by matrix-dependent variables and the pH of the specimen.

Much of the early literature pertains to the stability of cathinone (2-amino-1-phenyl-1-propanone) in seized drug material. In fact, the chemical instability of cathinone and the presence of a variety of degradation products were largely responsible for the delay in identifying the major pharmacologically active component of khat (Szendrei, 1980). Cathinone itself is an

unstable drug that can degrade after harvesting the plant. Just as the cathinones undergo reduction of the keto functional group to a hydroxylated metabolite in-vivo, similar transformations can occur in seized plant material to produce cathine or (+)-norpseudoephedrine from cathinone. Moisture is reported to increase the rate of degradation in seized plant material, so simple drying techniques prior to evidence storage have proven effective (Chappell, 2010). Transformations of cathinone into other species are also possible, notably cyclization to 3,6-dimethyl-2,5-diphenyldihydropyrazine with subsequent oxidation to 3,6-dimethyl-2,5-diphenylpyrazine (Berrang, 1982). Almost four decades later the landscape of cathinone use is inherently more complex, and the stability of cathinones in biological evidence rather than seized drug evidence, must be considered.

Reports of cathinone instability in biological specimens is not new. Morad was the first to report that cathinone was unstable in plasma (Morad, 1989) and the pH dependent degradation of cathinone and methcathinone in urine was reported by Paul and Cole thereafter. The stability of cathinone and methcathinone was assessed in urine at -18°C and 4°C over three months. Both drugs were stable for three days at 4°C and for two months when stored at -18°C. At the end of three months, a 79% decrease was observed for both compounds at 4°C (Paul, 2001).

More recently, issues associated with quantitative reproducibility and stability of the newer designer cathinones have emerged. Marinetti describe the lack of reproducibility of methylone and methedrone in toxicological samples in a series of published case reports (Marinetti, 2013). Soon thereafter, Johnson and Botch-Jones investigated the stability of MDPV and mephedrone at 1,000 ng/mL in blood, plasma and urine over 14 days of storage (Johnson, 2013). Mephedrone was considerably less stable than MDPV, demonstrating a 60% loss after 14 days at room temperature in urine, and complete (100%) loss after 7 days at room temperature in blood. Both drugs were stable under frozen storage conditions for the entire two-week period. Based on the considerable difference between mephedrone and MDPV, the authors emphasized the need for additional research and the potential for chemical instability to impact the interpretation of forensic toxicology casework.

More recently, Li investigated the stability of eleven synthetic cathinones in equine plasma over various time intervals. Samples were stored at 25°C for 24 hours, 4°C for 7 days, -20°C for 4 weeks, and -70°C for 24 weeks (6 months). The authors concluded that the eleven cathinones were stable for 30 days at -20°C, and 6 months in -70°C. Most were stable at room temperature for 24 hours, with the exception of 4-fluormethcathinone (4-FMC), 3-fluoromethcathinone (3-FMC), and 3-methyoxymethcathinone (Li, 2014). Soh and Elliott also described the stability of 4-MEC in blood and plasma at ambient temperature (Soh, 2013). 4-MEC, originally fortified at 2,000 ng/mL was undetectable within 14 days, although plasma was reported to have greater stability. Busardo investigated the stability of mephedrone in antemortem and postmortem blood over six months, concluding that preserved blood should be stored at -20$^{o}$C to prevent significant loss (Busardo, 2015).

A study by Tsujikawa investigated the stability of five synthetic cathinones in aqueous solutions over a range of pH (4 - 12). Cathinones were more stable at acidic pH and significant differences in decomposition rates were noted between drugs. *L*-ascorbic acid and sodium sulfite abated degradation to some degree, suggesting that the mechanism of the degradation was indeed oxidative. In this preliminary study, it was concluded that the stability of the cathinones was highly substituent-dependent. 3-Fluoro and 2-fluoromethcathinone were by far the most unstable analogs investigated, having half-lives as low as 0.5 to 3.4 h at pH 12 and pH 7, respectively. The ortho-substituted regioisomer 4-fluoromethcathinone was considerably more stable. The authors suggested that tertiary amines, which do not undergo oxidative deamination, should be more stable than their secondary or primary counterparts, but this was not investigated. Sorensen also noted cathinone instability in blood, suggesting that stability could be improved by acidification of the matrix (Sorensen, 2011). Although this might be feasible in clinical or pharmacokinetic studies where the class of drugs are known, this approach is not feasible in routine forensic toxicology investigations.

In this study we describe a systematic and comprehensive evaluation of synthetic cathinone stability using twenty-two drugs in biological fluids that are commonly encountered in forensic toxicology investigations. Cathinone stability in blood and urine was assessed during six months of storage to determine analyte, pH, concentration, matrix, and temperature dependent effects.

**Rationale for Research**

The stability of synthetic cathinones in biological evidence is an important issue that deserves further investigation. Reliable toxicological interpretation of results depends on an inherent understanding of drug stability. We propose a comprehensive and systematic approach to evaluate stability in different biological matrices and describe the influence of pH, matrix, temperature, concentration, and structural characteristics of the cathinone species, in particular the influence of phenyl and amine substituents. A better understanding of the factors that influence stability will assist with the interpretation of forensic toxicology results in criminal and death investigation casework. Moreover, an increased knowledge with respect to structural characteristics may also provide much needed insight regarding the stability of future synthetic cathinones, that are yet to emerge.

At the inception of the study, twenty-two cathinones of forensic interest were commercially available (**Table 1**). Analytical methods were developed to quantitatively identify these compounds in blood and urine. Using solid phase extraction (SPE) and liquid chromatography-quadrupole/time of flight mass spectrometry (LC-Q/TOF-MS), methods were scientifically validated in accordance with recognized and published standards (SWGTOX, 2013). Drug concentrations in blood and urine were determined to evaluate short-term and long-term stability in biological evidence under a variety of conditions. Finally, experimental observations using fortified samples were compared with authentic specimens from cathinone users following specified periods of storage.

## II. Methods

### Chemicals and Reagents

Reference standards including 3,4-DMMC, 3-FMC, 4-EMC, 4-FMC, 4-MEC, α-PVP, buphedrone, butylone, ethcathinone, ethylone, eutylone, MDPBP, MDPV, methcathinone, methedrone, methylone, mephedrone, MPBP, naphyrone, pentedrone, pentylone, pyrovalerone, and internal standards (butylone-D3, ethylone-D3, naphyrone-D3, α-PVP-D8, pentylone-D3, eutylone-D5, methylone-D3, mephedrone-D3, and MDPV-D8) were purchased from Cerilliant Corp. (Round Rock, TX, USA). Reference materials were purchased as methanolic 1.0 mg/mL standards with the exception of deuterated analogs (0.1 mg/mL). Pooled drug-free urine purchased from Utak Laboratories (Valencia, CA, USA) was preserved with 1% sodium fluoride prior to use. Bovine blood preserved with 1% sodium fluoride and 0.2% potassium oxalate was purchased from Quad Five (Ryegate, Montana, USA).

Dichloromethane, isopropyl alcohol, and glacial acetic acid were obtained from Mallinckrodt Chemicals (St. Louis, MO, USA) and methanol (LCMS grade), concentrated hydrochloric acid, acetonitrile (LCMS grade), and dibasic sodium phosphate ($Na_2HPO_4$, ACS grade) were obtained from J.T. Baker (Center Valley, MA, USA). Hexane (Optima®) and ethyl acetate (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA) and formic acid (>95%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Concentrated ammonium hydroxide was obtained from Macron Fine Chemicals (Center Valley, MA, USA) and monobasic sodium phosphate ($NaH_2PO_4$, ACS grade) was obtained from VWR (Radnor, PA, USA). Deionized water was purified in-house using a Millipore Direct-Q® UV Water Purification system (Billerica, MA, USA). PolyChrom Clin II 3 cc (35 mg) solid phase extraction (SPE) columns were obtained from SPEware (Baldwin Park, CA, USA).

Working standards containing all twenty-two target compounds were prepared in methanol at 0.01, 0.1, and 1.0 µg/mL for the fortification of urine, and 0.02, 0.2, and 2.0 µg/mL for the fortification of blood. The combined internal standard solution consisted of nine isotopically labelled standards in methanol at 0.25 µg/mL and 0.5 µg/mL each for urine and blood,

respectively. Phosphate buffer (pH 6, 0.1 M) was prepared from 0.1 M solutions of mono and dibasic sodium phosphate, and acidic methanol consisted of concentrated hydrochloric acid diluted in methanol (2%, v/v). The elution solvent, which was prepared daily, consisted of 2% concentrated ammonium hydroxide in 95:5 dichloromethane/isopropyl alcohol (v/v).

**Instrumentation**

Nitrogen was generated using a Genius 3040 nitrogen generator (Peak Scientific, Billerica, MA, USA). SPE was performed using a JT Baker vacuum manifold and extracts were evaporated to dryness under nitrogen using a TurboVap LV® concentration workstation (Caliper Life Sciences, Hopkinton, MA, USA). An Agilent Technologies 6530 LC-Q/TOF MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 1290 Infinity autosampler was used to analyze samples. Separation was achieved using an Agilent Technologies Series 1200 LC system equipped with an Agilent Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 µm particle size) and an Agilent Poroshell 120 EC-C18 guard column (2.1 x 5 mm, 2.7 µm particle size) in a thermostatically controlled column compartment (35$^{o}$C).

The mobile phase consisted of 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B). A flow rate of 0.4 mL/min was maintained using the gradient elution profile as follows: 96% A and 4% B (0 – 0.5 mins); 10% B (0.5 – 5 mins); 40% B (5 – 11 mins); 100% B (12 mins). The column was rinsed with 100% B for 1 minute before re-equilibration. The total acquisition time was 13 mins and the target compounds eluted between 3 and 11 mins.

The LC-Q/TOF MS was equipped with an electrospray ionization (ESI) source (positive mode) with Jet Stream technology under the following conditions: drying gas ($N_2$), 13 L/min; drying gas temperature, 200$^{o}$C; nebulizer, 20 psi; sheath gas temperature, 250$^{o}$C; nitrogen sheath gas flow, 12 L/min; capillary voltage, 4000 V; nozzle voltage, 0 V; fragmentor, 150 V; skimmer, 65V. Agilent MassHunter software was used for acquisition, qualitative, and quantitative analysis. Following optimization of collision induced dissociation (CID) voltages, a minimum of two transition ions were selected with a mass tolerance of 5 ppm using targeted MS/MS acquisition. Precursor and

product ions, collision energies, retention times, and the internal standard for each drug are summarized in **Table 4**. Precursor ions were selected in the quadrupole using a 1 Dalton (Da) window. Data was acquired using a mass range of 40-1000 Da, with a MS scan rate of 8 spectra/sec and a MS/MS scan rate of 3 spectra/sec. During the selection of product ions, greater emphasis was placed on specificity rather than sensitivity. Non-specific losses (including water loses) that have been utilized in publications elsewhere were not considered acceptable.

**Table 4.** Transition ions, collision energies (CE), retention time (RT), and internal standard selection. Analytes are listed in retention time order and ion ratios are indicated in parentheses.

| Cathinone | Precursor Ion (m/z) | Product Ions (m/z) | CE (V) | RT (min) | Internal Standard |
|---|---|---|---|---|---|
| Methcathinone | 164.1075 | 131.0731 (100%)<br>105.0703 (25%) | 20 | 3.394 | Mephedrone-D3 |
| 3-FMC | 182.0976 | 149.0634 (100%)<br>123.0605 (15%) | 20 | 3.938 | Mephedrone-D3 |
| 4-FMC | 182.0976 | 149.0636 (100%)<br>123.0605 (22%) | 20 | 4.094 | Mephedrone-D3 |
| Methylone | 208.0968 | 160.0757 (100%)<br>132.0807 (37%) | 20 | 4.133 | Methylone-D3 |
| Ethcathinone | 178.1226 | 131.0721 (100%)<br>117.0586 (34%)<br>105.0700 (50%) | 20 | 4.302 | Butylone-D3 |
| Ethylone | 222.1125 | 174.1222 (100%)<br>146.0958 (79%) | 30 | 5.153 | Ethylone-D5 |
| Methedrone | 194.1176 | 161.0833 (100%)<br>146.0598 (41%)<br>135.0803 (22%) | 20 | 5.291 | Mephedrone-D3 |
| Buphedrone | 178.1226 | 131.0731 (100%)<br>91.0549 (58%)<br>145.0880 (14%) | 20 | 5.442 | Mephedrone-D3 |
| Butylone | 222.1125 | 174.0914 (100%)<br>146.0964 (84%) | 30 | 6.259 | Butylone-D3 |
| Mephedrone | 178.1226 | 145.0889 (100%)<br>119.0853 (14%) | 20 | 6.444 | Mephedrone-D3 |
| Eutylone | 236.1281 | 188.1069 (100%)<br>174.0547 (104%)<br>161.0598 (26%)* | 30 | 6.901 | Eutylone-D5 |
| 4-MEC | 192.1383 | 145.0886 (100%) | 20 | 7.185 | Mephedrone-D3 |

| | | | | | |
|---|---|---|---|---|---|
| | | 159.1041 (33%)*<br>131.0738 (30%) | | | |
| MDPBP | 262.1438 | 161.0597 (100%)<br>191.0704 (80%)<br>112.1125 (96%) | 20 | 7.225 | Eutylone-D5 |
| Pentedrone | 192.1383 | 132.0810 (100%)<br>91.0546 (68%) | 20 | 7.505 | Mephedrone-D3 |
| Pentylone | 236.1281 | 188.1070 (100%)<br>175.0682 (40%) | 30 | 7.813 | Pentylone-D3 |
| 3,4-DMMC | 192.1383 | 159.1043 (100%)<br>144.0802 (24%) | 20 | 8.055 | Methylone-D3 |
| α-PVP | 232.1696 | 161.0954 (100%)<br>91.0549 (367%) | 20 | 8.159 | α-PVP-D8 |
| 4-EMC | 192.1383 | 145.0889 (100%)<br>105.0701 (10%) | 20 | 8.232 | Mephedrone-D3 |
| MPBP | 232.1696 | 161.0960 (100%)<br>133.1010 (48%)<br>112.1120 (61%) | 20 | 8.410 | Naphyrone-D5 |
| MDPV | 276.1594 | 205.0857 (100%)<br>126.1277 (137%)<br>175.0756 (116%) | 20 | 8.444 | MDPV-D8 |
| Pyrovalerone | 246.1852 | 175.1110 (100%)<br>126.1280 (63%)<br>105.0701 (212%) | 20 | 9.450 | Naphyrone-D5 |
| Naphyrone | 282.1852 | 211.1122 (100%)<br>126.1280 (37%)<br>141.0701 (143%) | 20 | 10.774 | Naphyrone-D5 |

*Transitions indicated with an asterisk were included for urine only.

**Isolation of Cathinones from Urine and Blood**

Internal standard solution (0.25 μg/mL) was added to 1.0 mL urine to achieve a final concentration of 25 ng/mL. Urine was diluted with 2 mL of pH 6.0 phosphate buffer (0.1 M) and briefly vortexed. Samples were transferred to PolyChrom Clin II SPE columns (3 cc columns, 35 mg) and allowed to flow through under gravity or sufficient vacuum to maintain constant flow (approximately 1 mL/min). Columns were rinsed with 1 mL deionized water followed by 1 mL of 1 M acetic acid. After drying columns for five mins on full vacuum, samples were washed successively using hexane (1 mL), ethyl acetate (1 mL), and methanol (1 mL). Cathinones were eluted using two 0.5 mL aliquots of elution solvent. Acidic methanol (30 μL) was added to each

extract prior to evaporation under nitrogen at 50°C. Extracts were reconstituted in 25 μL of a 50:50 mixture of Mobile Phase A/B and 1 μL was injected onto the LC-Q/TOF MS for analysis.

Cathinones were isolated from blood using a slightly modified procedure. Internal standard solution (0.5 μg/mL) was added to 2.0 mL blood to achieve a final concentration of 25 ng/mL. A protein precipitation was performed with the addition of 4 mL of cold acetonitrile while vortex mixing, followed by centrifugation at 4000 rpm for 5 mins. The supernatant was decanted and diluted with 6 mL of pH 6.0 phosphate buffer (0.1 M) and briefly vortexed. Samples were transferred to SPE columns (3 cc columns, 35 mg) and extracted in a manner analogous to urine. One additional SPE wash step using dichloromethane (1 mL) was included prior to the elution step. Extracts were reconstituted in 25 μL of a 50:50 mixture of Mobile Phase A/B and 1 μL was injected onto the LC-Q/TOF MS for analysis.

**Assay Validation**

Assay performance was evaluated in terms of extraction efficiency, calibration model, precision, bias, limit of detection (LOD), limit of quantification (LOQ), matrix effects, interference, ion suppression, carryover, processed sample stability, and dilution integrity (Glicksberg, 2016) in accordance with published recommendations (SWGTOX, 2013).

The extraction efficiency in blood (100 ng/mL) and urine (25 ng/mL) was determined by direct comparison of extracted and non-extracted samples. Urine and blood containing internal standard (25 ng/mL) was extracted in the presence and absence of the target compounds. Samples extracted without target compounds were fortified with equivalent drug post-extraction (prior to evaporation and reconstitution). Analytical recovery was calculated by comparing the relative peak area (drug/IS) for extracted samples (n=4) with the mean relative peak are for the non-extracted samples (n=4).

Limits of detection and quantitation were established using drug-free blood and urine fortified with reference materials. Three sources of drug-free matrix were analyzed in duplicate over three

independent runs. The LOD was the lowest concentration of drug that produced a reportable result (signal to noise ratio of 3:1 or more; retention time ±2% of the standard; ion ratios ±20%). Limit of quantitation was determined contemporaneously and was defined as the lowest concentration of drug to produce a quantitative value within 20% of the expected value, a S/N ratio of 10:1 or more, retention time ±2% of the standard, ion ratios within 20%, and acceptable precision and bias.

Precision and bias were evaluated in urine (10, 100, and 1,000 ng/mL) and blood (20, 100, and 1,000 ng/mL) using three samples of pooled fortified matrix at three concentrations (low, medium, and high) over five runs. Within-run precision was calculated for each concentration (n=3) over each of the five runs. Between-run precision was calculated for each concentration over all five runs (n=15). Bias was evaluated contemporaneously with precision using the same concentrations over five days. Tolerance for bias and precision was 20%.

The calibration model was established over five independent runs. Calibration models were evaluated visually and analytically using the correlation coefficient ($R^2$), standardized residual plots, and the F-test to determine the significance of the quadratic term ($\alpha$=0.05). A total of seven non-zero calibrators were prepared in urine (5, 10, 50, 100, 250, 500, and 1,000 ng/mL) and eight in blood (5, 10, 50, 100, 250, 500, 750, and 1,000 ng/mL).

Interferences associated with the biological matrix, isotopically labeled internal standards, common drugs, and structurally related compounds were systematically evaluated. Matrix interferences were evaluated using ten drug-free blood and urine samples from independent sources in the absence of internal standard. Ion contributions arising from the use of stable isotope internal standards were evaluated by fortifying drug-free urine and blood with internal standard (25 ng/mL) and monitoring the signal of the target analytes. In a similar fashion, ion contributions associated with high concentrations of drug (1,000 ng/mL) were evaluated in the absence of internal standard. Drug interferences were evaluated using four categories of compounds: common drugs, other amphetamines, structurally related designer drugs, and other

therapeutic drugs of significance**.** Twenty-two common drugs, ten common amphetamine-type drugs, and fifteen structurally related designer drugs were selected. Diethylpropion (amfepramone, Tenuate®) and bupropion (Wellbutrin®, Zyban®) are therapeutically used cathinones. These were included in the interference study, together with hydroxybupropion (metabolite) and ropivacaine (Naropin®) (due to its mass spectral similarity to MDPV). Putrefactive amines were also included for the interference study in blood. Interferences were assessed using negative and positive controls. A 10- to 100-fold excess of interferent (relative to the target drug) was employed for interference testing. The negative control consisted of drug-free urine or blood fortified with internal standard (25 ng/mL) and 1,000 ng/mL of interferent; positive controls contained internal standard (25 ng/mL), interferent (1,000 ng/mL), and target cathinones at a ten-fold and hundred-fold lower concentration (100 and 10 ng/mL, respectively). Interferences associated with more than fifty drugs were evaluated in total and these are summarized in **Table 5**.

**Table 5.** Summary of compounds included in the interference study.

| Common Drugs | Amphetamine-Like Drugs | Designer Drugs* | Other Drugs | Putrefactive Amines (Blood Only) |
|---|---|---|---|---|
| Alprazolam | Amphetamine | 2C-B | Bupropion | Putrescine |
| Amitriptyline | Methamphetamine | 2C-C | Diethylpropion | Phenethylamine |
| Caffeine | MDA | 2C-D | Hydroxy-bupropion | Tryptamine |
| Cocaine | MDEA | 2C-E | Ropivacaine | Tyramine |
| Codeine | MDMA | 2C-H | | |
| Cotinine | MBDB | 2C-I | | |
| Cyclobenzaprine | Ephedrine | 2C-T-2 | | |
| Dextromethorphan | Pseudoephedrine | 2C-T-4 | | |
| Diazepam | Phentermine | 2C-T-7 | | |
| Diphenhydramine | Phenylpropanolamine | 4-MTA | | |
| Hydrocodone | | DOB | | |
| Ketamine | | DOC | | |
| Methadone | | DOI | | |
| Morphine | | DOET | | |
| Nicotine | | DOM | | |
| Nordiazepam | | | | |
| Oxazepam | | | | |
| Oxycodone | | | | |

| | | | | |
|---|---|---|---|---|
| Phencyclidine<br>Propoxyphene<br>Tramadol<br>Zolpidem | | | | |

*4-bromo-2,5-dimethoxyphenethylamine (2C-B), 2,5-dimethoxy-4-chlorophenethylamine (2C-C), 2,5-dimethoxy-4methylphenethylamine (2C-D), 2,5-dimethoxy-4-ethylphenethylamine (2C-E), 2,5-dimethoxyphenethylamine (2C-H), 2,5-dimethoxy-4-iodophenethylamine (2C-I), 2,5-dimethoxy4-ethylthiophenethylamine (2C-T-2), 2,5-dimethoxy-4-isopropylthiophenethylamine (2C-T-4), 2,5-dimethoxy-4-propylthiophenethylamine (2C-T-7), 4-methylthioamphetamine (4-MTA), 2,5-dimethoxy-4-bromoamphetamine (DOB), 2,5-dimethoxy-4-chloroamphetamine (DOC), 2,5-dimethoxy-4-ethylamphetamine (DOET), 2,5-dimethoxy-4-iodoamphetamine (DOI), 2,5-dimethoxy-4-methylamphetamine (DOM).

Matrix effects were quantitatively assessed using post-extraction addition at two concentrations (20 ng/mL and 200 ng/mL for urine; 50 ng/mL and 500 ng/mL for blood). Ten drug-free matrices from independent sources (analyzed in duplicate) were extracted in the absence of drug and fortified with drug post-extraction. Ion suppression or enhancement was calculated by comparing the mean peak areas of drug in matrix with the drug in mobile phase (no matrix).

Carryover was assessed by analyzing a negative control immediately following the injection of a high control (1,000, 2,500, and 5,000 ng/mL). Carryover was present when the negative control produced a reportable result (signal to noise ratio of 3:1 or more, retention time ±2% and ion ratios within 20% of expected). The influence of sample dilution was evaluated using urine or blood fortified at 1,000 ng/mL. Dilution integrity for urine was determined using two- and four-fold dilutions in 0.1 M pH 6.0 phosphate buffer (to achieve final volume of 1 mL) prior to extraction. Dilution integrity for blood using two- and four-fold dilutions was determined by direct precipitation of 0.5 mL or 1 mL blood with 4 mL of cold acetonitrile. Quantitative results were evaluated and calculated concentrations within 20% of the expected concentration were deemed acceptable. The stability of processed samples was assessed by extracting samples (25 ng/mL and 350 ng/mL) in triplicate and analyzing the same extracts over a period of up to 48 hours. The samples were stored in the refrigerated autosampler tray and were considered stable until the quantitative result produced a bias exceeding ±20%.

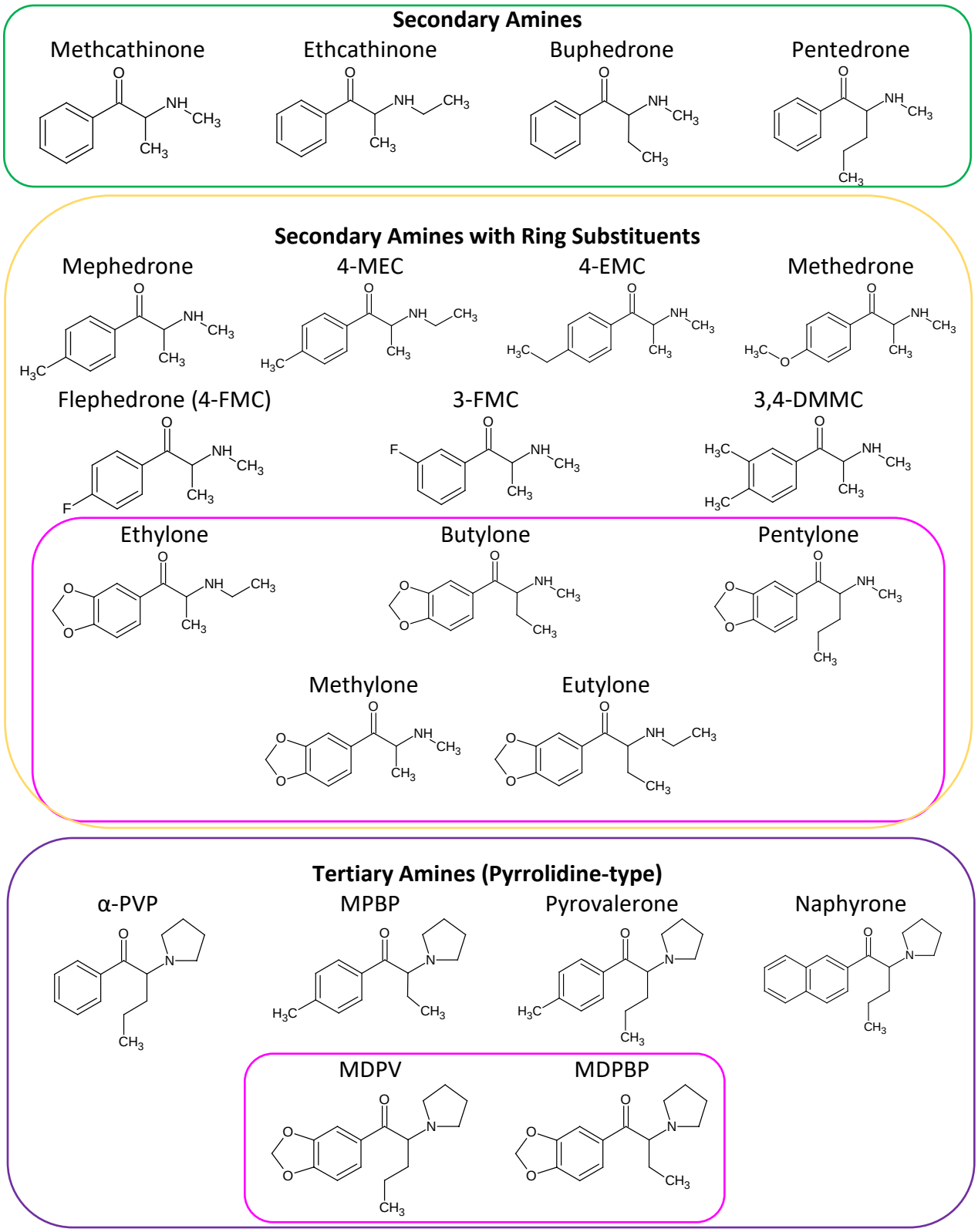
**Cathinone Stability in Fortified Biological Matrices**

The twenty-two cathinones selected for the study include analogs with a variety of benzylic and amine substitutions. These include secondary and tertiary (pyrrolidinyl) amines, ring-substituted analogs (including methylenedioxy type cathinones) and non-ring substituted drugs (**Figure 2**). Structural features were color-coded as follows: $2^o$ amines without aromatic substituents ($R_1$ and $R_2$ = H) - green; $2^o$ amines with benzylic substituents ($R_1$ or $R_2 \neq$ H) - yellow; $3^o$ amines (pyrrolidine-type) – purple; and methylenedioxy-substituted cathinones – magenta. These structural characteristics are also significant from a mass spectral standpoint, since they largely determine fragmentation patterns and characteristic ions. These target analytes not only represent a diverse number of cathinone analogs, but also the most widely abused drugs within the class at the time of the study. Substitutions on the aromatic ring, amino group, or alkyl terminus ($R_1$-$R_5$) are summarized in **Table 6**.

**Table 6.** Summary of cathinone substituents ($R_1$-$R_5$).

| Name | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| 3,4-DMMC | $CH_3$ | $CH_3$ | $CH_3$ | H | $CH_3$ |
| 4-EMC | H | $C_2H_5$ | $CH_3$ | H | $CH_3$ |
| 3-FMC | F | $CH_3$ | $CH_3$ | H | $CH_3$ |
| 4-FMC | H | F | $CH_3$ | H | $CH_3$ |
| 4-MEC | H | $CH_3$ | $CH_3$ | H | $C_2H_5$ |
| Buphedrone | H | H | $C_2H_5$ | H | $CH_3$ |
| Butylone | 3,4-Methylenedioxy | | $C_2H_5$ | H | $CH_3$ |
| Ethcathinone | H | H | $CH_3$ | H | $C_2H_5$ |
| Ethylone | 3,4-Methylenedioxy | | $CH_3$ | H | $C_2H_5$ |
| Eutylone | 3,4-Methylenedioxy | | $C_2H_5$ | H | $C_2H_5$ |
| MDPBP | 3,4-Methylenedioxy | | $C_2H_5$ | Pyrrolidinyl | |
| MDPV | 3,4-Methylenedioxy | | $C_3H_7$ | Pyrrolidinyl | |
| Mephedrone | H | $CH_3$ | $CH_3$ | H | $CH_3$ |
| Methcathinone | H | H | $CH_3$ | H | $CH_3$ |
| Methedrone | H | $OCH_3$ | $CH_3$ | H | $CH_3$ |
| Methylone | 3,4-Methylenedioxy | | $CH_3$ | H | $CH_3$ |
| MPBP | H | $CH_3$ | $C_2H_5$ | Pyrrolidinyl | |
| Naphyrone | Naphthyl | | $C_3H_7$ | Pyrrolidinyl | |
| Pentedrone | H | H | $C_3H_7$ | H | $CH_3$ |
| Pentylone | 3,4-Methylenedioxy | | $C_3H_7$ | H | $CH_3$ |
| α-PVP | H | H | $C_3H_7$ | Pyrrolidinyl | |
| Pyrovalerone | H | $CH_3$ | $C_3H_7$ | Pyrrolidinyl | |

**Figure 2.** Chemical structures of cathinones included in the study. Structures circled in pink indicate a methylenedioxy substitution.



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Cathinone stability was evaluated in blood and urine under a variety of experimental conditions over six months. Samples were stored in specimen containers that are typically encountered in forensic toxicology casework. Urine, preserved with 1% sodium fluoride (w/v), was stored in non-sterile polypropylene specimen cups. Whole blood, containing 1% (w/v) sodium fluoride and 0.2% (w/v) potassium oxalate was stored in commercial evacuated glass tubes (gray-top Vacutainer™ tubes).

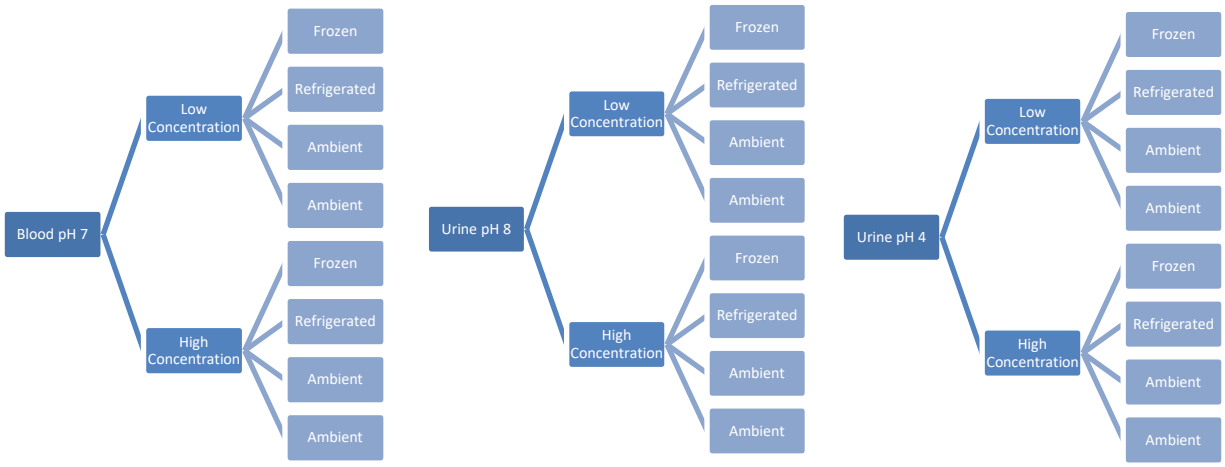
Stability was evaluated at four temperatures. These were chosen to reflect frozen (-20°C) and refrigerated (4°C) long- and short-term storage temperatures at the laboratory; exposure to ambient (20°C) or room temperature during routine processing and handling; and finally, potential exposure to elevated temperatures during shipping and transport to the laboratory (32°C, the median daytime temperature in Houston, Texas during the summer months).

In addition to blood (pH 7), pooled certified drug-free urine was evaluated at two pH values (pH 4 and pH 8) to evaluate stability at the low and high end of urinary pH values observed in authentic casework samples. Matrices were fortified with cathinones at low (100 ng/mL) and high concentrations (1,000 ng/mL) for each condition tested. Cathinone concentrations were determined for a period of six months to determine analyte, matrix, pH, concentration, and temperature dependence. The experimental design is summarized in **Figure 3**. Samples were analyzed in duplicate using the validated procedure described earlier. Calibrators (10, 25, 100, 250, 350, and 500 ng/mL) and controls were included in each run. Where necessary, statistical tests, including analysis of variance (ANOVA), were used to determine statistical significance ($\alpha$=0.05).

Drug-free urine was adjusted to pH 8 and pH 4 using the minimum volume of concentrated ammonium hydroxide or concentrated hydrochloric acid to achieve the desired pH. A total of 1 L of urine was prepared at each pH. Positive displacement pipettes were used to fortify 0.5 L of urine with cathinones at 1,000 and 100 ng/mL. Urine was immediately aliquoted into specimen containers and stored under the specified conditions. Blood was prepared in an a similar manner without modification of pH. Immediately following fortification, blood and urine samples were

analyzed to verify initial concentrations (time 0). Sampling frequency was variable throughout the study. During the initial 48 hours, quantitative analysis was performed every 2-6 hours. Sampling remained frequent (4 assays/week) throughout the initial month, decreasing to monthly thereafter.

**Figure 3.** Experimental design.




Authentic Specimens from Cathinone Users

Authentic unpreserved urine samples (n=151) from cathinone users were reanalyzed using the LC-Q/TOF method in accordance with an Institutional Review Board (IRB) approved study. Specimens and original quantitative results were provided by Redwood Toxicology Laboratories (RTL). Specimens were stored at refrigerated temperatures at the reference laboratory, shipped on ice, and immediately refrigerated upon receipt. Samples were reanalyzed and concentrations were compared to original quantitative results. Due to the pH-dependent nature of stability, the urinary pH of each specimen was also determined. Initial quantitative results obtained from the reference laboratory were compared and investigated further.



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## III. Results and Discussion

### Assay Performance

Synthetic cathinones are known to thermally degrade during GC-MS analysis. This oxidative degradation is characterized by the loss of two hydrogens to produce a 2,3-enamine or an imine (DeRuiter, 1994; Kerrigan, 2016). As part of the routine ionization optimization process, the possibility of thermal degradation was considered. Data was acquired using a non-targeted (full scan) method. Precursor ions for known (-2 Da) degradation products were not present for any of the drugs, demonstrating that heated conditions inside the ESI source did not result in thermal degradation of cathinones.

Extraction Efficiency and Calibration Model

An overlaid chromatogram depicts the chromatographic separation of all twenty-two cathinones (**Figure 4**). Extraction efficiencies were 84-104% in urine at 25 ng/mL (n=4) and 81-93% in blood at 100 ng/mL (n=4) (**Table 7**). Following visual, analytical, and statistical evaluation of calibration models, a weighted (1/x) quadratic model was selected for all analytes in both matrices. The correlation coefficients were all above 0.99 or 0.98 for all models. Upon visual assessment using residual plots, the data did not appear to be randomly dispersed, indicating a non-linear (quadratic) model should be used. Statistical evaluation further indicated a weighted (1/x) curve was optimal.

**Figure 4.** Chromatographic separation of cathinones in urine (100 ng/mL). Methcathinone (3.295); 3-FMC (3.821); 4-FMC (3.978); methylone (4.036); ethcathinone (4.171); ethylone (5.038); methedrone (5.171); buphedrone (5.298); butylone (6.141); mephedrone (6.325); eutylone (6.829); 4-MEC (7.071); MDPBP (7.142); pentedrone (7.402); pentylone (7.773); 3,4-DMMC (7.995); α-PVP (8.031); 4-EMC (8.167); MPBP (8.308); MDPV (8.371); pyrovalerone (9.329); naphyrone (10.626).

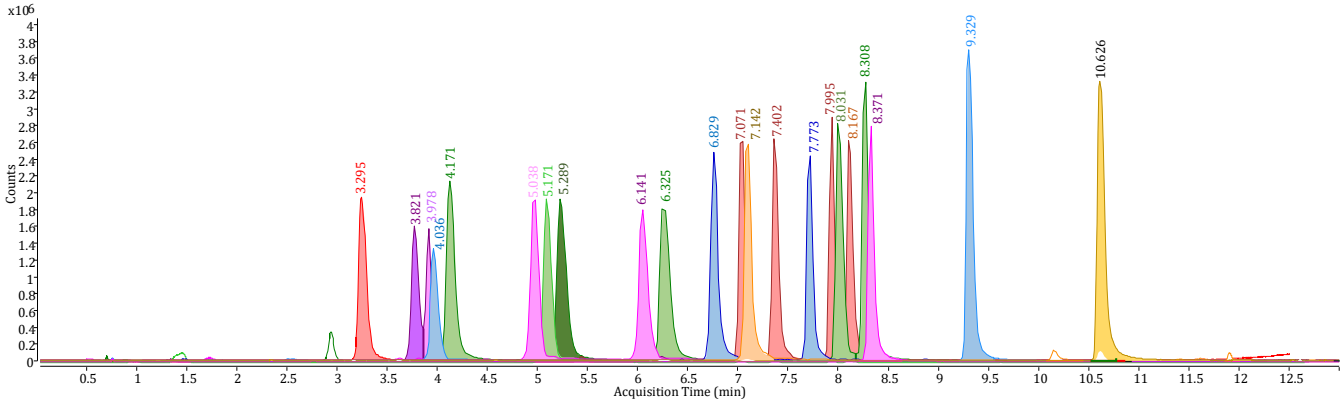


**Table 7.** Extraction efficiencies in urine (25 ng/mL) and blood (100 ng/mL) using replicate analyses (n=4).

| Cathinone | Mean Extraction Efficiency (%) | |
|---|---|---|
| | Blood | Urine |
| 3,4-DMMC | 83 ± 35 | 96 ± 7 |
| 4-EMC | 87 ± 17 | 97 ± 4 |
| 3-FMC | 81 ± 19 | 84 ± 12 |
| 4-FMC | 86 ± 12 | 90 ± 9 |
| 4-MEC | 85 ± 10 | 101 ± 4 |
| Buphedrone | 85 ± 11 | 95 ± 5 |
| Butylone | 87 ± 9 | 98 ± 3 |
| Ethcathinone | 87 ± 15 | 89 ± 4 |
| Ethylone | 87 ± 11 | 98 ± 3 |
| Eutylone | 93 ± 10 | 98 ± 3 |
| MDPBP | 87 ± 9 | 94 ± 3 |
| MDPV | 88 ± 20 | 95 ± 4 |
| Methcathinone | 83 ± 20 | 93 ± 10 |
| Methedrone | 84 ± 13 | 104 ± 6 |
| Methylone | 83 ± 20 | 99 ± 4 |
| Mephedrone | 82 ± 24 | 97 ± 7 |
| MPBP | 91 ± 7 | 93 ± 4 |
| Naphyrone | 88 ± 12 | 95 ± 4 |
| Pentedrone | 88 ± 11 | 95 ± 5 |
| Pentylone | 88 ± 17 | 100 ± 5 |
| α-PVP | 84 ± 21 | 94 ± 4 |
| Pyrovalerone | 90 ± 12 | 92 ± 4 |



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Limits of Detection and Quantification

Limits of detection and quantitation for the twenty-two synthetic cathinones in blood ranged from 1-5 ng/mL (n=18), significantly lower than previously published literature using high resolution mass spectrometry (HRMS) (50-100 ng/mL) (Pasin, 2015). Limits of detection in urine ranged from 0.25-5 ng/mL (n=18). Extracted ion chromatograms (EICs) for all drugs at the limit of quantitation in urine and blood are shown in **Figures 5-6**, respectively. Bias, precision, and signal to noise (S/N) ratios at the limits of detection and quantitation are summarized in **Tables 8-9**.

Precision and Bias

Precision and bias were evaluated at low, medium, and high concentrations in triplicate over five days. Intra-assay CVs were 0.5 – 10.8% (10 ng/mL), 0.2 – 7.3% (100 ng/mL), and 0.2 – 8.6% (800 ng/mL) for urine and 0.2 – 17.0% (20 ng/mL), 0.2 – 8.7% (100 ng/mL), and 0.8 – 13.8% (800 ng/mL) for blood. Inter-assay CVs over the same concentration ranges were 4.4 - 12.1%, 1.7 - 11.5%, and 2.5 - 8.6% in urine (n=15) and 3.3 – 11.7%, 2.7 – 7.0%, and 3.4 – 10.1% in blood (n=15). Bias and precision at all concentrations tested were within acceptable ranges (±20%) and are summarized in **Tables 10 and 11**.

**Figure 5.** Extracted ion chromatograms in urine at the limit of quantitation.

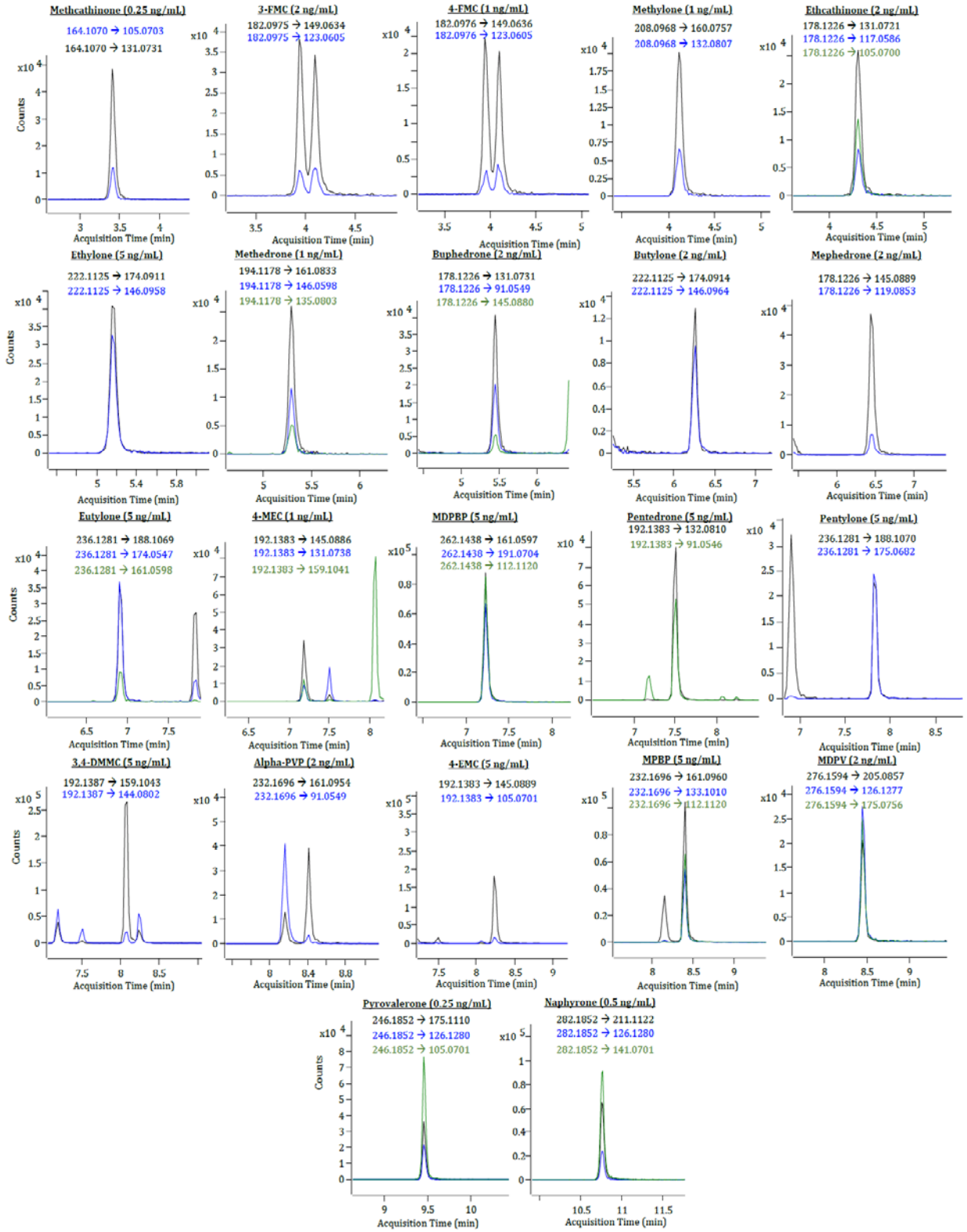

**Figure 6.** Extracted ion chromatograms in blood at the limit of quantitation.

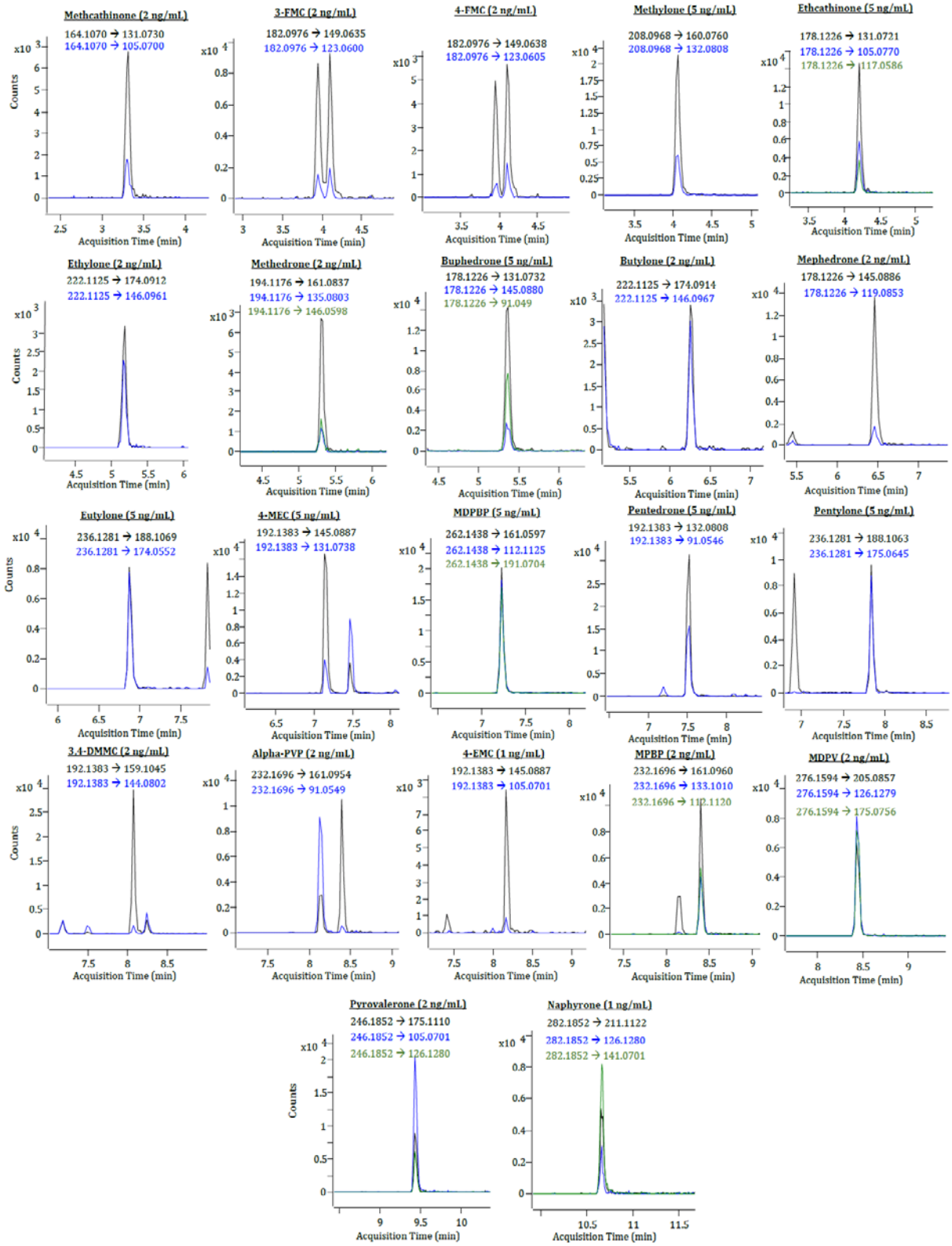

**Table 8.** Limits of detection and quantitation in urine. The mean, standard deviation (SD), signal to noise ratio (S/N), bias, and CV (%) at the LOQ are summarized for each drug (n=18).

| Cathinone | LOD (ng/mL) | LOQ (ng/mL) | Mean ± SD (ng/mL) | CV (%) | S/N | Bias (%) |
|---|---|---|---|---|---|---|
| 3,4-DMMC | 5 | 5 | 5.0 ± 0.3 | 6.4 | 23:1 | -0.7 |
| 4-EMC | 2 | 5 | 5.1 ± 0.3 | 5.3 | 43:1 | 2.3 |
| 3-FMC | 1 | 2 | 2.1 ± 0.2 | 9.1 | 349:1 | 2.7 |
| 4-FMC | 1 | 1 | 0.9 ± 0.1 | 5.9 | 358:1 | -8.5 |
| 4-MEC | 1 | 1 | 0.9 ± 0.05 | 5.3 | 1438:1 | -8.1 |
| Buphedrone | 2 | 2 | 2.0 ± 0.1 | 6.3 | 159:1 | 1.4 |
| Butylone | 1 | 2 | 2.0 ± 0.2 | 8.3 | 44:1 | 1.6 |
| Ethcathinone | 1 | 2 | 2.0 ± 0.1 | 6.6 | 578:1 | 0.0 |
| Ethylone | 1 | 5 | 4.9 ± 0.4 | 7.1 | 623:1 | -0.7 |
| Eutylone | 5 | 5 | 4.9 ± 0.3 | 5.5 | 338:1 | -1.1 |
| MDPBP | 0.5 | 5 | 5.2 ± 0.2 | 4.4 | 556:1 | 4.3 |
| MDPV | 1 | 2 | 2.1 ± 0.1 | 6.9 | 967:1 | 4.0 |
| Mephedrone | 2 | 2 | 2.1 ± 0.1 | 5.3 | 95:1 | 3.5 |
| Methcathinone | 0.25 | 0.25 | 0.24 ± 0.02 | 9.9 | 241:1 | -2.3 |
| Methedrone | 1 | 1 | 0.9 ± 0.1 | 14.2 | 102:1 | -2.0 |
| Methylone | 0.25 | 1 | 0.9 ± 0.1 | 9.4 | 1323:1 | -0.8 |
| MPBP | 1 | 5 | 4.9 ± 0.4 | 7.7 | 239:1 | -1.0 |
| Naphyrone | 0.5 | 0.5 | 0.55 ± 0.04 | 7.4 | 666:1 | 10.4 |
| Pentedrone | 5 | 5 | 5.1 ± 0.3 | 6.4 | 489:1 | 1.5 |
| Pentylone | 1 | 5 | 4.7 ± 0.3 | 5.3 | 125:1 | -5.4 |
| α-PVP | 2 | 2 | 2.0 ± 0.2 | 9.4 | 103:1 | -1.7 |
| Pyrovalerone | 0.25 | 0.25 | 0.27 ± 0.02 | 8.0 | 235:1 | 8.7 |

**Table 9.** Limits of detection and quantitation in blood. The mean, standard deviation (SD), signal to noise ratio (S/N), bias, and CV (%) at the LOQ are summarized for each drug (n=18).

| Cathinone | LOD (ng/mL) | LOQ (ng/mL) | Mean ± SD (ng/mL) | CV (%) | S/N | Bias (%) |
|---|---|---|---|---|---|---|
| 3,4-DMMC | 2 | 2 | 1.87 ± 0.13 | 6.9 | 196:1 | -6.4 |
| 4-EMC | 1 | 1 | 1.02 ± 0.06 | 5.9 | 70:1 | 1.0 |
| 3-FMC | 2 | 2 | 2.01 ± 0.19 | 9.2 | 69:1 | 0.0 |
| 4-FMC | 5 | 5 | 5.07 ± 0.43 | 8.4 | 128:1 | 1.5 |
| 4-MEC | 5 | 5 | 4.98 ± 0.37 | 7.4 | 72:1 | -0.5 |
| Buphedrone | 5 | 5 | 4.94 ± 0.39 | 7.8 | 117:1 | -1.2 |
| Butylone | 2 | 2 | 1.92 ± 0.17 | 8.8 | 63:1 | -4.0 |
| Ethcathinone | 5 | 5 | 4.82 ± 0.39 | 8.2 | 155:1 | -3.6 |
| Ethylone | 2 | 2 | 1.96 ± 0.19 | 10.0 | 67:1 | -3.0 |
| Eutylone | 5 | 5 | 4.80 ± 0.39 | 8.2 | 46:1 | -4.0 |
| MDPBP | 5 | 5 | 4.80 ± 0.25 | 5.2 | 84:1 | -4.0 |
| MDPV | 2 | 2 | 1.86 ± 0.14 | 7.4 | 55:1 | -7.3 |
| Mephedrone | 2 | 2 | 1.99 ± 0.13 | 6.6 | 181:1 | -0.4 |
| Methcathinone | 2 | 2 | 1.91 ± 0.16 | 8.6 | 155:1 | -4.8 |
| Methedrone | 2 | 2 | 1.90 ± 0.17 | 8.9 | 104:1 | -6.6 |
| Methylone | 2 | 2 | 1.92 ± 0.09 | 4.5 | 305:1 | -4.9 |
| MPBP | 2 | 2 | 1.87 ± 0.16 | 8.3 | 75:1 | -6.5 |
| Naphyrone | 1 | 1 | 1.00 ± 0.07 | 6.9 | 33:1 | 0.7 |
| Pentedrone | 5 | 5 | 5.05 ± 0.35 | 6.8 | 195:1 | 0.9 |
| Pentylone | 5 | 5 | 4.77 ± 0.42 | 8.9 | 61:1 | -4.6 |
| α-PVP | 2 | 2 | 1.85 ± 0.16 | 8.6 | 16:1 | -7.6 |
| Pyrovalerone | 1 | 2 | 1.86 ± 0.13 | 6.8 | 152:1 | -6.9 |

**Table 10.** Precision and bias (n=15) in urine at low (10 ng/mL), medium (100 ng/mL) and high (800 ng/mL) concentrations.

| Cathinone | Intra-assay CV (%) n=3 | | | Inter-assay CV (%) n=15 | | | Bias (%) n=15 | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10 ng/mL | 100 ng/mL | 800 ng/mL | 10 ng/mL | 100 ng/mL | 800 ng/mL | 10 ng/mL | 100 ng/mL | 800 ng/mL |
| 3,4-DMMC | 2.9-6.1 | 0.3-6.3 | 0.4-4.9 | 11.7 | 8.6 | 5.5 | -1 | -3 | 3 |
| 4-EMC | 1.3-4.1 | 0.3-2.2 | 2.7-5.5 | 6.8 | 2.2 | 3.5 | 8 | 2 | 3 |
| 3-FMC | 1.2-10.8 | 1.1-5.1 | 0.7-5.3 | 8.9 | 4.7 | 5.9 | 9 | 0 | 2 |
| 4-FMC | 0.5-3.8 | 1.8-6.9 | 0.3-3.1 | 5.6 | 4.5 | 9.2 | 7 | 1 | 4 |
| 4-MEC | 1.0-8.9 | 1.0-3.4 | 0.6-4.1 | 12.1 | 11.5 | 4.3 | 1 | 1 | 4 |
| Buphedrone | 0.8-5.6 | 0.9-4.1 | 0.7-5.3 | 8.3 | 2.8 | 4.7 | 10 | 2 | 6 |
| Butylone | 1.7-7.0 | 0.2-6.2 | 1.4-5.0 | 4.6 | 4.1 | 3.5 | 6 | 0 | 4 |
| Ethcathinone | 1.3-4.5 | 3.4-7.3 | 0.9-4.1 | 9.3 | 6.3 | 7.5 | 12 | 1 | 8 |
| Ethylone | 0.6-3.4 | 1.7-4.3 | 0.2-4.6 | 6.9 | 3.0 | 4.6 | 7 | 2 | 1 |
| Eutylone | 1.8-6.0 | 1.1-3.1 | 1.1-4.2 | 6.7 | 2.4 | 5.8 | 3 | 2 | 2 |
| MDPBP | 1.6-7.2 | 0.6-3.0 | 0.4-5.3 | 7.1 | 4.4 | 5.7 | 7 | 2 | 1 |
| MDPV | 0.8-6.8 | 1.5-3.4 | 1.1-4.6 | 6.1 | 5.0 | 5.1 | 7 | 2 | 1 |
| Mephedrone | 0.5-6.8 | 0.9-2.1 | 1.5-5.5 | 4.8 | 2.0 | 3.3 | 7 | 2 | 2 |
| Methcathinone | 0.9-6.4 | 0.9-3.9 | 1.4-5.8 | 7.0 | 3.0 | 3.5 | 8 | 1 | 5 |
| Methedrone | 3.6-7.2 | 0.4-1.4 | 2.0-8.6 | 4.7 | 1.7 | 6.4 | 8 | 1 | 2 |
| Methylone | 0.8-5.7 | 0.6-2.3 | 3.2-7.4 | 4.4 | 2.4 | 2.5 | 6 | 1 | 2 |
| MPBP | 2.4-4.5 | 2.5-4.2 | 0.4-4.7 | 9.4 | 4.3 | 3.2 | 6 | 2 | 5 |
| Naphyrone | 3.9-7.2 | 0.5-2.5 | 0.4-4.2 | 6.0 | 1.8 | 3.3 | 8 | 3 | 3 |
| Pentedrone | 1.0-3.2 | 1.2-4.6 | 1.9-7.3 | 7.8 | 3.6 | 4.1 | 8 | 1 | 5 |
| Pentylone | 2.9-8.5 | 1.3-3.8 | 1.1-3.9 | 11.6 | 3.6 | 5.8 | 3 | 4 | 3 |
| α-PVP | 1.5-3.9 | 0.2-3.6 | 1.6-4.7 | 6.7 | 4.2 | 8.9 | 9 | 0 | 6 |
| Pyrovalerone | 1.6-3.9 | 1.3-2.5 | 0.7-3.5 | 8.7 | 2.3 | 3.4 | 7 | 2 | 3 |

**Table 11.** Precision and bias (n=15) in blood at low (20 ng/mL), medium (100 ng/mL) and high (800 ng/mL) concentrations.

| Cathinone | Intra-assay CV (%) n=3 | | | Inter-assay CV (%) n=15 | | | Bias (%) n=15 | | |
|---|---|---|---|---|---|---|---|---|---|
| | 20 ng/mL | 100 ng/mL | 800 ng/mL | 20 ng/mL | 100 ng/mL | 800 ng/mL | 20 ng/mL | 100 ng/mL | 800 ng/mL |
| 3,4-DMMC | 2.3-9.5 | 1.2-7.2 | 2.1-13.8 | 6.9 | 4.9 | 10.1 | -3 | 1 | 2 |
| 4-EMC | 1.5-5.8 | 0.9-7.2 | 4.6-5.6 | 4.9 | 5.0 | 8.3 | -1 | -2 | -2 |
| 3-FMC | 1.1-17.0 | 0.3-6.2 | 1.8-9.8 | 9.1 | 5.6 | 8.1 | 0 | 11 | 4 |
| 4-FMC | 0.8-11.7 | 1.3-5.8 | 5.1-8.5 | 6.0 | 3.8 | 7.6 | 2 | 11 | 2 |
| 4-MEC | 0.7-5.4 | 1.3-4.0 | 2.1-7.3 | 5.7 | 3.7 | 7.7 | 3 | 1 | 2 |
| Buphedrone | 1.3-9.7 | 0.8-5.5 | 2.1-8.7 | 6.8 | 5.6 | 7.4 | 4 | 9 | 4 |
| Butylone | 0.9-8.1 | 2.4-3.8 | 3.8-5.2 | 4.6 | 4.0 | 5.5 | -6 | 1 | -1 |
| Ethcathinone | 1.6-14.0 | 0.5-7.4 | 5.5-9.4 | 8.8 | 6.0 | 7.2 | 6 | 11 | 4 |
| Ethylone | 1.1-7.5 | 1.5-2.6 | 1.7-6.3 | 3.3 | 4.3 | 3.6 | 0 | -1 | 0 |
| Eutylone | 1.3-5.6 | 2.2-6.0 | 6.3-10.0 | 6.6 | 5.9 | 5.1 | -1 | 3 | 1 |
| MDPBP | 1.1-5.5 | 1.5-5.4 | 1.0-6.7 | 5.7 | 5.1 | 3.9 | 9 | -2 | 1 |
| MDPV | 1.2-5.8 | 0.2-6.1 | 1.5-6.4 | 3.6 | 4.1 | 4.9 | -7 | 5 | 3 |
| Mephedrone | 1.3-6.6 | 1.0-2.2 | 4.2-4.6 | 3.6 | 3.7 | 6.2 | -5 | 3 | 1 |
| Methcathinone | 0.6-8.6 | 0.8-3.9 | 1.7-5.5 | 6.2 | 4.6 | 8.0 | 3 | 6 | 3 |
| Methedrone | 0.2-10.7 | 1.1-3.9 | 2.3-5.7 | 6.6 | 2.9 | 6.3 | -2 | -6 | -4 |
| Methylone | 1.4-5.5 | 0.5-3.7 | 2.4-4.8 | 3.9 | 3.7 | 3.4 | -6 | 1 | 1 |
| MPBP | 0.2-12.6 | 0.9-6.2 | 0.8-6.5 | 11.7 | 4.9 | 9.4 | 3 | 3 | 1 |
| Naphyrone | 1.1-9.1 | 0.7-2.4 | 1.4-3.0 | 6.8 | 2.7 | 3.8 | -1 | 1 | 0 |
| Pentedrone | 1.9-11.5 | 2.0-3.7 | 2.5-5.9 | 6.3 | 4.8 | 7.2 | 4 | 7 | 6 |
| Pentylone | 0.7-6.2 | 0.9-6.7 | 8.4-8.4 | 6.6 | 7.0 | 8.3 | -5 | -2 | 1 |
| α-PVP | 3.1-8.8 | 0.8-8.7 | 7.0-10.8 | 7.2 | 5.4 | 7.6 | -3 | 4 | -4 |
| Pyrovalerone | 0.3-10.6 | 0.6-4.0 | 3.7-7.3 | 5.3 | 3.9 | 6.8 | 7 | 5 | 3 |

Interferences and Matrix Effects

Interferences from matrix, isotopically labeled internal standards, and other drugs were systematically evaluated. Ten drug-free urine matrices from independent sources did not reveal interferences and there were no interfering ion contributions associated with the deuterated analogs. Furthermore, there were no qualitative interferences from more than fifty other compounds, including common drugs, amphetamine-like drugs, designer drugs, or therapeutically used cathinones, and putrefactive amines (**Table 5**). Negative and positive controls (10 ng/mL and 100 ng/mL) were analyzed in the presence of a 10- and 100-fold higher

concentration of potential interferents (1,000 ng/mL). No qualitative interferences were present for any of the compounds tested.

The potential for ion suppression or enhancement was evaluated using ten independently sourced blood and urine samples. Matrix effects were evaluated quantitatively using the post-extraction addition technique for all twenty-two analytes and nine internal standards. Ionization suppression in urine was -17 to -1% at 20 ng/mL and -21 to -4% at 200 ng/mL. Corresponding CVs were 2.4-13.7% and 3.5-7.5%, respectively. Ionization suppression in blood was -15 to 7% at 50 ng/mL and -3 to 3% at 500 ng/mL. Corresponding CVs in blood were 2.5-7.6% and 0.9–3.2%, respectively. Although some ion suppression was evident, matrix effects were well-within tolerable limits (±20%) and CVs were <15% (**Table 12**).

Dilution integrity was evaluated using two and four-fold dilutions of matrix at 1,000 ng/mL. All quantitative measurements were within 20% of the expected value. No carryover was present at 1,000 or 2,500 ng/mL for any of the analytes. However, at 5,000 ng/mL, carryover was observed for naphyrone in both matrices. Finally, processed samples were stable for 48 hours at 25 and 350 ng/mL.

**Table 12.** Matrix effect (%) and associated CVs in urine (20 and 200 ng/mL) and blood (50 and 500 ng/mL) (n=10).

| Cathinone | Urine | | | | Blood | | | |
|---|---|---|---|---|---|---|---|---|
| | CV (%) n=10 | | Matrix Effect (%) | | CV (%) n=10 | | Matrix Effect (%) | |
| | 20 ng/mL | 200 ng/mL | 20 ng/mL | 200 ng/mL | 50 ng/mL | 500 ng/mL | 50 ng/mL | 500 ng/mL |
| 3,4-DMMC | 10.5 | 6.0 | -17 | -18 | 7.0 | 1.0 | 5 | 2 |
| 4-EMC | 9.8 | 5.2 | -14 | -5 | 7.6 | 1.3 | 4 | 1 |
| 3-FMC | 13.2 | 3.5 | -15 | -21 | 4.0 | 3.2 | -5 | 0 |
| 4-FMC | 7.6 | 7.5 | -2 | -16 | 3.1 | 0.9 | 2 | 2 |
| 4-MEC | 4.0 | 4.9 | -10 | -16 | 5.9 | 1.8 | 4 | -2 |
| Buphedrone | 6.9 | 7.2 | -6 | -7 | 5.1 | 1.7 | 3 | 3 |
| Butylone | 4.0 | 6.9 | -10 | -18 | 5.2 | 1.9 | 6 | 3 |
| Ethcathinone | 5.5 | 8.9 | -5 | -9 | 3.9 | 1.2 | -5 | -3 |
| Ethylone | 4.7 | 6.1 | -5 | -13 | 4.0 | 1.5 | 4 | 1 |
| Eutylone | 8.7 | 5.5 | -14 | -9 | 4.7 | 2.2 | 1 | 2 |
| MDPBP | 4.9 | 4.8 | -8 | -12 | 3.3 | 1.5 | -2 | 2 |
| MDPV | 8.6 | 4.4 | -6 | -7 | 5.3 | 1.5 | -11 | 2 |
| Methcathinone | 9.9 | 5.8 | -13 | -14 | 5.4 | 1.5 | 5 | 2 |
| Methedrone | 7.0 | 5.7 | -12 | -9 | 3.4 | 1.4 | -3 | 0 |
| Methylone | 5.4 | 5.7 | -6 | -4 | 4.9 | 1.2 | 7 | 2 |
| Mephedrone | 3.8 | 6.6 | -12 | -15 | 4.6 | 1.2 | -2 | 0 |
| MPBP | 6.6 | 4.1 | -9 | -10 | 2.5 | 1.0 | -8 | 0 |
| Naphyrone | 2.4 | 4.8 | -8 | -11 | 3.3 | 1.4 | -15 | 1 |
| Pentedrone | 5.9 | 5.9 | -5 | -9 | 3.7 | 2.4 | -1 | 0 |
| Pentylone | 13.7 | 5.7 | -8 | -11 | 4.4 | 1.5 | 0 | 3 |
| α-PVP | 8.0 | 6.9 | -1 | -10 | 4.6 | 1.6 | -9 | -1 |
| Pyrovalerone | 3.5 | 4.6 | -4 | -10 | 2.7 | 0.9 | -10 | 2 |
| Butylone-D3 | 3.6 | 3.3 | -7 | -10 | 4.9 | 2.6 | 8 | -1 |
| Ethylone-D5 | 4.8 | 2.9 | -7 | -6 | 3.7 | 2.3 | 4 | 1 |
| Eutylone-D5 | 11.0 | 3.0 | -6 | -7 | 4.8 | 4.6 | 2 | 1 |
| MDPV-D8 | 8.0 | 4.3 | -22 | -6 | 2.2 | 2.4 | -12 | 2 |
| Methylone-D3 | 5.1 | 3.2 | -16 | -6 | 5.3 | 3.3 | 9 | -1 |
| Mephedrone-D3 | 3.6 | 2.5 | -8 | -7 | 2.8 | 3.1 | 4 | 0 |
| Naphyrone-D5 | 2.3 | 4.2 | -14 | -10 | 3.2 | 1.9 | -25 | -1 |
| Pentylone-D3 | 13.2 | 3.5 | -6 | -6 | 6.2 | 3.9 | -1 | 1 |
| α-PVP-D8 | 5.4 | 3.8 | -4 | -2 | 3.6 | 2.6 | -8 | 0 |

## Stability of Cathinones

The study was designed to evaluate concentration, temperature, matrix, pH, and analyte-dependent stability. One-way ANOVA was used to determine statistical significance in the means of samples ($\alpha$=0.05). Concentration dependence was assessed by comparing the percentage of drug remaining (% target) at 100 ng/mL and 1,000 ng/mL to normalize the data. Absolute concentration (ng/mL) was used to evaluate temperature, pH, matrix, and analyte dependence. No significance testing was performed if drug concentrations were within 20% of the initial (target) concentration for the entire duration of the study. Cathinone stability in blood (pH 7) and urine (pH 4 and 8) are summarized in **Figures 7-9**.

### Half-Life Estimation

Half-lives ($t_{1/2}$) for each drug were determined over the six-month period using blood and urine specimens that had been fortified with 1,000 ng/mL drug. Assuming first-order decay, rate constants (*k*) and half-lives were calculated from duplicate measurements at each time interval ($t_{1/2}=\ln 2/k$). Rate plots were only generated if a significant decrease in concentration (>20%) was evident over three consecutive measurements. In whole blood, half-lives were estimated for all drugs at elevated and ambient temperatures. In contrast, when frozen, all drugs were stable for the entire six month period, with the exception of 3-FMC. (**Figure 10**). Rate plots for urine at pH 4 and 8 are shown in **Figure 11**. Due to the rapid degradation of some of the cathinones under some conditions, sampling intervals were very short (every two hours). Due to the analyte-dependent variability, an expanded view of the data for the least stable condition tested (pH 8 at elevated temperature) is also shown. The estimated half-lives under each condition tested are summarized in **Tables 13-15** and the analyte, temperature and pH dependence of cathinone stability are discussed in more detail below.

**Figure 7.** Cathinone stability in blood (1,000 ng/mL) at pH 7.

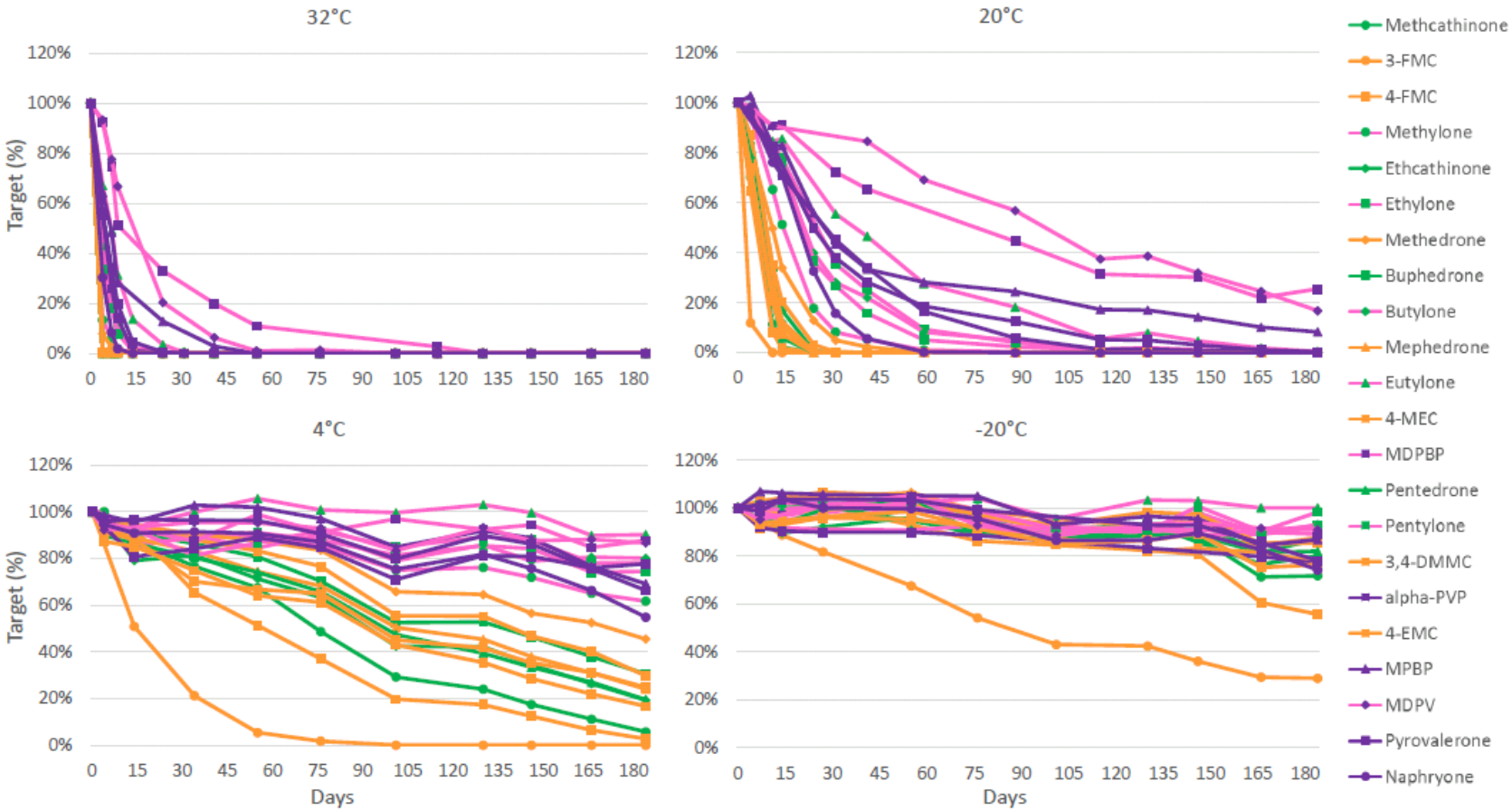

**Figure 8.** Cathinone stability in urine (1,000 ng/mL) at pH 8.

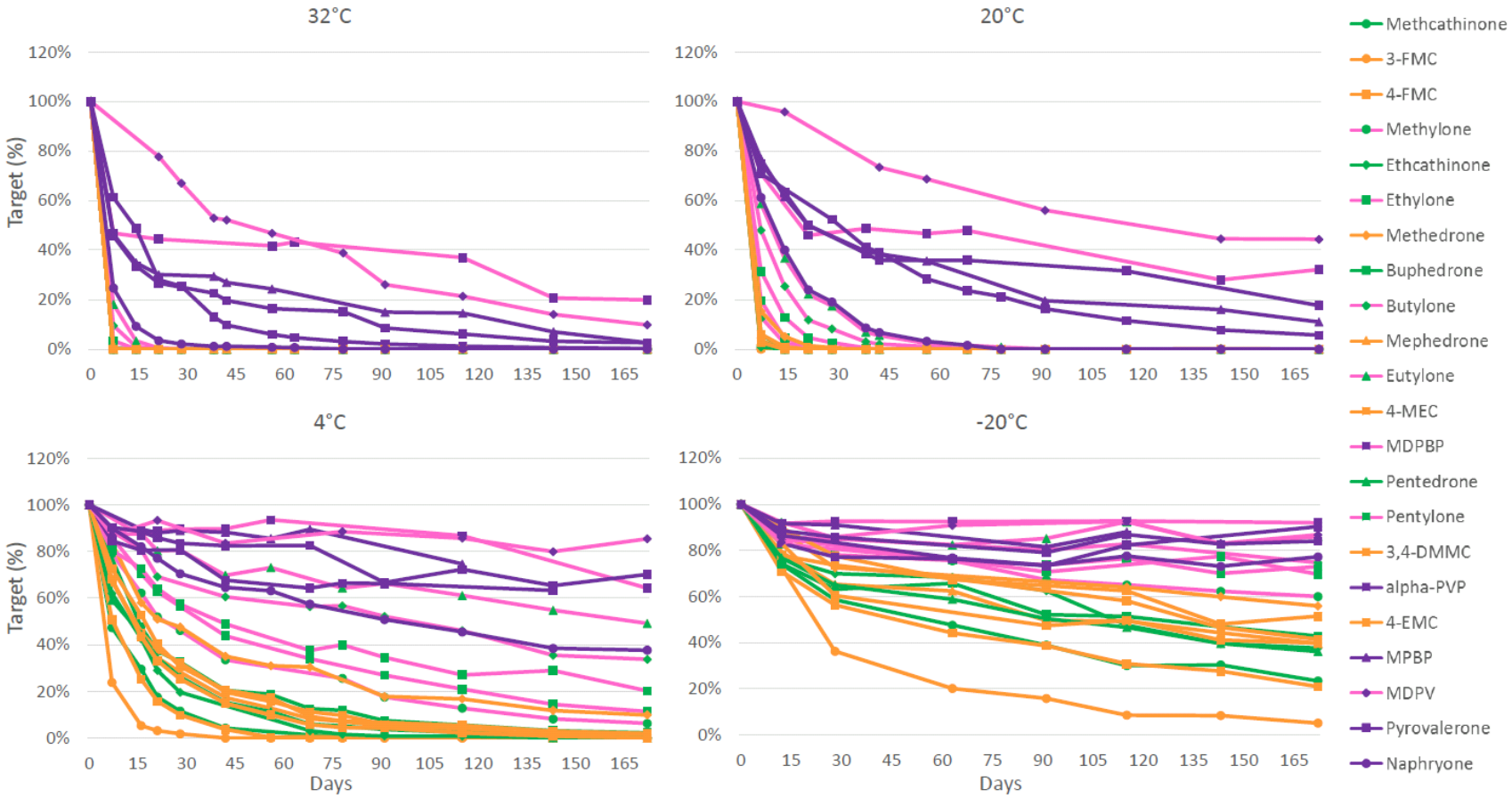




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**Figure 9.** Cathinone stability in urine (1,000 ng/mL) at pH 4.

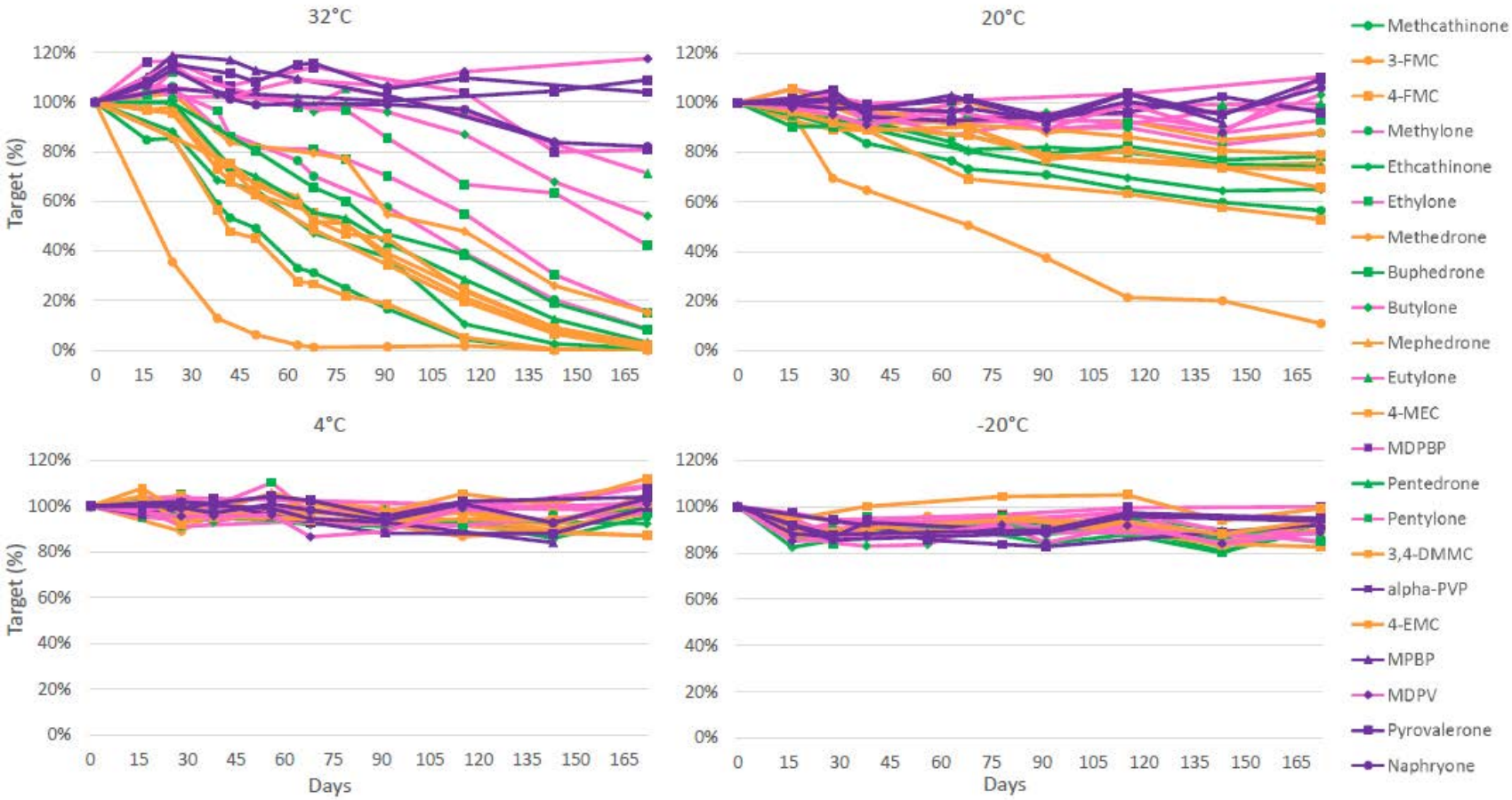




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**Figure 10**. Rate plots and estimation of $t_{1/2}$ in blood.

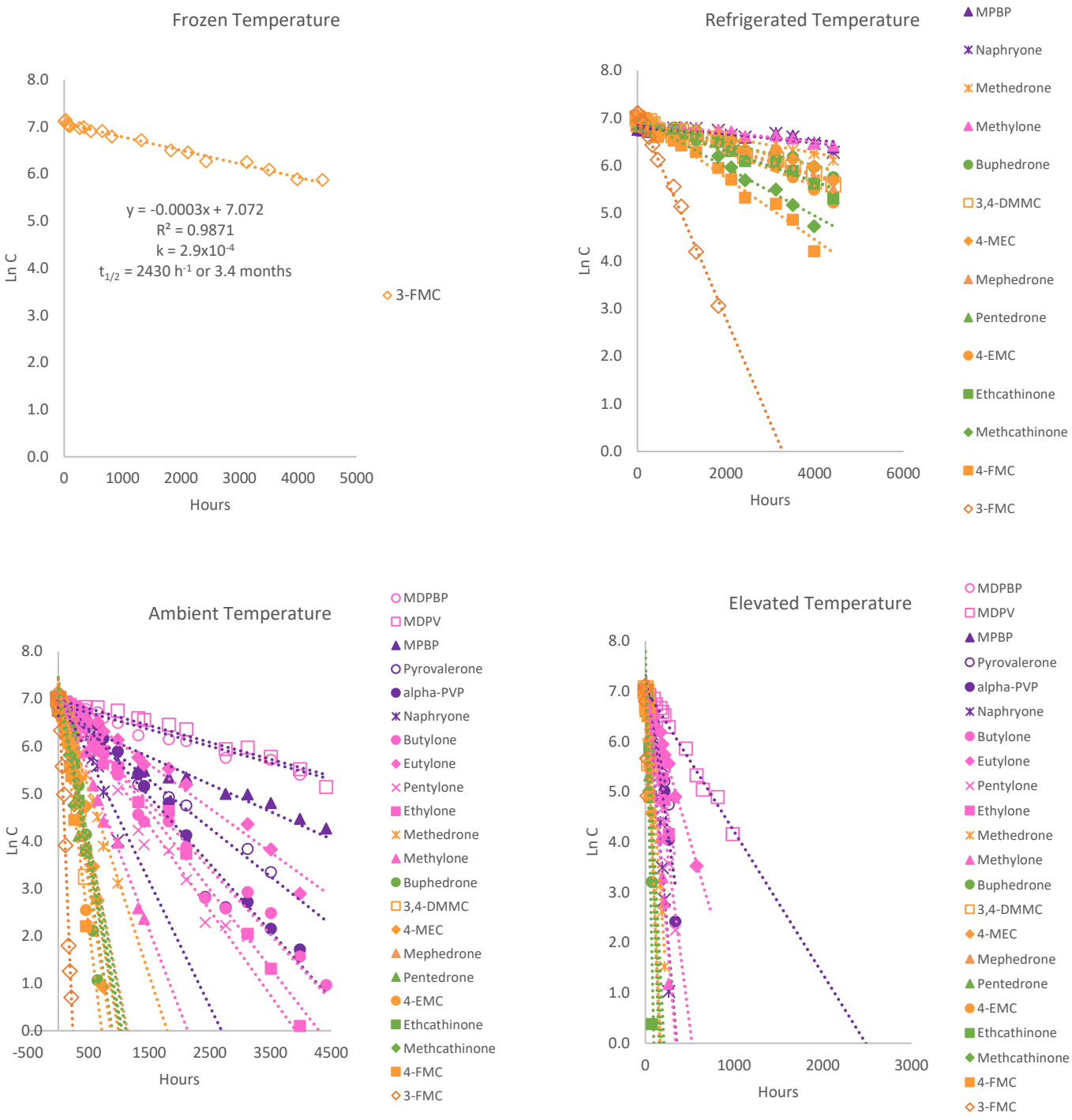


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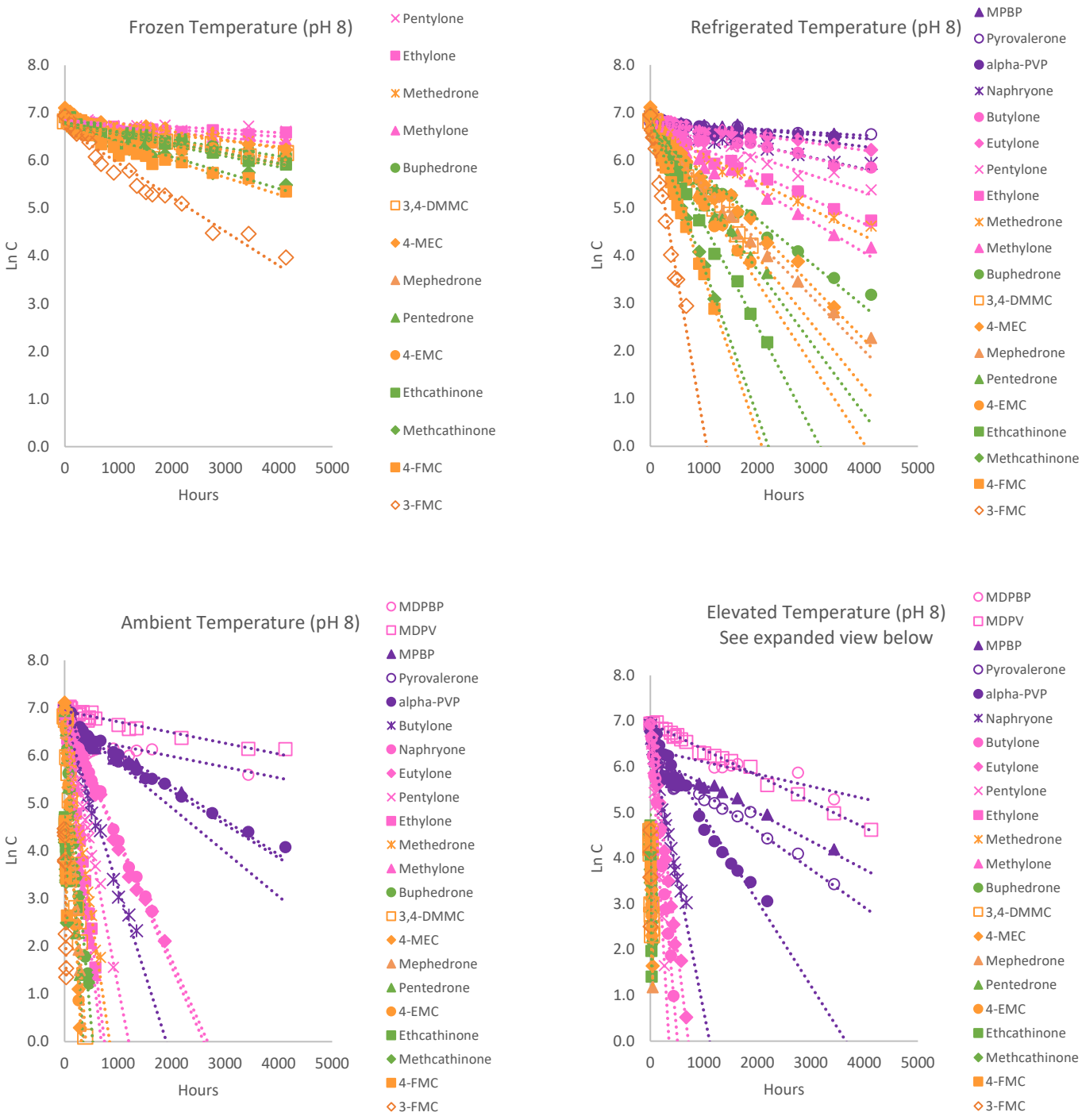
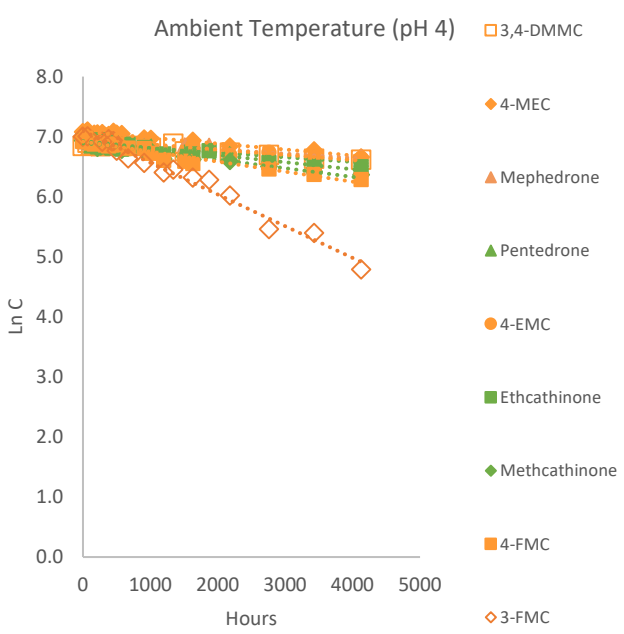
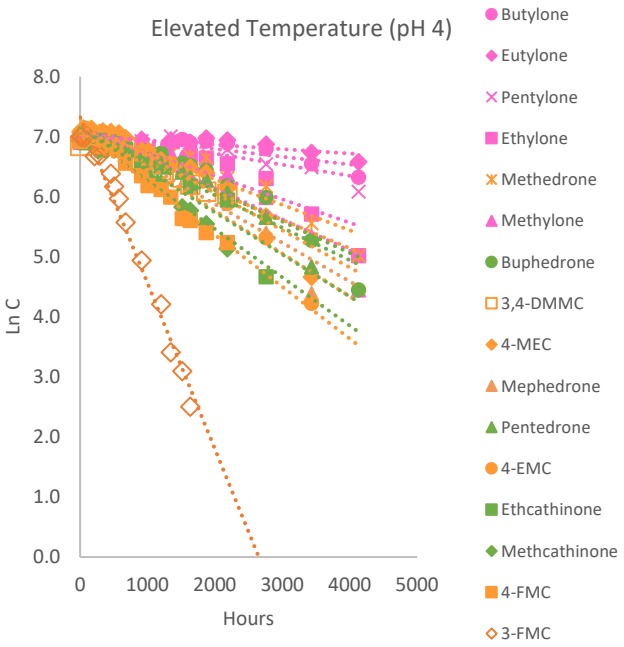
**Figure 11**. Rate plots and estimation of $t_{1/2}$ in urine.





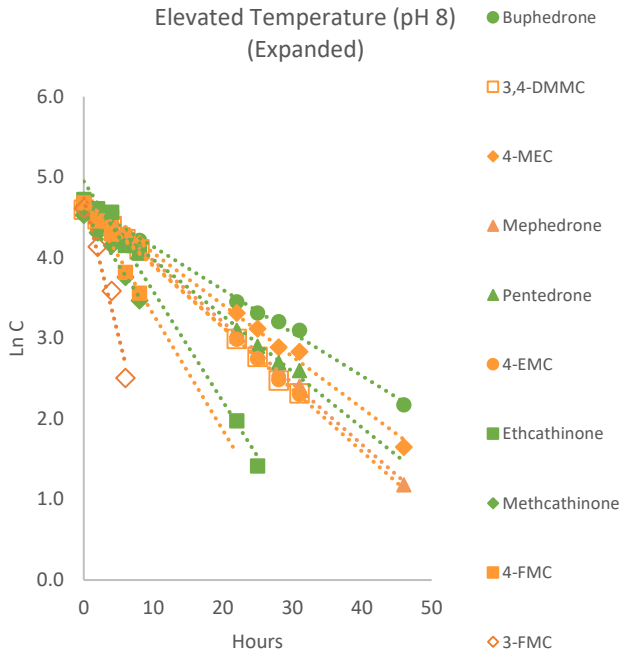






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**Table 13.** Estimated $t_{1/2}$ in blood. Drugs are listed in alphabetical order.

| Cathinone | Frozen (months) | Refrigerated (months) | Ambient (days) | Elevated (hours) |
|---|---|---|---|---|
| 3,4-DMMC | - | 3.4 | 4.0 | 22 |
| 4-EMC | - | 2.7 | 3.4 | 21 |
| 3-FMC | 2.6 | 13 d | 22 h | 8 |
| 4-FMC | - | 1.5 | 2.8 | 16 |
| 4-MEC | - | 4.1 | 4.1 | 13 |
| Buphedrone | - | 4.0 | 3.4 | 14 |
| Butylone | - | - | 21 | 4.1 d |
| Ethcathinone | - | 2.9 | 4.5 | 8 |
| Ethylone | - | - | 18 | 2.8 d |
| Eutylone | - | - | 31 | 4.8 d |
| MDPBP | - | - | 2.7 m | 21 d |
| MDPV | - | - | 2.7 m | 10 d |
| Mephedrone | - | 3.3 | 4.6 | 29 |
| Methcathinone | - | 1.9 | 4.2 | 17 |
| Methedrone | - | 5.9 | 7.3 | 28 |
| Methylone | - | 9.6 | 8.6 | 1.4 d |
| MPBP | - | 15 | 1.7 m | 8.2 d |
| Naphyrone | - | 10 | 11 | 1.4 d |
| Pentedrone | - | 3.0 | 4.3 | 20 |
| Pentylone | - | - | 16 | 2.1 d |
| α-PVP | - | - | 20 | 2.4 d |
| Pyrovalerone | - | - | 28 | 3.3 d |

**Table 14.** Estimated $t_{1/2}$ in urine (pH 4.0). Drugs are listed in alphabetical order.

| Cathinone | Frozen (months) | Refrigerated (months) | Ambient (months) | Elevated (months) |
|---|---|---|---|---|
| 3,4-DMMC | - | - | 14 | 2.0 |
| 4-EMC | - | - | 11 | 1.3 |
| 3-FMC | - | - | 1.8 | 10 d |
| 4-FMC | - | - | 5.8 | 1.1 |
| 4-MEC | - | - | 9.7 | 1.6 |
| Buphedrone | - | - | - | 1.8 |
| Butylone | - | - | - | 7.4 |
| Ethcathinone | - | - | 8.2 | 1.4 |
| Ethylone | - | - | - | 2.5 |
| Eutylone | - | - | - | 13 |
| MDPBP | - | - | - | - |
| MDPV | - | - | - | - |
| Mephedrone | - | - | 14 | 1.5 |
| Methcathinone | - | - | 6.6 | 1.2 |
| Methedrone | - | - | - | 2.3 |
| Methylone | - | - | - | 1.9 |
| MPBP | - | - | - | - |
| Naphyrone | - | - | - | - |
| Pentedrone | - | - | 13 | 1.8 |
| Pentylone | - | - | - | 5.2 |
| α-PVP | - | - | - | - |
| Pyrovalerone | - | - | - | - |

**Table 15.** Estimated $t_{1/2}$ in urine (pH 8.0). Drugs are listed in alphabetical order.

| Cathinone | Frozen (months) | Refrigerated (months) | Ambient (days) | Elevated (hours) |
|---|---|---|---|---|
| 3,4-DMMC | 5.3 | 21 d | 1.7 | 11 |
| 4-EMC | 4.4 | 17 d | 1.3 | 9 |
| 3-FMC | 1.3 | 4.5 d | 9 h | 2 |
| 4-FMC | 2.7 | 8.8 d | 20 h | 5 |
| 4-MEC | 5.5 | 25 d | 1.3 | 9 |
| Buphedrone | 5.6 | 1 | 2.3 | 20 |
| Butylone | - | 3.8 | 8 | 2.1 d |
| Ethcathinone | 4.5 | 14 d | 23 h | 8 |
| Ethylone | 13 | 1.8 | 3.2 | 19 |
| Eutylone | - | 6.2 | 11 | 3 d |
| MDPBP | - | - | 4.3 m | 3.6 m |
| MDPV | - | - | 4.3 m | 1.7 m |
| Mephedrone | 4.7 | 25 d | 1.5 | 10 |
| Methcathinone | 2.9 | 9.3 d | 18 h | 5 |
| Methedrone | 7.6 | 1.7 | 3.7 | 19 |
| Methylone | 8.7 | 1.4 | 3 | 1 d |
| MPBP | - | 15 | 1.4 m | 1.6 m |
| Naphyrone | - | 3.9 | 11 | 4.8 d |
| Pentedrone | 4.3 | 19 d | 1.4 | 10 |
| Pentylone | 15 | 2.6 | 5 | 1.4 d |
| α-PVP | - | 7.1 | 1.3 m | 18 d |
| Pyrovalerone | - | 11 | 1.0 m | 1.2 m |

Concentration Dependence

Cathinone stability was evaluated at 100 and 1,000 ng/mL in each matrix, under a variety of conditions. Concentration dependence was evaluated statistically using a one-way ANOVA to compare the means (% target at each sampling interval) at each temperature in blood, and at each temperature and pH in urine. Statistical significance was evaluated for each drug under all twelve conditions (four in blood, eight in urine).

Due to the large number of drugs, graphical representations of the data were color-coded to facilitate interpretation (**Table 6, Figure 2**). Cathinones ($2^o$ amines) without aromatic substituents ($R_1$ and $R_2$ = H) were identified in green; $2^o$ amines with benzylic substituents ($R_1$ or $R_2 \neq$ H) were yellow; cathinones with a methylenedioxy (MD) groups were indicated with a magenta line (for both $2^o$ and $3^o$ amines); and $3^o$ amines (pyrrolidine-type) cathinones were shown in purple.

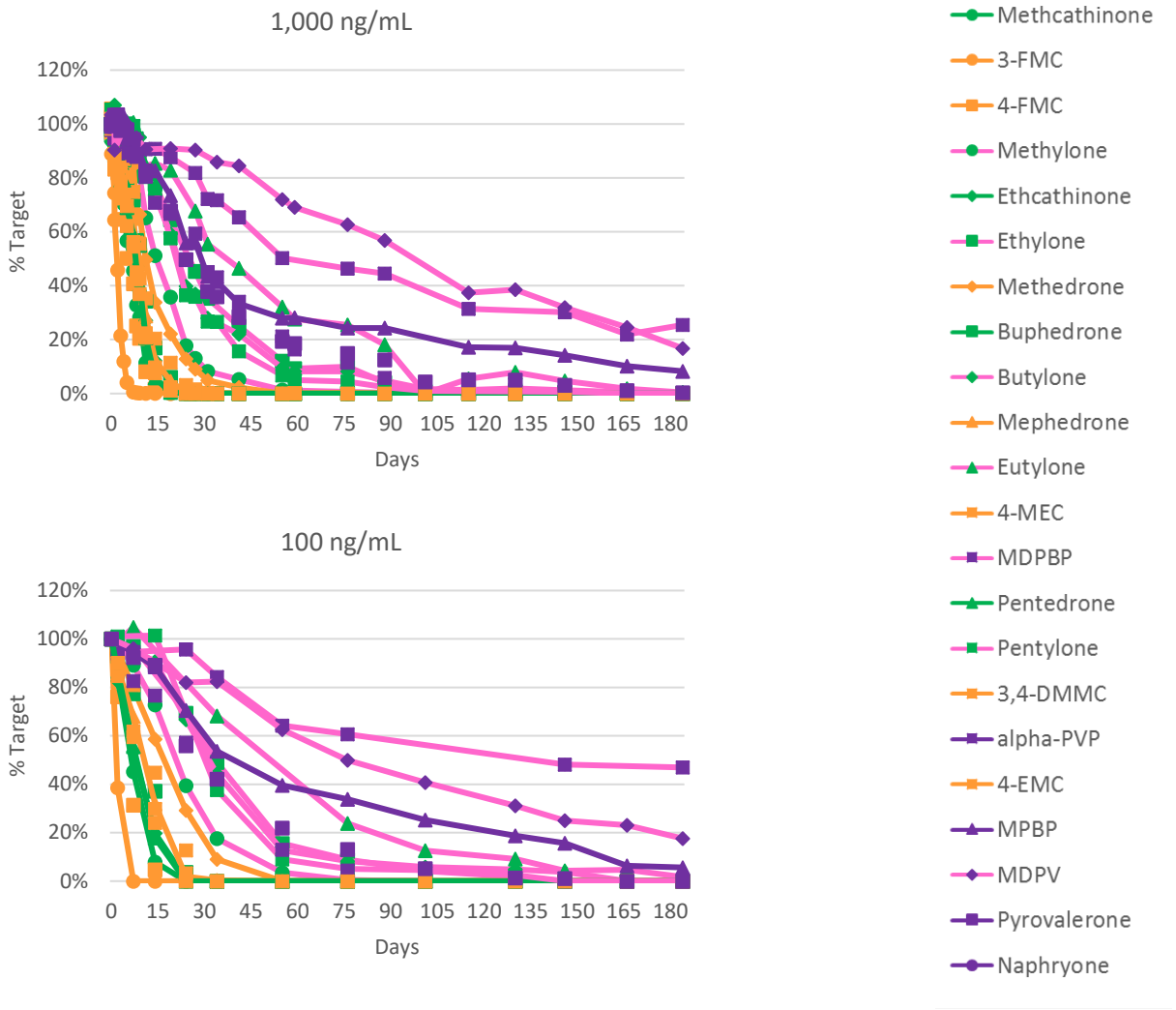
No concentration dependent instability was observed for any of the drugs. **Figure 12** depicts representative stability data in refrigerated blood at both concentrations tested. **Table 16** summarizes actual stability data (normalized to % target concentration) for methcathinone in blood at elevated temperature demonstrating lack of significance ($\alpha$=0.05), $F(1,58)=0.004$, $p$=0.95. Due to the absence of concentration dependent stability for any of the drugs, all subsequent statistical evaluations of temperature, pH, matrix, and analyte dependence were undertaken at 1,000 ng/mL.

**Table 16.** Stability of methcathinone in blood at elevated temperature (100 ng/mL and 1,000 ng/mL). Data is normalized to % Target concentration, based on replicate measurements at each sampling interval (time).

| | **Methcathinone in Blood Elevated Temperature** | |
|---|---|---|
| **Time (Days)** | **% Target (100 ng/mL)** | **% Target (1000 ng/mL)** |
| 0 | 102% | 102% |
| 0.3 | 95% | 102% |
| 0.6 | 92% | 93% |
| 1 | 87% | 86% |
| 1.5 | 67% | 68% |

| | | |
|---|---|---|
| 2 | 39% | 15% |
| 3 | 2% | 0% |
| 4 | 0% | 0% |

**Figure 12**. Cathinone stability in blood at refrigerated temperature at 100 and 1,000 ng/mL.




Analyte Dependence

In order to evaluate the significance of analyte dependence, ANOVA was used to examine the variances *within* and *between* populations. For example, before determining the significance of the methylenedioxy group, it was necessary to show that difference *within* the group were not significant ($F < F^{CV}$ or F-crit). Due to notable differences in stability for some of the fluorinated cathinones, it was necessary to exclude them from some of the comparisons.



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Among the secondary amines, there were no significant differences in stability between unsubstituted and ring substituted cathinones at elevated and ambient temperatures in blood due to their very rapid degradation under these conditions. No significant differences were observed in refrigerated or frozen blood when 3-FMC and 4-FMC were excluded. 3-FMC was consistently the least stable drug. Similar trends were observed in urine. No significant differences in stability were observed between substituted and unsubstituted cathinones in urine at pH 8 at elevated, ambient, or refrigerated temperatures. However, due to increased overall stability of the drug in acidic urine (pH 4), statistical comparisons could not be made at this pH.

Addition of the methylenedioxy (MD) group had a significant stabilizing effect. Among the secondary amines, MD substituted cathinones were more stable than their unsubstituted counterparts at all temperatures in pH 4 urine, and at ambient and refrigerated temperatures at pH 8. Furthermore, under the most unfavorable alkaline conditions, methylenedioxy substituted cathinones (ethylone, butylone, pentylone, methylone, and eutylone) were significantly more stable than their ring substituted counterparts (mephedrone, 4-MEC, 4-EMC, 3-FMC, 4-FMC, methedrone, and 3,4-DMMC) at all temperatures tested including refrigerated, $F(11,273)=8.74$, $p<0.0001$. The stabilizing effect of the methylenedioxy group was also evident for the tertiary amines (pyrrolidines). MD substituted pyrrolidines were generally observed to be more stable that their non-MD substituted counterparts, although in all but a few instances, the differences were not statistically significant. Comparisons between these groups were not always possible due to within group variability among the pyrrolidinyl analogs, notably naphyrone (the least stable among the group).

The stabilizing effect of the methylenedioxy group was evident throughout, most notably between unsubstituted secondary amines and the methylenedioxy substituted tertiary amines ($F(5,131)=24.4$, $p<0.0001$) and $F(5,125)=4.7$, $p<0.0001$ in refrigerated urine (pH 8) and blood, respectively). Similar results were obtained with substituted secondary amines ($F(8,218) = 24.1$, $p<0.0001$) in urine (pH 8) and blood, $F(8,252)=3.1$, $p=0.002$). Similar trends were observed in

urine (pH 4), although the magnitude of the significance was variable, due to changes in overall stability (i.e. temperature and pH).

An even greater stabilizing influence was observed between the secondary and tertiary amines. Tertiary amines were consistently more stable than their secondary amine counterparts. This stabilizing effect was even evident when comparing the most stable MD substituted secondary amines with their pyrrolidinyl counterparts. MD substituted tertiary amines were more stable than MD substituted secondary amines and this was particularly evident under unfavorable conditions such as alkaline pH, $F(6,147)=10.0$, $p<0.00001$, even when refrigerated.

As expected, stability was highly analyte dependent. **Table 17** highlights how structural characteristics influence cathinone stability, from least to most stable at elevated temperature in blood. Pyrrolidinyl-type cathinones with tertiary amines were notably more stable than their secondary amine counterparts. The inability of the tertiary amines to undergo oxidative deamination is a likely explanation. Although not within the scope of this report, degradation products are currently under investigation using HRMS. Although significant differences between unsubstituted and ring substituted secondary amines were not observed, substitution with a methylenedioxy group had a notable stabilizing effect for all drugs. Among the twenty-two drugs tested, methylenedioxy substituted pyrrolidinyl cathinones were the most stable, followed by tertiary amines and methylenedioxy substituted secondary amines. Substituted and unsubstituted secondary amines were the least stable, with 3-FMC exhibiting the greatest instability of all. The notable difference in stability between 3-FMC and its substituted counterparts (including 4-FMC) were most apparent under conditions which favored overall stability (i.e. frozen blood or acidic urine). These analyte dependent differences in stability are summarized for all matrices in **Table 18**.

**Table 17.** Analyte dependent stability of synthetic cathinones (least to most stable) at elevated temperature in blood.

| Cathinone | Ring Substituents | Amine | Blood $t_{1/2}$ (h) |
|---|---|---|---|
| 3-FMC | Substituted | Secondary | 8 |
| Ethcathinone | Unsubstituted | Secondary | 8 |
| 4-MEC | Substituted | Secondary | 13 |
| Buphedrone | Unsubstituted | Secondary | 14 |
| 4-FMC | Substituted | Secondary | 16 |
| Methcathinone | Unsubstituted | Secondary | 17 |
| Pentedrone | Unsubstituted | Secondary | 20 |
| 4-EMC | Substituted | Secondary | 21 |
| 3,4-DMMC | Substituted | Secondary | 22 |
| Methedrone | Substituted | Secondary | 28 |
| Mephedrone | Substituted | Secondary | 29 |
| Methylone | MD-substituted | Secondary | 33 |
| Naphyrone | Substituted | Tertiary | 33 |
| Pentylone | MD-substituted | Secondary | 51 |
| PVP | Unsubstituted | Tertiary | 58 |
| Ethylone | MD-substituted | Secondary | 68 |
| Pyrovalerone | Substituted | Tertiary | 78 |
| Butylone | MD-substituted | Secondary | 98 |
| Eutylone | MD-substituted | Secondary | 116 |
| MPBP | Substituted | Tertiary | 197 |
| MDPV | MD-substituted | Tertiary | 244 |
| MDPBP | MD-substituted | Tertiary | >365 |

**Table 18.** Influence of structure on cathinone half-life in blood and urine.

| Half Life in Blood | | | | | |
|---|---|---|---|---|---|
| Amine | Ring Substitution | Elevated | Ambient | Refrigerated | Frozen |
| Tertiary | MD-substituted | 10 - 21d | 2.7m | - | - |
| Tertiary | - | 1 - 8d | 0.3 – 1.7m | ≥10m | - |
| Secondary | MD-substituted | 1 - 5d | 9 - 31d | ≥10m | - |
| Secondary | - | 8 - 29h | 1 - 7d | 0.4 - 6m | 2.6m (3-FMC) |
| **Half-Life in Urine (pH 8)** | | | | | |
| Amine | Ring Substitution | Elevated | Ambient | Refrigerated | Frozen |
| Tertiary | MD-substituted | 2 - 4m | 4m | - | - |
| Tertiary | - | 5 - 46d | 0.4 - 1.4m | ≥4m | - |
| Secondary | MD-substituted | 19 - 72h | 3 - 11d | 1.4 - 6m | ≥8m |
| Secondary | - | 2 - 20h | 0.4 - 4d | 4 - 25d | ≥1m |
| **Half-Life in Urine (pH 4)** | | | | | |
| Amine | Ring Substitution | Elevated | Ambient | Refrigerated | Frozen |
| Tertiary | MD-substituted | - | - | - | - |
| Tertiary | - | - | - | - | - |
| Secondary | MD-substituted | ≥2m | - | - | - |
| Secondary | - | 0.3 - 2.3m | ≥2m | - | - |

Temperature Dependence

Cathinone stability was also highly temperature dependent (**Figures 7-9** and **13-14**). Temperature dependent differences were significant ($p<0.001$) for all twenty-two cathinones at both 100 ng/mL and 1,000 ng/mL ($\alpha=0.05$). For the most unstable drug (3-FMC) in the most unfavorable matrix (urine at pH 8), half-lives ranged from 2 hours at elevated temperature to 40 days when frozen. These results highlight how low temperatures significantly reduced degradation, even for the most unstable cathinones.

The influence of storage temperature on stability range, or the time period during which the drug was stable (< 20% loss) is shown graphically in **Figure 15**. These charts depict the range for the most stable drug within the class and **Table 19** provides the range of stability for each of the cathinone groups. This data not only highlights the importance of storage temperature on cathinone stability, but also analyte dependent variables discussed earlier. At elevated temperature (32°C) in blood, significant losses were observed for unsubstituted and ring



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substituted cathinones within hours and within 8 days for the pyrrolidines. Unless protected from heat, it is not uncommon for specimens to be exposed to elevated temperatures during shipping and transport to the laboratory, particularly during summer months. Although the range of stability at elevated temperature in urine at pH 8 was comparable to blood (hours to days), the influence of urinary pH on stability was striking for all temperatures tested. Cathinones were equally stable in acidic (pH 4) urine for the entire duration of the study at both refrigerated and frozen temperatures. In contrast, significant losses were observed even in frozen urine at alkaline pH (pH 8) for many of the cathinones, in particular the unsubstituted and ring substituted secondary amines. In urine (pH 8) and blood, at refrigerated temperature, significant losses were observed for all cathinones within 30 days, with the exception of the pyrrolidines. This is significant because the majority of forensic toxicology laboratories utilize refrigeration for short-term storage of biological specimens. Similar trends were observed in blood. At refrigerated temperature, with the exception of 3-FMC, all of the cathinones were stable or exhibited only moderate losses (<40% loss) in blood after 30 days. Cathinones stabilized by the pyrrolidine or methylenedioxy groups were more stable and several did not experience significant loss for the entire duration of the study.

With the exception of alkaline urine (pH 8) and 3-FMC, half-lives for cathinones under frozen conditions were precluded, due to their overall stability. As expected, half-lives decreased significantly with increasing temperature, ranging from hours to days in blood and alkaline urine at elevated temperature for most drugs.

**Figure 13**. Temperature dependent stability of cathinones (tertiary amines) in blood and urine. Unlabeled data indicates a half-life of >365 days or no measurable half-life due to stability.

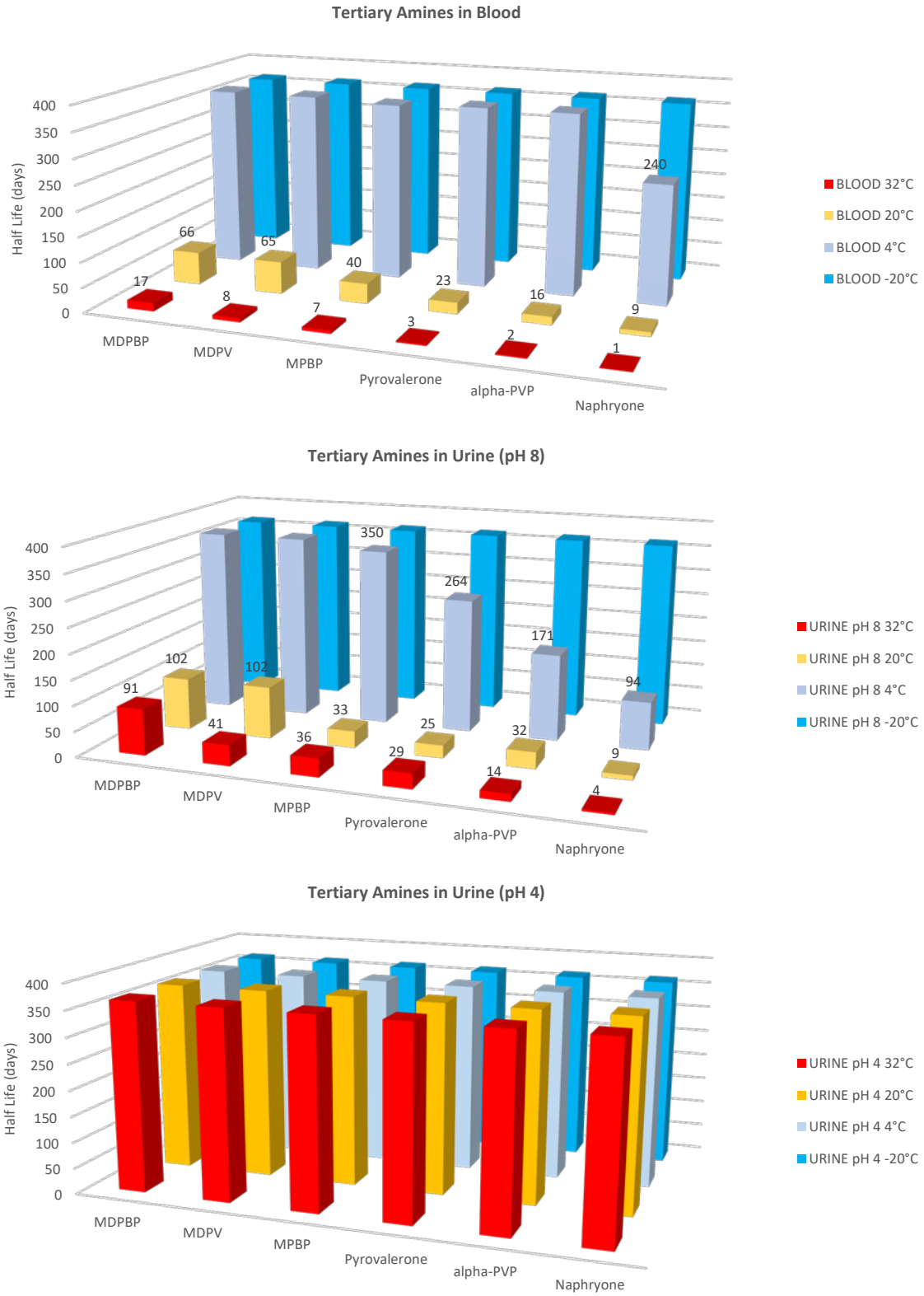




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**Figure 14**. Temperature dependent stability of cathinones (secondary amines) in blood and urine. Unlabeled data indicates a half-life of >365 days or no measurable half-life due to stability.

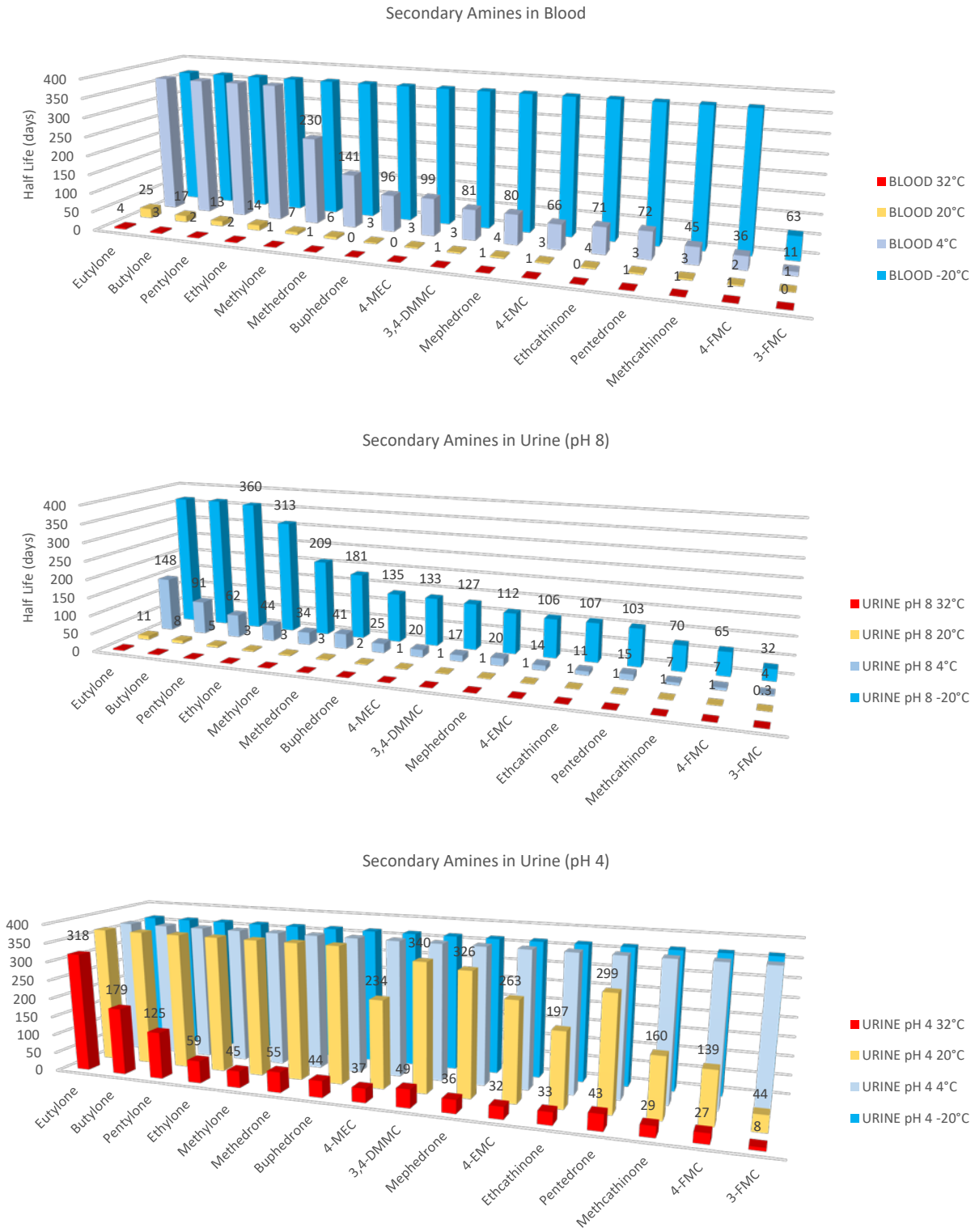




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**Figure 15.** Range of stability in blood and urine (pH 8 and pH 4).

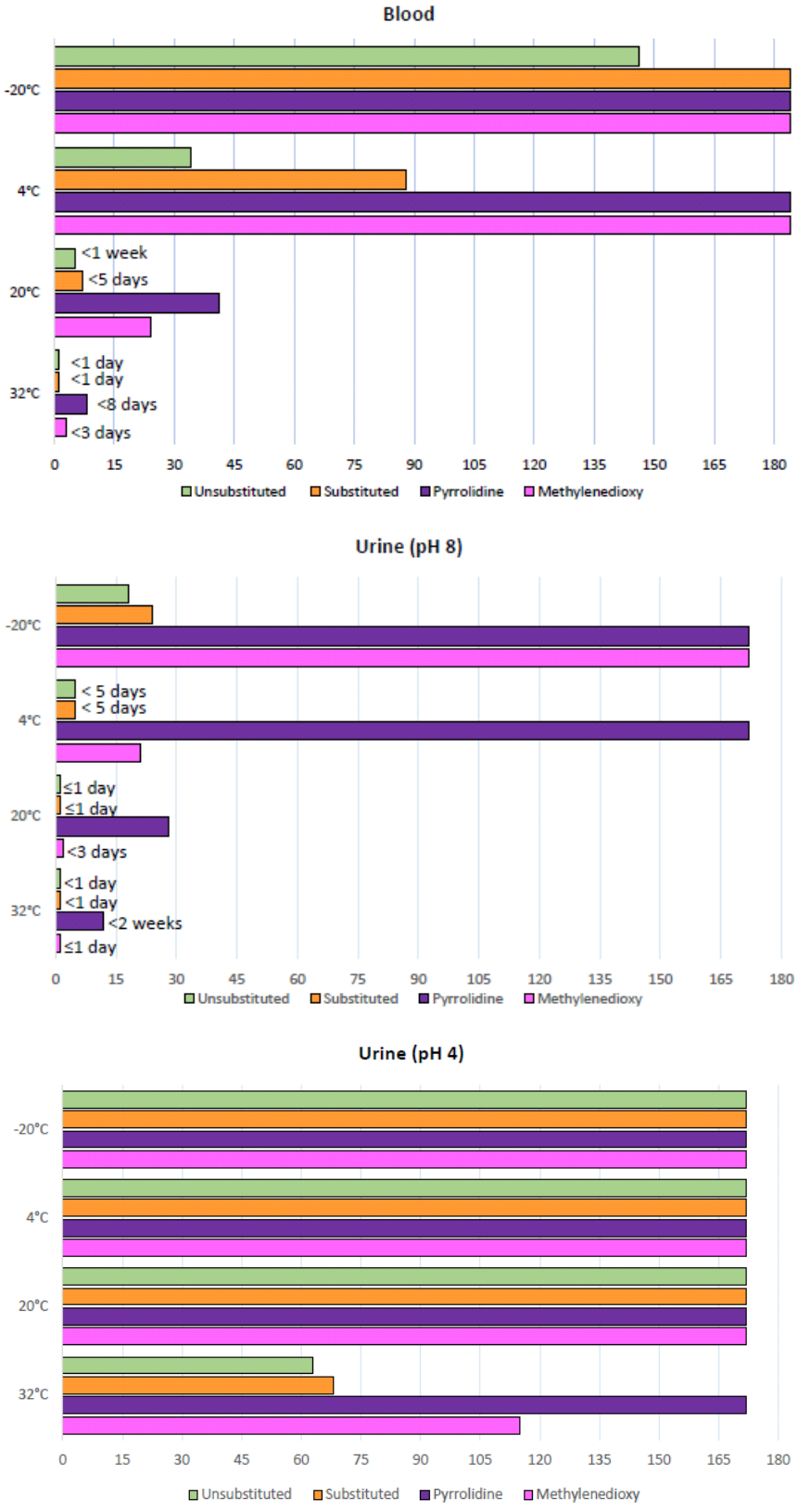

**Table 19.** Range of stability (no significant change in concentration) in blood and urine at elevated, ambient, refrigerated, and frozen temperatures at 100 ng/mL.

| **Stability Range in Blood** | | | | | |
|---|---|---|---|---|---|
| Amine | Ring Substitution | Elevated | Ambient | Refrigerated | Frozen |
| Tertiary | MD-substituted | 9 – 14d | 55d | >6m | >6m |
| Tertiary | - | 3 – 7d | 14 – 24d | 4.3 - >6m | 3.4 - >6m |
| Secondary | MD-substituted | 2 – 4d | 11 – 27d | 3.3 - >6m | >6m |
| Secondary | - | ≤2d | <1 – 8d | 4 – 101d | 0.5 - >6m |
| **Stability Range in Urine (pH 8)** | | | | | |
| Amine | Ring Substitution | Elevated | Ambient | Refrigerated | Frozen |
| Tertiary | MD-substituted | 5 – 13d | 28d | >6m | >6m |
| Tertiary | - | 1 – 3d | 3 – 7d | 18 – 91d | 0.5 - >6m |
| Secondary | MD-substituted | ≤ 1d | 1 – 5d | 5 – 21d | 0.5 - >6m |
| Secondary | - | <1d | ≤ 1d | 1 – 5d | 3 – 19d |
| **Stability Range in Urine (pH 4)** | | | | | |
| Amine | Ring Substitution | Elevated | Ambient | Refrigerated | Frozen |
| Tertiary | MD-substituted | >6m | >6m | >6m | >6m |
| Tertiary | - | 4.8 - >6m | >6m | >6m | >6m |
| Secondary | MD-substituted | 68 – 143d | >6 m | >6m | >6m |
| Secondary | - | 21 – 78d | 1.4 - >6m | >6m | >6m |

pH and Matrix Dependence

Although matrix dependent differences were observed, these were likely due to differences in pH, which were shown to be highly significant. In general, specimen pH appeared to have far greater influence on cathinone stability than the composition of the biological matrix (blood pH 7 versus urine at pH 8) (**Figure 16**). **Figure 17** summarizes half-life ranges graphically by matrix pH, from most alkaline (unstable) to most acidic (stable) over the range of temperatures tested. This shows that under most conditions, cathinone stability in blood (pH 7) followed similar trends as pH 8 urine; and most importantly, stability in alkaline urine (pH 8) was markedly different from acidic urine (pH 4). Under acidic conditions, cathinones (including 3-FMC) were stable at refrigerated and frozen temperatures. This is a significant finding because although steps can be taken to preserve biological evidence during shipping, handling and storage, specimen pH is not within the control of the laboratory. The influence of specimen pH on cathinone stability not only applies to blood and urine as described here, but to other biofluids that have a tendency towards

acidity or alkalinity (e.g. oral fluid, breast milk, amniotic fluid) (Kerrigan, 2010). **Figure 18** also depicts pH dependence of the tertiary and secondary amines in each matrix.

In addition to the determination of half-lives in each matrix and pH (**Tables 13-15**), the number of days to produce a significant (20%) or complete (100%) loss of drug was also determined (**Table 20**). With the exception of the methylenedioxy- or pyrrolidine-stabilized analogs, significant losses were observed in blood within hours to days in alkaline urine, compared with approximately one month or more at acidic pH (with the exception of 3-FMC). Significant losses in blood were observed on the order of days for all drugs (except 3-FMC) at both elevated and ambient temperatures. These results highlight the need to limit exposure to elevated or ambient temperatures where practical, refrigerate evidence upon receipt and interpret results within the context of evidence handling protocols.

While significant losses occurred on the order of days in many conditions, complete losses did not occur until weeks or months later. This data shows that despite changes in concentration over time, cathinones were detectable for extended periods at refrigerated and frozen temperatures. Although all temperatures were evaluated for a period of six months, biological samples are rarely exposed to elevated temperatures for extended periods. Biological samples transferred to the laboratory without protection form environmental conditions may be exposed to elevated temperatures for days, rather than weeks. Once in the laboratory, exposure to ambient temperatures are limited during the accessioning and evidence handling processes (typically hours). Thereafter, samples are maintained at refrigerated or frozen temperatures for short or long term storage.

The analyte, pH, and temperature dependent variables associated with cathinone stability must be carefully considered when interpreting quantitative results. Under optimal conditions (acidic pH and low temperature), losses can be minimized for all drugs, including the most unstable cathinones.

**Figure 16.** Cathinone stability on blood and urine (pH 8).




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**Figure 17**. Influence of matrix pH on cathinone stability ($t_{1/2}$).

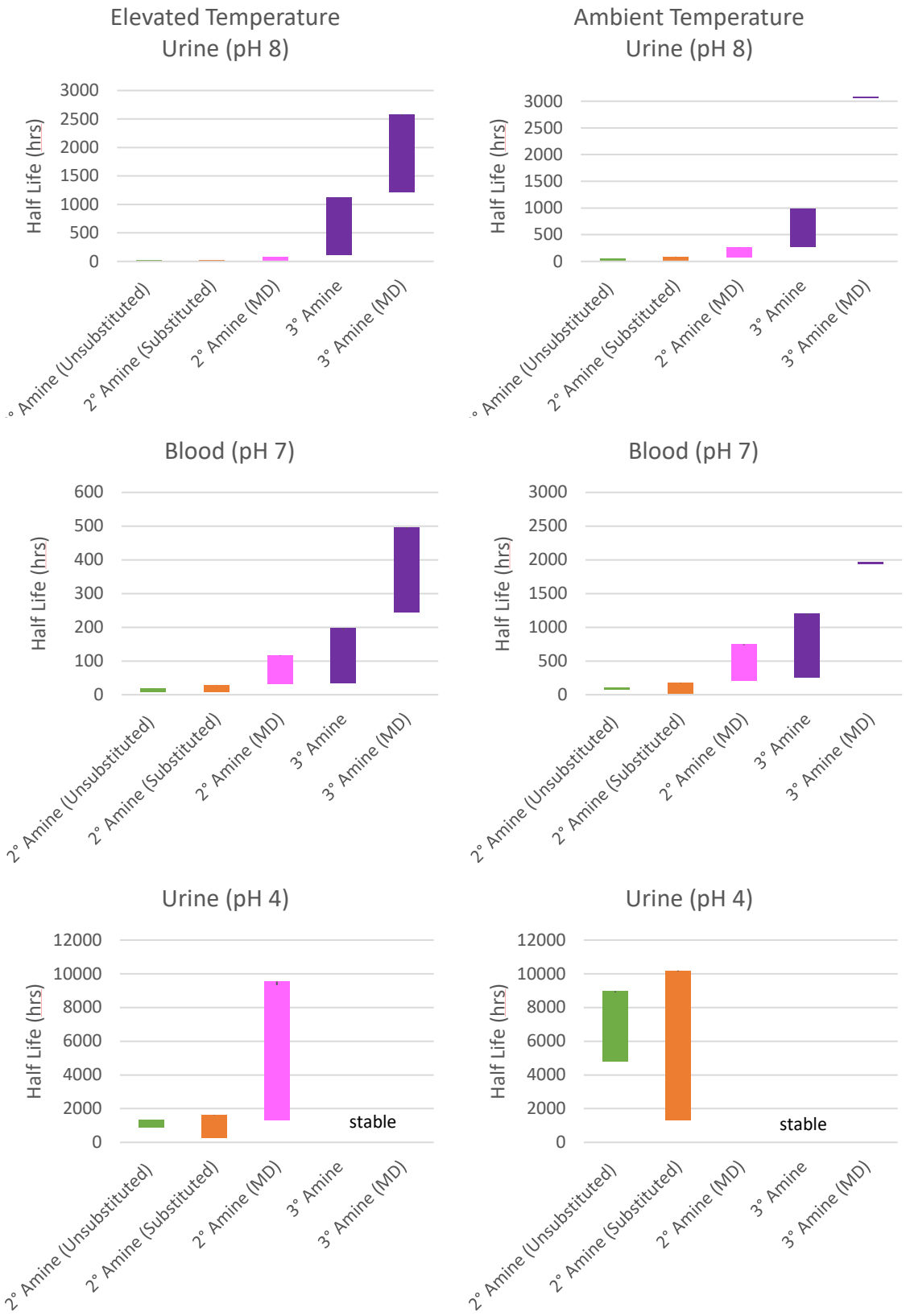




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**Figure 17 continued**. Influence of matrix pH on cathinone stability ($t_{1/2}$).

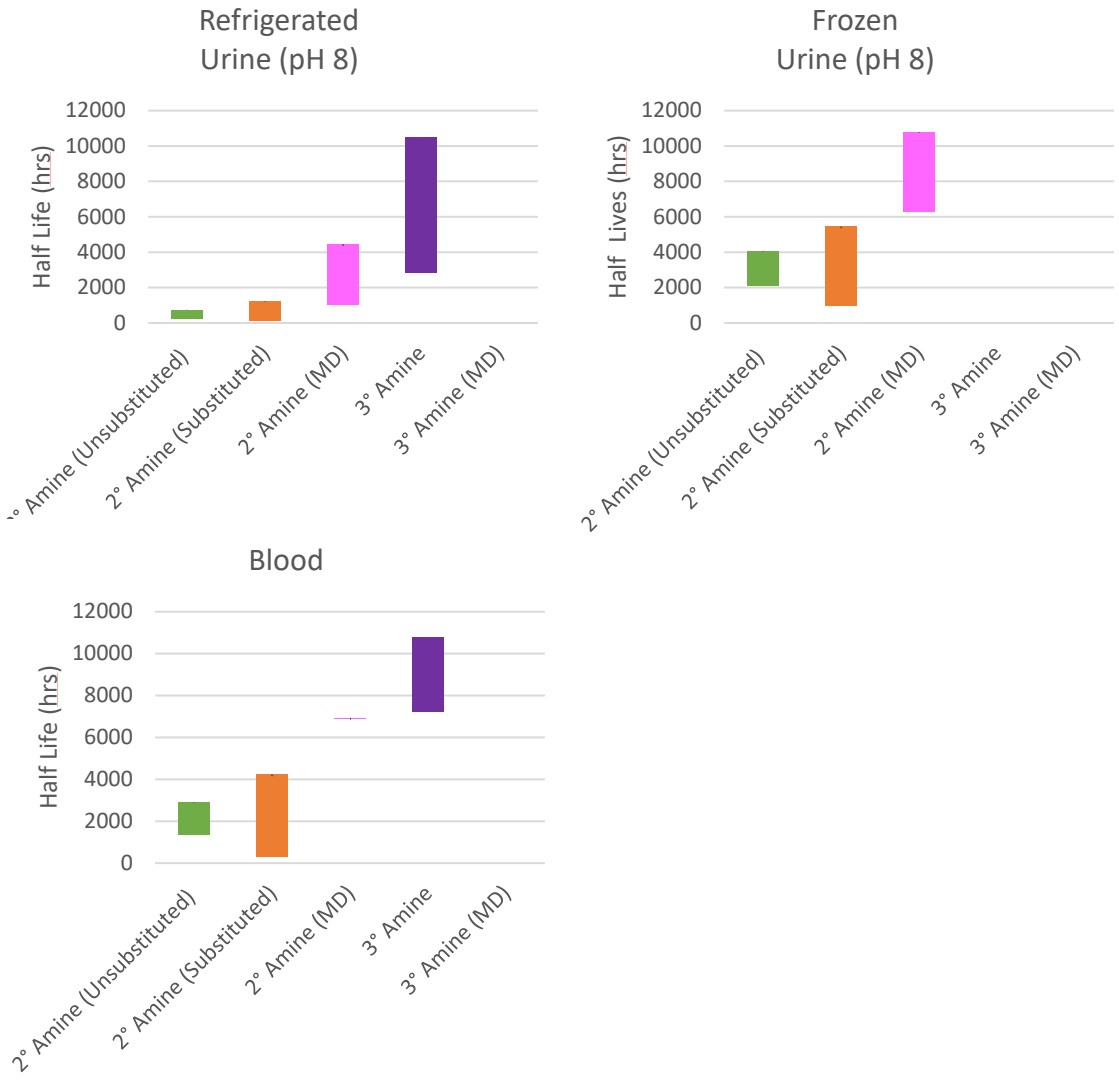

**Figure 18.** Matrix and pH dependent stability ($t_{1/2}$) of pyrrolidinyl cathinones (tertiary amines). Influence of matrix pH on cathinone stability ($t_{1/2}$). Unlabeled data indicates a half-life of >365 days or no measurable half-life due to stability.

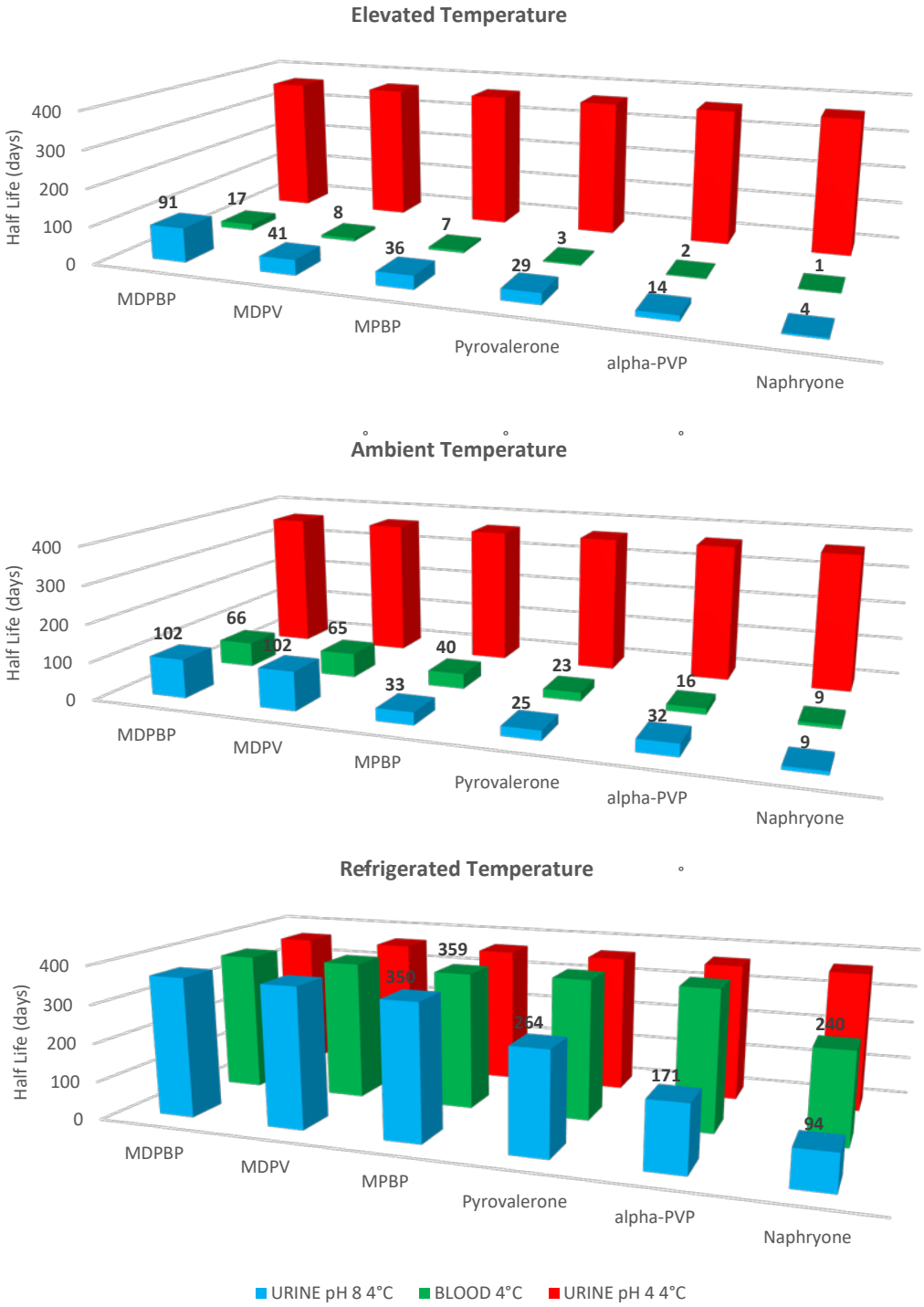




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**Figure 19.** Matrix and pH dependent stability ($t_{1/2}$) of cathinones with secondary amines. Influence of matrix pH on cathinone stability ($t_{1/2}$). Unlabeled data indicates a half-life of >365 days or no measurable half-life due to stability.

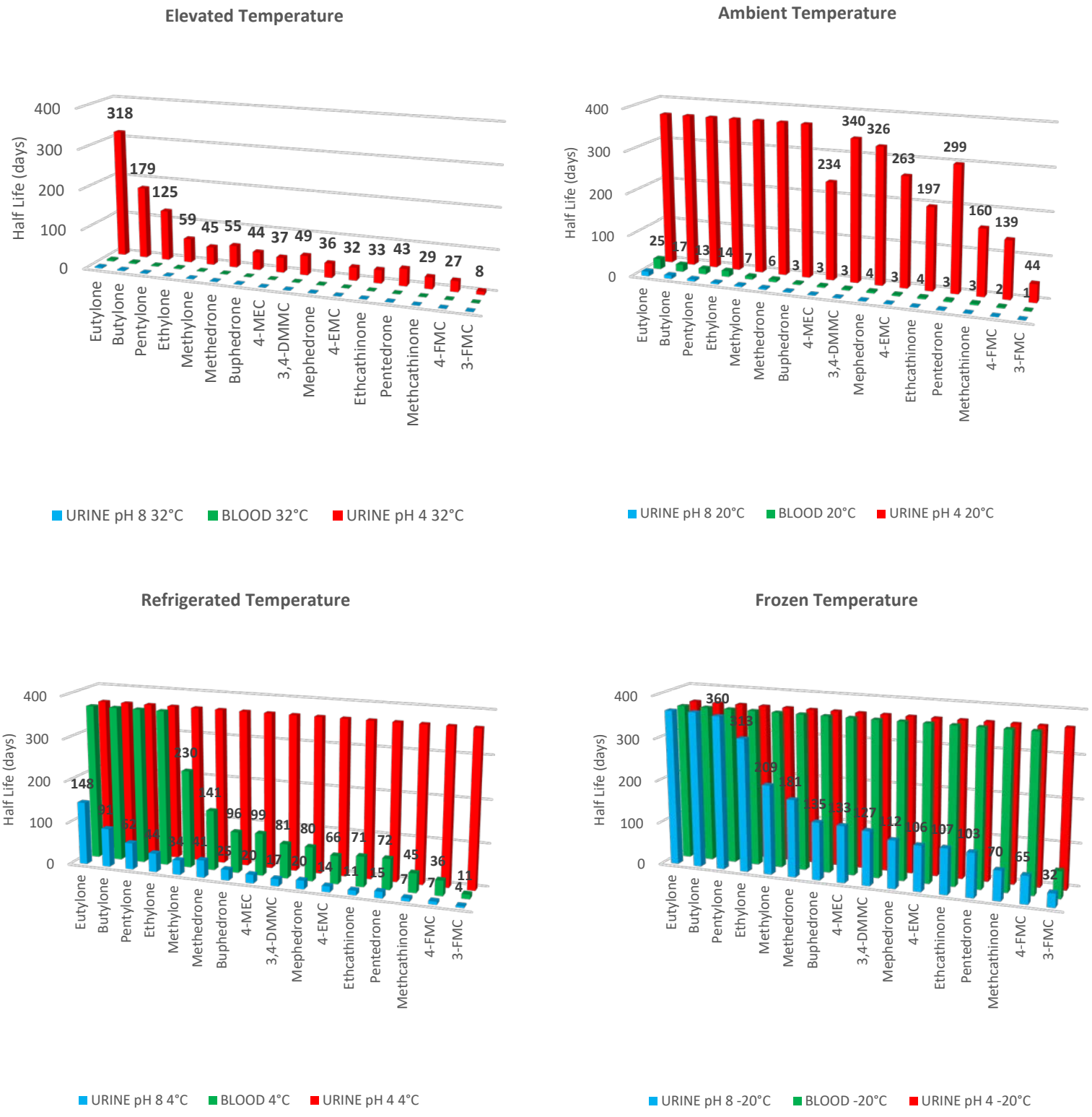




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**Table 20.** Number of days to significant (20%) and complete (100%) loss of drug.

| Blood (1,000 ng/mL) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Cathinone** | **Elevated** | | **Ambient** | | **Refrigerated** | | **Frozen** | |
| | 20% Loss | 100% Loss | 20% Loss | 100% Loss | 20% Loss | 100% Loss | 20% Loss | 100% Loss |
| 3,4-DMMC | 1 | 3 | 3 | 24 | 34 | -- | 184 | -- |
| 4-EMC | 1 | 3 | 3 | 24 | 34 | -- | 166 | -- |
| 3-FMC | 5.5h | 2 | 1 | 7 | 7 | 88 | 34 | -- |
| 4-FMC | 1 | 3 | 3 | 24 | 19 | -- | 46 | -- |
| 4-MEC | <2 | 4 | 5 | 31 | 76 | -- | -- | -- |
| Buphedrone | <2 | 4 | 5 | 27 | 76 | -- | -- | -- |
| Butylone | 3 | 27 | 14 | 166 | 146 | -- | -- | -- |
| Ethcathinone | <2 | 3 | 5 | 24 | 41 | -- | 166 | -- |
| Ethylone | 3 | 19 | 14 | 115 | 166 | -- | -- | -- |
| Eutylone | 3 | 31 | 27 | 184 | -- | -- | -- | -- |
| MDPBP | 7 | 130 | 31 | -- | -- | -- | -- | -- |
| MDPV | 7 | 88 | 55 | -- | -- | -- | -- | -- |
| Mephedrone | <2 | 4 | 5 | 24 | 55 | -- | -- | -- |
| Methcathinone | 1.5 | 3 | 3 | 19 | 19 | -- | 166 | -- |
| Methedrone | <2 | 9 | 8 | 55 | 88 | -- | -- | -- |
| Methylone | 2 | 11 | 11 | 88 | 101 | -- | -- | -- |
| MPBP | <4 | 55 | 19 | -- | 166 | -- | -- | -- |
| Naphyrone | <3 | 11 | 11 | 59 | 88 | -- | 184 | -- |
| Pentedrone | 1 | 3 | 3 | 19 | 34 | -- | -- | -- |
| Pentylone | <3 | 19 | 11 | 166 | 166 | -- | -- | -- |
| α-PVP | 3 | 24 | 11 | 184 | 88 | -- | 166 | -- |
| Pyrovalerone | 3 | 27 | 19 | 184 | 166 | -- | 184 | -- |
| **Urine pH 8 (1,000 ng/mL)** | | | | | | | | |
| **Cathinone** | **Elevated** | | **Ambient** | | **Refrigerated** | | **Frozen** | |
| | 20% Loss | 100% Loss | 20% Loss | 100% Loss | 20% Loss | 100% Loss | 20% Loss | 100% Loss |
| 3,4-DMMC | 5h | 4 | 1 | 16 | 5 | -- | 28 | -- |
| 4-EMC | 5h | 3 | 1 | 11 | 5 | 143 | 11 | -- |
| 3-FMC | 2h | <1 | 4h | 3 | <1 | 42 | 5 | -- |
| 4-FMC | 4h | <3 | 6h | 7 | 1 | 56 | 9 | -- |
| 4-MEC | 5h | 3 | <1 | 11 | 3 | 172 | 9 | -- |
| Buphedrone | 6h | 5 | <1 | 14 | 3 | -- | 5 | -- |
| Butylone | 1 | 18 | 3 | 63 | 18 | -- | 38 | -- |
| Ethcathinone | 5h | 3 | <1 | 9 | 2 | 143 | 12 | -- |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ethylone | 8h | 7 | 1 | 24 | 9 | -- | 28 | -- |
| Eutylone | 1 | 21 | 4 | 91 | 21 | -- | -- | -- |
| MDPBP | 1 | -- | 4 | -- | 143 | -- | -- | -- |
| MDPV | 18 | -- | 28 | -- | -- | -- | -- | -- |
| Mephedrone | 5h | 4 | <1 | 12 | 3 | -- | 12 | -- |
| Methcathinone | 4h | <2 | 6h | 5 | 2 | 56 | 7 | -- |
| Methedrone | 8h | 7 | 1 | 38 | 8 | -- | 16 | -- |
| Methylone | 6h | 7 | 1 | 24 | 5 | -- | 38 | -- |
| MPBP | 1 | 172 | 7 | -- | 91 | -- | -- | -- |
| Naphyrone | 1 | 78 | 4 | 78 | 18 | -- | 38 | -- |
| Pentedrone | 5h | 4 | <1 | 11 | 3 | 143 | 12 | -- |
| Pentylone | <1 | 11 | 2 | 38 | 7 | -- | 38 | -- |
| α-PVP | 3 | 172 | 3 | -- | 18 | -- | -- | -- |
| Pyrovalerone | <2 | -- | 6 | -- | 91 | -- | -- | -- |
| **Urine pH 4 (1,000 ng/mL)** | | | | | | | | |
| | **Cathinone** | | **Elevated** | | **Ambient** | | **Refrigerated** | |
| | 20% Loss | 100% Loss | 20% Loss | 100% Loss | 20% Loss | 100% Loss | 20% Loss | 100% Loss |
| 3,4-DMMC | 42 | -- | 172 | -- | -- | -- | -- | -- |
| 4-EMC | 38 | 172 | 91 | -- | -- | -- | -- | -- |
| 3-FMC | 9 | 143 | 21 | -- | -- | -- | -- | -- |
| 4-FMC | 28 | 143 | 63 | -- | -- | -- | -- | -- |
| 4-MEC | 42 | -- | 91 | -- | -- | -- | -- | -- |
| Buphedrone | 63 | -- | 91 | -- | -- | -- | -- | -- |
| Butylone | 143 | -- | -- | -- | -- | -- | -- | -- |
| Ethcathinone | 38 | -- | 63 | -- | -- | -- | -- | -- |
| Ethylone | 78 | -- | -- | -- | -- | -- | -- | -- |
| Eutylone | 172 | -- | -- | -- | -- | -- | -- | -- |
| MDPBP | 143 | -- | -- | -- | -- | -- | -- | -- |
| MDPV | -- | -- | -- | -- | -- | -- | -- | -- |
| Mephedrone | 38 | -- | 91 | -- | -- | -- | -- | -- |
| Methcathinone | 28 | 143 | 63 | -- | -- | -- | -- | -- |
| Methedrone | 68 | -- | -- | -- | -- | -- | -- | -- |
| Methylone | 63 | -- | -- | -- | -- | -- | -- | -- |
| MPBP | -- | -- | -- | -- | -- | -- | -- | -- |
| Naphyrone | 172 | -- | -- | -- | -- | -- | -- | -- |
| Pentedrone | 42 | -- | 115 | -- | -- | -- | -- | -- |
| Pentylone | 115 | -- | -- | -- | -- | -- | -- | -- |
| α-PVP | -- | -- | -- | -- | -- | -- | -- | -- |
| Pyrovalerone | -- | -- | -- | | | | | |

## Authentic Urine Specimens from Cathinone Users

In accordance with an IRB-approved study, authentic urine samples from cathinone users were investigated following a specified time of storage at refrigerated temperature. A total of 188 urine specimens were received and quantitatively analyzed using the validated procedure described earlier. The initial drug concentration and date of analysis was provided by the reference laboratory. The original 188 specimens yielded a total of 197 cathinone positive results for α-PVP (n=114), ethylone (n=61), methylone (n=11), MDPV (n=1), butylone (n=1), pentylone (n=1), 4-FMC (n=2), 4-MEC (n=2) and pentedrone (n=4). No quantitative comparison was made between data if the original date of analysis was unknown or if results were reported qualitatively during either assay. Of the 197 positive findings, it was possible to make quantitative comparisons on 162 results. Quantitative comparisons are summarized in **Tables 21-24**. Qualitative data is summarized in **Table 25**.

Specimens were stored for a period of 5 to 59 months at refrigerated temperature and specimen pH upon reanalysis ranged from 4.5 to 10. Alpha-PVP, ethylone, and methylone were further investigated due to their relatively large populations. Not surprisingly, α-PVP exhibited the best correlation due to its overall stability (**Figure 20**). Conversely, a sample containing 7,316 ng/mL methylone was undetectable upon reanalysis. However, this specimen had been stored for 55 months and had a pH of 9.3, which is highly unfavorable.

Among the α-PVP positive samples (n=114), specimen pH ranged from 4.5 to 10 and storage time ranged from 5 to 52 months. Quantitative reanalysis produced results between 0% and 119% of the original result (**Table 21**). Original α-PVP concentrations in urine were 25 – 104,111 ng/mL, with mean and median concentrations of 4,571 and 1,068 ng/mL, respectively. Upon reanalysis α-PVP concentrations were in the range 0 – 19,926 ng/mL, with mean and median concentrations of 1,890 and 100 ng/mL, respectively. The percent of drug remaining was investigated as a function of pH and storage time (**Figure 21**). Although it was not possible to determine a correlation as a function of storage time (due to clustering), the influence of specimen pH was evident. Upon reanalysis, alkaline urine specimens resulted in much lower drug concentrations,

relative to acidic urine samples. In general, for α-PVP, the lowest % drug remaining was found in urine samples with urinary pHs of 8.5 and above.

Among the ethylone positive samples (n=61), the pH of the specimen ranged from 5 to 10 and storage time ranged from 5 to 16 months. Quantitative reanalysis produced results between 0% and 102% of the original result (**Table 22**). Original ethylone concentrations in urine were 30 – 167,973 ng/mL, with mean and median concentrations of 9,119 and 206 ng/mL respectively. Upon reanalysis ethylone concentrations were in the range 0 – 146,124 ng/mL, with mean and median concentrations of 4,243 and 12 ng/mL, respectively. The percent of drug remaining was investigated as a function of pH and storage time (**Figure 22**). As with α-PVP, specimen pH rather than storage time exerted the greatest influence on cathinone stability. Some samples were confirmed within ±20% of the initial drug concentrations following 16 months of storage, attributable to the acidity of the urine sample (pH 6). Although it was not possible to determine a correlation as a function of storage time (due to clustering), the influence of specimen pH was clearly evident. Upon reanalysis, alkaline urine specimens resulted in much lower drug concentrations, relative to acidic urine samples. In general, as for α-PVP, the lowest % drug remaining was found in urine samples with urinary pHs of 7.5 and above.

Methylone consisted of a relatively small population of samples (n=10). Specimen pH ranged from 5 to 9 and storage time ranged from 5 to 55 months. Quantitative reanalysis produced results between 0% and 81% of the original result (**Table 23**). Original methylone concentrations in urine were 32 – 7,316 ng/mL, with mean and median concentrations of 1,361 and 131 ng/mL respectively. Upon reanalysis methylone concentrations were in the range 2 – 922 ng/mL, with mean and median concentrations of 149 and 41 ng/mL, respectively. Consistent with other drugs, quantitative values were well correlated for acidic urine specimens, even after extended periods of storage (7 months), while drug was undetectable after extended periods of storage at alkaline pH (9.3) (**Figure 23**).

**Figure 20.** Comparison of initial and final cathinone concentrations following specified periods of storage.

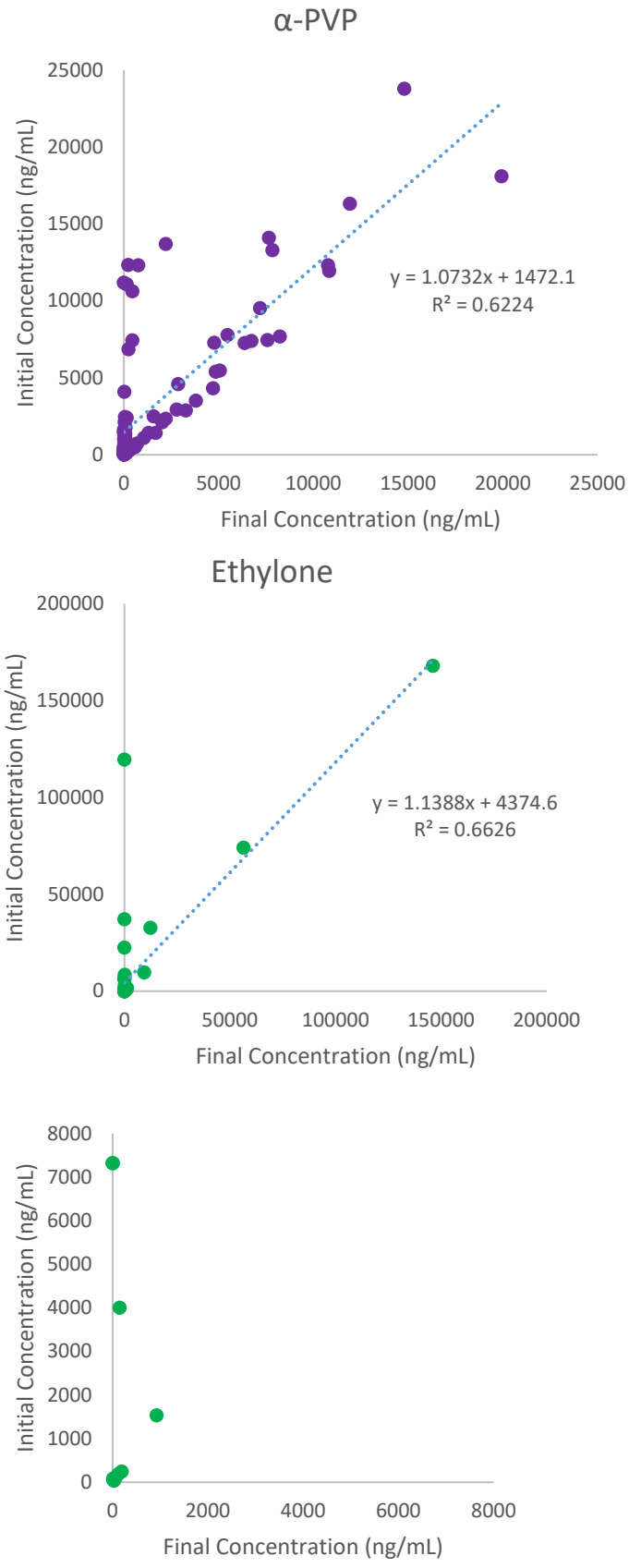




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**Table 21.** Concentrations of α-PVP in authentic urine samples following refrigerated storage.

| Unique ID | Initial Concentration (ng/mL) | Final Concentration (ng/mL) | % Drug Remaining | pH | Storage Time (months) |
|---|---|---|---|---|---|
| R054 | 9523 | 7,189 | 75% | 4.5 | 14.7 |
| R123 | 67 | 70 | 104% | 5.0 | 14.5 |
| R135 | 7,465 | 7,580 | 102% | 5.0 | 14.3 |
| R046 | 11,943 | 10,826 | 91% | 5.0 | 17.2 |
| R063 | 12,314 | 10,765 | 87% | 5.0 | 17.0 |
| R079 | 16,316 | 11,921 | 73% | 5.0 | 16.8 |
| R121 | 7,435 | 446 | 6% | 5.0 | 14.5 |
| R065 | 500 | 593 | 119% | 5.5 | 14.5 |
| R086 | 1,427 | 1,671 | 117% | 5.5 | 14.7 |
| R003 | 729 | 688 | 94% | 5.5 | 39.8 |
| T201205765 | 810 | 102 | 13% | 5.5 | 52.2 |
| R012 | 1,429 | 1,301 | 91% | 5.6 | 12.7 |
| R111 | 2,887 | 3,261 | 113% | 6.0 | 14.6 |
| R55 | 18,094 | 19,926 | 110% | 6.0 | 17.1 |
| 2R004 | 100 | 110 | 110% | 6.0 | 7.5 |
| R119 | 4,328 | 4,711 | 109% | 6.0 | 14.5 |
| R039 | 5,480 | 5,057 | 92% | 6.0 | 14.8 |
| R070 | 5,396 | 4,838 | 90% | 6.0 | 14.5 |
| R139 | 540 | 72 | 13% | 6.0 | 14.2 |
| 2R021 | 87 | 100 | 115% | 6.5 | 5.4 |
| 2R020 | 101 | 103 | 102% | 6.5 | 5.6 |
| R011 | 2,114 | 2,014 | 95% | 6.5 | 12.7 |
| R058 | 7,396 | 6,239 | 84% | 6.5 | 14.5 |
| 2R015 | 68 | 0 | 0% | 6.5 | 5.2 |
| R147 | 95 | 103 | 108% | 7.0 | 14.0 |
| R137 | 3,524 | 3,805 | 108% | 7.0 | 14.3 |
| R041 | 93 | 99 | 106% | 7.0 | 14.8 |
| R094 | 129 | 135 | 105% | 7.0 | 14.6 |
| R031 | 1,094 | 1,058 | 97% | 7.0 | 14.9 |
| R036 | 330 | 318 | 96% | 7.0 | 14.9 |
| R033 | 270 | 257 | 95% | 7.0 | 14.9 |
| R052 | 2,948 | 2,782 | 94% | 7.0 | 14.7 |
| R004 | 2,347 | 2,201 | 94% | 7.0 | 13.9 |
| R113 | 13,292 | 10,787 | 81% | 7.0 | 16.3 |
| R110 | 7,774 | 5,473 | 70% | 7.0 | 14.6 |

| | | | | | |
|---|---|---|---|---|---|
| R029 | 7,277 | 4,766 | 65% | 7.0 | 15.0 |
| R084 | 2,501 | 1,562 | 62% | 7.0 | 14.7 |
| R088 | 14,112 | 7,644 | 54% | 7.0 | 16.7 |
| R081 | 11,187 | 0 | 0% | 7.0 | 16.8 |
| R093 | 104,111 | 0 | 0% | 7.0 | 17.0 |
| R014 | 4,599 | 2,866 | 62% | 7.5 | 12.7 |
| R069 | 13,703 | 2,206 | 16% | 7.5 | 17.0 |
| R032 | 2,407 | 145 | 6% | 7.5 | 14.9 |
| R059 | 10,627 | 450 | 4% | 7.5 | 17.0 |
| R017 | 797 | 31 | 4% | 7.5 | 12.7 |
| R064 | 7,679 | 8,221 | 107% | 8.0 | 14.5 |
| R097 | 324 | 287 | 89% | 8.0 | 14.6 |
| R117 | 7,255 | 6,368 | 88% | 8.0 | 14.5 |
| R157 | 23,792 | 14,802 | 62% | 8.0 | 15.5 |
| R115 | 25 | 9 | 35% | 8.0 | 14.6 |
| R151 | 40 | 10 | 26% | 8.0 | 13.8 |
| R146 | 12,311 | 755 | 6% | 8.0 | 15.8 |
| R053 | 329 | 10 | 3% | 8.0 | 14.7 |
| R083 | 375 | 7 | 2% | 8.0 | 14.7 |
| R116 | 11,095 | 144 | 1% | 8.0 | 16.3 |
| R150 | 1,013 | 5 | 0% | 8.0 | 13.8 |
| R120 | 471 | 2 | 0% | 8.0 | 14.5 |
| R030 | 147 | 0 | 0% | 8.0 | 14.9 |
| R051 | 352 | 0 | 0% | 8.0 | 14.7 |
| R091 | 26 | 0 | 0% | 8.0 | 14.6 |
| R062 | 37 | 5 | 13% | 8.5 | 14.5 |
| R037 | 1,105 | 74 | 7% | 8.5 | 14.8 |
| R061 | 131 | 6 | 5% | 8.5 | 14.5 |
| R038 | 375 | 18 | 5% | 8.5 | 14.8 |
| R056 | 1,770 | 80 | 5% | 8.5 | 14.6 |
| R068 | 114 | 5 | 4% | 8.5 | 14.5 |
| R009 | 200 | 7 | 3% | 8.5 | 12.7 |
| R107 | 703 | 22 | 3% | 8.5 | 14.7 |
| R035 | 36 | 1 | 3% | 8.5 | 14.9 |
| R049 | 1,686 | 45 | 3% | 8.5 | 14.7 |
| R050 | 520 | 5 | 1% | 8.5 | 14.7 |
| R034 | 77 | 0 | 0% | 8.5 | 14.9 |
| R152 | 26 | 12 | 45% | 9.0 | 13.8 |
| R136 | 392 | 58 | 15% | 9.0 | 14.3 |

| | | | | | |
|---|---|---|---|---|---|
| R159 | 764 | 107 | 14% | 9.0 | 13.6 |
| R066 | 1,042 | 61 | 6% | 9.0 | 14.5 |
| R109 | 285 | 16 | 6% | 9.0 | 14.6 |
| R010 | 31 | 2 | 5% | 9.0 | 12.7 |
| R133 | 6,864 | 246 | 4% | 9.0 | 14.3 |
| R096 | 85 | 2 | 3% | 9.0 | 14.6 |
| R129 | 46 | 1 | 2% | 9.0 | 14.3 |
| R102 | 2,466 | 49 | 2% | 9.0 | 14.5 |
| R148 | 12,333 | 231 | 2% | 9.0 | 15.8 |
| R018 | 2,126 | 37 | 2% | 9.0 | 12.7 |
| R114 | 1,628 | 16 | 1% | 9.0 | 14.6 |
| R078 | 4,091 | 24 | 1% | 9.0 | 14.7 |
| R099 | 247 | 0 | 0% | 9.0 | 14.5 |
| R101 | 1,477 | 0 | 0% | 9.0 | 14.5 |
| R016 | 1,201 | 45 | 4% | 9.3 | 12.7 |
| R015 | 450 | 71 | 16% | 9.5 | 12.7 |
| R124 | 1,348 | 65 | 5% | 10.0 | 14.5 |
| R122 | 27 | 1 | 4% | 10.0 | 14.5 |
| R125 | 570 | 5 | 1% | 10.0 | 14.4 |
| R138 | 392 | 3 | 1% | 10.0 | 14.3 |

**Table 22.** Concentrations of ethylone in authentic urine samples following refrigerated storage.

| Unique ID | Initial Concentration (ng/mL) | Final Concentration (ng/mL) | % Drug Remaining | pH | Storage Time (months) |
|---|---|---|---|---|---|
| 2R001 | 379 | 255 | 67% | 4.5 | 7 |
| 2R006 | 36 | 22 | 61% | 4.5 | 6 |
| R027 | 127 | 69 | 55% | 5.0 | 14 |
| R104 | 74,050 | 56,418 | 76% | 5.0 | 16 |
| R156 | 252 | 171 | 68% | 5.0 | 13 |
| 2R011 | 1,542 | 1,162 | 75% | 5.0 | 6 |
| R045 | 1,312 | 23 | 2% | 5.5 | 14 |
| R080 | 97 | 88 | 91% | 5.5 | 14 |
| R085 | 1,059 | 1,037 | 98% | 5.5 | 14 |
| R106 | 308 | 273 | 89% | 5.5 | 14 |
| 2R002 | 98 | 66 | 67% | 5.5 | 7 |
| 2R018 | 189 | 150 | 79% | 5.5 | 5 |
| R023 | 206 | 144 | 70% | 6.0 | 14 |

| | | | | | |
|---|---|---|---|---|---|
| R025 | 110 | 68 | 61% | 6.0 | 14 |
| R026 | 75 | 22 | 30% | 6.0 | 14 |
| R040 | 131 | 132 | 101% | 6.0 | 14 |
| R077 | 38 | 21 | 56% | 6.0 | 14 |
| R089 | 32,661 | 12,284 | 38% | 6.0 | 16 |
| R090 | 195 | 198 | 102% | 6.0 | 14 |
| R103 | 167,973 | 14,6124 | 87% | 6.0 | 16 |
| R105 | 9,584 | 9,368 | 98% | 6.0 | 16 |
| R008 | 275 | 240 | 87% | 6.5 | 12 |
| R022 | 2,257 | 493 | 22% | 7.0 | 14 |
| R071 | 433 | 3 | 1% | 7.0 | 14 |
| R092 | 195 | 94 | 48% | 7.0 | 14 |
| R095 | 42 | 31 | 74% | 7.0 | 14 |
| R118 | 2,788 | 153 | 6% | 7.0 | 14 |
| R131 | 59 | 5 | 9% | 7.0 | 14 |
| R140 | 312 | 7 | 2% | 7.0 | 14 |
| 2R005 | 91 | 0 | 0% | 7.0 | 14 |
| R043 | 272 | 0 | 0% | 7.0 | 5.2 |
| R028 | 6,416 | 0 | 0% | 7.5 | 14 |
| R73 | 119,535 | 0 | 0% | 7.5 | 16 |
| R87 | 22,512 | 0 | 0% | 7.5 | 16 |
| R024 | 62 | 0 | 0% | 8.0 | 14 |
| R047 | 1,144 | 0 | 0% | 8.0 | 14 |
| R072 | 237 | 0 | 0% | 8.0 | 14 |
| R112 | 110 | 6 | 6% | 8.0 | 14 |
| R126 | 39 | 0 | 0% | 8.0 | 14 |
| R145 | 105 | 1 | 1% | 8.0 | 13 |
| R149 | 30 | 0 | 0% | 8.0 | 13 |
| R154 | 41 | 0 | 0% | 8.0 | 13 |
| R155 | 35 | 0 | 0% | 8.0 | 13 |
| R005 | 487 | 0 | 0% | 8.5 | 12 |
| R006 | 121 | 0 | 0% | 8.5 | 12 |
| R013 | 642 | 0 | 0% | 8.5 | 12 |
| R075 | 37,080 | 0 | 0% | 8.5 | 16 |
| R076 | 8,423 | 17 | 0% | 9.0 | 14 |
| R098 | 695 | 0 | 0% | 9.0 | 14 |
| R100 | 69 | 0 | 0% | 9.0 | 14 |
| R141 | 123 | 1 | 1% | 9.0 | 13 |
| R144 | 33 | 0 | 0% | 9.0 | 13 |

| | | | | | |
|---|---|---|---|---|---|
| R108 | 6,311 | 0 | 0% | 9.5 | 14 |
| R130 | 102 | 0 | 0% | 9.5 | 14 |
| R153 | 61 | 0 | 0% | 10.0 | 13 |

**Table 23.** Concentrations of methylone in authentic urine samples following refrigerated storage.

| Unique ID | Initial Concentration (ng/mL) | Final Concentration (ng/mL) | % Drug Remaining | pH | Storage Time (months) |
|---|---|---|---|---|---|
| 2R001 | 87 | 56 | 64% | 4.5 | 7 |
| 2R011 | 175 | 110 | 63% | 5.0 | 6 |
| 2R016 | 1,535 | 922 | 60% | 5.0 | 5 |
| 2R002 | 32 | 26 | 81% | 5.5 | 7 |
| 2R018 | 246 | 191 | 78% | 5.5 | 5 |
| 2R004 | 75 | 9 | 12% | 6.0 | 7 |
| 2R020 | 84 | 15 | 18% | 6.5 | 5 |
| 2R021 | 56 | 12 | 21% | 6.5 | 5 |
| T201209784 | 4,000 | 149 | 4% | 7.0 | 47 |
| R002 | 7,316 | 2 | 0% | 9.3 | 55 |

**Table 24.** Concentrations of cathinones in authentic urine samples following refrigerated storage.

| Unique ID | Cathinone | Initial Concentration (ng/mL) | Final Concentration (ng/mL) | % Drug Remaining | pH | Storage Time (months) |
|---|---|---|---|---|---|---|
| R001 | Butylone | 385 | 50 | 13% | 6.5 | 57 |
| R010 | Pentylone | 585 | 434 | 74% | 6.5 | 6 |
| R049 | MDPV | 6,626 | 479 | 7% | 8.5 | 14 |

**Table 25.** Qualitative findings in authentic urine samples following refrigerated storage.

| Unique ID | Cathinone | Initial Result | Final Result | pH | Storage Time (months) |
|---|---|---|---|---|---|
| 2R008 | 4-FMC | Positive | ND | 4.5 | 6.8 |
| 2R007 | 4-FMC | Positive | <LOQ | 7 | 6.8 |
| 2R003 | 4-MEC | Positive | <LOQ | 6 | 7.5 |
| 2R019 | 4-MEC | Positive | ND | 7 | 5.6 |
| 2R002 | Pentedrone | Positive | < LOQ | 5.5 | 7.8 |
| 2R001 | Pentedrone | Positive | ND | 4.5 | 6.8 |

| | | | | | |
|---|---|---|---|---|---|
| R003 | Pentedrone | Positive | < LOQ | 5.5 | 39.8 |
| 2R016 | Pentedrone | Positive | 176 ng/mL | 5 | 5.9 |

**Figure 21.** Influence of pH and storage time on α-PVP concentrations in cathinone users (n=114).

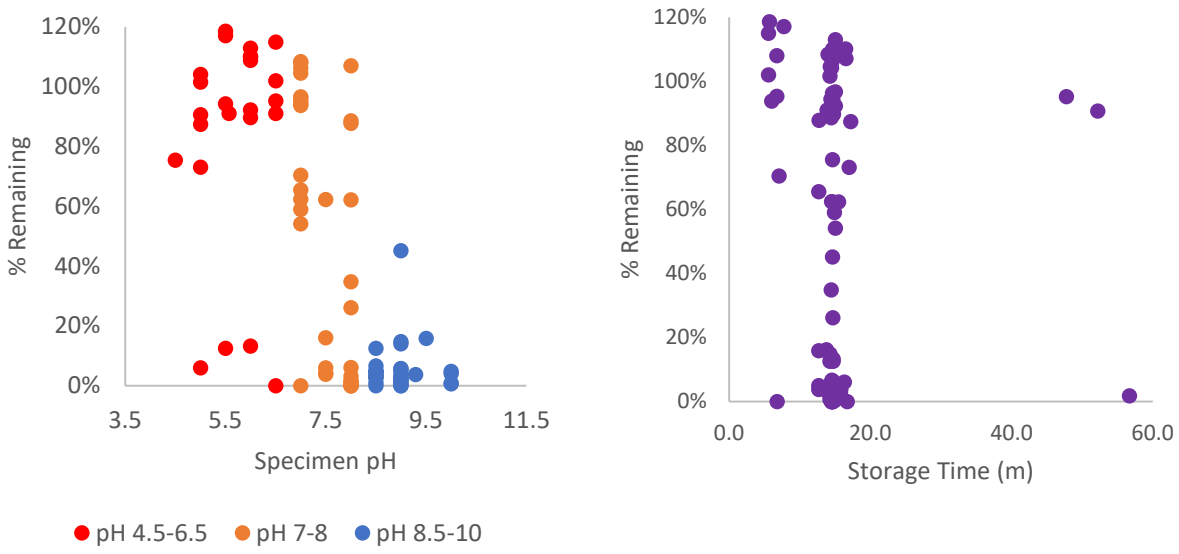



**Figure 22.** Influence of pH and storage time on ethylone concentrations in cathinone users (n=61).

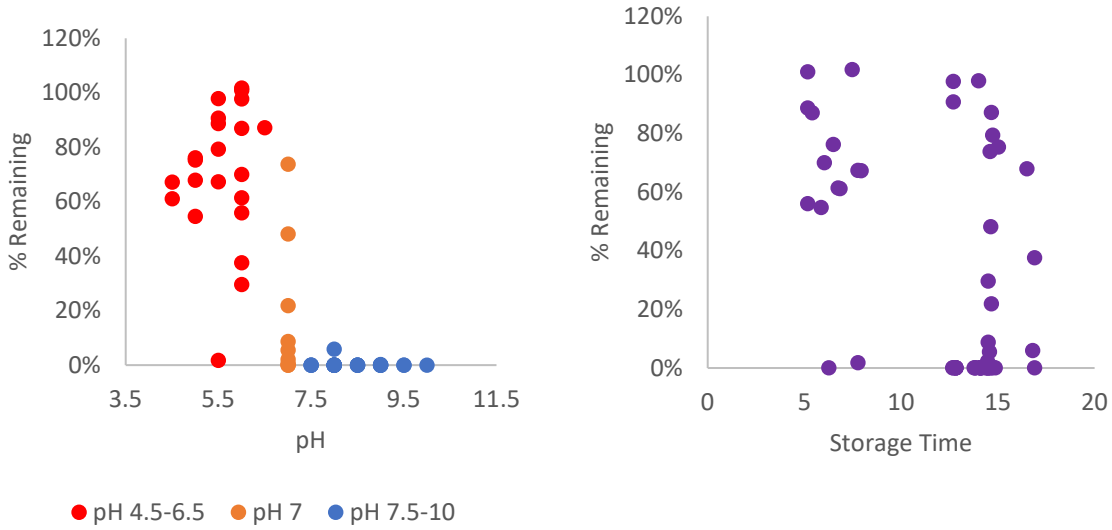
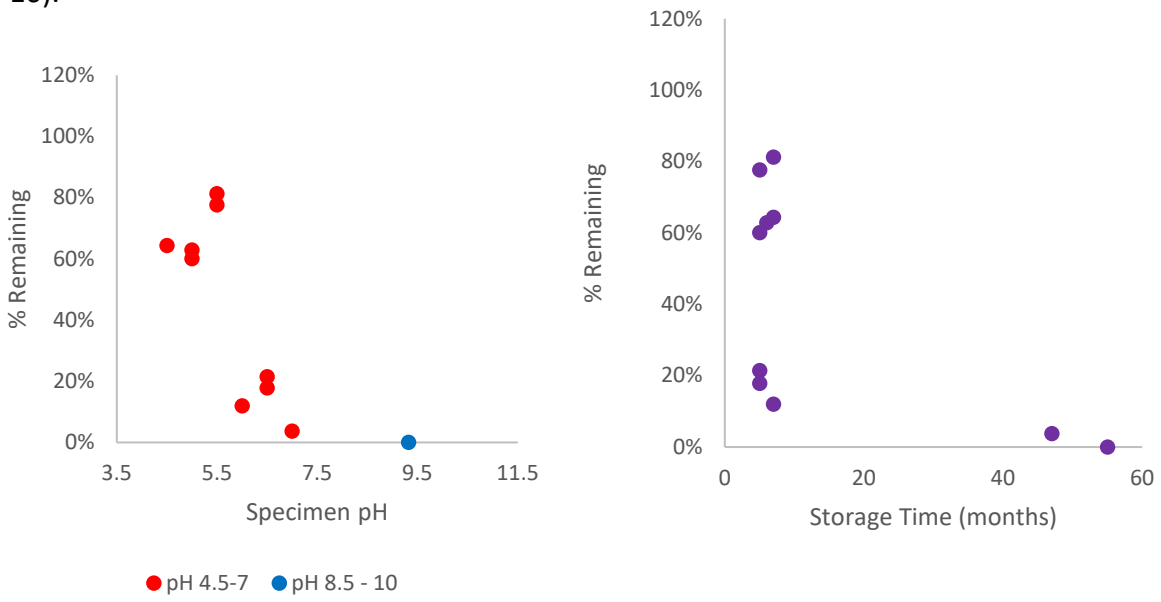


**Figure 23.** Influence of pH and storage time on methylone concentrations in cathinone users (n=10).




Quantitative comparisons using authentic urine samples from cathinone users were in good agreement with experimentally determined stability data using fortified matrix. These results also reinforced the importance of specimen pH on overall drug stability. The limited degradation of some drugs following extended periods of storage suggest that pH dependent variables were equally as important as conventional time dependent interpretation.



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## IV. Conclusions

### Discussion of Findings

Cathinone stability was systematically evaluated to determine temperature, pH, matrix, concentration, and analyte dependence. Although no concentration dependence was observed, cathinone stability was greatly influenced by pH, temperature, and cathinone structure. Matrix dependent differences between blood (pH 7) and urine (pH 8) were largely attributed to differences in specimen pH.

Pyrrolidine-type cathinones (tertiary amines) were significantly more stable than their secondary amine counterparts under all conditions tested. Among the secondary amines, no significant differences were observed between unsubstituted and ring substituted cathinones. However, addition of the methylenedioxy group had a significant stabilizing effect on both secondary and tertiary analogs. As a result, cathinones containing both a pyrrolidine and a methylenedioxy group exhibited the greatest stability.

Stability was highly pH dependent for all of the cathinones, including the most stable pyrrolidines. Cathinone stability increased with decreasing pH. Half-lives decreased significantly in alkaline biological samples (pH 8 urine). In contrast, under acidic conditions (pH 4 urine), significant improvements in stability were observed.

Temperature dependent stability was observed for all cathinones in blood and urine. Significant degradation was observed for all drugs (except methylenedioxy substituted pyrrolidines) within hours following exposure to elevated temperatures (32$^{o}$C). Cathinones were most stable in blood when stored at frozen temperature (-20$^{o}$C), and in acidic urine (pH 4), cathinones were equally stable at both refrigerated and frozen temperatures.

With the exception of the fluorinated cathinones (3-FMC and 4-FMC), cathinones were detected in blood and urine following several months of storage when refrigerated or frozen. Drug instability and the magnitude of the loss was heavily influenced by temperature, pH, and

structural characteristics. Increased temperatures and pHs were highly unfavorable and produced significant changes in concentration over time. As a result, concentrations at the time of testing may not always reflect those at the time of interest, for example the time of death or time of driving. Although drugs may still be detectable, significant losses are possible. Results using fortified biological matrix were compared with authentic urine samples from actual cathinone users. Results were in good agreement and particularly reinforced the pH and analyte dependent nature of stability.

Given that biological evidence is sometimes exposed to unfavorable conditions in both postmortem and antemortem toxicology investigations, toxicological findings related to synthetic cathinones should be interpreted cautiously and within the full context of evidence disposition. Finally, a greater understanding of analyte dependent variables (specifically functional groups that have stabilizing effects) will help predict the stability of future synthetic cathinones, as these designer drugs continue to evolve.

**Implications for Policy and Practice**

Cathinones are an important class of designer drug and their use has increased in the United States since the mid 2000s. Numerous cathinones have been reported in death and criminal investigations and forensic toxicology laboratories are often required to determine their presence in biological evidence. Conditions under which these drugs are unstable will improve the reliability of interpretive toxicology findings in criminal and death investigation casework. Moreover, greater understanding regarding structural influences will provide valuable insight concerning the stability of future analogs, yet to be developed.

**Implications for Further Research**

Synthetic cathinones are powerful psychostimulants that produce sought-after effects in recreational drug users. In 2011, the National Drug Intelligence Center of the U.S. Department of Justice described synthetic cathinones (“bath salts”) as an emerging domestic threat (NDIC,

2011). Although twenty-two cathinones were evaluated at the time of this study, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) now reports as many as eighty synthetic cathinone derivatives *via* the EU Early Warning System (EMCDDA, 2015). The proliferation of novel psychoactive substances is expected to continue for cathinones and the many other classes of designer drugs that have emerged during the past two decades. As this trend continues, the properties, characteristics, and toxicological impact of these drugs will require ongoing study.

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## VI. Dissemination of Research Findings

Lindsay Glicksberg, Kelsie Bryand and Sarah Kerrigan. Identification and Quantification of Synthetic Cathinones in Blood and Urine using Liquid Chromatography-Quadrupole/Time of Flight (LC-Q/TOF) Mass Spectrometry. Journal of Chromatography B, 1035, 91-103 (2016).

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Lindsay Glicksberg and Sarah Kerrigan. Short-Term Stability of Synthetic Cathinones in Blood. In preparation at the time of submission of this report.

Lindsay Glicksberg and Sarah Kerrigan. Short-Term Stability of Synthetic Cathinones in Urine. In preparation at the time of submission of this report.

Lindsay Glicksberg and Sarah Kerrigan. Long-Term Stability of Synthetic Cathinones in Blood and Urine. In preparation at the time of submission of this report.

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