Abstract

Over the past decade, synthetic cathinones have emerged as an important class of designer drugs within the United States. The proliferation of these new psychostimulants and their sought after effects among recreational drug users presents a formidable challenge for forensic toxicology laboratories. These drugs have been associated with impaired driving, intoxications and fatalities. Nevertheless, not all laboratories are capable of testing for these drugs despite the fact that their use may have very serious public health and safety consequences.

Despite the emergence of more sophisticated and sensitive instrumentation, the most widely used technique in forensic toxicology laboratories is gas chromatography/mass spectrometry (GC/MS). Cathinones, and in particular the pyrrolidine-type (or tertiary amine) drugs within this class, undergo extensive fragmentation in electron impact (EI) ionization to yield relatively poor quality mass spectra. Derivatization is commonly used to address this problem and in this study we investigated a number of novel derivatization schemes that might improve mass spectral quality by GC/MS analysis. However, the absence of an active hydrogen on the pyrrolidine-type cathinones prevents traditional derivatization reagents from being used. The purpose of this study was to investigate a number of alternative approaches involving derivatization of the ketone functional group. The cathinones were resistant to derivatization and although some ketone-reactive schemes showed promise, they suffered from poor yields, very limited improvements in mass spectral quality, or multiple products due to the formation of stereoisomers.

Most importantly however, during the investigation of novel derivatives important observations were made related to their thermal instability. Thermal decomposition products for all eighteen synthetic cathinones included in the study were characterized chromatographically and spectroscopically. These drugs underwent oxidative decomposition in-situ during GC/MS analysis with loss of hydrogen. Cathinone decomposition products for all eighteen compounds were characterized by this 2H loss. Spectroscopically their degradation products were characterized by base peaks two Daltons lower than the parent drug. Small

retention time differences and almost identical mass spectra for secondary amine degradation products could present a significant challenge during GC/MS analysis. In contrast, degradation products of cathinones bearing a tertiary amine were well resolved from the parent drug and were characterized by intense molecular ions (-2 Da) in addition to the pyrrolidinium ion.

Factors influencing the thermal degradation of synthetic cathinones were investigated. In-situ degradation was minimized using lower temperatures, decreasing residence time in the inlet and eliminating active sites. Although thermal degradation was minimized, these factors should be carefully considered during method development, validation and routine testing of cathinones by GC/MS.

In conclusion, synthetic cathinones are thermally labile and may undergo oxidative decomposition in-situ during GC/MS analysis. Although derivatization is a common approach to improve thermal stability and mass spectral properties, the chemistry of these polyfunctional drugs is inherently more complex than their non-ketone counterparts. Although functionalization of the ketone was possible, the products suffered from a number of drawbacks. In light of the potential for thermal instability during GC/MS and the need for sensitivity in forensic toxicology determinations, alternative analytical techniques such as LC/MS, LC/MS/MS or LC-q-TOF might be preferable for the determination of synthetic cathinones in biological evidence.

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Executive Summary

Statement of the Problem

Synthetic cathinones are derivatives of cathinone, the principal psychoactive component of the khat plant. These designer drugs are potent psychostimulants that have grown in popularity among recreational drug users. From a forensic standpoint they are important because they have been widely associated with criminal and death intoxications. State and federal efforts to curb their use have met with some success, but the proliferation of new synthetic cathinones presents an ongoing challenge to the forensic community, whose analytical methods must keep pace with illicit drug trends.

Chemically, the synthetic cathinones are small amphetamine-like compounds bearing a beta-keto functional group. Like their amphetamine counterparts, derivatization is commonly used to improve chromatographic and spectroscopic properties. These small polyfunctional compounds contain a ketone and an amine functional group. However, many of the forensically important cathinones contain a pyrrolidine moiety. These tertiary amines do not have the active hydrogen that is most often exploited with common derivatization approaches. Although acetylation using reagents such as TFAA (trifluoroacetic anhydride), PFPA (pentafluoropropionic anhydride) or HFBA (heptafluorobutyric anhydride) are among the most widely used for other amphetamines and some of the cathinones, these are inactive towards the pyrrolidine-type cathinones.

Purpose of the Study

In this study we investigated the derivatization chemistry, thermal stability and GC/MS analysis of synthetic cathinones, including secondary amines (methcathinone, ethcathinone, buphedrone, pentedrone, flephedrone, mephedrone, 4-MEC, 4-EMC, methedrone, methylone, ethylone, butylone, pentylone) and tertiary amines (MDPV, PVP, pyrovalerone, naphyrone, MDPBP and MDPB). The purpose of this study was to investigate new derivatives, suitable for

use in routine forensic toxicology testing by GC/MS, which is still the most widely used technique. Ideally the new derivatization would improve the quality of electron impact (EI) mass spectra, proceed quantitatively to yield a single product under mild to moderate conditions, and be universally reactive towards the pyrrolidine and non-pyrrolidine-type cathinones, independent of the active hydrogen. Once identified, the new derivative would be compared with non-derivatized approach for forensic toxicology purposes.

Cathinone Derivatization

Non-derivatized cathinones undergo extensive fragmentation via alpha cleavage to yield mass spectra that are heavily dominated by the nitrogen-containing iminium ion. The extensive fragmentation leaves very few characteristic ions to choose from, and this presents a challenge if selected ion monitoring is to be used. The mass to charge ratio of the iminium ion base peak is determined exclusively by the side chain substituents, so different drugs that are positional or regioisomers of each other can share very similar, or almost identical mass spectra. As a result, chromatographic separation is critically important during forensic analysis.

In this study, a number of ketone reactive derivatizations were proposed including cyanosilylation, oxime, and hydrazone formation. Trimethylsilylcyanide (TMSCN) was selected as a candidate for cyanosilylation, but efforts to functionalize the ketone using this reagent were unsuccessful. Oximes have proven useful for other polyfunctional drugs of abuse including keto opioids and keto steroids. Although traditional approaches using methoxylamine, hydroxylamine, and hydroxylamine followed by silylation to form a trimethylsilyl oximes were unsuccessful, *O*-pentafluorobenzyl oximes were produced using pentafluorobenzylhydroxylamine. Although this approach significantly increased molecular weight and improved mass spectral properties, they formed two products, corresponding with the *syn* (E) and *anti* (Z) isomers. Although this is consistent with oximes, multiple products (peaks) presents a real drawback in terms of quantitative assay performance and reproducibility. This is a significant concern in light of the fact that isotopically labeled internal standards are not yet available for all of the synthetic cathinones.

Keto-enol tautomerization can significantly complicate the chemistry and subsequent derivatization of the cathinones. Given the apparent difficulty associated with ketone derivatization of the cathinones, some more aggressive approaches were investigated. Despite significant potential for detector fouling, pentafluorophenylhydrazones were also evaluated without success, in addition to silylating reagents such as MSTFA:DTE:TMIS (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide: dithioerythritol: trimethyliodosilane) which have proven successful for other sterically hindered ketones. Synthetic cathinones were surprisingly resistant to derivatization of the ketone, even using these highly reactive derivatives. Reductive silylation and reductive acylation were then pursued with mixed success. Using a two-step derivatization, simple reduction was used to convert the ketone to the hydroxyl. Having introduced an active hydrogen, silylation was achieved using TMSI (trimethylsilylimidazole), a selective silylating reagent which is not reactive towards amines. Unlike more traditional silylating reagents that are reactive towards hydroxyls and amines, this TMSI permitted derivatization of these polyfunctional compounds to produce just one product. Although the two-step reductive silylation was successful, their mass spectral properties were not significantly improved. Fragmentation was still extensive and was dominated by the unchanged pyrrolidinium ion. Reductive acylation was readily accomplished, although steric hindrance was a factor when larger fluorinated derivatives such as PFPA or HFBA were used. Although all of the synthetic cathinones were capable of forming trifluoroacetyl derivatives at the ketone following simple reduction, multiple products were formed for cathinones bearing a secondary amine.

Thermal stability

During the optimization of the forensic toxicology assay, some important observations were made regarding the thermal instability of the synthetic cathinones. Artifact peaks were identified for all of the cathinones that were targeted in the assay. Most of the artifacts produced very similar mass spectra to the parent compound, with the notable exception of the base peak, which was 2 Da lower. Since the m/z of the base peak originates from the iminium

ion, this 2 Da loss was attributed to the loss of 2H. However, the pyrrolidine-containing cathinones produced very stable molecular ions (-2 Da) in addition to the iminium ions (-2 Da). Based on the quality of their mass spectra and deuterium labeling, the artifacts of the pyrrolidine-type cathinones indicated degradation to form an enamine. Thermal oxidation in-situ during GC analysis was first documented more than two decades ago by DuRuiter and Noggle using methcathinone. Degradation products for all eighteen cathinones were identified chromatographically and spectroscopically.

Instrumental factors that influence degradation were explored. Oxidative degradation was minimized by lowering temperatures, reducing residence time, and eliminating active sites in the inlet. Although it was possible to minimize in-situ degradation, thermal instability of the cathinones presents a significant drawback in terms of GC/MS analysis, particularly if quantitative assays are to be used.

Identification of cathinones in biological matrices

A mixed mode solid phase extraction was used to isolate synthetic cathinones from biological matrices. Recoveries in urine and blood were 65-98% and 50-73%, respectively. Recoveries of the six pyrrolidine-containing cathinones were higher and more reproducible than their secondary amine counterparts. Using full scan acquisition, limits of detection in urine were 25 ng/mL for 4-EMC, 4-MEC, buphedrone, butylone, ethcathinone, ethylone, flephedrone, MDPBP, MDPV, mephedrone, MPBP, naphyrone, pentedrone, pentylone, α-PVP, pyrovalerone, and 50 ng/mL for methylone. Limits of detection in blood were 25 ng/mL for 4-MEC, buphedrone, MDPBP, MDPV, MPBP, naphyrone, pentedrone, α-PVP and pyrovalerone, 50 ng/mL for 4-EMC, butylone, ethylone, flephedrone, mephedrone, methcathinone, and pentylone, and 100 ng/mL for ethcathinone, methedrone and methylone. Based on the potential for in-situ degradation, the assay was validated for qualitative use only. However, precision and bias were evaluated for investigational purposes. Intra-assay precision in urine (N=6) yielded CVs between 1.2-3.6% and accuracy in the range 86-95%. Interferences from biological matrix, isotopically labeled internal standard (MDPV-D8), common drugs, structurally related compounds and endogenous bases

were evaluated in the interference study. No interferences were present in drug free urine or blood from different sources (N=10), endogenous bases or the MDPV-D8 internal standard. The interference study included more than forty compounds including ten common amphetamine-like drugs, fifteen designer drugs of the DO-, 2C and 2CT-series, fifteen common basic drugs, in addition to bupropion and diethylpropion. No false positives or false negatives were present in any of the negative controls or cathinone-positive (100 ng/mL) controls. The optimized assay produced clean extracts and excellent separation of all nineteen target analytes.

The GC/MS assay was used to evaluate forty authentic urine specimens form cathinone users. Despite some differences in the scope of testing between the reference laboratory and the GC/MS assay, results were in excellent agreement. Of the sixty four confirmed positive results by the reference laboratory, fifty-five were confirmed by the GC/MS assay within three months of the original test. Methylone presented the greatest challenge, attributed to the apparent instability of the drug in the biological matrix. Although the stability of the synthetic cathinones has not yet been systematically evaluated, there is growing evidence that some drugs within this class are unstable in biological evidence and this deserves additional study.

Summary

Synthetic cathinones are important psychostimulants that have been associated with impaired driving, intoxications, and fatal overdoses. Forensic toxicology laboratories must be able to identify and determine the presence of these drugs in biological evidence. Many challenges exist, not least the proliferation of analogs and the availability of isotopically labelled reference standards and metabolites. After analytical testing has concluded, forensic toxicologists are often required to interpret those findings and determine their role in death investigations as well as criminal and civil proceedings. In order for a forensic toxicologists to provide an interpretation of the results it is important to understand how concentrations of these drugs might be influenced during analysis or storage. The chemistry of the synthetic cathinones is inherently more complicated than their amphetamine counterparts. These polyfunctional

compounds are subject to keto-enol tautomerization which significantly complicates their derivatization chemistry, and many of the forensically important drugs contain pyrrolidine groups that are not amenable to traditional approaches to derivatization. In this study we characterize thermal degradation products for all of the synthetic cathinones tested. Although thermal degradation can be minimized during analysis, in-situ degradation should be evaluated during method development, optimization and during routine analysis by GC/MS. Thermal stability is an important consideration when considering analytical approaches and most suitable techniques for analysis. Hyphenated liquid chromatography-mass spectrometry techniques such as LC/MS/MS or LC-Q-TOF may be highly advantageous form this standpoint, and provide much needed sensitivity for the toxicological detection of synthetic cathinones in biological evidence.

I. Introduction

Statement of the Problem

Despite the growing popularity of the synthetic cathinone designer drugs, many forensic toxicology laboratories performing routine casework do not test for these substances in antemortem and postmortem casework. Nevertheless, these drugs have been associated with impaired driving and postmortem death intoxications. These designer drugs are synthetic derivatives of cathinone, the principal psychoactive component of khat (*Catha edulis*). As potent modulators of the monoamine transporters, their combination of stimulant and mood-altering sensations have contributed to their popularity among recreational drug users.

Over the past five years the United States government has exercised emergency and permanent scheduling authority to control the proliferation of synthetic cathinone abuse. The abuse of synthetic cathinones in the United States has escalated considerably (NFLIS, 2014) and these drugs now receive widespread attention. 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 these changes may also circumvent existing drug legislation. Demand from recreational drug users, and the clandestine supply and effective "marketing" of designer drugs via the Internet, significantly outpaces the ability of government to regulate, legislate and enforce those actions. As a result, it is an ongoing challenge for laboratories to keep pace with the analytical demands placed upon them by such an expansive array of designer drugs.

Synthetic cathinones present a variety of issues, not only for law enforcement and public safety officials, but also for toxicologists who encounter these drugs in forensic investigations. Although identification of these substances is somewhat routine in controlled substance casework involving drug offenses (e.g. seized pills, powders), toxicological detection is more challenging. First, pharmacokinetic studies in humans are still somewhat limited and as a consequence, metabolic reference materials are not commercially available for all the synthetic

cathinones. Most laboratories rely upon the ability to detect the parent drug in toxicological specimens and this may present a challenge for laboratories that rely upon gas chromatography/mass spectrometry (GC/MS), the most widespread analytical technique.

The synthetic cathinones are small amphetamine-like compounds bearing a beta-keto functional group. Like the amphetamines, toxicological detection can be improved by derivatization to enhance both the chromatographic and spectroscopic properties. Although common derivatization techniques have been used for some but not all of the drugs of interest, many of the forensically important cathinones are tertiary amines that do not readily derivatize due to the absence of an active hydrogen. The relatively poor quality of the mass spectra in the underivatized form can be a limiting factor for GC/MS analysis.

In this study a number of novel derivatization schemes are investigated. Additionally, observations regarding the thermal instability and in-situ degradation of synthetic cathinones during GC/MS analysis are documented and investigated.

Literature Citations and Review

Designer Drugs

Synthetic cathinones are a rapidly evolving class of designer drugs that are structurally related to cathinone, the principal psychoactive component of khat (*Catha edulis*). As beta-keto analogs of phenethylamine, these drugs can be classified as sympathomimetic amines. Many are potent inhibitors 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.

Recreational and Therapeutic Use of Synthetic Cathinones

Interest in the recreational use of cathinones has been attributed to their relatively low cost, psychostimulant effects similar to cocaine and methamphetamine and ready availability, particularly prior to scheduling actions (Kelly 2011). They have been associated with a variety of toxicological investigations including impaired driving and fatal intoxications. In addition to their centrally acting stimulant effects, they produce distinct mood-altering and psychoactive effects. The sought after effects of increased energy, openness, empathy and libido can be accompanied by distinct psychiatric, cardiac and neurological effects (Prosser, 2012). Deceptive labeling of synthetic cathinones is a common practice. These drugs have been presented to consumers as bath salts, plant food, and research chemicals. Although packaging may state "not for human consumption", the clear intention is for these products to be administered for recreational purposes. Very often, these products contain a mixture of cathinones, often diluted with lidocaine, caffeine and other common adulterants and synthetic byproducts of their clandestine synthesis.

Methcathinone (ephedrone) and 4-methylmethcathinone (mephedrone) were first synthesized in the 1920s. Mephedrone was marketed as an antidepressant in the USSR in the 1930s and 1940s, followed by the appetite suppressant diethylpropion by Parke Davis in the United States

in the 1950s. Methcathinone abuse was widespread in the USSR in the 1970s but did not become popular in the US, Asia, Europe and Australia until the 1990s. Although methylone was patented as an antidepressant and for the treatment of Parkinson's disease in 1996, it was not marketed for that purpose. Currently there are only two synthetic cathinones that are approved for medical use in the United States, bupropion and diethylpropion. Bupropion (Wellbutrin, Zyban) is prescribed as an antidepressant and for smoking cessation in doses of 200-450 mg/day, while diethylpropion (Tenuate, Tepanil) is an appetite suppressant that is prescribed in doses of 25 mg for the clinical management of obesity. More recently, bupropion has been suggested for the treatment of methamphetamine and cathinone withdrawal (Coppola, 2012; Lev-Ran, 2012).

Methcathinone was the first synthetic cathinone to be federally scheduled in October of 1993 as a Schedule I substance. Mephedrone, methylone, and MDPV were temporarily placed into Schedule I in October 2011 (DEA, 2011). Mephedrone and MDPV were added to Schedule I in April 2012 and methylone was added in April 2013. In 2014, 4-MEC, 4-MePP, α-PVP, butylone, pentedrone, pentylone, 4-FMC, 3-FMC, naphyrone, and α-PBP were temporarily placed into Schedule I (DEA, 2014). At the time of this report, at least 43 states have enacted legislation to ban synthetic cathinones. While some states have chosen to schedule specific cathinones, others have enacted general class bans, whereby similar compounds or substances that produce the same pharmacological effects are also prohibited.

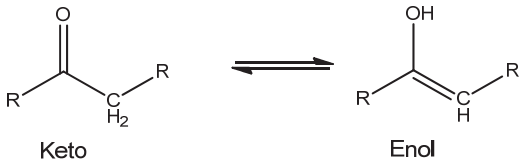
Chemistry

The synthetic cathinones described in this study are arylamino ketones that can be broadly sub-classified into alkylamines (secondary amines) and pyrrolidines (tertiary amines). Despite the popularity of the synthetic cathinones, relatively little is understood of their inherent chemistry (Zawilska, 2013). Their chemical behavior is dominated by two functional groups: the ketone and the amine. As a result, cathinones may undergo keto-enol tautomerization (**Figure 1**) and although enols are typically thermodynamically unstable relative to their carbonyl isomers, the enol form can be stabilized by conjugation, aromaticity and hydrogen bonding. Tauteromeric equilibria in mass spectrometry have been explored (Allegretti, 2007). Ionization in the ion

source is reported to have no effect on the position of the keto-enol equilibrium, such that the results reflect the tautomers in the gas phase, prior to ionization. The same may not be true for mass spectroscopic techniques where molecular ions are associated with solvent ions during their formation (Allegretti, 2007).

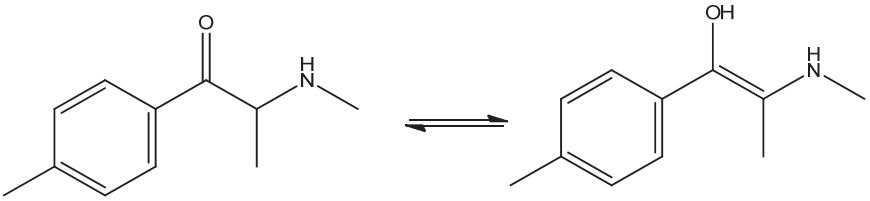
Keto-enol equilibria can complicate derivatization reactions by producing incomplete reactions and multiple products. This is well recognized for other drug classes including the *keto opioids*. For example, hydromorphone typically forms both mono- and di-TMS derivatives due to derivatization of the enol form of the drug in addition to the phenolic group.

Figure 1. Keto-enol tautomerization.



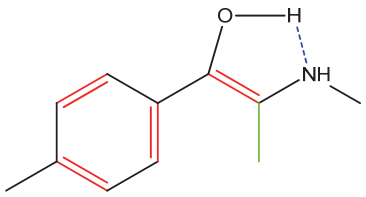
The position of the keto-enol equilibrium is influenced by several factors, particularly pH, electronic and steric effects of the substituents and the nature of the solvent. Solvent effects are due to the strong tendency of the enol form to hydrogen bond intramolecularly, while the keto form may hydrogen bond with protic solvents, providing stabilization (Allegretti, 2007). Keto-enol tautomers of mephedrone are shown below in **Figure 2**.

Figure 2. Tautomers of mephedrone.



Aromaticity, intramolecular hydrogen bonding or intramolecular hydrogen bonding with solvents and other species may stabilize the enol. The rate of keto-enol conversion may also be enhanced by acid. Acid makes the carbonyl group more electrophilic, increasing the acidity of alpha-protons, facilitating the formation of the enol. Whereas the keto form is a hydrogen bond acceptor due to the lone pair of electrons on the oxygen, the enol form has both hydrogen bond donor and acceptor abilities, is nucleophilic and now contains an acidic functional group. It can involve itself in hydrogen bonding via the OH group and it differs from its keto form in its polarity, acidity and nucleophilicity. If a Lewis base is present, hydrogen bonding further stabilizes the enol form and this influence can shift the keto-enol equilibrium significantly to the right. The fact that the enol double bond is still in conjugation with the phenyl, increases its stability, as does the C-substitution. Since enols are alkenes, the more substituted, the greater the stability. Stabilization of the enol form of mephedrone due to intramolecular hydrogen bonding, conjugation and increased substitution is depicted in **Figure 3**.

Figure 3. Stabilization of the enol form of mephedrone due to intramolecular hydrogen bonding (blue), conjugation (red) and increased substitution (green).



The syntheses of drugs within this class are well documented. Whereas reduction of ephedrine (and pseudoephedrine) produces methamphetamine, oxidation of ephedr*ine* produces methcathinone or "ephedr*one*". This synthetic route is the basis of the common naming system that is used for many of these compounds. Chemical and common names for the target analytes described in this study are summarized in **Table 1**. 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 4**). The individual structures of the synthetic cathinone species discussed in this report are shown in **Figure 5**. Although they are broadly categorized by their

benzylic, methylenedioxy and pyrrolidine substituents, although some share more than one of these characteristic features (e.g. MDPV and MDPBP).

Figure 4. General structure of the synthetic cathinones.

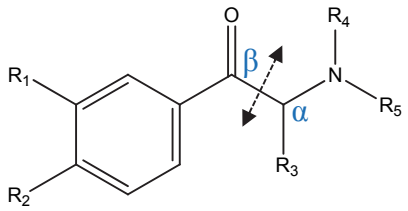
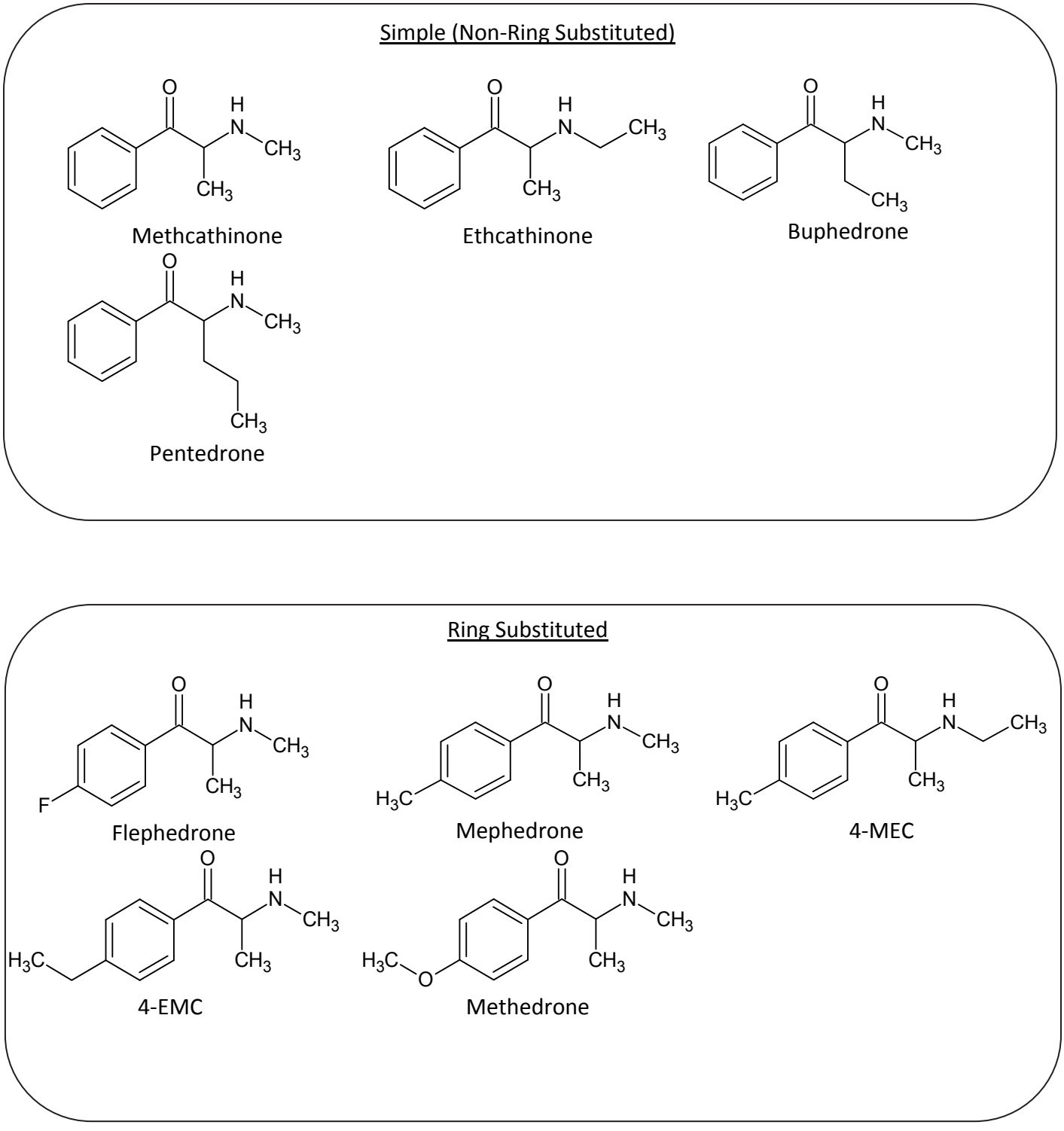


Table 1. Common names and chemical names of synthetic cathinones included in this study.

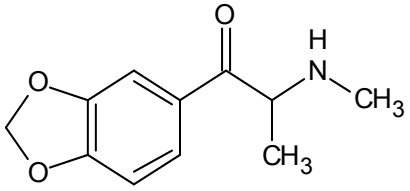
Common Name	IUPAC Name	Other Name(s)
4-EMC	1-(4-ethylphenyl)-2-methylaminopropan-1-one	4-ethylmethcathinone
4-MEC	1-(4-methylphenyl)-2-ethylaminopropan-1-one	4-methylethcathinone
Buphedrone	2-(methylamino)-1-phenylbutan-1-one	α-methylaminobutyrophenone
Butylone (bk-MBDB)	2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one	β-keto-*N*-methylbenzodioxolylbutanamine
Ethcathinone	2-ethylamino-1-phenyl-propan-1-one	*N*-ethylcathinone
Ethylone (bk-MDEA)	1-(1,3-benzodioxol-5-yl)-2-(ethylamino)-propan-1-one	3,4-methylenedioxy-n-ethylcathinone
Flephedrone, 4-FMC	1-(4-fluorophenyl)-2-(methylamino)-propan-1-one	4-fluoromethcathinone
MDPBP	1-(3,4-methylenedioxyphenyl)-2-(1-pyrrolidinyl)-1-butanone	3,4-methylendioxy-α-pyrrolidinobutiophenone
MDPV	1-(1,3-benzodioxol-5-yl)-2-(pyrrolidinyl)-pentan-1-one	3,4-methylenedioxypyrovalerone
Mephedrone	1-(4-methylphenyl)-2-methylaminopropan-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-methoxymethcathinone
Methylone (bk-MDMA)	2-methylamino-1-(3,4-methylenedioxyphenyl)-propan-1-one	3,4-methylenedioxy-*N*-methylcathinone

MPBP	1-(4-methylphenyl)-2-(1-pyrrolidinyl)-1-butanone	4-methyl-α-pyrrolidinobutiophenone
Naphyrone	1-(2-naphthyl)-2-(1-pyrrolidinyl)-pentan-1-one	naphthylpyrovalerone
Pentedrone	2-methylamino-1-phenyl-pentan-1-one	α-methylaminovalerophenone
Pentylone	1-(1,3-benzodioxol-5-yl)-2-(methylamino)-pentan-1-one	β-keto-methylbenzodioxolylpentanamine
PVP	1-phenyl-2-(1-pyrrolidinyl)-pentan-1-one	α-pyrrolidinovalerophenone
Pyrovalerone	1-(4-methylphenyl)-2-(1-pyrrolidinyl)-pentan-1-one	-

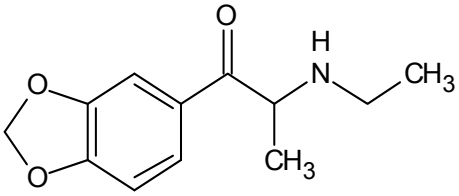
Figure 5. Chemical structures of the synthetic cathinones.



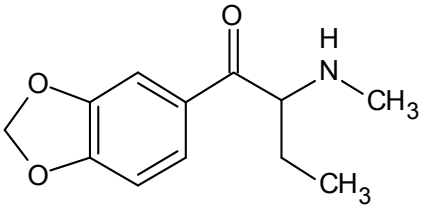
Methylenedioxy-Substituted



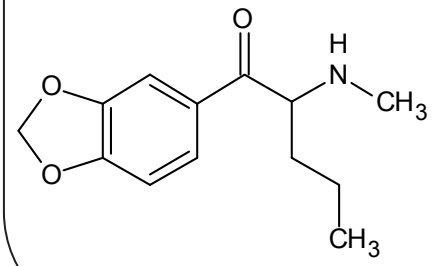
Methylone



Ethylone

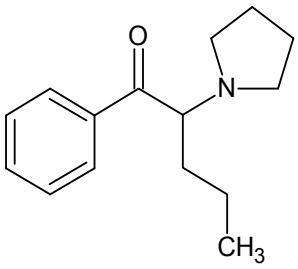


Butylone

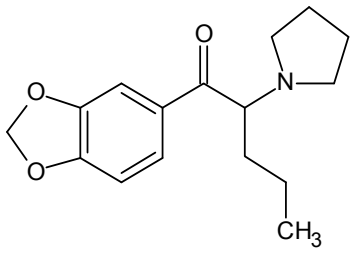


Pentylone

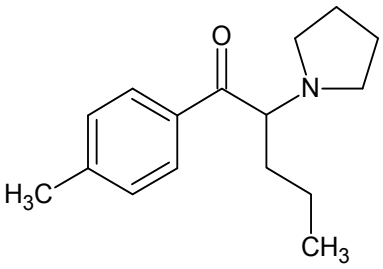
Pyrrolidine-Type (Tertiary Amines)



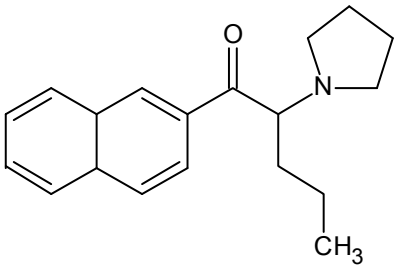
PVP



MDPV



Pyrovalerone



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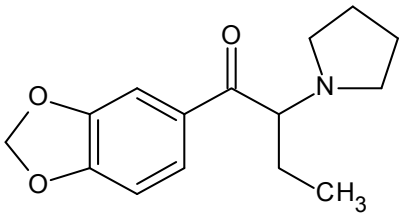
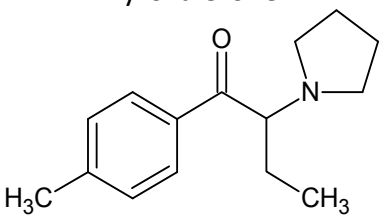


MDPBP



MDPB

Dosage and Effect

Synthetic cathinones are most commonly administered via oral ingestion and nasal insufflation. "Bombing" (whereby powdered drug wrapped in paper is ingested) is popular due to the nasal irritation that occurs following insufflation. Illicit doses vary significantly depending upon the mode of administration, tolerance and specific cathinone. Recreational doses, onset and duration of action are summarized in **Table 2**. Following oral ingestion of cathinone, peak concentrations were achieved within about 1.5 hours (Brenneisen, 1990). The ketone group is responsible for the increased polarity of the cathinones relative to their amphetamine counterparts. This in turn decreases the lipophilicity of the drug and ultimately its distribution within the body. Moreover, synthetic cathinones that contain the pyrrolidine moiety, are less polar than their secondary amine counterparts and have higher permeability and typically lower dosages among recreational drug users (Hill and Thomas, 2011).

Cathinones are capable of producing an array of central, peripheral and psychiatric effects. These include elevations in heart rate, blood pressure and respiration, alertness, psychomotor effects, euphoria, sensory stimulation, hyperthermia, agitation, tremors, insomnia and anorexia. However, in addition to their classical psychostimulant effects, many produce hallucinogenic effects resembling those of MDMA.

Table 2: Typical single dosages, modes of administration, and onset and duration times for various synthetic cathinones.

Synthetic Cathinone	Typical Dosage	Onset of Action	Duration of Effects	Reference (s)
Mephedrone	150-250 mg (PO)	45-120 mins	2-4 hrs	Dargan, 2011; Rosenbaum, 2012; Schifano, 2011; Valente, 2014
	5-75 mg (IN)	-	0.5-1 hr	
	50-75 mg (IV)	-	0.25-0.5 hr	

MDPV	3-20 mg (PO)	15-30 mins	2.5-3 hrs	Rosenbaum, 2012; Valente, 2014; Zawilska and Wojcieszak, 2013
MDPV	3-20 mg (IN)	< 30 mins	6-8 hrs	Valente, 2014
Methylone	100-250 mg (PO)	15-30 mins	2-5 hrs	Rosenbaum, 2012; Valente, 2014
4-MEC	100-300 mg (PO)	30-45 mins	2-4 hrs	Gil, 2013
Butylone	100-250 mg (PO)	15-30 mins	4-6 hrs	Valente, 2014
Methedrone	50-500 mg (PO)	-	0.75-2 hrs	Wikström, 2010
Buphedrone	5-30 mg (IN)	2-4 mins	0.5-1 hr	Zuba, 2013
	80-150 mg (PO)	-	2.5-4 hrs	
Methcathinone	60-250 mg (IV, IN, PO)	-	-	Kelly, 2011
Pyrovalerone	20 mg	-	-	Miotto, 2013
Ethylone	175 mg	-	-	

IN, intranasal; IV, intravenous; PO, oral.

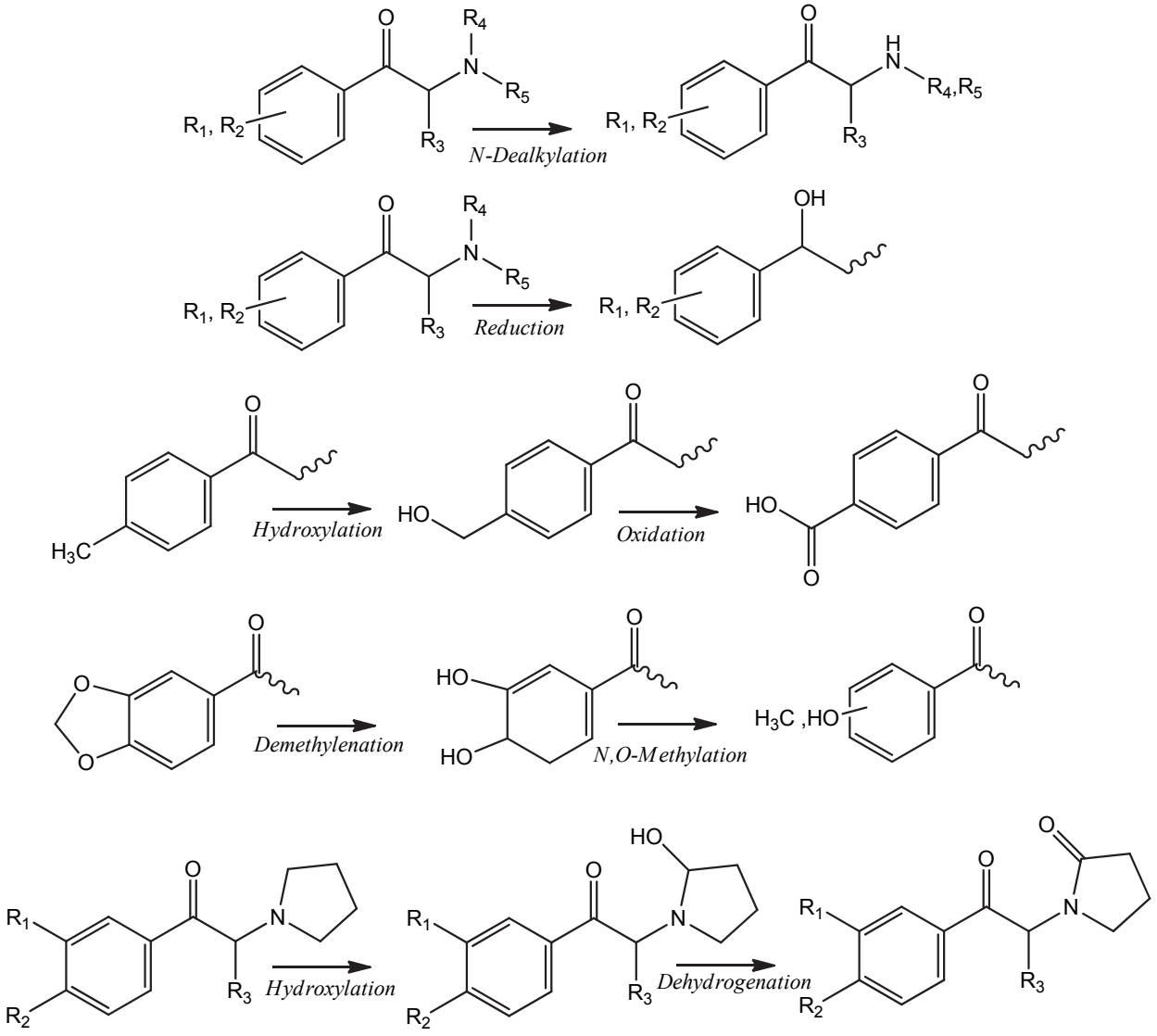
Pharmacology

Although the toxic effects of the cathinones are well recognized, pharmacological studies in humans are still relatively limited. The cathinones can produce a wide array of metabolites. Phase I biotransformations are summarized in **Figure 6** (Kamata, 2006; Kelly, 2011; Meyer, 2010; López-Arnau, 2013; Meyer, 2013; Prosser and Nelson, 2012; Valente, 2014; Zaitsu, 2014; Strano-Rossi, 2010; Zawilska and Wojcieszak, 2013). For most cathinones this involves *N*-dealkylation and carbonyl reduction to the corresponding amino alcohol. The pyrrolidino cathinones may undergo hydroxylation followed by dehydrogenation to form the lactam and methylenedioxy-type cathinones can undergo demethylenation followed by *N*- or *O*-methylation. 4-Methyl derivatives can also undergo hydroxylation and subsequent oxidation to

the carboxylic acid. During phase II, phenolic metabolites can undergo subsequent conjugation to glucuronides and sulfates prior to elimination.

The sympathomimetic toxicity of the cathinones is attributed to their influence on monoamine reuptake or release. Their influence on dopaminergic, adrenergic and serotonergic systems is complex and widely variable. In-vitro studies suggest that while mephedrone, methylone, ethylone, butylone and naphyrone are non-selective monoamine uptake inhibitors (cocaine-like), methcathinone and flephedrone act as preferential DA and NE uptake inhibitors (methamphetamine-like). Mephedrone, methylone, ethylone and butylone also induce the release of 5-HT, similar to MDMA and other entactogens. Pyrovalerone and MDPV are highly potent and selective DA and NE transporter inhibitors, but unlike amphetamines, do not evoke the release of monoamines (Simmler, 2012). In addition to acute neurochemical changes, chronic use can produce dopaminergic, and possibly serotonergic dysfunction for some drugs (Baumann, 2013; German, 2014; Hadlock, 2011; Motbey, 2012). To date there are limited reports concerning the distribution of cathinones in humans (Cawrse, 2012; Wyman, 2013), although Marinetti and Antonides determined the average heart to peripheral blood ratio to be approximately 1.5 for MDPV, consistent with the increased lipophilicity of the drug.

Figure 6. Summary of phase I biotransformations of synthetic cathinones.



Analytical Detection

Traditional immunoassays have not proven effective for synthetic cathinones due the speed at which new analogs have appeared on the illicit drug market. Although the detection of synthetic cathinones using amphetamine immunoassays are often negative (Swortwood, 2014), results are highly variable between manufacturers (Regester, 2014) and cross reactivity for some synthetic cathinones may occur with traditional amphetamine, methamphetamine or MDMA immunoassays (Toennes, 2002; Truscott, 2013; deCastro, 2014). The structural diversity of the synthetic cathinones presents a practical limitation for immunogen design and ultimately antibody-based screening, suggesting that either chromatographic or mass spectrometry-based screening techniques may be more appropriate. Although liquid chromatography-mass spectrometry (LC/MS) and other hyphenated LC techniques are becoming more popular, gas chromatography-mass spectrometry (GC/MS) remains the most widely used technique in routine forensic toxicology laboratories. **Table 3** summarizes instrumental analyses for cathinones to date.

Table 3. Summary of published toxicological analyses (by publication year).

Drug(s)	Matrix	Internal Standard	Extraction Method	Detection Method	Reference
Methcathinone	Serum, urine	Not specified	Not specified	HPLC-UV	Belhadj-Tahar, 2005
Methylone	Urine	ß-Phenethylamine	LLE	GC/MS, LC/MS, LC/MS/MS	Kamata, 2006
Butylone, ethylone	Urine	Not specified	LLE	GC/MS	Zaitsu, 2009
MDPV	Urine	Diphenylamine	LLE	GC/MS, LC-TOF/MS	Strano-Rossi, 2010
Mephedrone	Blood	MDA-D5	SPE	GC/MS	Torrance, 2010
Mephedrone	Urine, serum	Not specified	Not specified	GC/MS, LC/MS/MS	Wood, 2010
Mephedrone	Blood, urine	Methamphetamine-D14, mephedrone-D3	LLE	GC/MS	Dickson, 2010
Mephedrone, butylone, methylone	Urine	Not specified	SPE, LLE	GC/MS	Meyer, 2010
Methedrone	Blood, hair	Amphetamine-D5, MDMA-D5	LLE	GC/MS, LC/MS/MS	Wikström, 2010
Methylone	Urine	ß-Phenethylamine	LLE	LC-/MS, GC/MS	Katagi, 2010
Mephedrone	Blood, urine, hair	MDA-D5	SPE	GC/MS	Torrance, 2010
MDPV	Blood	MDEA-D5	SPE	LC/MS/MS	Kriikku, 2011
MDPV	Urine	MDPV-D8	LLE	PCI-GC/MS	Ojanperä, 2011
MDPV	Blood, urine	Phencyclidine, methcathinone	LLE	GC/MS	Spiller, 2011
Mephedrone	Urine	MBDB-D5	LLE	LC/MS/MS	Grueninger,

					2011
Mephedrone	Femoral blood	Oxazepam-D5	None	LC/MS/MS	Lusthof, 2011
Naphyrone	Plasma	Not specified	Not specified	GC/MS	Derungs, 2011
Mephedrone	Blood	Cinchonine	LLE	HPLC-UV	Maskell, 2011
Flephedrone, MDPV	Serum, urine	Not specified	Not specified	LC-TOF/MS	Thornton, 2012
MDPV	Serum, urine	MDPV-D8	Protein precipitation	LC/MS/MS	Murray, 2012
Methylone	Blood	Methylone-D3	LLE	GC/MS	Pearson, 2012
Methylone, MDPV, mephedrone	Blood, tissues, urine	Methylone-D3	SPE	GC/MS	Cawrse, 2012
Mephedrone	Hair	MDMA-D5	LLE	GC/MS	Martin, 2012
Methylone	Blood	Methylone-D3	SPE	GC/MS	McIntyre, 2013
MDPV	Blood	Mepivacaine	LLE	GC/MS	Kesha, 2013
4-MEC	Blood, urine	Mephedrone-D3	LLE	LC/MS/MS	Gil, 2013
Mephedrone	Blood, urine	Prazepam	LLE	LC/MS	Cosbey, 2013
MDPV	Serum	MDPV-D8	Protein precipitation	LC/MS/MS	Truscott, 2013
MDPV, α-PVP	Blood, hair	PVP-D8	SPE	GC/MS, LC/MS	Namera, 2013
MDPV	Blood	Mephedrone-D3	Protein precipitation	LC/MS/MS	Adamowicz, 2013a
MDPV, methylone, pyrovalerone, pentylone, α-PVP methedrone,	Blood, urine, tissues	MDPV-D8, methylone-D3, butylone-D3, mephedrone-D3	LLE	LC/MS/MS	Marinetti, 2013
Buphedrone	Blood	Mephedrone-D3	Protein precipitation	LC/MS/MS	Zuba, 2013
Methylone	Blood, urine	Not specified	SPE	GC/MS	Bauer, 2013
MDPV	Blood	SKF525A	LLE	GC/MS	Wright, 2013

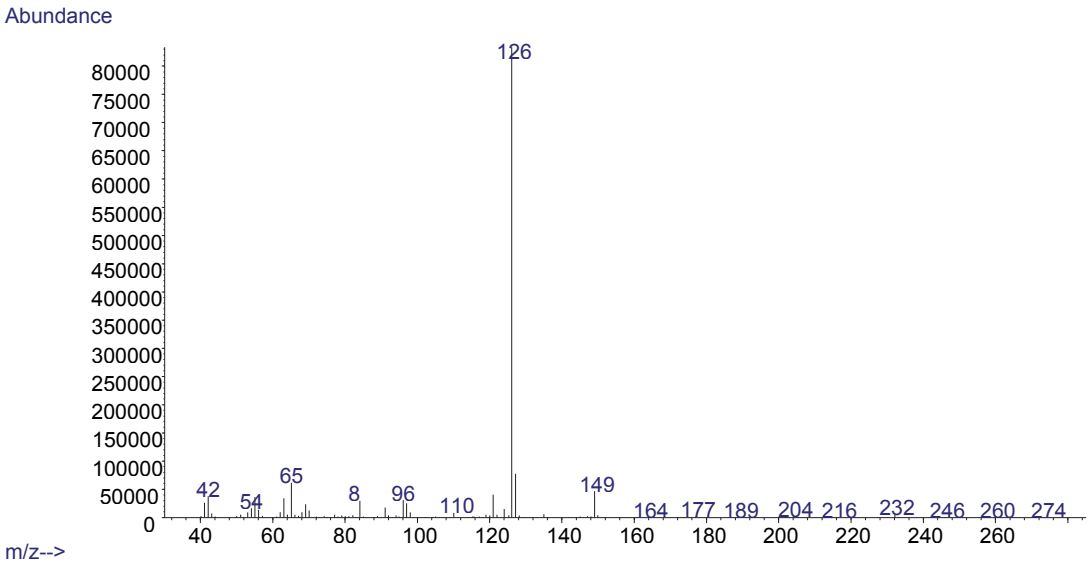
MDPV	Blood, tissues, hair	MDPV-D8,	SPE	GC/MS, LC/MS/MS	Wyman, 2012
α-PVP	Blood	α-PBP	LLE, SPE	LC/MS/MS	Hasegawa, 2014
α-PVP, methylone	Blood	PCP-D5, MDA-D5, norketamine-D4, Atropine-D3, MDMA-D5, Ketamine-D4	LLE	LC-TOF/MS	Knoy, 2014
4-MEC	Meconium Hair	Pentazocine	SPE	LC/MS/MS	Pichini, 2014

GC/MS, gas chromatography-mass spectrometry; HPLC-UV, High performance liquid chromatography-UV detection; LC/MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LC-TOF/MS, liquid chromatography-time of flight mass spectrometry; LLE, liquid-liquid extraction; MBDB, *N*-Methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine; MDA-D5, 3,4-methylenedioxyamphetamine-D5; MDEA, methylenedioxyethylamphetamine; MDEA-D5, 3,4-methylenedioxy-*N*-ethylamphetamine-D5; MDMA-D5, 3,4-methylenedioxymethamphetamine-D5; α-PBP, α-pyrrolidinobutiophenone; PCI-GC/MS, positive chemical ionization, gas chromatography-mass spectrometry; PFPA, pentafluoropropionic anhydride; SPE, solid phase extraction; TFAA, trifluoroacetic anhydride.

Cathinone Mass Spectra

Confirmation of unknown drugs using hyphenated mass spectroscopic techniques such as GC/MS or LC/MS rely upon reproducible chromatographic separation (retention time) and characteristics fragmentation (mass spectra). As a general rule, ions with a higher m/z ratio are preferred due to increased specificity, although abundance (intensity) of the ion is highly significant from the standpoint of detectability. When selected ion monitoring (SIM) is used in place of full scan acquisition in GC/MS, at least three characteristic ions should be selected and ion ratios must be critically evaluated (ABFT, 2013). This approach presents a challenge due to the relatively poor mass spectral quality of many of the synthetic cathinones, particularly the pyrrolidine species. **Figure 7** depicts the electron impact (EI) mass spectrum of MDPV which is heavily dominated by the m/z 126 base peak. The extensive fragmentation leaves very few qualifier ions to choose from. An additional challenge is that due to the tertiary amine, the pyrrolidine-type cathinones lack the active hydrogen necessary for commonly used or widely accepted approaches to derivatization.

Figure 7. Mass spectrum of MDPV.



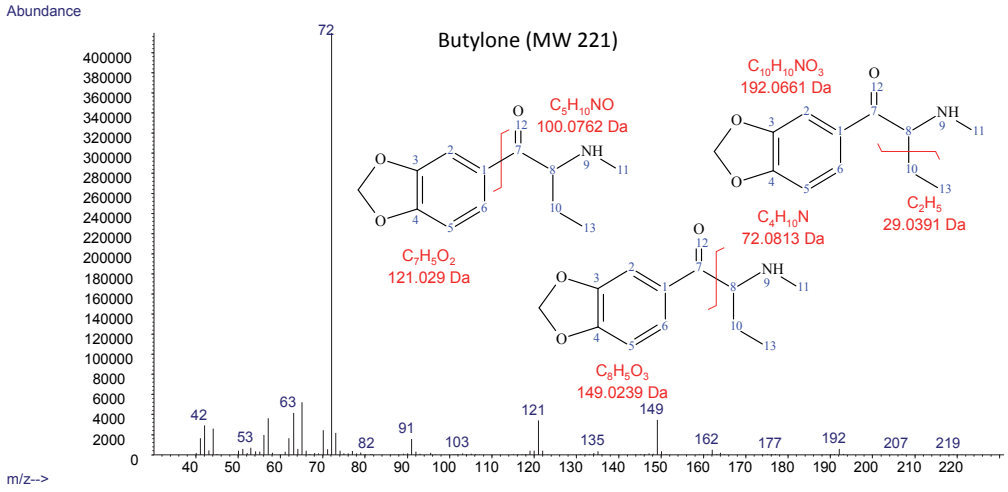
The electron impact (EI) mass spectra of synthetic cathinones are dominated by the formation of iminium and acylium ions, with relatively minor secondary and tertiary fragmentations. The principal fragmentation occurs by dissociation of the α and β-carbon bond, which results in relatively few diagnostic ions and minimal or no molecular ion. The base peak is determined by the R_3, R_4 and R_5 substituents, which means that positional isomers with the same side chain constituents produce very similar mass spectra (**Table 4**). The straight chain (alkylamino) derivatives are dominated by a $C_nH_{2n+2}N^+$ base peaks (i.e. m/z 44, 58, 72, 86, 100 *etc*.). **Figure 8** shows the mass spectral similarity between the positional isomers ethylone and butylone. Both isomers share identical prominent fragments at m/z 72, 149 and 121. Only small differences in fine spectra are observed in the intensity of the m/z 44 ion, which due to its small size, is poorly diagnostic. The tertiary amines such as MDPV, naphyrone and pyrovalerone, tend to be even more limited because their spectra are dominated by the highly stable pyrrolidinium ion ($C_nH_{2n}N^+$) (e.g. m/z 126 for MDPV). As a consequence, chromatographic resolution and characteristic retention times are of paramount importance and must be closely monitored during analysis.

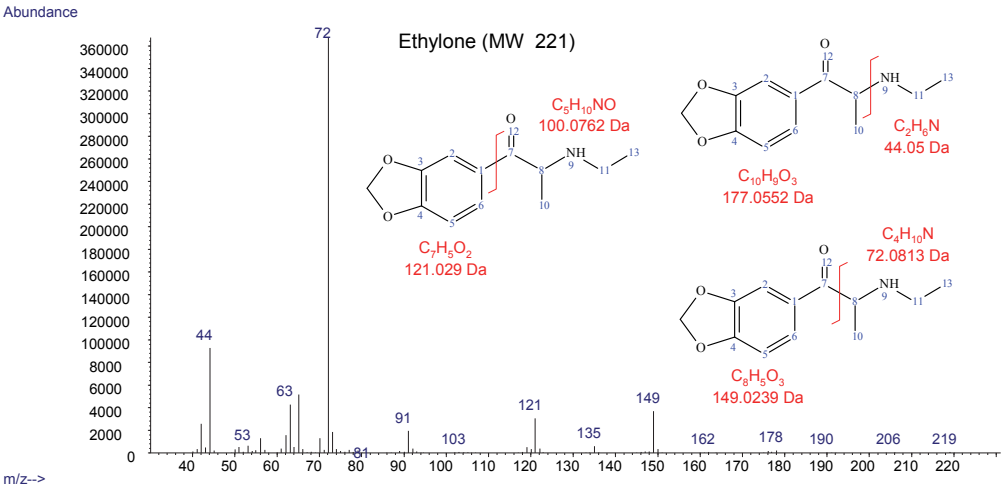
Table 4. Relationship between functional group substituents and base peak.

Name	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**	**Base Peak m/z**	**MW**
Methcathinone	H	H	CH_3	H	CH_3	58	163
Mephedrone	H	CH_3	CH_3	H	CH_3	58	177
Methedrone	H	OCH_3	CH_3	H	CH_3	58	193
Methylone	3,4-Methylenedioxy		CH_3	H	CH_3	58	207
Flephedrone	H	F	CH_3	H	CH_3	58	181
4-EMC	H	C_2H_5	CH_3	H	CH_3	58	191
Ethcathinone	H	H	CH_3	H	C_2H_5	72	177
Buphedrone	H	H	C_2H_5	H	CH_3	72	177
4-MEC	H	CH_3	CH_3	H	C_2H_5	72	191
Ethylone	3,4-Methylenedioxy		CH_3	H	C_2H_5	72	221
Butylone	3,4-Methylenedioxy		C_2H_5	H	CH_3	72	221
Pentedrone	H	H	C_3H_7	H	CH_3	86	191
Pentylone	3,4-Methylenedioxy		C_3H_7	H	CH_3	86	235
MPBP	H	CH_3	C_2H_5	Pyrrolidinyl		112	231
MDPBP	3,4-Methylenedioxy		C_2H_5	Pyrrolidinyl		112	261
MDPV	3,4-Methylenedioxy		C_3H_7	Pyrrolidinyl		126	275

α-PVP	H	H	C_3H_7	Pyrrolidinyl	126	231
Pyrovalerone	H	CH_3	C_3H_7	Pyrrolidinyl	126	245
Naphyrone	Naphthyl		C_3H_7	Pyrrolidinyl	126	281

Figure 8. Mass spectra of positional isomers butylone and ethylone.





Toxicological Analyses

Detection of cathinones in forensic toxicology samples can be accomplished using liquid-liquid or solid phase extraction methods in combination with hyphenated chromatographic-mass spectrometric techniques (**Table 3**). Although isotopically labelled standards are preferred (particularly for quantitative purposes) a wide variety of internal standards have been employed. In biological matrices, cathinone concentrations have been reported over a wide range in both impaired driving and postmortem toxicology investigations. However, until the stability of synthetic cathinones is fully understood, quantitative determinations must be interpreted with caution.

The instability of cathinone in plasma was first reported by Morad in 1989 (Morad, 1989) and the pH dependent degradation of cathinone and methcathinone in urine was reported by Paul and Cole more than a decade ago (Paul, 2001). Issues associated with quantitative reproducibility and stability of the newer designer cathinones have emerged relatively recently and are still the being investigated (Maskell, 2011; Sørensen, 2011; Tsujikawa, 2012; Marinetti, 2013; Johnson, 2013; Soh, 2013). Forensic toxicologists are required to go to considerable lengths to ensure that the analytical methods used are of sufficient quality to produce reliable quantitative results (SWGTOX, 2013). Drug stability and concentration changes that might occur during handling, storage or analysis must be well understood if results are to be reliably interpreted in antemortem or postmortem forensic investigations.

Although toxicological data in humans in limited, synthetic cathinones are now an important consideration during antemortem or postmortem forensic toxicology investigations. Quantitative reports throughout the literature are summarized in **Tables 5 and 6**. Antemortem concentrations in blood are reported in tens to hundreds of nanograms per milliliter, and often much higher in postmortem investigations. As with many drugs, the concentration ranges in antemortem and postmortem cases overlap.

Table 5: Antemortem concentrations of synthetic cathinones quantitatively reported in the literature.

Drug	Concentration	Reference
4-MEC	46 ng/mL (blood)	Gil, 2013
	0.7 ng/g (meconium)	Pichini, 2014
	4.3 ng/mg (hair)	Pichini, 2014
A-PVP	63 ng/mL (blood)	Knoy, 2014
Buphedrone	3 ng/mL (blood)	Zuba, 2013
Flephedrone	346 ng/mL (serum); 257 ng/mL (urine)	Thornton, 2012
MDPV	24 - 241 ng/mL (blood/serum); 34-1,386 ng/mL (urine)	Spiller, 2011
	<10 - 368 ng/mL (blood)	Marinetti, 2013
	124, 306 ng/mL (blood); N=2	Adamowicz, 2013a
	75 ng/mL (serum)	Truscott, 2013
	186 ng/mL (serum); 136 ng/mL (urine)	Thornton, 2012
	20 ng/mL – 8.4 mg/L (blood); N=25	Kriikku, 2011
	24 ng/mL (blood)	Marinetti, 2013
Mephedrone	10 - 740 ng/mL (blood); N=32	Cosbey, 2013
	150 ng/mL (serum)	Wood, 2010
	0.2 – 313 ng/mg; N=13	Martin, 2012
Methcathinone	500 ng/mL (serum); 17.24 mg/L (urine)	Belhadaj-Tahar, 2005
Methylone	7 ng/mL (blood)	Marinetti, 2013
	6 ng/mL	Knoy, 2014

Table 6: Postmortem concentrations of synthetic cathinones quantitatively reported in the literature.

Drug	Concentration	Reference
4-MEC	152 ng/mL (blood); 122 ng/mL (urine)	Gil, 2013
	56 ng/mL (blood)	Gil, 2013
α-PVP	654 ng/mL (femoral blood); 458 ng/mL (heart blood); 11,200 ng/mL (urine);	Hasegawa, 2014
Buphedrone	127 ng/mL (blood)	Adamowicz, 2013a
	127 ng/mL (blood)	Zuba, 2013
MDPV	82 ng/mL (serum)	Miotto, 2013
	39 ng/mL (blood); 760 ng/mL (urine)	Wright, 2013
	130 ng/mL (blood); 3800 ng/mL (urine)	Wright, 2013
	170 ng/mL (blood); 1400 ng/mL (urine)	Spiller, 2011
	700 ng/mL (heart blood); 1000 ng/mL (peripheral blood)	Kesha, 2013
	17 ng/mL (blood)	Adamowicz, 2013a
	440 ng/mL (peripheral blood); 500 ng/mL (heart blood)	Wyman, 2013
	1,200 ng/mL (blood)	Namera, 2013
	670 ng/mL (urine); 82 ng/mL (serum)	Murray, 2012
	56 ng/mL (blood)	Marinetti, 2013
	38 ng/mL (blood)	Adamowicz, 2013a
	470 ng/mL (heart blood)	Cawrse, 2012
Mephedrone	500 ng/mL (blood); 198 mg/L (urine)	Dickson, 2010
	3 - 2,100 ng/mL (femoral blood); N=12	Cosbey, 2013
	1.2, 3.3, 5.7, 22 mg/L (blood); N=4	Torrance, 2010
	130 – 2,240 ng/mL (femoral blood); N=4	Maskell, 2011
	5.1 mg/L (blood); 186 mg/L (urine)	Lusthof, 2011
	55 mg/L (blood); 7.1 mg/L (vitreous humor)	Adamowicz, 2013b
Methedrone	13.3 µg/g (antemortem blood); 8.4 µg/g (postmortem blood)	Wikström, 2010
Methylone	60 – 1,120 ng/mL (blood); N=4 0.22 – 38 mg/L (urine); N=3	Cawrse, 2013

	126 ng/mL (femoral blood)	Bauer, 2013
	0.84 mg/L (iliac blood); 1.0 mg/L (heart blood); 1.4 mg/L (vitreous humor); 0.55 mg/L (urine); 12 mg/L (stomach contents)	Pearson, 2012
	729 ng/mL (blood)	Marinetti, 2013
	3.4 mg/L (central blood); 3.4 mg/L (peripheral blood); 4.3 mg/L (vitreous)	McIntyre, 2013

Stability of Cathinones

In-situ degradation of the cathinones during GC analysis is not a new phenomenon and was first documented two decades ago by Noggle and DeRuiter. Prior to this, 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 *Catha edulis* several decades ago (Szendrei, 1980). Cathinone itself is an unstable drug that can degrade after harvesting the plant (Chappell, 2010). Just as the cathinones undergo reduction of the keto functional group to a hydroxyl in-vivo, similar transformations can occur in seized plant material to produce cathine or (+)-norpseudoephedrine from cathinone. Moisture may increase the rate of degradation in seized plant material, so simple drying techniques prior to evidence storage have proven to be effective. Transformations of cathinone into other species are also possible. The tendency of cathinone to cyclize to 3,6-dimethyl-2,5-diphenyldihydropyrazine with subsequent oxidation to 3,6-dimethyl-2,5-diphenylpyrazine is documented in the early literature (Berrang, 1982). Dimerization has been reported to occur at room temperature (Coppola, 2012) and following base extraction and evaporation to dryness (Chappell, 2010).

Other studies on seized drug evidence have identified additional compounds such as *iso*-cathinones. These refer to the phenylacetone isomers of drugs like mephedrone. The presence of iso-cathinones in seized materials were attributed to the synthesis of the drug, rather than spontaneous formation (McDermott, 2011). Fortunately, studies using meth- and ethcathinones as well as pentedrone have shown that iso-cathinones produce strikingly different mass spectra and are readily separated from the parent drug (McDermott, 2011; Westphal, 2012).

Thermal degradation of cathinones during GC analysis was first documented several decades ago (Noggle 1994; DeRuiter, 1994). However, in-situ degradation of cathinones has not received

widespread attention, perhaps due to their proliferation over such a short period. A very small number of recent reports however have drawn attention to this issue (Archer, 2009; Leffler, 2014; Tsujikawa, 2012; Tsujikawa, 2013a). Although stability in biological evidence is not within the scope of this report, a small number of recent publications have suggested that some synthetic cathinones including methcathinone, ethcathinone, mephedrone, flephedrone, methylone, and MDPV may degrade during sampling or storage (Sørensen, 2011; Maskell, 2011; Tsujikawa, 2012; Johnson, 2013;).

Chemical Derivatization

Derivatization is often necessary because conventional GC techniques are not designed for non-volatile or highly polar species. Derivatization procedures for xenobiotics and drugs of abuse have been extensively reviewed elsewhere (Segura, 1998). Hydroxyls, amines and carboxylic acids are among the most commonly derivatized functional groups in forensic toxicology. Derivatization can enhance mass spectral quality, increase specificity, impart volatility, improve chromatography, and decrease polarity. Derivatization is also useful when the target drug has poor diagnostic ions, which is common to many of the cathinone species. Derivatization can significantly alter the fragmentation pathway which in turn may significantly improve both the sensitivity and specificity of the assay. Derivatization can also be useful for thermolabile drugs. The reduction in polarity can improve chromatographic properties of the drug by decreasing non-specific adsorption to the column, improving peak shape and reducing the appearance of artifact peaks.

Ideally, a derivatization should be robust, rapid, proceed under relatively mild conditions, and should produce a high and reproducible yield of a single derivative which is readily distinguishable from the starting material and from the derivative. Multiple derivatives are possible, particularly in polyfunctional compounds. This is relevant for the cathinones because they contain both the carbonyl (keto) and the amine functionality.

Silylation and acylation are among the most widely used derivatization techniques in forensic toxicology. Silylation is effective for alcohols, phenols, carboxylic acids as well as primary and secondary amines due to active hydrogen displacement. Of the silylation procedures, the most common approach involves trimethylsilylation (TMS). Typically, TMS derivatives are easy to prepare and highly conducive to GC/MS. The wide variety of commercial TMS reagents (including trimethylhalosilanes, TMS-amines, TMS-esters and TMS-amides) make them a popular choice. *N,O*-bistrimethylsilyltrifluoroacetamide (BSTFA) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) are among some of the most frequently used TMS reagents in forensic toxicology. However, silylation must be carried out under anhydrous conditions because both reagents and products can be moisture sensitive.

Acylation reactions are equally important derivatization reactions in forensic toxicology and have been widely exploited for the amphetamine class. It is therefore not surprising that it is also the most widely used approach for the synthetic cathinones to date (**Table 7**). However, although this approach works well for cathinones that contain an active hydrogen (primary or secondary amines), common acylating reagents are incapable of derivatizing tertiary amines, such as MDPV, naphyrone, pyrovalerone, MDPBP, MPBP or α-PVP (**Figure 9**). Alcohols, primary and secondary amines, amides, thiols, phenols, enols, sulfonamides and aromatic rings are all functional groups that can be acylated. Acyl halides and acid anhydrides are the most commonly used reagents for this purpose. However, excess derivatization reagent should be removed prior to injection to prevent column damage and detector fouling. Fluorinated anhydrides such as pentafluoropropionic anhydride (PFPA), trifluoroacetic anhydride (TFAA) and heptafluorobutyric anhydride (HFBA), are among the most frequently used reagents for acylation of the amphetamines. The *N*-perfluoracylimidazoles (e.g. heptafluobutyrylimidazole or HFBI) are also reactive towards alcohols and amines. Their reactions are typically quantitative and do not produce acidic byproducts that should be removed prior to injection. Instead the byproduct is imidazole which is relatively inert.

Figure 9. Acylation of synthetic cathinones using TFAA via the active hydrogen (shown in red).

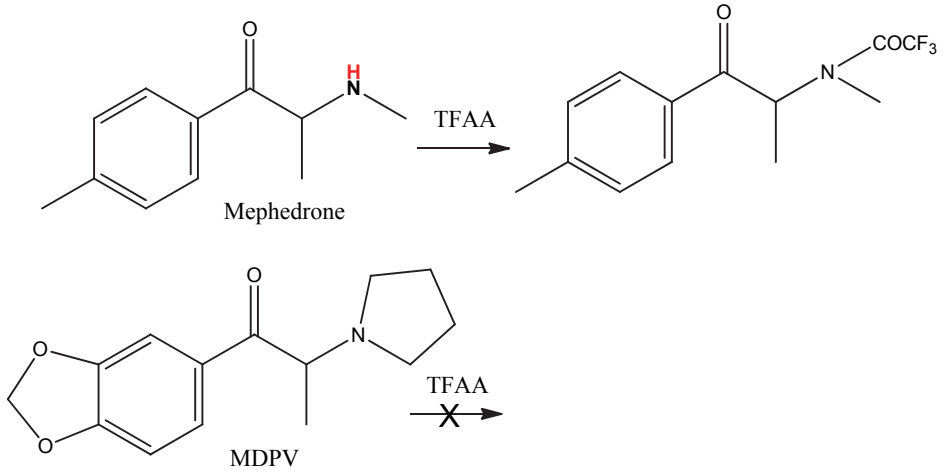


Table 7. GC/MS analysis of synthetic cathinones in forensic toxicology.

Cathinone(s)	IS	Derivatization	Refs
Cathinone, diethylpriopion, methcathinone	Amphetamine-D5, methamphetamine-D5	TFAA, PFPA, HFBA	Valentine, 2000
Methylone	Methylone-D3	HFBA	Pearson, 2012
MDPV, α-PBP, α-PVP	α-PVP-D8	None	Namera, 2013
Mephedrone	Mephedrone-D3	None	Hadlock, 2011
Mephedrone	Oxazepam-D5	BSTFA	Lusthof, 2011
Mephedrone	MDMA-D5	None	Martin, 2012
MDPV	Methapyrilene	None	Wyman, 2013
Mephedrone	Mephedrone-D3, methamphetamine-D14	PFPA	Dickson, 2010
Mephedrone	MDMA-D5	None	Wood, 2010
Cathinone, methcathinone	Amphetamine-D8, methamphetamine-D11, MDA-D5, MDMA-D5, MDEA-D6	TFAA	Kim, 2010
Cathinone	Ephedrine-D3	HFBA	Sporkert, 2003
Methylone	-	TFAA	Katagi, 2010
Butylone, ethylone	Dibenzylamine	TFAA	Zaitsu, 2009
DMMC	Dibenzylamine	TFAA	Shima, 2013
Methedrone	Amphetamine-D5, MDMA-D5	TFAA	Wikström, 2010

BSTFA, *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide; HFBA, heptafluorobutyric anhydride; LLE, liquid-liquid extraction; SPE, solid phase extraction; MDA-D5, 3,4-methylenedioxyamphetamine-D5; MDEA-D5, 3,4-methylenedioxy-*N*-ethylamphetamine-D5; MDMA-D5, 3,4-methylenedioxymethamphetamine-D5; PFPA, pentafluropropionic anhydride.

Ketone Derivatization

Although significantly more complicated in nature, the ketone moiety presents an alternative site for derivatization. Although all of the synthetic cathinones contain an amine and a ketone, the absence of an active hydrogen on the pyrrolidine species renders them inactive towards conventional acetylation reagents. Although keto-enol tautomerization must be considered, the keto is perhaps the most likely site of reaction for a universal derivative. Cyanosilanes, which are reactive to carbonyl compounds including ketones, may be suitable for this purpose. Since silyl derivatives are already used in forensic toxicology applications, cyanotrimethylsilane, or trimethylsilyl cyanide (TMSCN) is of particular interest.

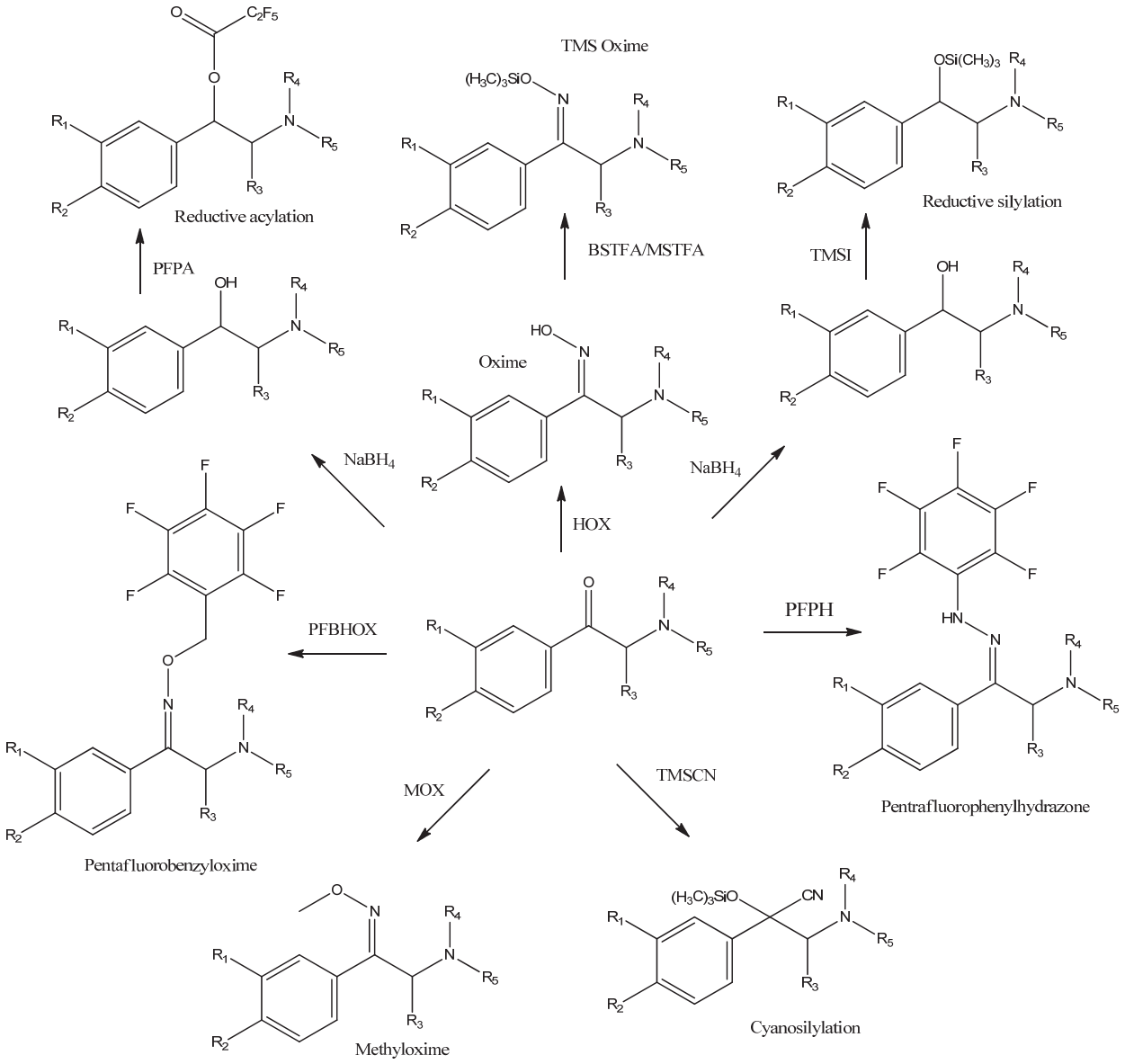
Complications associated with the derivatization of polyfunctional drugs have proven problematic for other drug classes including the keto opioids and the keto steroids. Catalysts such as trimethyliodosilane (TMIS) and antioxidants like dithioerythritol (DTE) are sometimes combined with the silylation reagent to facilitate the derivatization of sterically hindered active hydrogens or ketone groups (Fang, 2010; Saraiva, 2011).

Oximes have also proven effective for drugs that contain ketones. The use of hydroxylamine to convert keto opioids such as hydromorphone, hydrocodone, oxymorphone and oxycodone into oximes, with subsequent trimethylsilylation using BSTFA to form the *O*-trimethylsilyl (oxime) ether was first described by Broussard and Cremese (Broussard, 1997; Cremese, 1998). Subsequent to this approach, others exploited *O*-alkylhydroxylamine reagents such as methoxylamine, which does not require a two-step derivatization. Methoxylamine is a common derivatizing reagent for keto opioids and keto steroids and these reactions typically occur under mild conditions (St. Germain, 1997; Meatherall, 1999; Ropero-Miller, 2002; Tiscione, 2011). Additional oxime forming reagents such as *O*-(2,3,4,5,6-pentaflurobenzyl)hydroxylamine have

been less widely utilized but may offer significant benefits in terms of increased molecular mass of the pentafluorobenzyloxime derivative (Strassnig, 2000; Charlton, 2000; Li, 2005; Yu, 1995; Luo, 1995; Sugaya, 2004; Cancho, 2002).

Hydrazines are typically highly reactive reagents that can convert aldehydes and ketones into their respective hydrazones (Vogel, 2000). However, thermal instability, water insolubility, accessibility of the functional group and the formation of isomers (multiple products) are concerns associated with the use of hydrazines (Dong, 2004; Stashenko, 1997; Tougas, 1987; Hoshika, 1978; Li, 2009). While 2,4-dinitrophenylhydrazine (DNPH) is the most well-known hydrazine reagent, their hydrazones are thermal unstable and lack sufficient volatility for GC analysis. An alternative hydrazine for use with GC/MS is pentafluorophenylhydrazine (PFPH). The presence of five fluorine atoms increases the thermal stability, volatility and detectability (Dong, 2004; Sheen, 2004). Although pentafluorophenylhydrazine reacts with many carbonyls at room temperature, it requires strongly acidic conditions which can influence the analytes of interest (Stashenko, 1997). Although PFPH can produce multiple (E and Z) isomeric products, it has been used successfully for keto steroids, including nabumetone and testosterone (Sheen, 2004). A schematic showing potential derivatization schemes involving hydroxylamine (HOX) and MSFTA, methoxylamine (MOX), pentafluorobenzylhydroxylamine (PFBHOX), pentafluorophenylhydrazine (PFPH) and trimethylsilyl cyanide (TMSCN) is depicted in **Figure 10**.

Figure 10. Potential keto-reactive derivatization pathways for cathinones involving the formation of oximes, pentafluorohydrazones, pentafluorobenzyloximes and cyanosilylation.



Rationale for the Research

The aim of this study was to explore the mass spectra of the synthetic cathinones and determine whether their mass spectral properties could be improved by the use of novel derivatization techniques that were applicable to all synthetic cathinones, including those without active hydrogens (e.g. MDPV, naphyrone, pyrovalerone, MDPBP, MPBP or α-PVP). Derivatization of the ketone functional group should provide a universal derivatization approach and facilitate the modification of the pyrrolidine-containing cathinones (tertiary amines) of forensic interest. Unlike the more traditional approach of derivatizing the active hydrogen, the ketone group is present on all of the synthetic cathinones. This study evaluates the applicability of alternative and less common derivatization methods that might improve mass spectral characteristics of the cathinones and discusses the in-situ degradation of the non-derivatized drugs. Greater understanding of the instrumental factors that influence their detection by GC/MS can improve overall understanding and analytical approaches for the synthetic cathinone class as a whole.

II. Methods

Reagents and Chemicals

Cathinone reference standards of flephedrone, methcathinone, ethcathinone, buphedrone, mephedrone, pentedrone, 4-MEC, 4-EMC, methedrone, methylone, ethylone, α-PVP, butylone, MPBP, pentylone, pyrovalerone, MDPBP, MDPV, and naphyrone were obtained from Lipomed (Cambridge, MA) or Cerilliant (Round Rock, TX) at a concentration of 1 mg/mL in methanol. Internal standard reference standards of MDA-D5, mephedrone-D3, methylone-D3, MDPV-D8, and naphyrone-D5 were obtained from Cerilliant (Round Rock, TX) at a concentration of 1 mg/mL for MDA-D5 and 0.1 mg/mL for the remaining cathinone standards. A total of eighteen target cathinones were included in the study initially. However, when α-PVP became commercially available as a DEA exempt methanolic solution, it was included. For this reason, there are nineteen target cathinones described in the toxicology assay.

For the interference study, methanolic standards for amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), ephedrine, pseudoephedrine, phentermine, phenylpropanolamine, alprazolam, amitriptyline, bupropion, cocaine, codeine, diethylpropion, dextromethorphan, diazepam, hydrocodone, ketamine, meperidine, methadone, nordiazepam, oxycodone, phencyclidine (PCP), tramadol and zolpidem were obtained from Cerilliant (Round Rock, TX). Standards of phenethylamine, putrescine, tryptamine, tyramine were obtained from Sigma Aldrich (St. Louis, MO) and *N*-methyl-l-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB) was obtained from Lipomed (Cambridge, MA). Additional designer drugs of the DO-, 2C- and 2CT-series including 2,5-dimethoxy-4-bromophenethylamine (2C-B), 2,5-dimethoxyphenethylamine (2C-H), 2,5-dimethoxy-4-iodophenethylamine (2C-I), 2,5-dimethoxy-4-ethylthiophenethylamine (2C-T-2), 2,5-dimethoxy-4-isopropylthiophenethylamine (2C-T-4), 2,5-dimethoxy-4-propylthiophenethylamine (2C-T-7), 2,5-dimethoxy-4-bromoamphetamine (DOB), 2,5-dimethoxy-4-ethylamphetamine (DOET), 2,5-dimethoxy-4-methylamphetamine

(DOM) and 4-methylthioamphetamine (4-MTA) were obtained from Lipomed (Cambridge, MA) in 1 mg/mL solutions. The 2,5-dimethoxy-4-iodoamphetamine (DOI) was obtained from Sigma-Aldrich (St. Louis, MO) and the Drug Enforcement Agency (DEA) Special Testing and Research Laboratory (Dulles, VA) provided solid reference standards for 2,5-dimethoxy-4-chlorophenethylamine (2C-C), 2,5-dimethoxy-4-methylphenethylamine (2C-D), 2,5-dimethoxy-4-ethylphenethylamine (2C-E), and 2,5-dimethoxy-4-chloroamphetamine (DOC).

Additional chemicals, acids, inorganic salts (for buffers), and solvents were purchased from VWR International (Houston, TX) and Fisher Scientific (Pittsburg, PA). Deionized water was generated using a Millipore Milli Q water system (Billerica, MA). All solvents were LC grade and all other chemicals were ACS grade or higher.

For derivatization, pentafluorophenylhydrazine (PFPH), sodium borohydride ($NaBH_4$), ethanol, dithioerythritol (DTE), trimethyliodosilane (TMIS), acetic anhydride (AA), trifluoroacetic acid anhydride (TFAA), *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHOX) hydrochloride, hydroxylamine hydrochloride (HOX), triethylamine (TEA), trichloroacetic acid (TCA) and trimethylsilylcyanide (TMSCN), pyridine, dimethylformamide (DMF) and pyridine were obtained from Sigma Aldrich (St. Louis, MO). Pentafluoropropionic acid anhydride (PFPA), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), trimethylsilylimidazole (TMSI), heptafluorobutyrylimidazole (HFBI), 2% methoxylamine in pyridine (MOX) reagent and chloroform were obtained from Thermo Scientific (Waltham, MA).

Working standards of the cathinones were prepared by simple dilution in methanol. A sodium acetate buffer (pH 4.5) was prepared by adding glacial acetic acid drop-wise to a 0.1 M solution of sodium acetate. The working hydroxylamine reagent consisted of 1 g hydroxylamine in 10 mL deionized water. A 0.1% *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine reagent was prepared by dissolving 1 mg in 1 mL pyridine. Pentafluorophenylhydrazine was utilized as a 10 mg/mL solution in methanol. Solutions of 1% (v/v) acidic and basic methanol were prepared by addition of 1 mL concentration HCl or TEA, respectively, to 100 mL methanol. A 2% (w/v)

solution of sodium borohydride was prepared by combining 0.02 g $NaBH_4$ with 1 mL ethanol. The MSTFA:DTE:TMIS reagent was prepared by combining 1 mL MSTFA, 5 mg DTE, and 2 µL of TMIS (Fang, 2010). Phosphate buffer (0.1 M, pH 6) was prepared by mixing dibasic and monobasic sodium phosphate solutions. The elution solvent consisted of 95:5 (v/v) dichloromethane/isopropyl alcohol containing 2% concentrated ammonium hydroxide. Acidic methanol consisted of a 2% solution of concentrated hydrochloric acid in methanol. Pooled human urine collected from healthy drug free volunteers was preserved with sodium fluoride (1% w/v) and drug free bovine blood containing sodium fluoride (1%) and potassium oxalate (0.2%) was purchased from Quad Five (Ryegate, MT).

Instrumentation

GC/MS analysis was performed using an Agilent HP 5975 MSD/7890 GC (Santa Clara, CA) equipped with a DB-5MS column (30 m x 0.25 mm x 0.25 µm) from Agilent Technologies (New Castle, DE). Unless stated otherwise, injector and interface temperatures were set at 260°C and 280°C, respectively. Injections (2 µL) were made in split mode with a 10:1 split ratio unless otherwise stated using a Sil-tek deactivated cyclosplitter® liner (4 x 6.3 x 78.5 mm) (Restek, Bellefonte, PA) and Ultra Inert Gold Seal (#5190-6149) (Agilent Technologies, Santa Clara, CA). A range of temperature programming conditions were utilized during the investigation of novel chemical derivatives to accommodate derivatives with different properties. All new derivatives were investigated using full scan acquisition (40-650 Da) at 70 eV using solvent delays and temperature programming to accommodate volatile (early eluting derivatives) as well as potentially late eluting compounds. As a result initial GC temperatures as low as 50°C were used, in addition to extended hold times at the final temperature of 290°C. Ramps of 30°C/min were used for all chromatographic assays involving derivatives and carrier gas flow (helium) was set at 1.3 mL/min. All extracts and derivatives were evaporated to dryness under nitrogen using a TurboVap LV from Caliper LifeSciences (Hopkinton, MA).

Optimum separation of non-derivatized cathinones was achieved using an inlet temperature of 175°C and a 25:1 split ratio. The initial oven temperature was 140°C (held for 0.50 min) followed by a 10°C/min ramp to a final temperature of 265°C. When analyzing biological extracts for cathinones, a post-run burnout at 290°C (2.5 minutes) was used for a total cycle time of 15.5 minutes. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. The MS was operated in the electron impact (EI) ionization mode (40-650 Da) at 70 eV and the interface was held at 280°C.

Ketone Derivatization

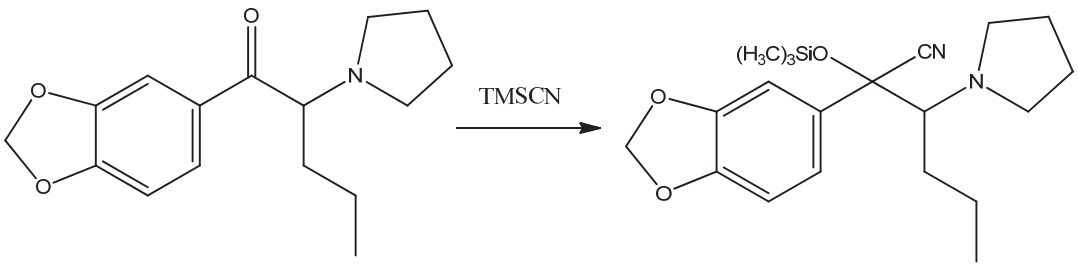
In order to derivatize the ketone group of the synthetic cathinones, multiple derivatizing reagents and conditions were employed. The derivatization schemes included cyanosilylation (trimethylsilylcyanide); single and multi-step oximes (including methoxylamine (MOX), hydroxylamine (HOX), *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHOX); phenylhydrazones using highly reactive pentafluorophenylhydrazine (PFPH); and finally reduction (using sodium borohydride), together with multi-step reductive silylation, and reductive acylation. In many instances, derivatization reagents were harsh and simple clean-up procedures involving evaporation or organic extractions were necessary to remove excess reagent and prevent detector fouling. Since derivatization of the pyrrolidine-containing species presented the major challenge, MDPV was selected during the initial investigation of each derivative. When derivatives of interest were successfully produced, additional cathinones were investigated and fragmentation pathways were explored.

Trimethylsilylcyanide

Cyanosilylation of MDPV was investigated using a method adapted from earlier reports involving aldehyde and ketone derivatization using TMSCN (Prakash, 2007; Rasmussen, 1991; Chen, 2003; Fuerst, 2005; Hamashima, 2001; Tian, 2003; Deng, 2002; Nishikawa, 1995). A schematic for the derivatization of MDPV is shown in **Figure 11**. Methanolic drug standard was gently evaporated to dryness at room temperature (1000 ng total). TMSCN (30 µL) was added,

vortex mixed, tightly capped and heated for 15 minutes at 75°C. Excess TMSCN reagent was then evaporated to dryness at room temperature and reconstituted in 30 µL of ethyl acetate. A total of 2 µL was injected onto the GC/MS in full scan mode. During the evaluation of TMSCN, reaction time (0 to 60 minutes), reaction temperature (room temperature to 100°C) and solvent (no solvent, DMF, ethyl acetate or pyridine, 15 µL) were investigated.

Figure 11. Derivatization of MDPV with TMSCN.

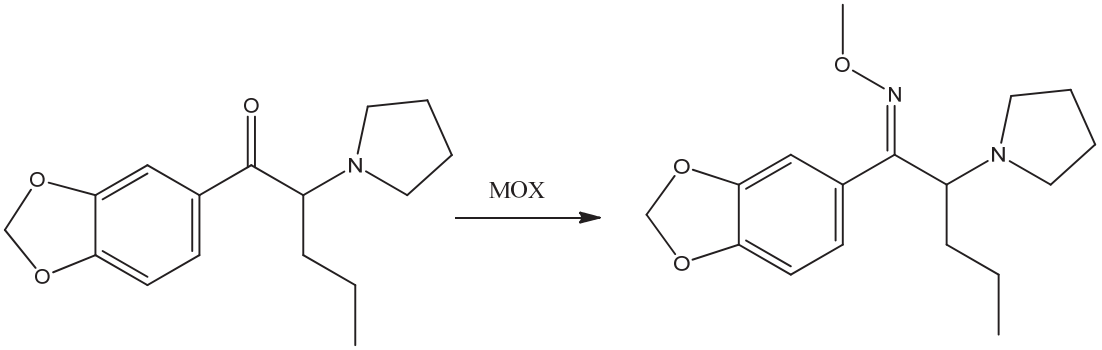


Methoxylamine Derivatives

Derivatization of the ketone group with methoxylamine (MOX) was adapted from earlier published reports (Broussard, 2001; Meatherall 1999; Chen, 2007). Methanolic drug standard was gently evaporated to dryness at room temperature (1000 ng total). MOX reagent consisting of 2% methoxylamine in pyridine (30 µL) was added, vortex mixed, tightly capped and reacted at temperatures ranging from room temperature to 70°C for 15 - 60 minutes. After cooling to room temperature samples were evaporated to dryness and excess reagent was removed using a simple clean-up procedure (Meatherall, 1999). A total of 1 mL hexane/chloroform (3:1) and 100 µL of 50% ammonium hydroxide were added, tubes were rotated for 5 minutes and centrifuged at 4,000 rpm for 5 minutes. The organic layer was removed, evaporated to dryness and the residue was reconstituted in ethyl acetate. Alternative clean-up procedures using either ethyl acetate or *N*-butyl chloride were also investigated, as well as 0.1 M sodium hydroxide in place of the ammonium hydroxide. Final products were reconstituted in 30 µL of ethyl acetate and injected (2 µL) onto the GC/MS in full scan mode using the GC temperature program

described earlier. A scheme for the derivatization of MDPV with methoxylamine is shown in **Figure 12**.

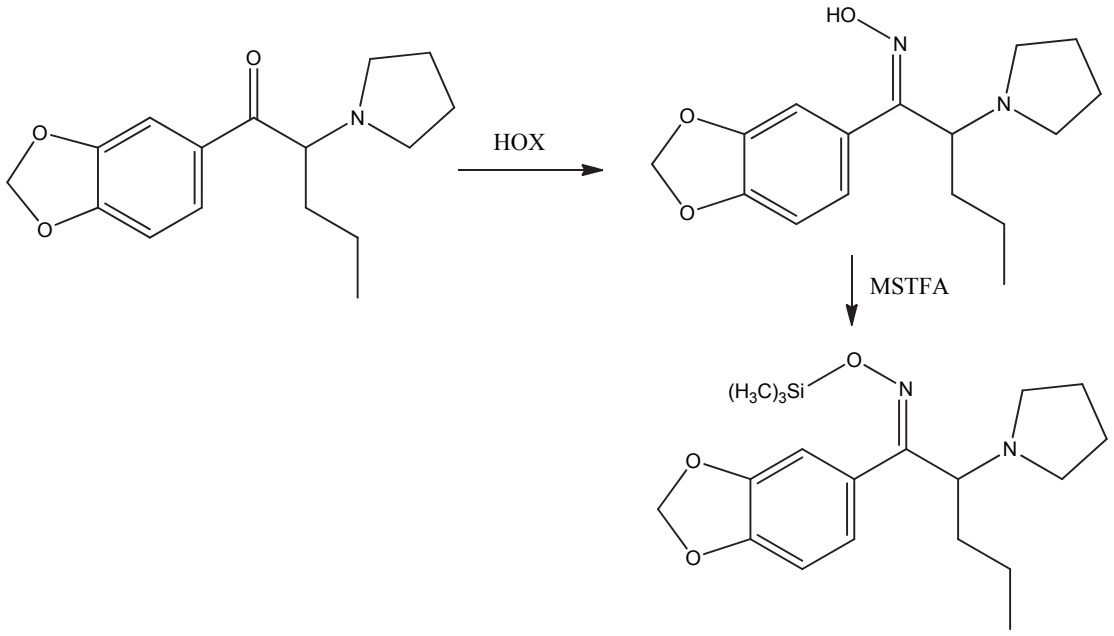
Figure 12. Derivatization of MDPV with methoxylamine (MOX).



Hydroxylamine Derivatives

Hydroxylamine (HOX) was also investigated using procedures adapted from earlier reports involving keto opioids and keto steroids (Broussard, 1997; Cremese, 1998; Andrási, 2011; Chen 2007; Tiscione 2011). A methanolic drug standard (1000 ng) was evaporated to dryness at room temperature followed by 0.5 mL of a 10% (w/w) hydroxylamine hydrochloride (HOX) in deionized water. Samples were tightly capped, vortex mixed and heated over a range of temperatures (room temperature to 80°C) for 15 to 60 minutes. After cooling to room temperature, samples were subjected to a simple base extraction to remove excess reagent (described above). After oxime formation using HOX, samples were silylated using 30 µL of MSTFA. The tubes were capped, vortex mixed and heated at 70°C for 15-30 minutes. TMS-oxime derivatives were injected onto the GC/MS in full scan mode. A schematic of the two-step preparation of the trimethylsilyl (oxime) ether of MDPV is shown in **Figure 13**. In a similar way, trimethylsilyl oximes were prepared using a solution of 2.5% (w/w) HOX in pyridine, as described by Andrási *et al*. (Andrási, 2011).

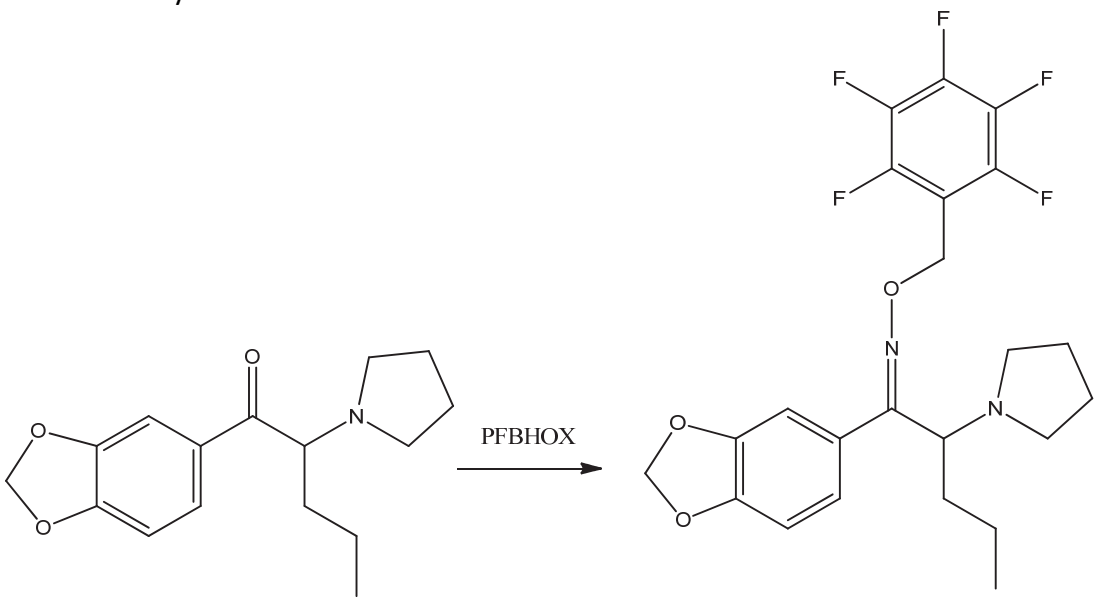
Figure 13. Two-step derivatization of MDPV with hydroxylamine (HOX) and trimethylsilylation using MSTFA.



O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine Derivatives

A PFBHOX derivatization procedure was adopted from published procedures involving volatile carbonyls and acyl homoserine lactones (Charlton, 2000; Li, 2005; Yu, 1995; Strassnig, 2000). PFBHOX reagent (30 µL) was added to 1000 ng of drug. Tubes were capped, vortex mixed, heated for 30 minutes at 90°C. After cooling to room temperature, derivatives were evaporated to dryness under nitrogen and subjected to a clean-up procedure to remove excess reagent (as described earlier). The organic layer was evaporated to dryness and reconstituted in 30 µL of ethyl acetate. The volume and concentration of the PFBHOX reagent, as well as the reaction temperature (room temperature to 90°C) were varied. Both 0.1% (1 mg/mL) and 2% (10 mg/mL) PFBHOX were used in quantities ranging from 10 to 30 µL.

Figure 14. Derivatization of MDPV with pentafluorobenzylhydroxylamine (PFBHOX) to form the *O*-pentafluorobenzyl-oxime.

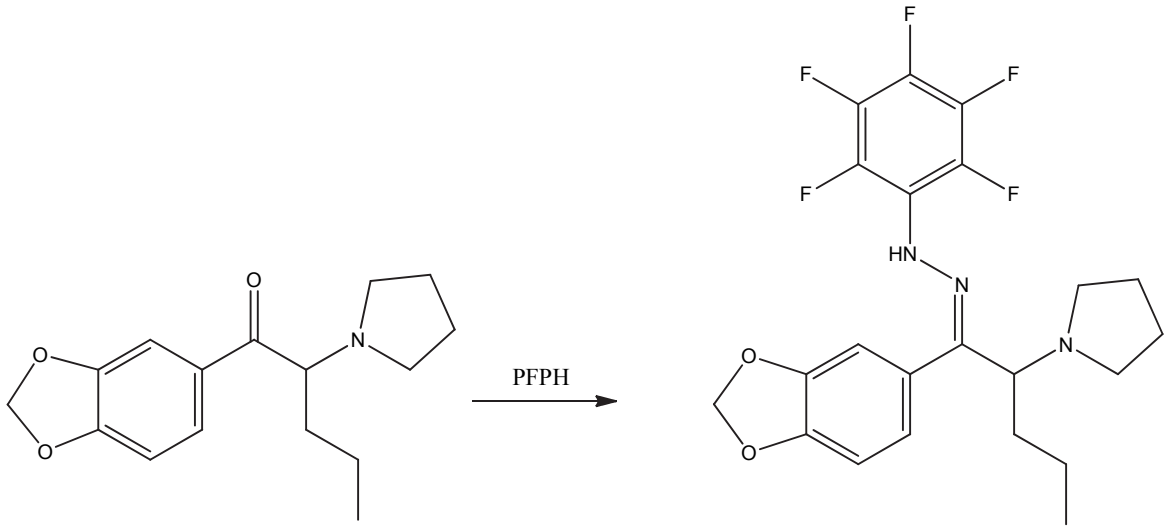


Pentafluorophenylhydrazones

The method for the derivatization of cathinones using PFPH was adapted from Sheen's derivatization of keto steroids (testosterone and nabumetone) as follows. Methanolic PFPH (100 µL of 10 mg/mL) and 100 µL of 0.05 M HCl were added to approximately 1000 ng of drug that had been evaporated to dryness. Tubes were tightly capped, vortexed and heated at 70°C for 30 minutes. After cooling, excess reagent was removed using the hexane/chloroform or *N*-butyl chloride clean-up procedure described earlier. After evaporation of the solvent, derivatives were reconstituted in 30 µL ethyl acetate and injected onto the GC/MS.

In an attempt to optimize the PFPH derivatization, reaction conditions and clean-up procedures were investigated. These included changing the concentration of acid (0.05 – 1M HCl), acid type (HCl, TCA, 1% acidic methanol, no acid), concentration of PFPH solution (10, 20 and 45 mg/mL), PFPH reagent solvent (methanol, 0.1M HCl, hexane, chloroform, dichloromethane) and clean-up solvents (ethyl acetate, hexane, *N*-butyl chloride). A scheme for the formation of the pentafluorophenylhydrazone derivative of MDPV is shown in **Figure 15**.

Figure 15. Derivatization of MDPV with pentafluorophenylhydrazine (PFPH).



Sterically Hindered Ketone Derivatization

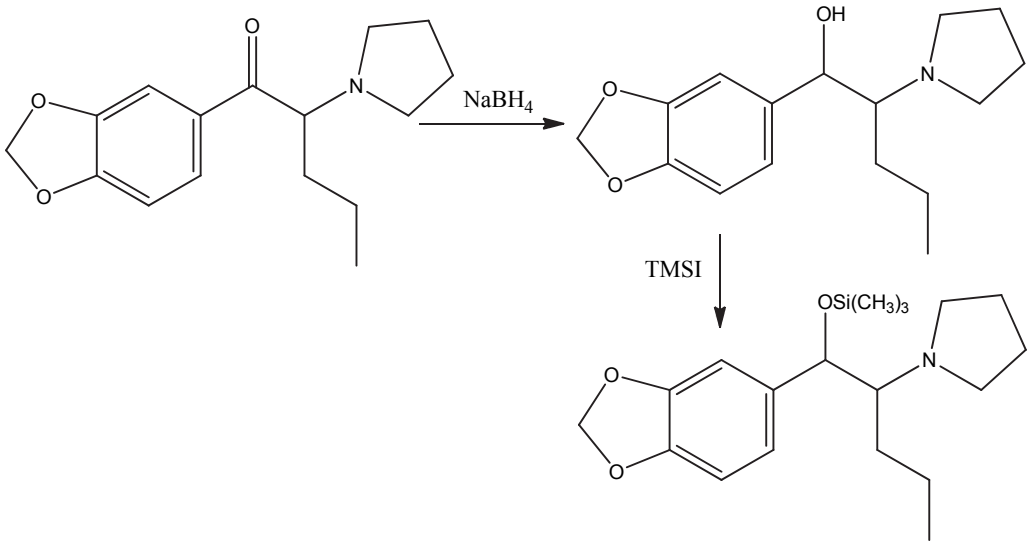
A MSTFA:DTE:TMIS derivatization procedure intended for use with sterically hindered ketones was adapted from Fang *et al*. MSTFA:DTE:TMIS (1000:2:5) reagent (75 µL) was added to approximately 1000 ng of drug. The tube was capped heated at 70°C for 30 minutes, 60°C for 1 hour, or placed in a microwave oven where the reaction occurred at 600 W for 1 minute. After cooling to room temperature, derivatives were evaporated to dryness and reconstituted in ethyl acetate prior to injection (2 µL) onto the GC/MS. An alternative preparation the derivatization mix consisting of MSTFA:DTE:TMIS (5ml: 10 mg; 10 µL) were investigated using a similar approach (Saraiva, 2011).

Ketone Reduction: Reductive Silylation and Reductive Acylation

As an alternative to the direct ketone derivatization, a two-step approach involving the reduction of the ketone, followed by conventional derivatization was also investigated. Sodium borohydride solution (2% w/v) in ethanol was added to drug (approximately 1000 ng) that had been evaporated to dryness from a methanolic standard. Tubes were capped, vortex mixed and heated at 70°C for 15 minutes and evaporated to dryness at room temperature. To remove excess reducing agent, hexane (30 µL) was added, vortex mixed and removed from the reaction

vial before being evaporated to dryness. Reduced forms of the drug were either injected directly onto the GC/MS (in ethyl acetate) or subjected to a second derivatization step as follows: For reductive silylation, 30 µl of MSTFA or TMSI was added to the evaporated hexane fraction. The solution was heated at 70°C for 15 minutes or microwaved at 600W for 60 seconds prior to being injected onto the GC/MS in full scan mode. A schematic for the reductive silylation of MDPV is shown in **Figure 16.** When subsequent cathinones were investigated using this method, alternative trimethylsilylating agents were evaluated. Although MSTFA is reactive towards hydroxyls, it is also reactive towards the secondary amine which can result in multiple derivatives for the polyfunctional cathinones that contain a secondary amine. Trimethylsilylimidazole (TMSI) was selected because of its selectivity towards the hydroxyl group.

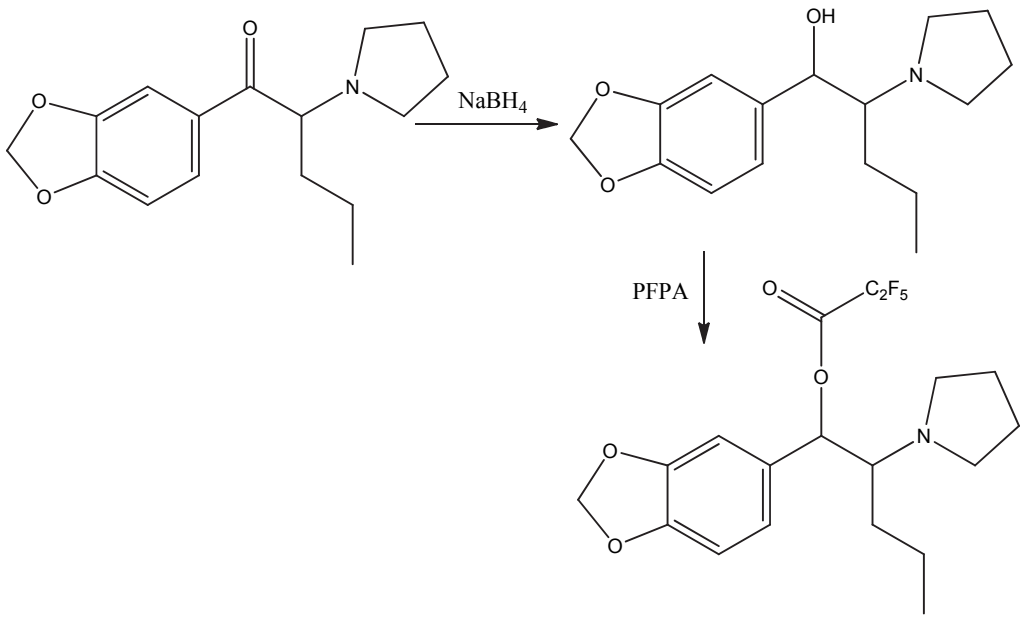
Figure 16. Reductive silylation of MDPV using sodium borohydride ($NaBH_4$) and trimethylsilylimidazole (TMSI).



In a similar manner, reductive acylation was performed by addition of 50 µL pentafluoropropionic acid anhydride (PFPA) to the evaporated hexane fraction. After heating the reagent in a conventional heating block or microwave derivatization (600W), excess reagent was removed by evaporation and samples were reconstituted in 30 µL of ethyl acetate prior to GC/MS analysis. A schematic for the reductive silylation of MDPV is shown in **Figures 17.**

Heptafluorobutyrylimidazole (HFBI) was also used in a similar fashion. Due to steric hindrance, a number of alternative acylating agents were used including trifluoroacetic acid anhydride (TFAA) and acetic anhydride (AA). These reagents were used in the manner described for PFPA.

Figure 17. Reductive acylation of MDPV using sodium borohydride ($NaBH_4$) and pentafluoropropionic acid anhydride (PFPA).



Analysis of Non-Derivatized Synthetic Cathinones

GC/MS Analysis

GC/MS analysis was performed using an Agilent HP 5975 MSD/7890 GC (Santa Clara, CA) equipped with a DB-5MS column (30 m x 0.25 mm x 0.25 µm) from Agilent Technologies (New Castle, DE). Although injector and interface were initially set at 260°C and 280°C, respectively, optimal conditions are described below. The inlet and interface temperatures were set at 175°C and 280°C, respectively. Injections (2 µL) were made in split mode with a 25:1 split ratio. Optimum separation was achieved using an initial oven temperature of 140°C (held for 0.50

min) followed by a 10°C/min ramp to a final temperature of 265°C. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. The MS was operated in the electron impact (EI) ionization mode (40-650 Da) at 70 eV.

When analyzing biological extracts for cathinones, a post-run burnout at 290°C (2.5 minutes) was used for a total cycle time of 15.5 minutes. The acceptance criteria for the assay was based on a retention time within 2% of the verified standard and mass spectral fit. In the absence of commercially available deuterated analogs for all of the target cathinones, mephedrone-D3, methylone-D3, naphyrone-D5, MDPV-D8 and MDA-D5 were evaluated as internal standards. MDA-D5 was considered representative of amphetamine-like drugs, mephedrone-D3 for cathinones with secondary amines; methylone-D3 for the methylenedioxy analogs; and naphyrone-D5 and MDPV-D8 both representative of the tertiary amines, with the latter also containing the methylenedioxy functional group.

Analysis of Cathinones in Biological Samples

Optimized extractions were performed using 2 mL aliquots of drug free urine fortified with cathinones over the concentration range of interest. Deuterated internal standard solution was added to achieve a final concentration of 250 ng/mL. Four milliliters of phosphate buffer were added to the urine and vortex mixed. The urine samples were then transferred to PolyChrom ClinII (3cc) SPE columns (SPEWare, Baldwin Park, CA) and allowed to flow under gravity. Columns were washed successively with deionized water (1 mL) and 1 M acetic acid (1 mL) before being dried for 5 minutes under full vacuum. Columns were then successively washed with 1mL aliquots of hexane, ethyl acetate and methanol. Finally, cathinones were eluted using 1 mL of elution solvent consisting of methylene chloride/isopropyl alcohol (95:5 v/v) containing 2% concentrated ammonium hydroxide. Acidic methanol (30 µL) was added prior to evaporation at 50°C under a stream of nitrogen. Extracts were then reconstituted in 20 µL of ethyl acetate injected (2 µL) onto the GC/MS.

Blood was extracted in a similar fashion as follows. Internal standard (250 ng/mL) was added to drug free blood that had been fortified with cathinones over the concentration range of interest. Chilled acetonitrile (4 mL) was added to blood with vortex mixing in a glass centrifuge tube. Samples were centrifuged at 4000 rpm for 5 minutes. The supernatant layer was removed and diluted with 6 mL phosphate buffer (pH 6, 0.1 M). Samples were added to SPE columns and allowed to flow under gravity, or minimal vacuum necessary to maintain constant flow. Columns were successively washed with 1 mL deionized water and 1 M acetic acid before drying under full vacuum for five minutes. Columns were then washed successively with 1 mL aliquots of hexane, ethyl acetate, methanol and dichloromethane. Finally, cathinones were eluted 1 mL of methylene chloride/isopropyl alcohol (95:5 v/v) containing 2% concentrated ammonium hydroxide. Acidic methanol (30 µL) was added prior to evaporation at 50°C under a stream of nitrogen. Extracts were then reconstituted in 20 µL of ethyl acetate injected (2 µL) onto the GC/MS.

Assay Performance

The GC/MS assay was validated in accordance with SWGTOX recommendations for qualitative confirmatory procedures (SWGTOX, 2013). Analytical recovery was determined by comparison of extracted and non-extracted replicates. Six urine samples containing 250 ng/mL of each drug and internal standard were extracted, together six drug free samples that contained only internal standard. After the elution step, drug (equivalent to 250 ng/mL) was added to the drug free replicates. The analytical recovery was calculated from the ratio of the relative peak area (drug/I.S.) for the extracted and non-extracted samples. Recovery from whole blood was determined in a similar fashion in triplicate. This approach mitigates some of the random error associated with absolute peak area measurements, injection volume and detector response.

The limit of detection (LOD) was determined empirically. Three sources of drug free matrix were fortified with analyte and analyzed in duplicate over three days. The limit of detection (LOD) was defined as the lowest concentration of analyte that met the following criteria for all eighteen determinations: signal-to-noise (S/N) ratio of at least 3:1 for the total ion

chromatogram; acceptable mass spectral fit with all major ions present and no interfering ions; and a retention time within 2% of the expected value. Analyte carryover was evaluated by analyzing drug free extracts immediately following injection of the highest calibrator (control).

Interferences were assessed from the biological matrix (blood and urine), stable isotope standard (MDPV-D8), common drugs and other structurally similar compounds. Matrix interferences were assessed using ten aliquots of drug free human urine and blood (from different sources) in the absence of internal standard. Interfering ions and possible contributions of non-deuterated drug from the internal standard reference material were evaluated by extracting drug free blood and urine in the presence of the internal standard (250 ng/mL) and monitoring the target analytes. In a similar fashion, drug free blood and urine fortified with cathinones at the upper range (1000 ng/mL) in the absence of internal standard was also evaluated.

Interferences from commonly encountered drugs or structurally related compounds were evaluated using drug free matrix (negative controls) and positive cathinone controls (100 ng/mL) in the presence of interfering compounds at a ten-fold higher concentration (i.e. 1000 ng/mL). Interferences were evaluated using a number of common drugs, structurally related substances, designer drugs, therapeutically used cathinones and endogenous bases. The potential interference of other abused amphetamine-like drugs was investigated. Negative and positive controls were assayed in the presence of amphetamine-like drugs (amphetamine, methamphetamine, MDA, MDMA, MDEA, MBDB, ephedrine, pseudoephedrine, phentermine, phenylpropanolamine); other designer drugs (2C-B, 2C-C, 2C-D, 2C-E, 4-MTA, DOC, DOET, DOI, DOM, 2C-T-2, 2C-T-4, 2C-T-7, 2C-H, 2C-I, and DOB); endogenous bases (phenethylamine, putrescine, tryptamine, tyramine); common basic drugs, including dextromethorphan, zolpidem, ketamine, diphenhydramine, cocaine, amitriptyline, diazepam, nordiazepam, oxycodone, hydrocodone, alprazolam, phencyclidine (PCP), methadone, tramadol and codeine; and finally, therapeutically used cathinones (bupropion and diethylpropion).

Although the assay was intended for qualitative use, intra-assay precision and bias was evaluated for investigational purposes only. Drug free urine was fortified with target drugs at 200 ng/mL (N=6) and linear regression analysis (0 – 1000 ng/mL) was used to determine the concentration of each target analyte.

Analysis of Authentic Specimens from Cathinone Users

Redwood Toxicology Laboratory (RTL) provided urine samples from drug users that had previously tested positive for cathinones or their metabolites. After analysis by the reference laboratory, samples were stored refrigerated for a period of time prior to shipping. Specimens were shipped by courier at ambient temperature and refrigerated prior to reanalysis. There were some notable differences in the scope of testing, and these are summarized in **Table 9**. Procedures for the evaluation of urine specimens from cathinone-users were subject to Institutional Review Board (IRB) review and approval by the Protection of Human Subjects Committee of Sam Houston State University.

Table 9. Scope of testing for authentic urine samples.

Cathinone	SHSU	Reference Laboratory
4-EMC	Parent	Parent
4-MEC	Parent	Parent and/or metabolites
Buphedrone	Parent	Parent and/or metabolites
Butylone	Parent	Parent
Ethcathinone	Parent	Parent
Ethylone	Parent	Parent
Flephedrone	Parent	Parent and/or metabolites
MDPBP	Parent	Parent
MDPV	Parent	Parent
Mephedrone	Parent	Parent and/or metabolites
Methcathinone	Parent	Parent
Methedrone	Parent	Parent
Methylone	Parent	Parent
MPBP	Parent	Parent
Naphyrone	Parent	Parent
Pentedrone	Parent	Parent and/or metabolites
Pentylone	Parent	Parent
Pyrovalerone	Parent	Parent
α-PVP	Parent	Parent

III. Results and Discussion

GC/MS Analysis of Chemical Derivatives

Cyanosilylation

Ketone derivatization with TMSCN proved to be unsuccessful for the cathinone species. Even after extensive and prolonged heating, parent MDPV was the only compound detected. The initial oven temperature and solvent delay were minimized to accommodate a very volatile derivative, but none was detected. Derivatization temperatures, reaction time and solvent produced no improvement, even using DMF which is reported to be most efficient (Prakash, 2007). Other (secondary amine) cathinones including mephedrone were also investigated with similar results.

Cyanosilanes are applicable to three major types of reactions: cyanosilylation, cyanation and silylation (Rasmussen, 1991). The largest reaction group, cyanosilylation, incorporates the cyano and silyl group into the synthesized product. Reports suggest however that ketone cyanosilylation may require a catalyst for increased yield, reaction rate (Rasmussen, 1991) and enantioselectivity (Chen, 2003; Fuerst, 2005; Hamashima, 2001). In the presence of catalysts reaction times spanned from 1 to 130 hours (Fuerst, 2005; Hamashima, 2001; Tian, 2003; Deng, 2002) and were highly analyte dependent. Efforts to functionalize the keto group of the cathinones using TMSCN were unsuccessful.

Oxime Formation

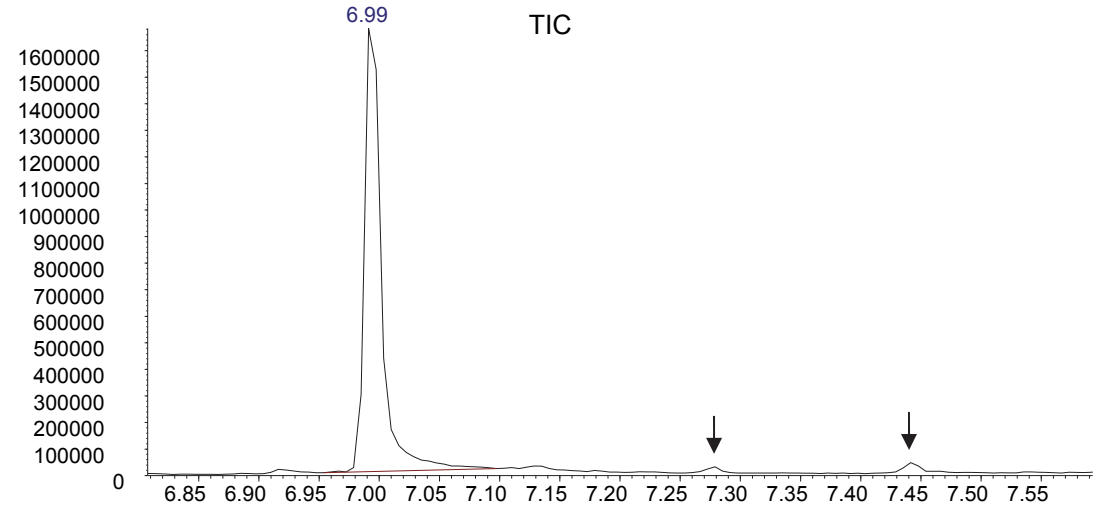
Published procedures involving methoxylamine (MOX) for keto opioid derivatization were adapted for synthetic cathinones. Typically, MOX is sufficiently reactive at room temperature to derivatize keto opioids (Meatherall, 1999; Broussard, 2001) or moderate (65°C) temperature for aldehydes (St. Germain, 1997). The initial ketone derivatization was performed at room temperature. No oximes were detected by GC/MS, even when elevated reaction temperatures were used (room temperature up to 90°C).

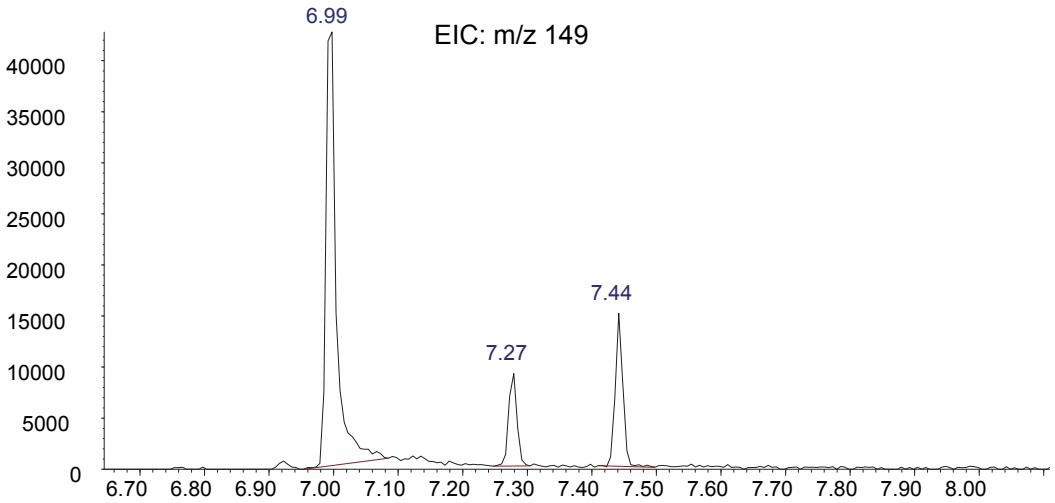
Hydroxylamine has also been used successfully for other ketone-containing drugs, often with a second silylation step to form a trimethylsilyl oxime. Although this two-step approach for carbonyl-containing compounds has been reviewed extensively, this produces two peaks corresponding to the *syn* (E) and *anti* (Z) isomers. When biological specimens are used, derivatization using hydroxylamine can be added to the matrix prior to extraction. Using this approach, ketone containing opioids and steroids have been derivatized at moderate temperatures (Broussard, 1997; Cremese, 1998; Andrási, 2011). However, cathinones were resistant to derivatization and parent drug remained under all reaction conditions.

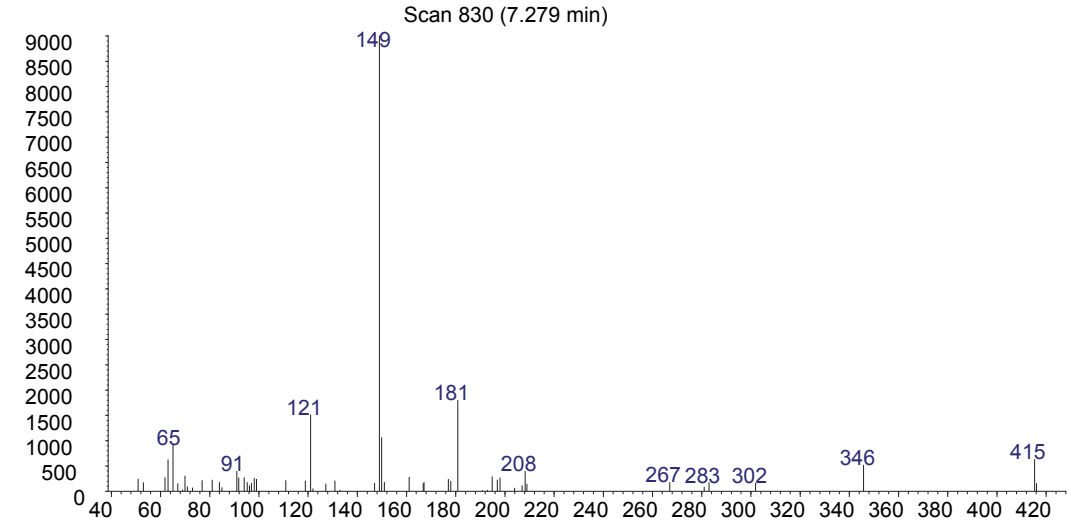
Oxime formation using the fluorinated derivative PFBHOX is theoretically advantageous due to its increased molecular weight and has been shown to be reactive towards carbonyls. Using a method adapted from Charlton (Charlton, 2000), *O*-Pentafluorobenzyl-oximes of the cathinones were produced. **Figure 18** depicts the total ion chromatogram and extracted ion chromatogram (m/z 149) for the pentafluorobenzyloxime derivative of MDPV with corresponding mass spectra. The derivative forms two peaks, corresponding with the *syn*- and *anti*- stereoisomers, which are formed during the conversion of ketones into oximes. Similar data for naphyrone is depicted in **Figure 19.** Oximes were separated using a GC temperature program as follows: Initial temperature 70°C (0.5 min), 30°C/min to 290°C (hold 15 mins). Using this program, parent drug and derivatized cathinones were well separated. Excess PFBHOX reagent eluted at 2.6 minutes and in order to reduce background and detector fouling, the post-derivatization clean-up procedure was optimized and *N*-butyl chloride was the preferred extraction solvent

for this purpose. Based on the difficulty associated with the ketone derivatization and the significant improvement in mass spectra quality relative to the non-derivatized form, numerous steps were taken to optimize the derivatization procedure. Optimum results were achieved at 90°C for 30 minutes. The significant improvement in the quality and specificity of the mass spectra are shown in **Figure 20**. However, the reaction did not go to completion, even when large excesses of reagent were used. This reluctance to derivatize confirmed our earlier observations with more traditional (non-fluorinated) oxime reagents. Despite improved mass spectral qualities, the pentafluorobenzyloxime derivatives were not pursued because incomplete derivatization and the formation of the *syn*- and anti- isomers would present a significant challenge (particularly for quantitative analysis of these compounds in toxicological samples). The challenges associated with the formation of both the Z and E geometric isomers are well documented in the literature (Charlton, 2000; Strassnig, 2000; Sugaya, 2004).

Figure 18. Total ion chromatogram (TIC) and extracted ion chromatogram (EIC, m/z 149) of residual (un-derivatized) MDPV (6.99 mins) and the *syn*- and *anti*- stereoisomers of the pentafluorobenzyloxime derivative of MDPV (7.27 and 7.44 min) (top) shown with mass spectra (bottom).







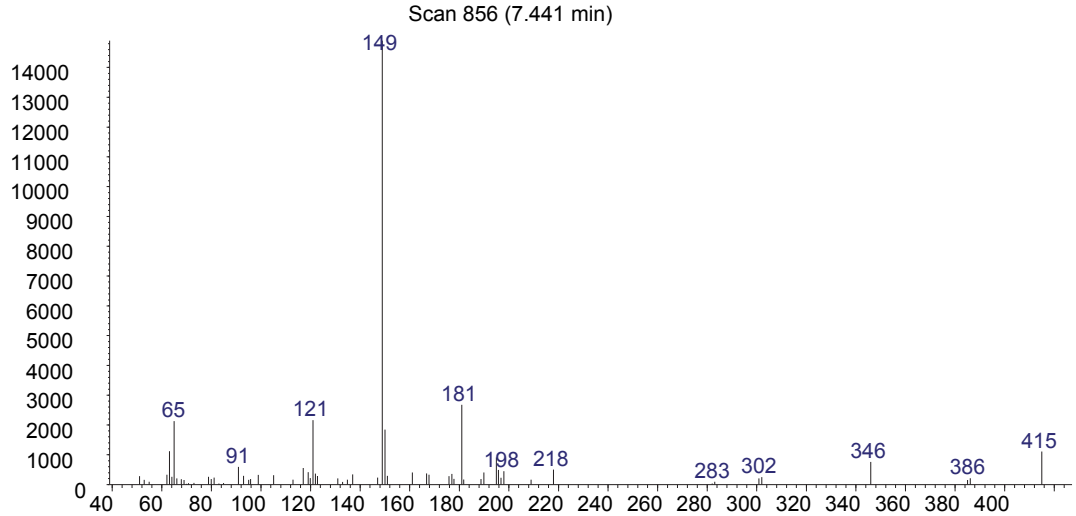
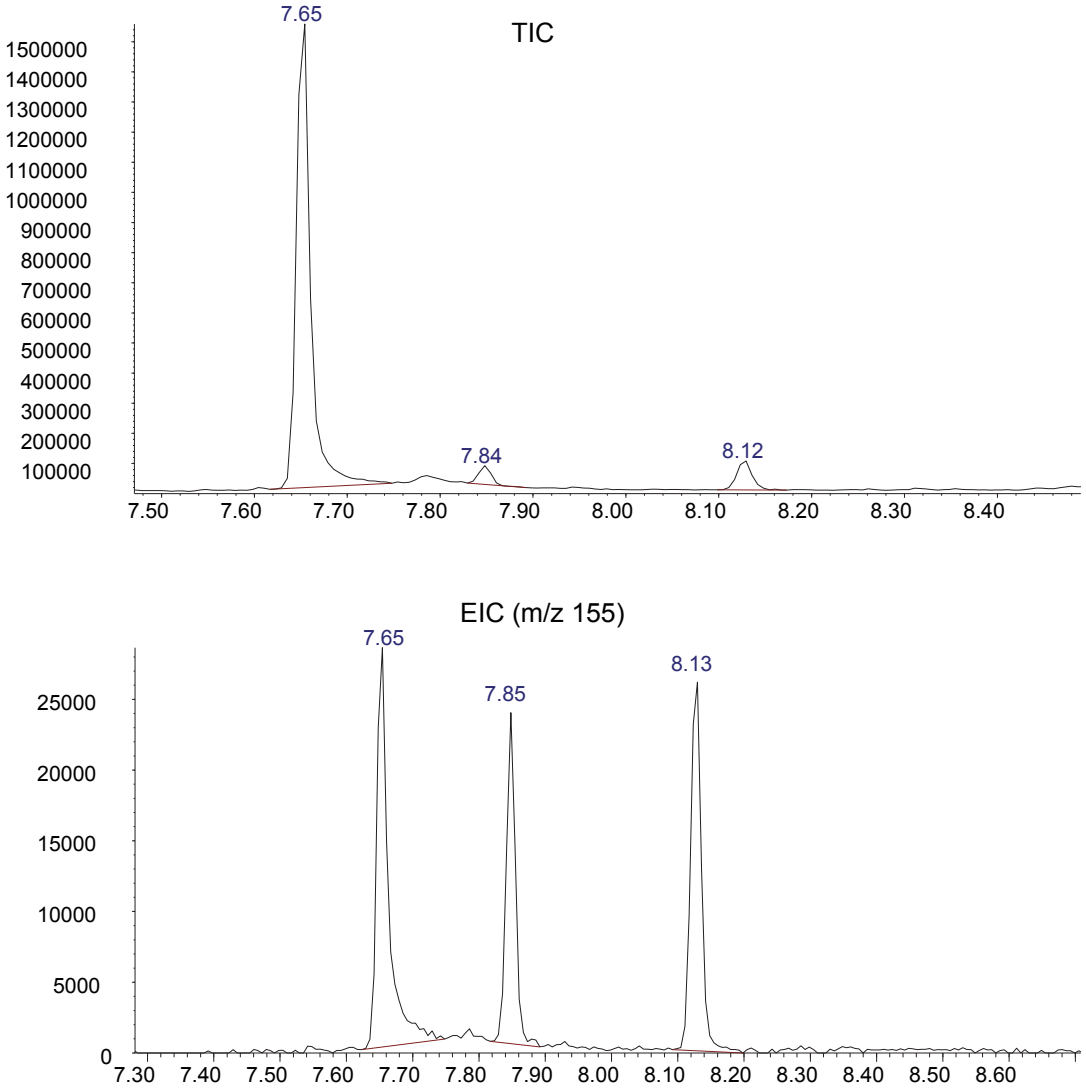
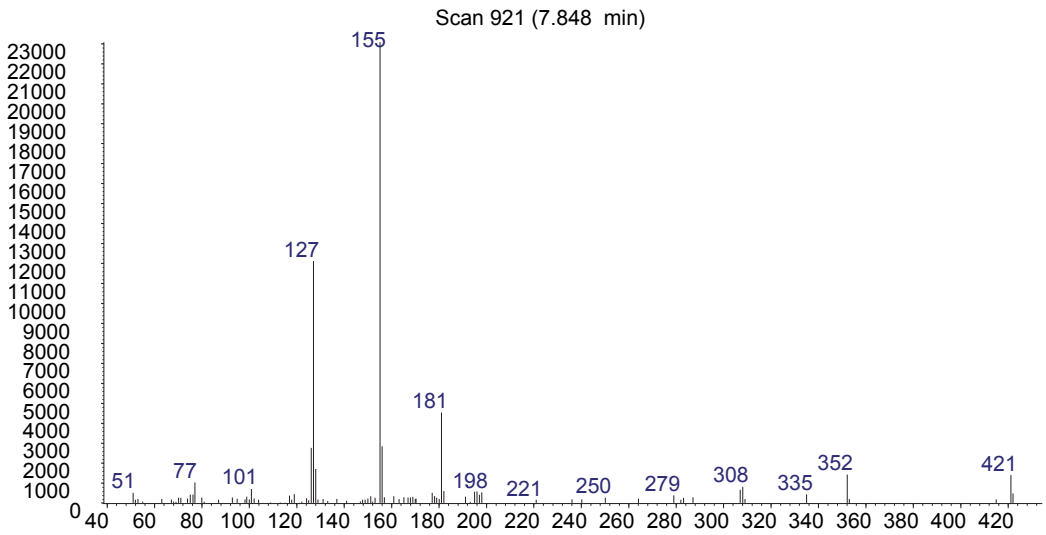


Figure 19. Total ion chromatogram (TIC) and extracted ion chromatogram (EIC, m/z 155) of residual (un-derivatized) naphyrone (7.65 mins) depicting the *syn*- and *anti*- stereoisomers of the pentafluorobenzyloxime derivative of naphyrone (7.85 and 8.13 min) (top), shown with mass spectra (bottom).





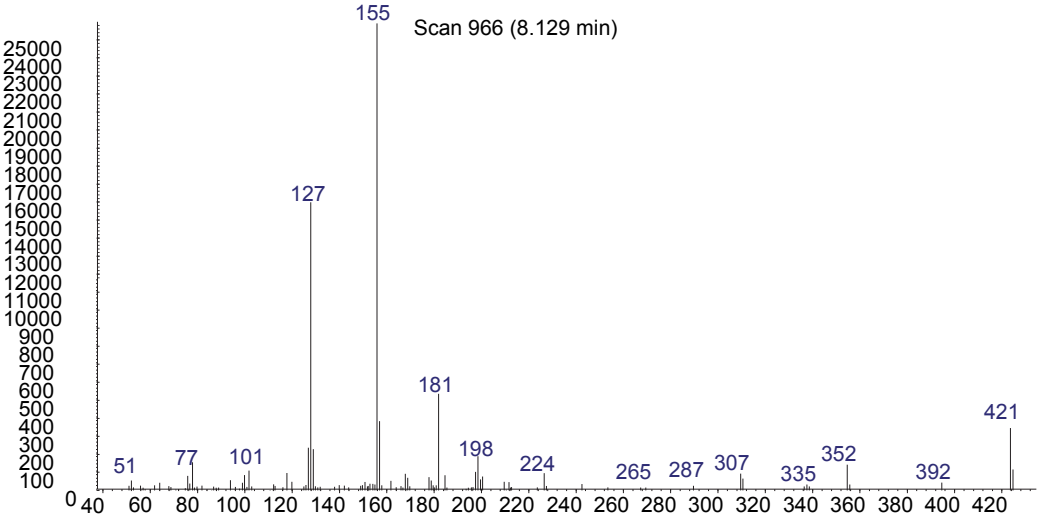
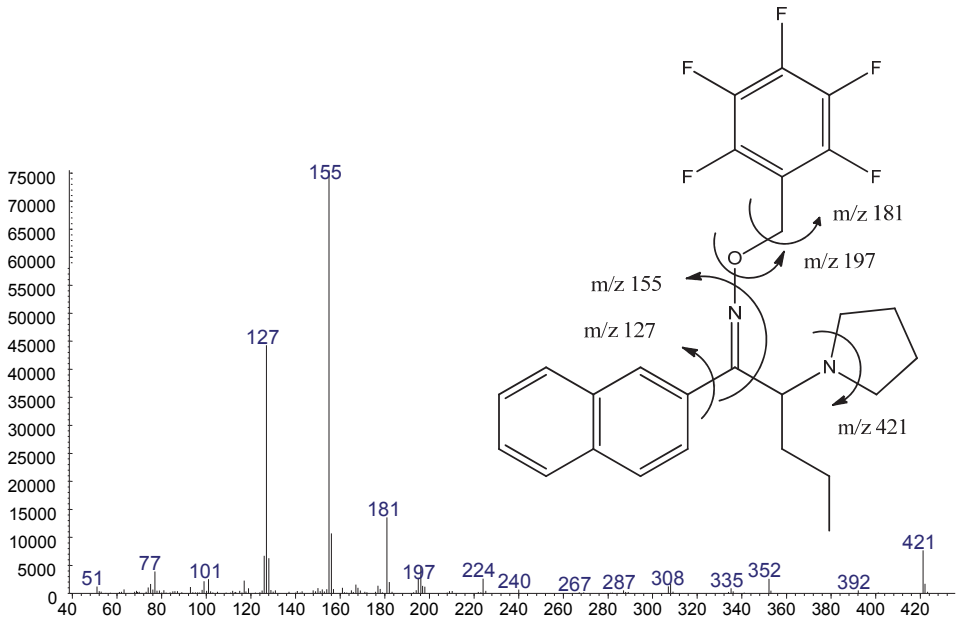
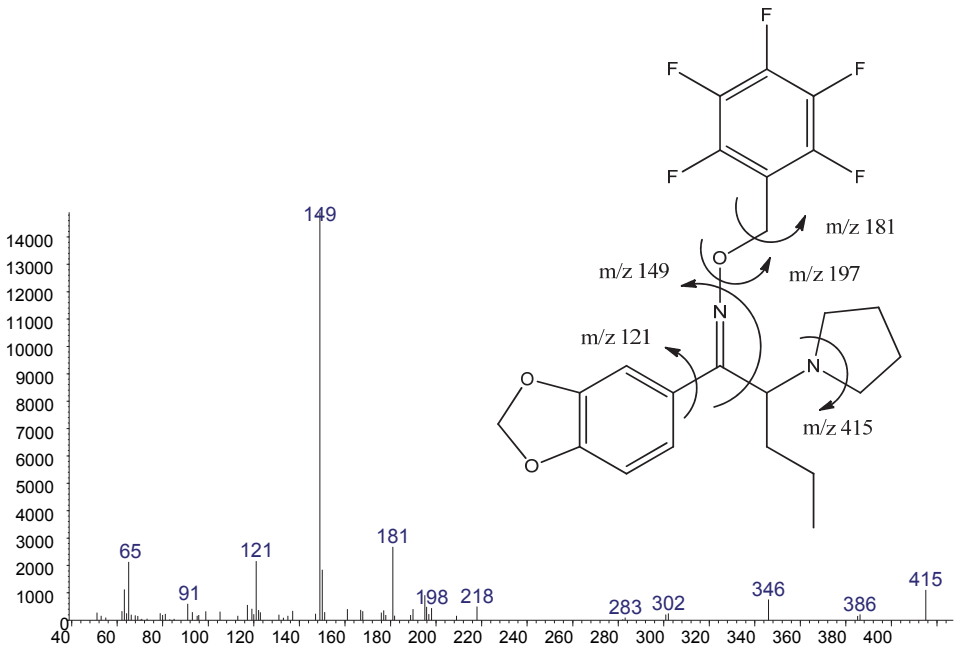


Figure 20. Mass spectra of *O*-pentafluorobenzyloximes of MDPV and naphyrone.





Hydrazones

Cathinones should react with PFPH to produce the respective pentafluorophenylhydrazone. However, none of the conditions tested were capable of producing hydrazones of cathinone. Since these reactions require strongly acidic conditions a variety of modifications were made, without success. Use of non-hydrogen bonding and non-polar solvents to minimize formation of the enol showed no derivatization improvement. Hydrazines are generally harsh reagents that can be used to derivatize the ketone functional group. Pentafluorophenylhydrazine (PFPH) is reported to produce derivatives that are thermally stable, sufficiently volatile, and the presence of fluorine in the reagent is favorable in terms of molecular weight. However, these reactions require strongly acidic conditions and typically produce two peaks, corresponding with the syn (E) and *anti* (Z) forms. Although PFPH has been used to derivatize volatile carbonyls (Stashenko, 1997; Pang, 2011), malondialdehyde (Yeo, 1994) nabumetone and testosterone (Sheen, 2004), pentafluorohydrazones of the cathinones were not detected under any of the conditions tested. This may be due to the hydrolytic instability of the hydrazones which are reported hydrolyze at a 1000-fold greater rate, compared to oximes (Kalia, 2008).

Silylation of Sterically Hindered Ketones

In an effort to introduce a silyl function group directly onto the ketone, MSTFA:DTE:TMIS was investigated using techniques described for cholesterols and steroid hormones (Fang, 2010; Saraiva, 2011). Trimethyliodosilane (TMIS) is a more potent trimethylsilyl donor and 1,4-dithioerythritol (DTE) acts as a catalyst (Saraiva, 2011). Significant detector fouling can occur with TMIS and its use should be minimized to prevent damage to the GC column. However, none of the conditions tested (room temperature to 90°C) produced direct silylation of the ketone functional group on any of the cathinones. Although this harsh reagent has been used to derivatize sterically hindered ketones directly, the cathinones were not amenable to this approach.

Reductive Silylation

All synthetic cathinones underwent rapid and complete reduction using the conditions described, with no parent drug detected. Hydroxylated (reduced) drug was further derivatized to exploit the newly created active hydrogen. Although common silylating agents such as MSTFA and BSTFA were effective for this purpose, their reactivity towards the amine functional group produced multiple products. Trimethylsilylimidazole (TMSI) is a more selective derivatizing reagent. It is highly reactive and selective towards hydroxyls and carboxylic acids, is used without a catalyst, and does not react with aliphatic amines. Its selectivity makes it useful in polyfunctional compounds and was capable of producing a single trimethylsilyl cathinone derivative for all nineteen drugs.

Although reductive silylation was accomplished, the TMS ethers underwent extensive alpha cleavage and did not produce high quality mass spectra. **Figure 21** depicts the mass spectrum for MDPV and MDPV-TMS. The mass spectrum of derivatized drugs were still heavily dominated by the iminium ion (e.g. m/z 126) and the m/z 73 ion is not diagnostic because it arises from the trimethylsilyl group.

The two-step reductive silylation of the synthetic cathinones was complete and easy to perform. However, their respective mass spectra still lacked the necessary diagnostic ions to warrant this approach. Reduction and silylation were performed using both conventional and microwave heating. Mass spectral properties of the mono-TMS derivatives following reductive silylation are summarized in **Table 8**.

Figure 21. Comparison of mass spectra for non-derivatized and MDPV-TMS following reductive silylation.

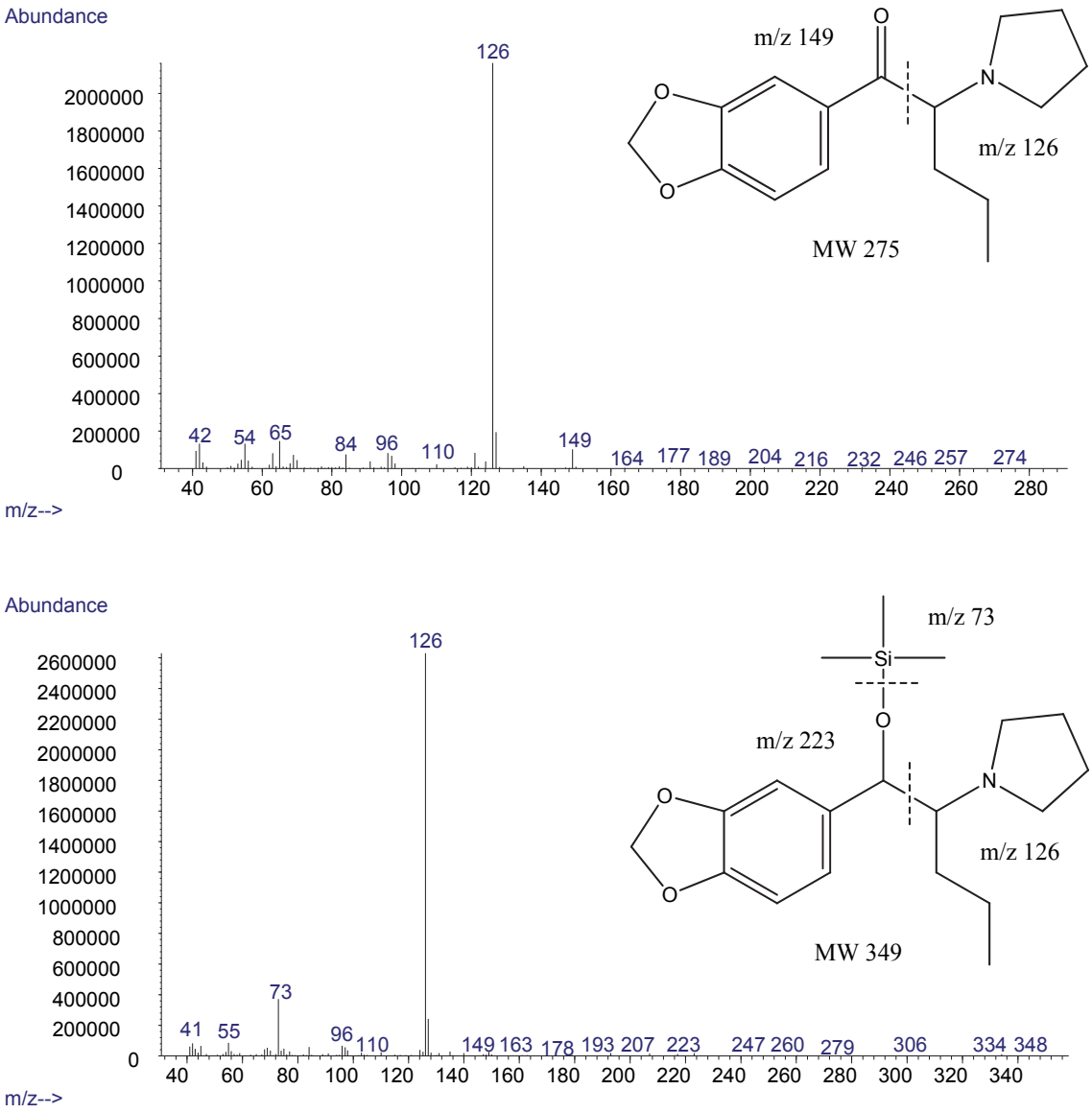


Table 8. Reductive silylation of the cathinones.

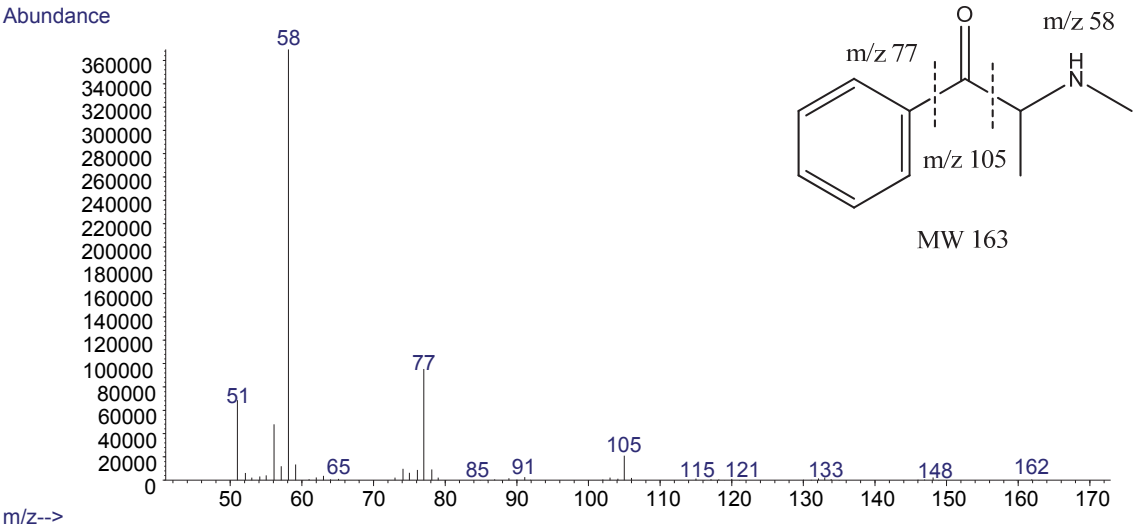
Cathinone	SIM Ions	Non-derivatized		Reductive Silylation (M+74)	
		MW	m/z	MW	m/z
Flephedrone	*58*, 95	181	**58**, 95, 123	255	**58**, 73, 125, 140
Methcathinone	*58*, 77	163	**58**, 77, 105	237	**58**, 73, 140
Ethcathinone	*72*, 44, 105	177	**72**, 44, 77, 105	251	**72**, 44, 73, 125, 140
Buphedrone	*72*, 105	177	**72**, 57, 77, 105	251	**72**, 72, 140
Mephedrone	*58*, 91	177	**58**, 91, 119	251	**58**, 73, 140, 163
Pentedrone	*86*, 44, 77	191	**86**, 44, 77, 105	265	**86**, 73, 140
4-MEC	*72*, 44, 91	191	**72**, 44, 91, 119	265	**58**, 140, 163
4-EMC	*58*, 77	191	**58**, 77	265	**58**, 73, 140
Methedrone	*58*, 77, 135	193	**58**, 135	267	**58**, 73, 209
Methylone	*58*, 121	207	**58**, 121, 149	281	**58**, 73, 223
Ethylone	*72*, 44, 149	221	**72**, 44, 149	295	**72**, 44, 73
A-PVP	*126*, 77	231	**126**, 77, 105	305	**126**, 73
Butylone	*72*, 149	221	**72**, 121, 149	295	**72**, 73, 140
MPBP	*112,* 44	231	**112**, 70, 91, 119	305	**112**, 73, 163
Pentylone	*86*, 44	235	**86**, 44, 121, 149	309	**86**, 44, 73
Pyrovalerone	*126*, 91	245	**126**, 91, 119	319	**126**, 73, 163
MDPBP	*112*, 70	261	**112**, 121, 149	335	**112**, 73
MDPV	*126*, 96	275	**126**, 121, 149	349	**126**, 73
Naphyrone	*126*, 96	281	**126**, 155	355	**126**, 73

Reductive Acylation

Preparation of trifluoroacetyl, pentafluoropropionyl and heptafluorobutyrl derivatives of cathinones is a commonly used approach (**Table 3**). This not only improves thermal stability of the drug, but significantly improves the mass spectral quality. This is illustrated in **Figure 22**, which shows the mass spectrum for methcathinone and its trifluoroacylamide derivative. However, derivatization of the active hydrogen on the amine is not possible for the pyrrolidine-containing cathinones, such as MDPV, α-PVP, pyrovalerone, naphyrone, MPBP and MDPBP.

These drugs are incapable of forming the perfluoroacyl amide. However, reductive acylation is an alternative approach that allows these tertiary amines to be acylated via the ketone functional group (**Figure 23**) to form the respective perfluoroacyl ester. However, primary and secondary amines can accept one or two acylated groups respectively, forming mono or di-derivatives, or a combination of both.

Figure 22. Mass spectrum of methcathinone in its non-derivatized (top) and trifluoroacetylated form (bottom).



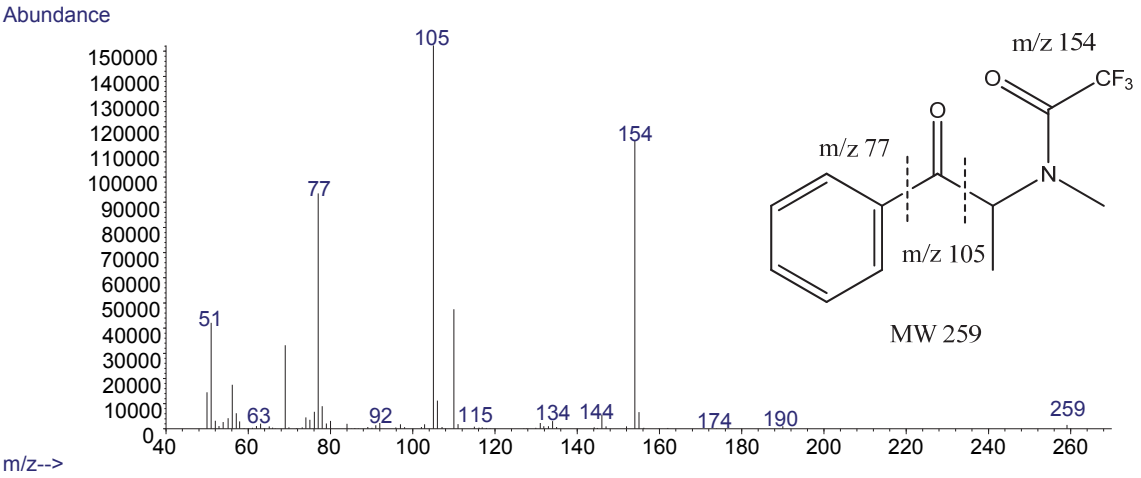
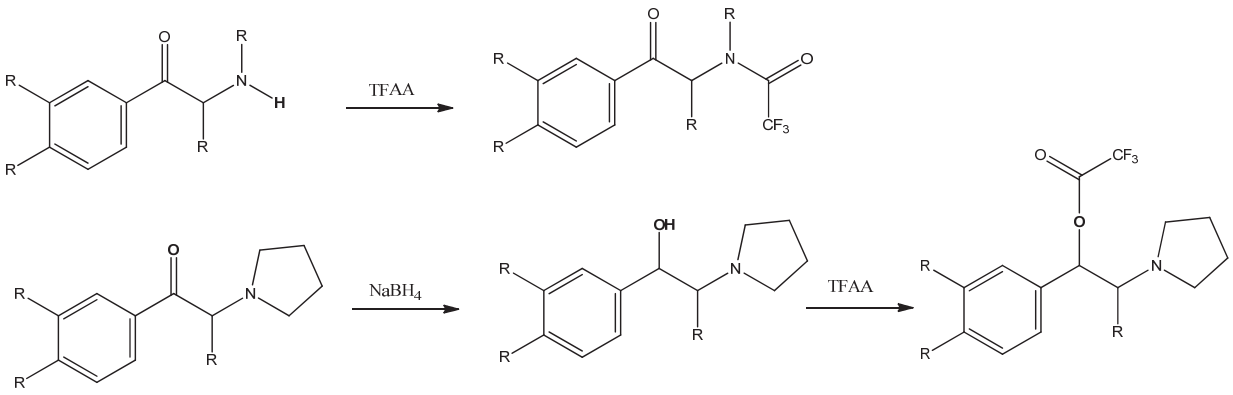


Figure 23. Acylation of the amine to produce a trifluoroacetyl amide (top) and reductive acylation to produce the trifluoroacetyl ester (bottom).



Pentafluoropropionyl and heptafluorobutyrl derivatives were prepared using PFPA and HFBI. Neither were capable of derivatizing the hydroxyl group following reduction, despite the fact that the reduction step was quantitative. In contrast, derivatization of the amine was always successful for the polyfunctional cathinones (secondary amines). The heptafluorobutyryl and pentafluoropropionyl groups are relatively bulky groups. Their inability to react at the hydroxyl, while efficiently reacting at the amine suggested steric hindrance. Acylation of the hydroxyl was successful using acetic anhydride (AA) and trifluoroacetic acid anhydride (TFAA) (**Figure 24**). Although MDPV could be differentiated from the reductive acylated derivative by its retention time (**Figure 25)**, their mass spectra were virtually identical due to the loss of the acetyl group during fragmentation (**Figure 26**). Additionally, unlike TMSI which is selective towards the hydroxyl group, TFAA and AA react at both the alcohol and amine, producing multiple derivatives which can be problematic from the standpoint of quantitation. This, together with the lack of diagnostic ions for the derivatized pyrrolidines-type cathinones made reductive acylation less attractive.

Figure 24. Reductive acylation of MDPV using acetic anhydride (AA) and trifluoroacetic anhydride (TFAA).

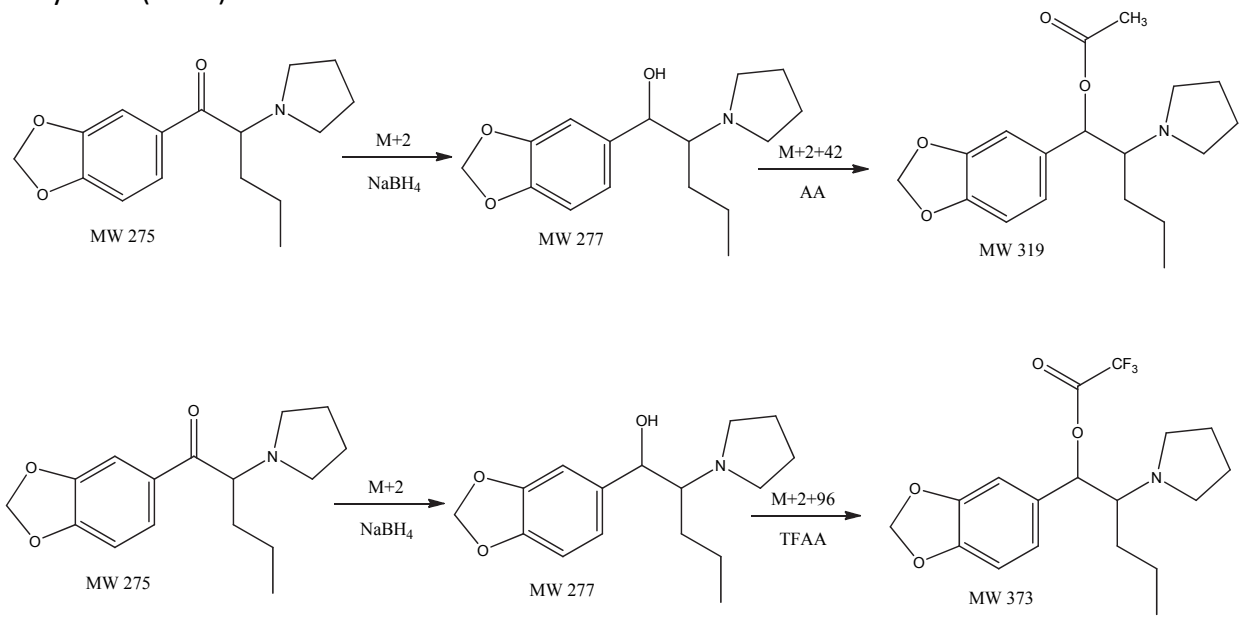


Figure 25. Retention time of MDPV before (top) and after reductive acylation (bottom).

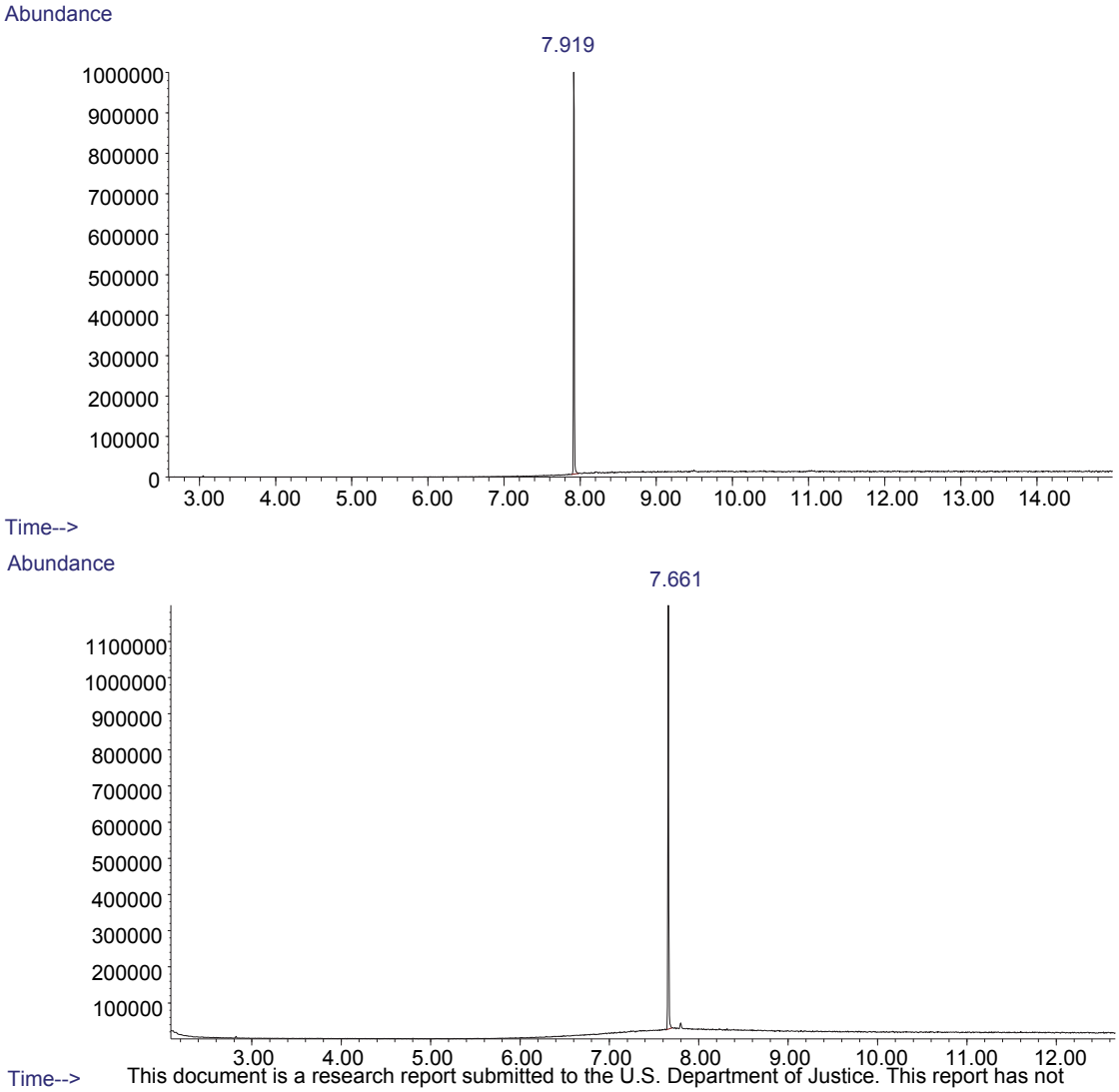
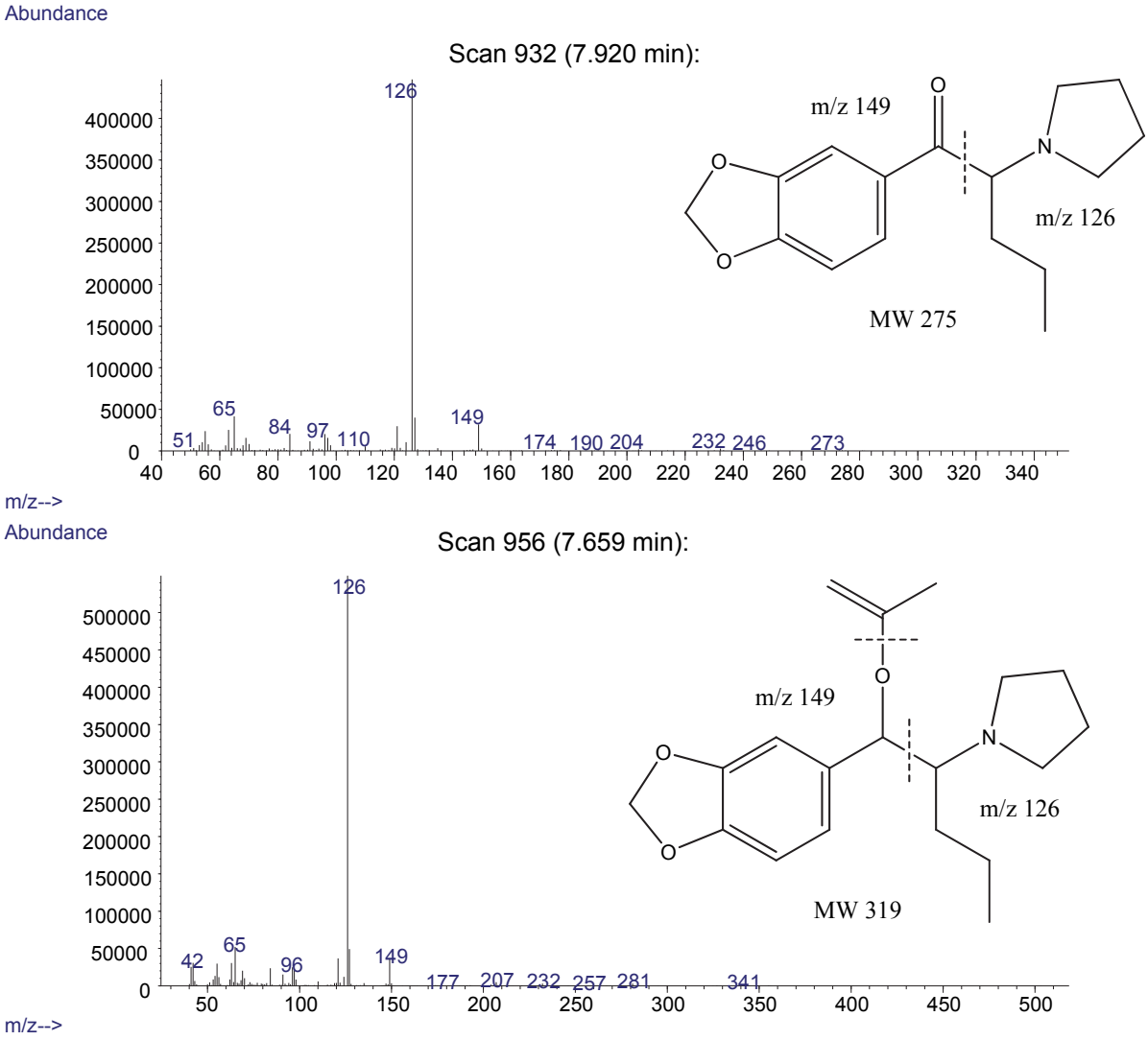


Figure 26. Mass spectra for MDPV before (top) and after reductive acylation (bottom).



GC/MS Analysis of Non-Derivatized Cathinones

The optimized conditions described earlier produced effective separation and peak shape for all eighteen non-derivatized cathinone standards (**Figure 27**). For these non-extracted standards, PCP was included as a retention time reference. Relative retention times for all target analytes are shown in **Table 10**.

Figure 27. Separation of target analytes by GC/MS.

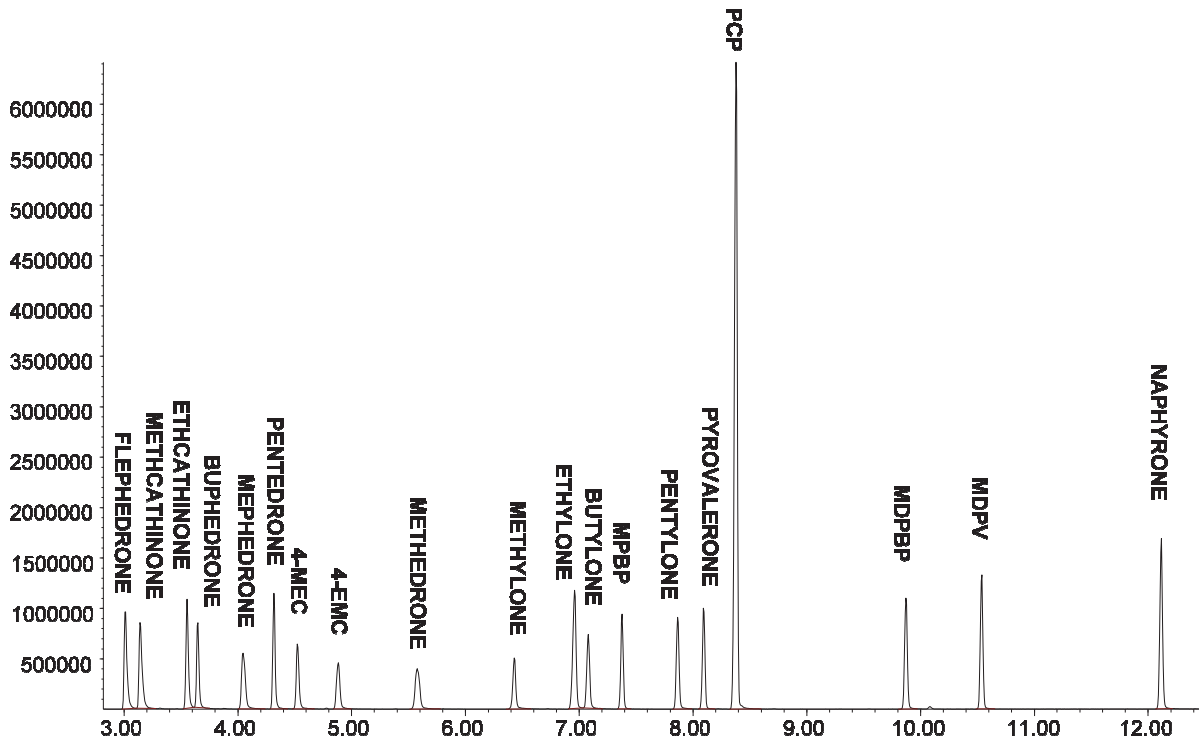


Table 10. Retention times of synthetic cathinones (relative to PCP).

Cathinone	Retention Time	Relative Retention Time
Flephedrone	3.012	0.360
Methcathinone	3.142	0.375
Ethcathinone	3.555	0.425
Buphedrone	3.65	0.436
Mephedrone	4.042	0.483
Pentedrone	4.322	0.516
4-MEC	4.527	0.540
4-EMC	4.875	0.582
Methedrone	5.548	0.662
Methylone	6.395	0.763
Ethylone	6.936	0.828
Butylone	7.07	0.844
MPBP	7.383	0.882
Pentylone	7.863	0.939

Pyrovalerone	8.102	0.967
MDPBP	9.879	1.179
MDPV	10.544	1.259
Naphyrone	12.123	1.448

Formation of GC artifacts

However, during the course of method development, we observed significant shouldering with subtle mass spectral changes, in what appeared to be an artifact peak. Temperature programming was optimized to maximize separation of these suspected "artifacts", which fell into three broad categories: mixed or unresolved (flephedrone, ethcathinone, buphedrone, pentedrone, 4-MEC, ethylone, butylone, pentylone); partially (near baseline) resolved (mephedrone, 4-EMC, methcathinone); and fully resolved artifact peaks (methylone, methedrone, MPBP, MDPV, MDPBP, pyrovalerone, naphyrone). Representative examples from each of these categories are shown in **Figure 28**. Extracted ion chromatograms (m/z 58 and 56) clearly show the flephedrone artifact, which shares the same ions as the parent drug, with the notable exception of the base peak (-2 Da). Since the m/z base peak originates from the iminium ion, this 2 Da loss is attributed to the loss of 2H (**Figure 29**). Partially and fully resolved species produced similar results, but were more readily identifiable due to chromatographic separation. **Figure 30** depicts the spectra and fragmentation for MDPV and the 2,3-enamine degradation product. The proposed structures were also verified using deuterated analogs. The mass spectrum, fragmentation pathway and structure for the MDPV-D8 2,3-enamine degradation product (MW 281) are shown in **Figure 31**. Chromatographic and mass spectral data for all eighteen drugs and their respective degradation products are summarized in **Table 11**. Small changes in absolute retention time were present due to routine column maintenance *etc*. The characteristic 2 Da shift was evident for all eighteen of the cathinone degradation products in this study, most prominently in the base peak, but also in the molecular ion (when present). Notably, the degradation products for the pyrrolidinyl-containing cathinones produced very stable molecular ions of high abundance, relative to their secondary amine counterparts.

Figure 28. Artifact peaks during GC analysis showing resolved (MDPV), partially resolved (methylone) and unresolved (flephedrone) species.

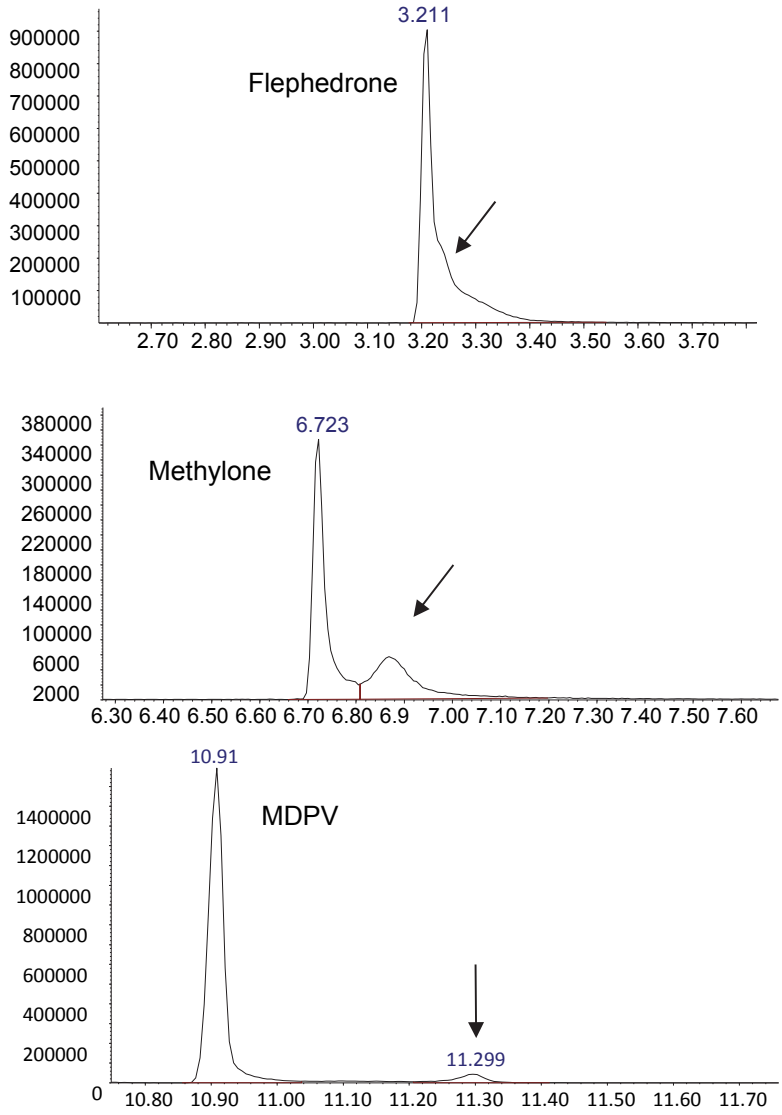


Figure 29. Mass spectra and proposed fragments for flephedrone (MW 181) and its artifact (MW 179).

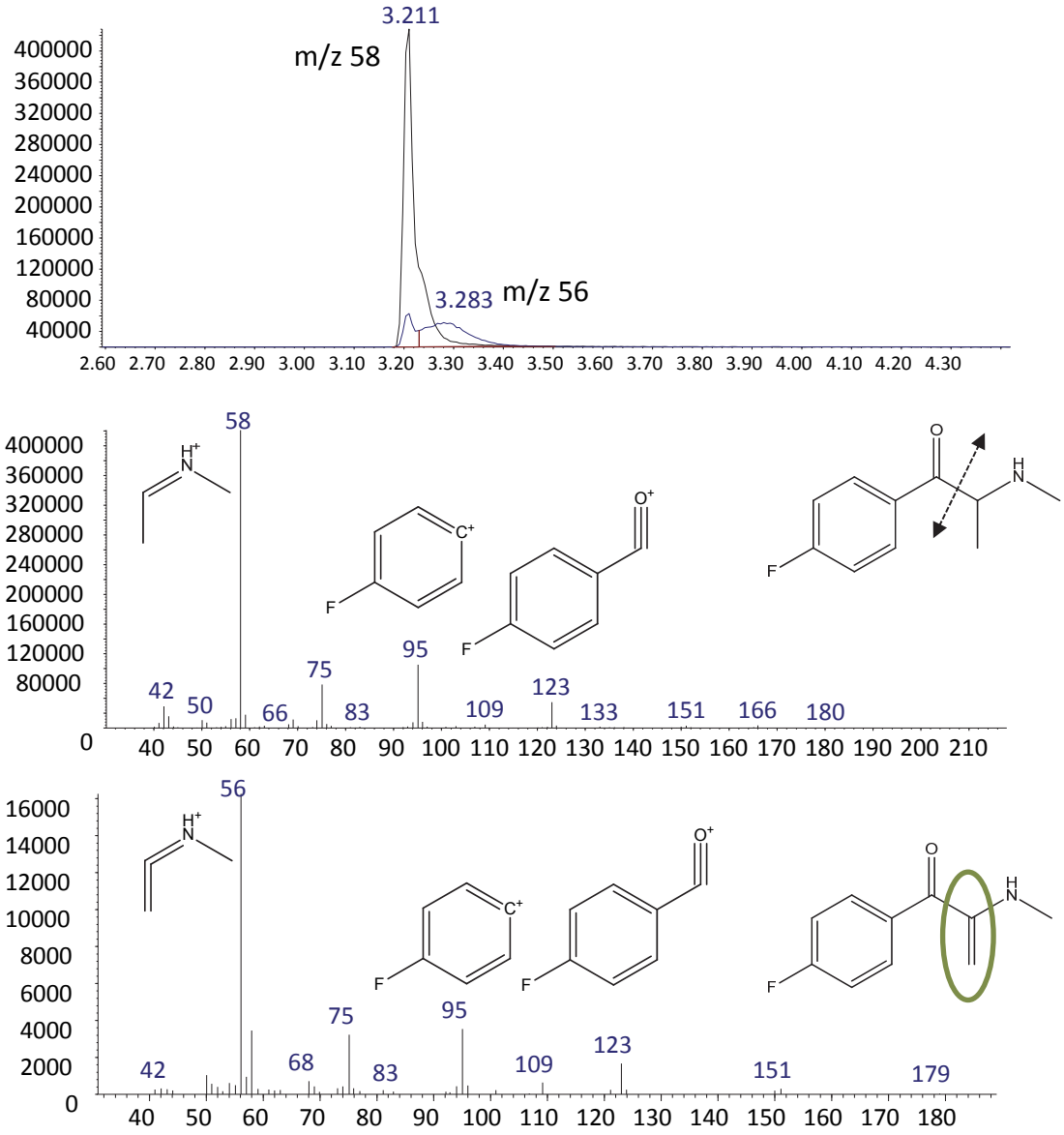


Figure 30. Mass spectra and fragmentation for MDPV (MW 275) and the 2,3-enamine degradation product (MW 273).

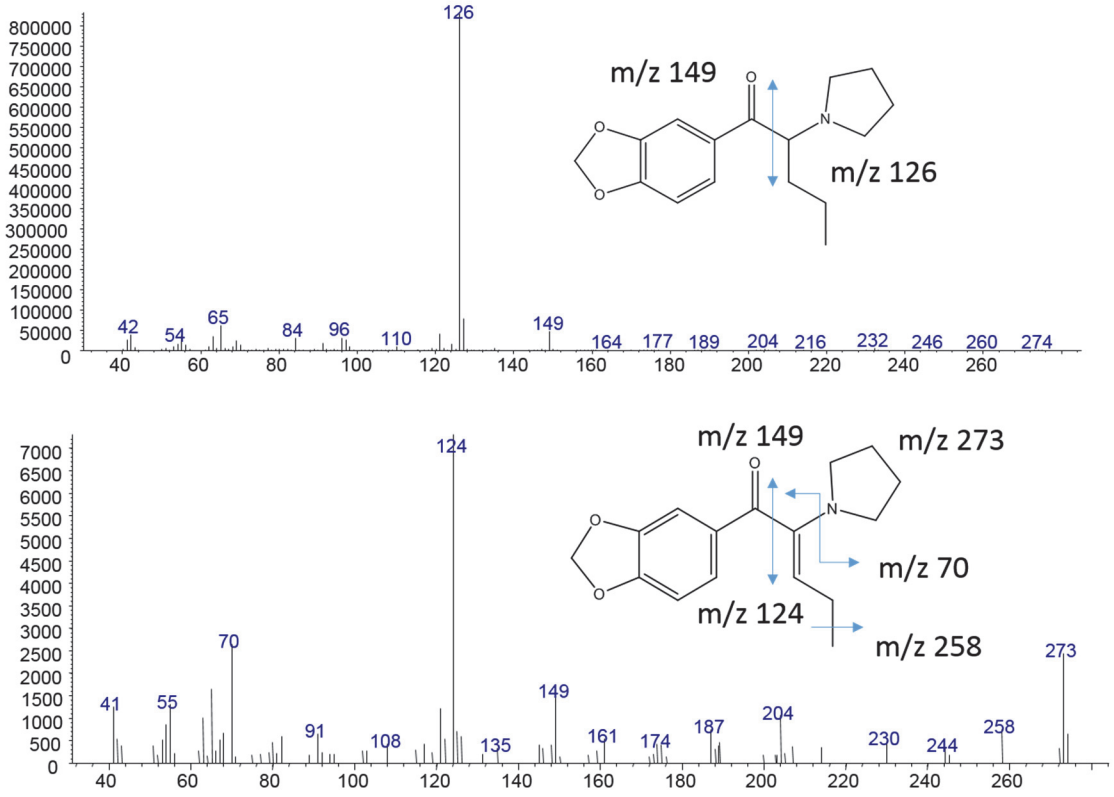


Figure 31. Verification of 2,3-enamine degradation product using MDPV-D8.

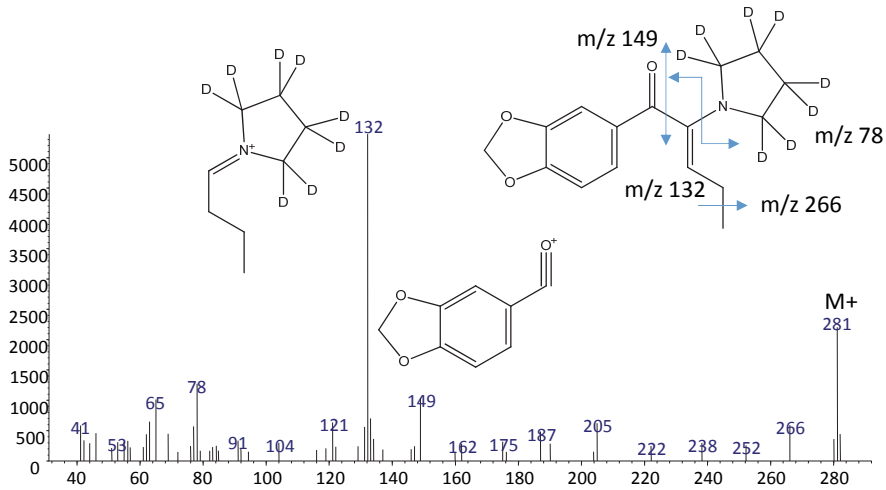


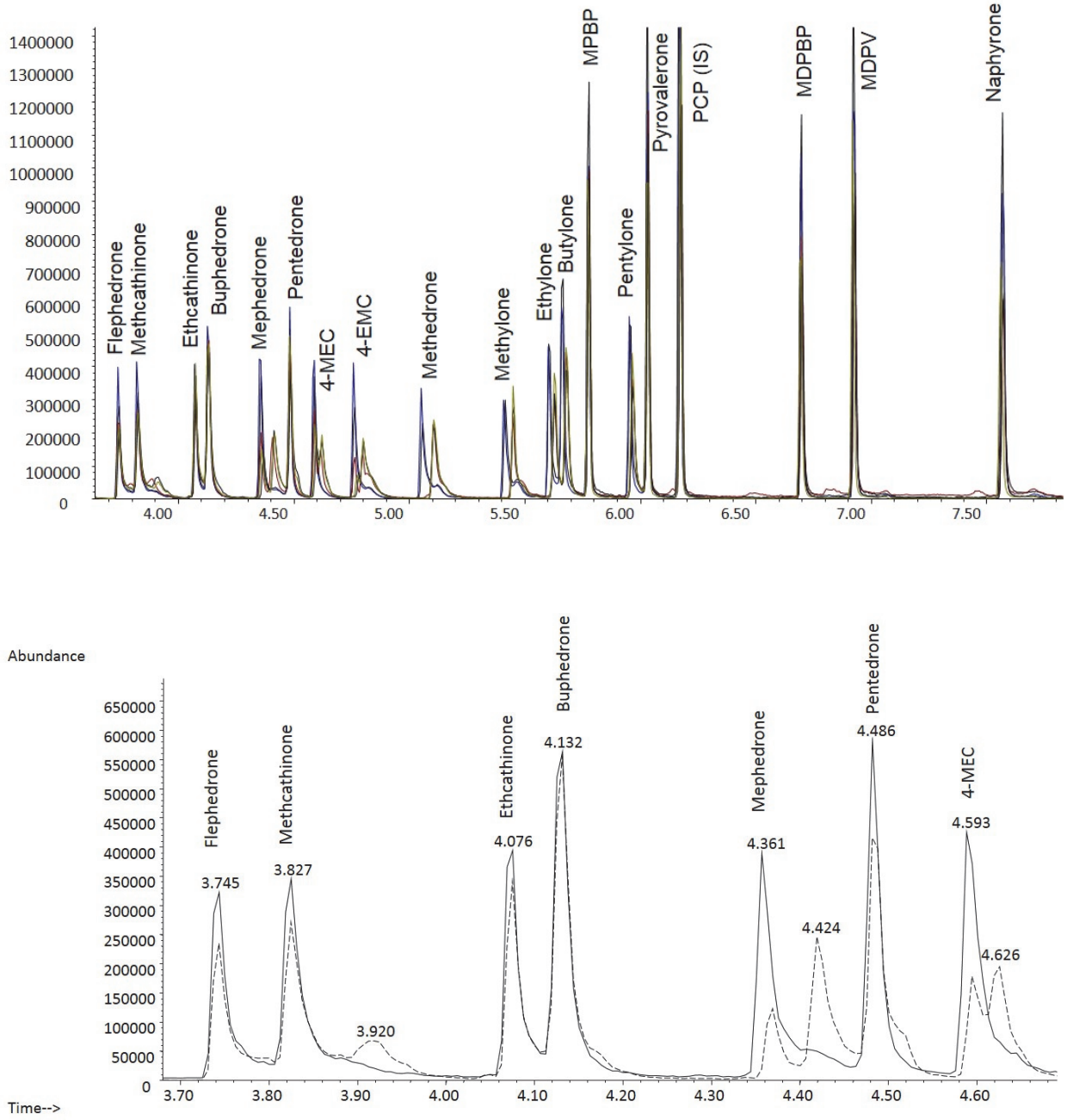
Table 11. Chromatographic and mass spectral data for the target analytes and their artifacts due to oxidative degradation. Cathinones are listed in retention time order. Base peaks are shown in bold and molecular ions are underlined. Molecular ions of high abundance are indicated by an asterisk.

Drug	Parent			Artifact		
	MW	RT	Base Peak (m/z)	MW	RT	m/z
Flephedrone	181	3.210	**58**	179	3.283	**56**, 75, 95, 123, 179
Methcathinone	163	3.348	**58**	161	3.446	**56**, 77, 105
Ethcathinone	177	3.764	**72**	175	3.766	42, **70**, 77, 105, 175
Buphedrone	177	3.866	**72**	175	3.821	**70**, 77, 105
Mephedrone	177	4.292	**58**	175	4.416	**56**, 119, 175
Pentedrone	191	4.554	**86**	189	4.554	42, 77, **84**, 105
4-MEC	191	4.782	**72**	189	4.839	42, 65, **70**, 91, 119, 189
4-EMC	191	5.155	**58**	189	5.301	**56**, 160, 189
Methedrone	193	5.844	**58**	191	6.000	**56**, 77, 92, 107, 135, 160, 191
Methylone	207	6.723	**58**	205	6.871	**56**, 65, 121, 149, 176, 205
Ethylone	221	7.259	**72**	219	7.314	42, 65, **70**, 91, 121, 149, 219
Butylone	221	7.396	**72**	219	7.358	**70**, 121, 149, 219
MPBP	231	7.718	**112**	229	8.361	41, 70, 91, **110**, 119, 145, 214, 229*
Pentylone	235	8.205	**86**	233	8.205	65, **84**, 121, 149
Pyrovalerone	245	8.427	**126**	243	8.849	70, 91, 119, **124**, 159, 228, 243*
MDPBP	261	10.244	**112**	259	10.875	41, 70, **110**, 121, 149, 190, 230, 259*
MDPV	275	10.910	**126**	273	11.299	70, **124**, 149, 204, 244, 258, 273*
Naphyrone	281	12.534	**126**	279	12.852	70, **124**, 155, 250, 264, 279*

Once artifacts for all eighteen cathinones had been identified, a series of time and temperature dependent variables were investigated. Total ion chromatograms of the methanolic drug standards (0.1 mg/mL) and internal standard, injected five times over five day intervals (25-days total) are shown in **Figure 32**. The total ion chromatogram indicated small but observable artifact peaks that increased over time. **Figure 32** also shows the phenomenon more clearly for

a representative portions of the chromatogram, injected ten days apart on the same instrument.

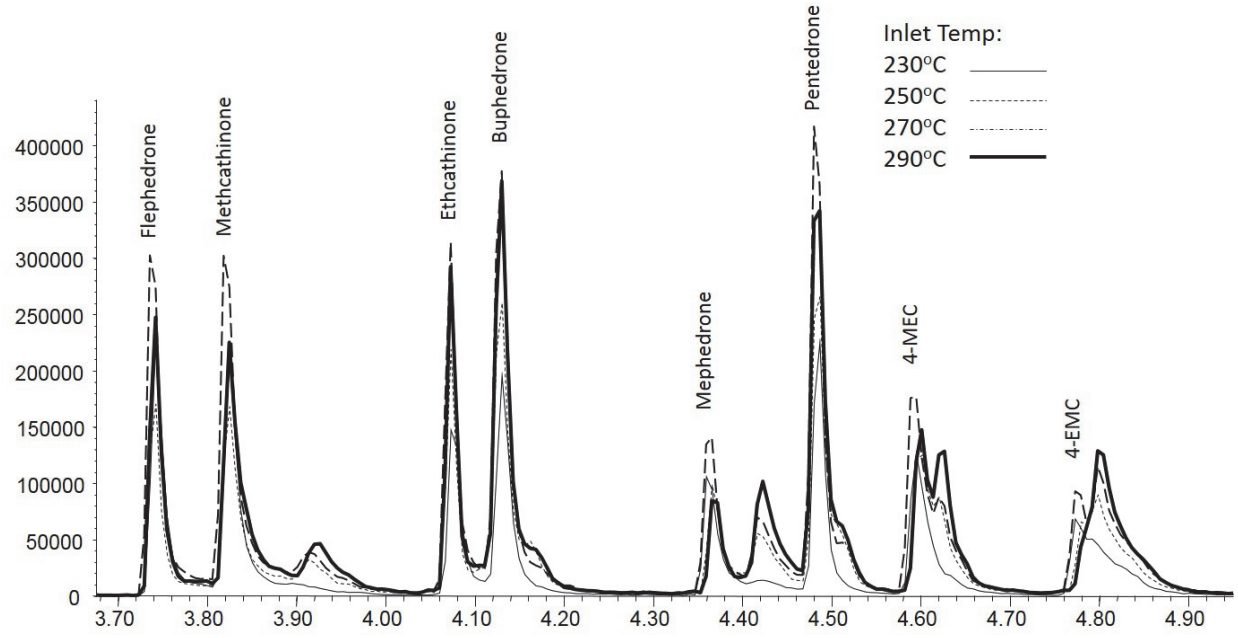
Figure 32. Increases in artifact peaks over 25 days at 5 day intervals (top) and a representative portion of the chromatogram showing the formation of artifacts 10 days apart (bottom).



Injection of the same drug standard mix on an instrument with a new deactivated liner, gold seal and column caused these artifacts to completely disappear, suggesting that these

phenomena were associated with thermal instability and active sites inside the GC. Based on these preliminary results, the injector temperature and residence time inside the injector were explored. Injection port temperature was found to have considerable influence on the appearance of thermal degradation products. **Figure 33** depicts a representative section of the total ion chromatogram using injector temperatures between 230 and 290°C. This data clearly shows the increasing abundance of the artifact peak with increasing temperature. The injection port temperature is highly relevant because it must be sufficiently high to completely and efficiently vaporize the sample, but not to cause thermal decomposition. Although the initial injector temperature was initially 260°C, it was optimally set at 185°C (**Figure 34**). Active sites produced by contaminants, or the loss of deactivated sites on inlet surfaces both have the potential to catalyze decomposition reactions and diminish assay performance. As a result, the influence of residence time inside the injector was evaluated using split and splitless injections.

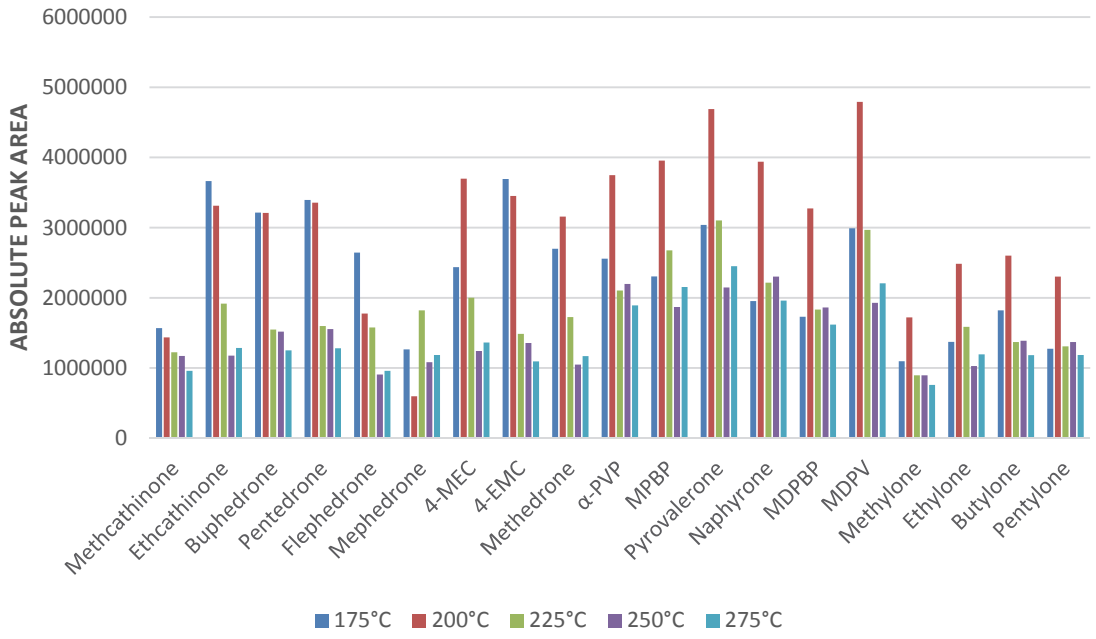
Figure 33. Influence of injector temperature on degradation.



During a split injection the residence time of the sample in the injection port is very short because of the very high carrier gas flow rate through the injection liner and out of the split vent. The split ratio influences not only the quantity of sample that is introduced, but its velocity. Residence time did influence in-situ degradation. This was quantitatively evaluated

using MDPV (RT 10.86 min) because it was well resolved from its enamine degradation product (RT 11.25 min). The peak area of the MDPV-enamine, relative to the combined peak area of the parent and degradation product was used to evaluate degradation. Using a splitless injection, the MDPV-enamine accounted for 10% of the total area, compared with 5% using split injections. Changes to the split ratio had limited influence (4.7 – 5.5%) and a split ratio of 1:25 was routinely used.

Figure 34. Influence of injector temperature on peak area.



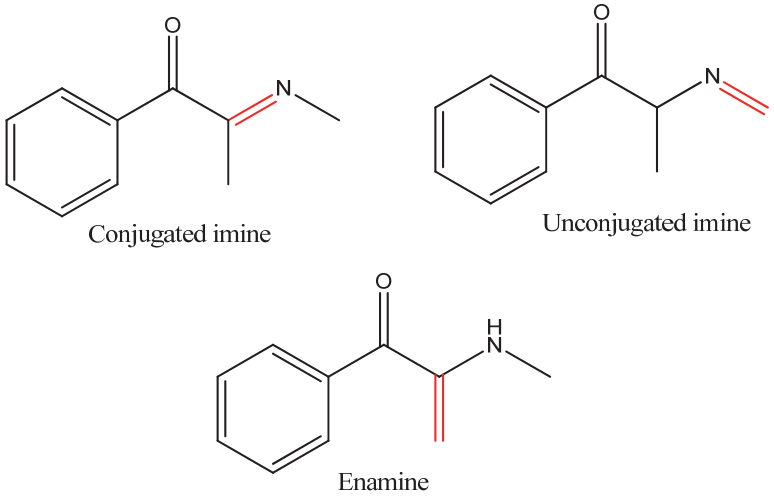
Oxidative degradation of cathinones

Although thermal decomposition products of cathinones following GC analysis were first documented two decades ago, this issue has received limited attention. Noggle (Microgram, 1994) and DeRuiter (DeRuiter, 1994) were the first to highlight the potential analytical problems that may arise when the decomposition product coelutes with the parent drug, producing a *hybrid* spectrum. This is particularly important given the low boiling point of many of the cathinones, their relatively short retention times and the fact that the mass spectra for the parent drug and degradation product are so similar and share similar ions. More recently

this phenomenon was reported by Archer using positional isomers of fluoromethcathinone (Archer, 2009) and by Tsujikawa using α-PVP (Tsujukawa, 2013a). These cathinones produced a major chromatographic peak accompanied by a second peak, or a shoulder that yielded a molecular ion 2 Da less than the parent drug.

The oxidative decomposition of a cathinone species may result in an enamine or imine (conjugated or unconjugated) as shown in **Figure 35**. The imine arises from the loss of hydrogen from the nitrogen, whereas the enamine arises from the loss of hydrogen from the carbon-carbon bond. Noggle and DeRuiter were the first to propose the structure of the breakdown product for methcathinone. In order to determine which was most likely, *N,N*-dimethylmethcathinone and diethylcathinone (diethylpropion) were investigated, because these tertiary amines lacked an available hydrogen and were incapable of forming the imine species. Therefore, it was postulated that should these drugs degrade in an analogous fashion, it must be attributed to the enamine. Results showed this to be the case, and the imines were considered unlikely candidate structures.

Figure 35. Oxidative decomposition of a cathinones to form imine or enamine species.



Tsujikawa (2013a) also suggested that the in-situ thermal degradation of α-PVP and its deuterated analog were attributed to the enamine. They proposed residence time and surface activity of the liner to be major factors. Under their conditions, lowering injection port temperatures (200-300°C) did not decrease the relative amount of enamine, but did improve absolute abundance of α-PVP. In our study, injector temperature did in fact play an important role for the 18 cathinones investigated. The SWGDRUG monograph for 2-fluoromethcathinone also includes the mass spectrum for the degradation product with a notable shift in base peak from m/z 58 to 56, which it attributes to the enamine (SWGDRUG, 2013). Archer (2009) reported in-situ degradation of synthetic cathinones by GC/MS, particularly 4-methylmethcathinone (mephedrone). Using regioisomers of fluoromethcathinone, the position of the fluorine atom was found to greatly influence stability by GC/MS. Analogs became progressively less stable when 4-fluoro, 3-fluoro and 2-fluoromethcathinone were evaluated. Instability was characterized by peak shouldering and mixed or hybrid spectra of the parent compound and degradation product, although this improved with derivatization (acetylation). Although derivatization is commonly found to increase thermal stability in general, the pyrrolidines (tertiary amines) do not derivatize using commonly used reagents due to the absence of an active hydrogen.

Although not the focus of this report, pH is known to play an important role. Bupropion was shown to be unstable under alkaline conditions (O'Byrne, 2010). Degradation followed first-order kinetics, with stability decreasing dramatically at high pH (>12). No degradation was observed in acidic conditions (<pH 5.0) when the drug was in its protonated state (pKa 7.9). Although hydrolysis and oxidation products were postulated, they were not investigated structurally or spectroscopically. Further, all proposed degradation pathways involved the loss of nitrogen from the molecule. A review of the mass spectra of degradation products showed that each contains a nitrogen (based on the odd mass of the molecular ion) so it is reasonable to assume that the pathways proposed for bupropion are not similar to those observed in our work. In a more recent article the same authors proposed a mechanism for the degradation of the cathinones via base-catalyzed tautomerism hydrolysis of the imine, followed by oxidation

and hydrolysis, although no data was presented (O'Byrne, 2013). In contrast, DeRuiter and Noggle (1994) used deuterium labeling experiments to demonstrate the thermal oxidation of the 2,3-carbon-carbon bond to yield the 2,3-enamine.

These drugs can also exhibit thermal degradation caused by instrumentation factors such as injection method, inlet temperature, and active surfaces on the inlet liner (Tsujikawa, 2013). Tsujikawa *et al* determined two degradation pathways for mephedrone in a basic solution (pH 12). The degradation was attributed to dissolved oxygen in solution, which was confirmed by the addition of antioxidants. The antioxidants suppressed the degradation of mephedrone. They also determined that under acidic conditions (pH 4) mephedrone, 4-FMC, 3-FMC, 2-FMC, methedrone, ethcathinone, and dimethylcathinone were stable, but degraded under neutral-to-basic pH. As the pH increased, the rate of degradation increased. MDPV was shown to be stable when refrigerated or frozen in whole blood, serum, and urine, but was unstable at room temperature over a 14 day period. Mephedrone however, was only stable in the three matrices when frozen, and showed significant degradation after 7 days when refrigerated. This is forensically relevant because it is possible that drug concentrations may decrease between collection and analysis.

Thermal instability of the arylaminoketones presents a real drawback in terms of GC/MS analysis. Although degradation may be minimal, thermal lability makes it questionable as to whether non-derivatized analogs can be accurately quantitated by GC/MS. This is particularly important given that deuterated internal standards, which may compensate for some of these losses, are not yet available for all of these cathinones. Although in-situ degradation can be minimized using lower inlet temperatures, minimizing residence time in the inlet, and by eliminating active sites, these factors must be properly investigated and evaluated during method development, validation and routine testing.

GC/MS Analysis of Cathinones in Blood and Urine

The toxicological assay was optimized for all nineteen synthetic cathinones. Based on the possibility of oxidative degradation in-situ and the absence of deuterated internal standards for all of the target analytes, a quantitative GC/MS assay was not pursued. Although five representative internal standards were evaluated, MDPV-D8 provided optimal results. Although the assay was not intended for quantitative purposes, R^2 values for calibration curves (0 to 1,000 ng/mL) were evaluated. Retention times, target ions and R^2 values for cathinones in the toxicology assay are summarized in **Table 12**. Total ion chromatograms for the positive (250 ng/mL) and negative urine controls are shown in **Figure 36** demonstrate the cleanliness of the extracts. An expanded view of the chromatogram in **Figure 37** shows more clearly the separation between ethylone and PVP in the optimized assay and the mass spectrum of the internal standard (MDPV-D8).

Table 12. Retention times of synthetic cathinones in the optimized toxicology assay.

Cathinone	Retention Time	R^2	Target Ion (m/z)
4-EMC	4.784	0.991	58
4-MEC	4.434	0.994	72
Buphedrone	3.574	0.995	72
Butylone	6.961	0.989	72
Ethcathinone	3.478	0.997	72
Ethylone	6.830	0.990	72
Flephedrone	2.947	0.998	58
MDPBP	9.757	0.987	112
MDPV	10.421	0.995	126
MDPV-D8 (I.S.)	10.381	-	134
Mephedrone	3.963	0.995	58
Methcathinone	3.076	0.995	58
Methedrone	5.456	0.991	58
Methylone	6.293	0.993	58
MPBP	7.274	0.989	112
Naphyrone	12.005	0.996	126
Pentedrone	4.231	0.995	86
Pentylone	7.751	0.987	86
α-PVP	6.859	0.990	126
Pyrovalerone	7.988	0.988	126

Figure 36. Total ion chromatogram of positive control (250 ng/mL) in urine (top) and negative control (bottom).

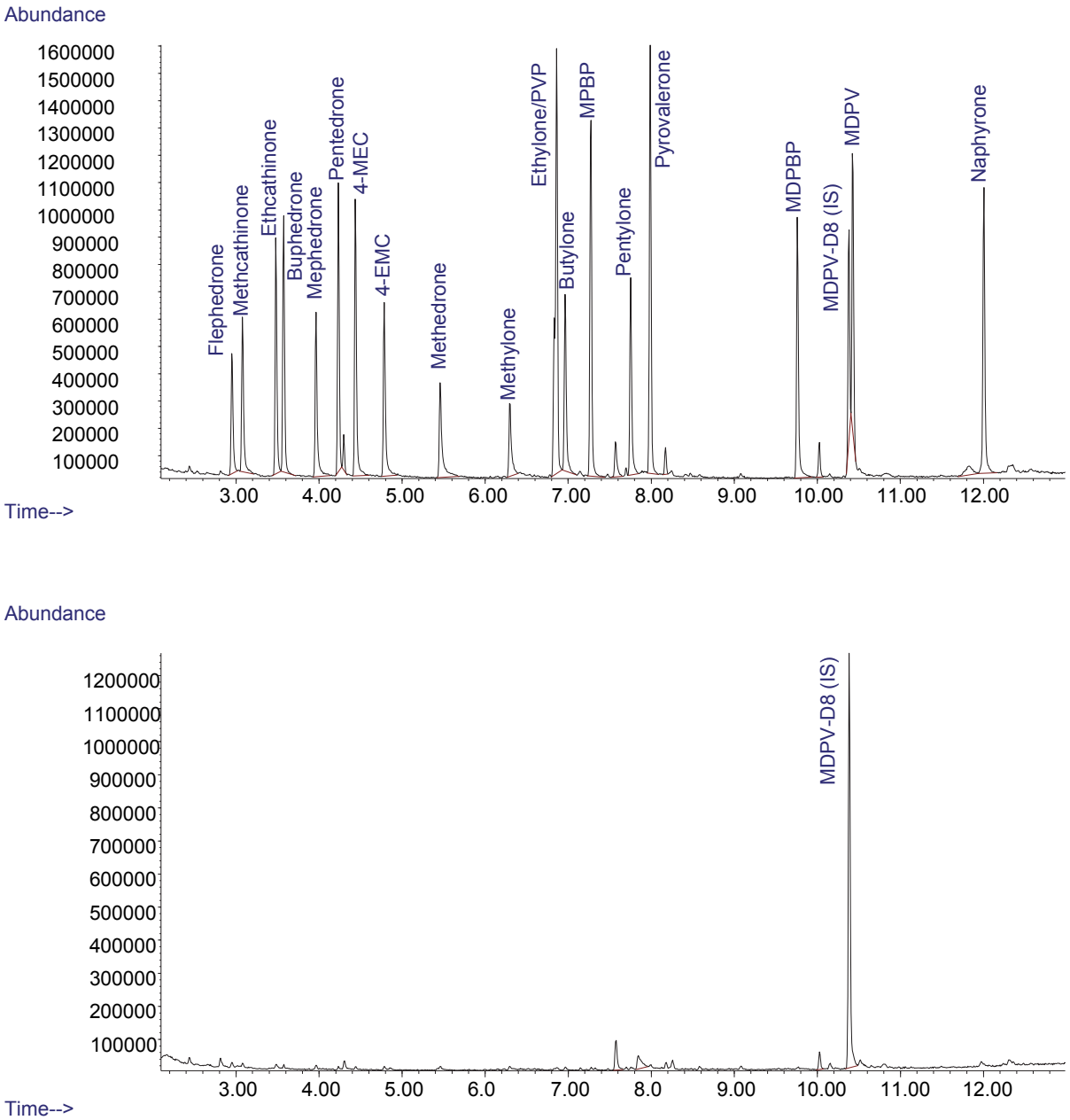
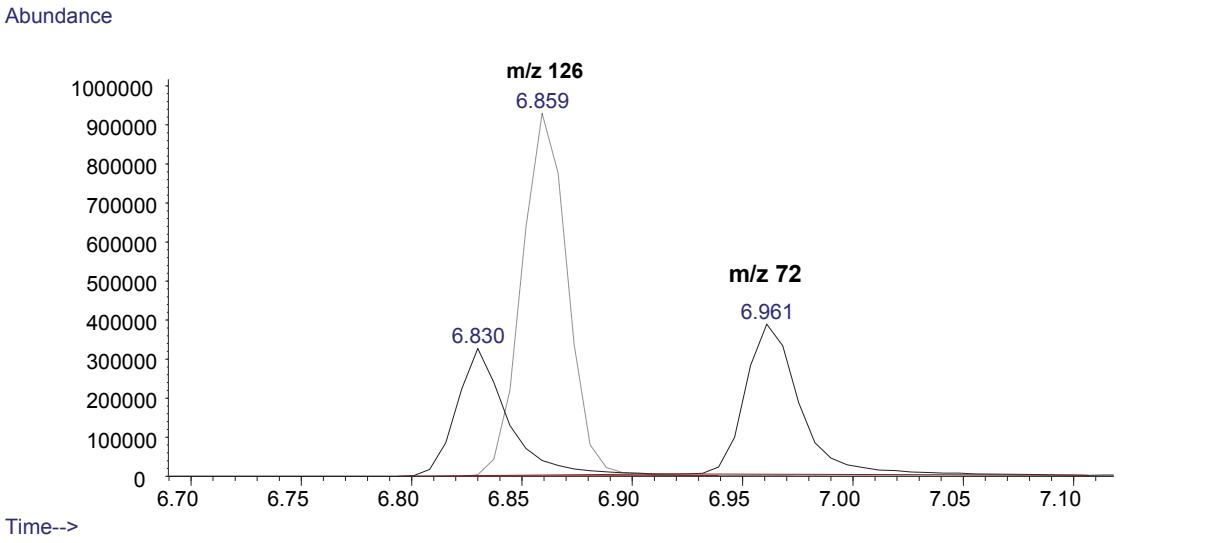
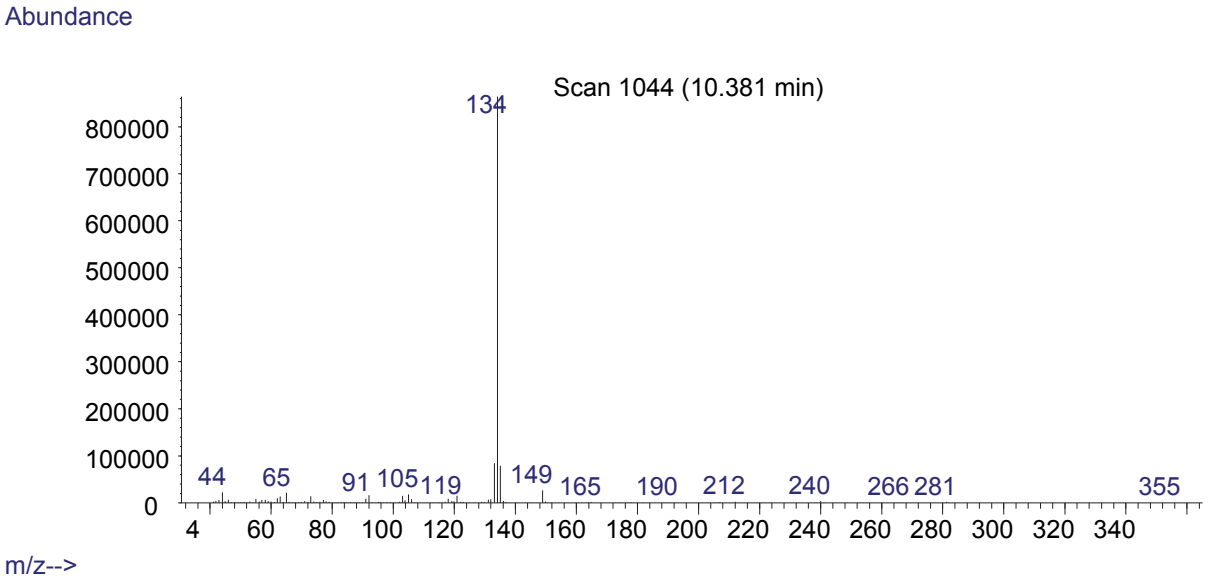


Figure 37. Extracted ion chromatograms showing separation of ethylone (m/z 72), α-PVP (m/z 126) and butylone (m/z 72) in the positive urine control (250 ng/mL) and full scan mass spectrum of the internal standard, MDPV-D8.





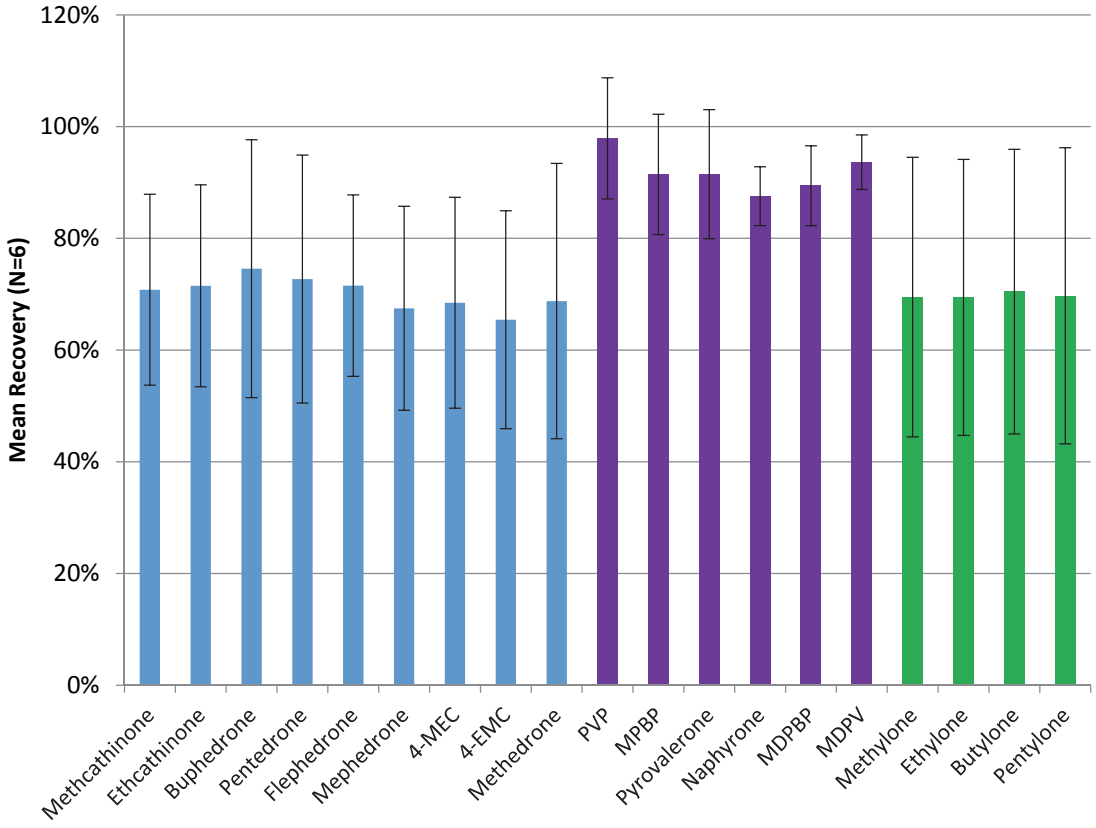
Analytical Recovery

Analytical recoveries for blood and urine are summarized in **Table 13**. However, there were noticeable difference in reproducibility between secondary and tertiary (pyrrolidinyl) species (**Figure 38**). Although some of the target cathinones fall into more than one category (i.e. pyrrolidine and methylenedioxy-type), recoveries were on average higher and more reproducible for the tertiary amines that contained the pyrrolidinyl group.

Table 13. Analytical recovery of synthetic cathinones from urine and blood.

Drug	Analytical Recovery	
	Urine (N=6)	Blood (N=3)
4-EMC	65%	58%
4-MEC	68%	70%
Buphedrone	75%	69%
Butylone	70%	61%
Ethcathinone	72%	71%
Ethylone	69%	63%
Flephedrone	72%	57%
MDPBP	89%	67%
MDPV	94%	73%
Mephedrone	67%	57%
Methcathinone	71%	59%
Methedrone	69%	47%
Methylone	69%	50%
MPBP	91%	71%
Naphyrone	88%	67%
Pentedrone	73%	71%
Pentylone	70%	62%
α-PVP	98%	73%
Pyrovalerone	91%	71%

Figure 38. Mean recovery (N=6) of synthetic cathinones from urine by chemical classification: Simple (secondary) cathinones (blue), pyrrolidinyl or tertiary amines (purple) and methylenedioxy analogs (green). Error bars represent one standard deviation.



Limit of Detection

Limits of detection (LOD) were evaluated using three sources of biological matrix, analyzed in duplicate over three days (**Table 14**). Using full scan acquisition, limits of detection in urine were 25 ng/mL for all cathinones except methylone (50 ng/mL). Limits of detection in blood were 25 or 50 ng/mL, with the exception of ethcathinone, methedrone and methylone (100 ng/mL). Although signal to noise (S/N) ratios were excellent, the limiting factor in LOD determination was the quality of the full scan spectra. **Table 15** summarizes S/N ratios at the LOD for all analytes in blood and urine using the total ion chromatogram (TIC) and the target ion.

Table 14. Limits of detection in urine and blood.

Analyte	LOD (ng/mL)	
	Urine	Blood
4-EMC	25	50
4-MEC	25	25
Buphedrone	25	25
Butylone	25	50
Ethcathinone	25	100
Ethylone	25	50
Flephedrone	25	50
MDPBP	25	25
MDPV	25	25
Mephedrone	25	50
Methcathinone	25	50
Methedrone	25	100
Methylone	50	100
MPBP	25	25
Naphyrone	25	25
Pentedrone	25	25
Pentylone	25	50
A-PVP	25	25
Pyrovalerone	25	25

Table 15. Signal to noise ratios in urine and blood at the LOD.

Analyte	Target Ion (m/z)	S/N Ratio at the LOD Urine		S/N Ratio at the LOD Blood	
		TIC	Target Ion	TIC	Target Ion
4-EMC	58	39:1	39:1	39:1	91:1
4-MEC	72	37:1	20:1	20:1	47:1
Buphedrone	72	56:1	26:1	26:1	316:1
Butylone	72	29:1	38:1	38:1	92:1
Ethcathinone	72	26:1	74:1	74:1	575:1
Ethylone	72	24:1	31:1	31:1	68:1
Flephedrone	58	8:1	42:1	42:1	90:1
MDPBP	112	78:1	35:1	35:1	72:1
MDPV	126	24:1	94:1	94:1	134:1
Mephedrone	58	40:1	39:1	39:1	104:1
Methcathinone	58	15:1	47:1	47:1	124:1
Methedrone	58	26:1	46:1	46:1	160:1
Methylone	58	33:1	68:1	68:1	110:1

MPBP	112	43:1	43:1	43:1	84:1
Naphyrone	126	22:1	31:1	31:1	67:1
Pentedrone	86	51:1	25:1	25:1	119:1
Pentylone	86	18:1	43:1	43:1	141:1
A-PVP	126	37:1	61:1	61:1	226:1
Pyrovalerone	126	17:1	28:1	28:1	97:1

Carryover

Under the conditions described, no detectable carryover was present in a drug free extract following injection of the highest blood or urine control (1,000 ng/mL).

Precision and Bias

Based on the potential for in-situ degradation during GC/MS analysis, the toxicological assay was intended for qualitative purposes only. Intra-assay precision and bias were evaluated for investigational purposes only and are summarized in **Table 16**. Linear regression over the entire calibration (0 – 1,000 ng/mL) yielded R^2 values of 0.99 or higher. Although replicate (N=6) measurements of the 200 ng/mL control were well within acceptable ranges, the stability of these drugs in biological evidence is not yet well understood.

Table 16. Intra-assay precision and bias in urine at 200 ng/mL (N=6).

Drug	CV	%Accuracy	Bias
4-EMC	1.7%	90%	-10.3%
4-MEC	1.7%	92%	-7.7%
Buphedrone	2.4%	91%	-8.8%
Butylone	2.3%	91%	-8.6%
Ethcathinone	1.9%	91%	-8.7%
Ethylone	2.3%	91%	-9.0%
Flephedrone	2.5%	88%	-11.8%
MDPBP	2.2%	93%	-7.3%
MDPV	1.9%	95%	-4.8%
Mephedrone	1.9%	91%	-9.4%
Methcathinone	2.8%	88%	-11.7%
Methedrone	3.6%	86%	-14.4%
Methylone	3.6%	86%	-13.6%
MPBP	1.2%	93%	-6.9%
Naphyrone	1.2%	93%	-7.1%

Pentedrone	2.3%	91%	-8.8%
Pentylone	2.2%	91%	-8.6%
A-PVP	1.7%	94%	-6.5%
Pyrovalerone	1.9%	94%	-6.5%

Interferences

The evaluation of interferences from drug free matrix, isotopically labeled internal standard, common drugs, structurally related compounds and endogenous bases yielded favorable results. No interferences were present in drug free urine or blood from different sources (N=10), endogenous bases or the MDPV-D8 internal standard. The interference study included more than forty compounds including ten common amphetamine-like drugs, fifteen designer drugs of the DO-, 2C and 2CT-series, fifteen common basic drugs, in addition to bupropion and diethylpropion. No false positives or false negatives were present in any of the negative controls or cathinone-positive (100 ng/mL) controls.

Analysis of Authentic Urine Samples From Cathinone Users

A total of forty authentic urine samples from cathinone users were evaluated. Results obtained using the qualitative toxicology assay described here were in excellent agreement with previously reported results from the independent reference laboratory (Redwood Toxicology Laboratory). Individual results for each sample tested are summarized in **Table 17**. Among the 40 urine samples, there were a total of 64 confirmed positive results (Reference Laboratory), of which we were able to confirm 55 during reanalysis. The time between the original test at the reference laboratory and the reanalysis by GC/MS did not exceed 12 weeks. Samples were refrigerated at all times except during transit between the laboratories.

A summary of the positive findings by drug is shown in **Table 18**. Although pentedrone was not identified in any of the retested samples, the reference laboratory reported pentedrone metabolite in its scope of testing (**Table 9**). This is a most likely explanation for the discrepant results for this cathinone. All of the samples with discrepant results were reanalyzed in our facility by liquid chromatography-quadrupole time of flight mass spectrometry (LC-Q-TOF)

which inherently more sensitive that the GC/MS assay. Among the samples that originally contained pentedrone and/or metabolites, it was possible to confirm pentedrone in 3 of the 5 samples by LC-Q-TOF (LOD 5 ng/mL).

Methylone was confirmed in 16 of the 19 samples that originally tested positive for the drug and consistently presented the greatest challenge for reconfirmation, possibly due to the stability of the drug. Reanalysis by LC-Q-TOF was able to confirm just one of the three unconfirmed methylones (Sample 15). During original testing, this sample contained 246 ng/mL methylone. Because the remaining two samples could not be confirmed by LC-Q-TOF (LOD 0.25 ng/mL), they were reanalyzed by the reference laboratory. Methylone was not detected in either sample during the retest. Originally, Sample 24 contained 327 ng/mL methylone and Sample 38 contained 183 ng/mL methylone and 88 ng/mL ethylone. LC-Q-TOF analysis was unable to detect ethylone in Sample 38 (LOD 1 ng/mL). Within six months, neither drug was detected, which lends support for the apparent instability of these drugs in biological matrices.

Representative extracts from cathinone users are shown in **Figures 39-41**. The total ion chromatogram for each extract and mass spectra for positively identified drugs are shown for Samples 7, 18 and 21. The PolyChrom Clin II SPE columns provided clean extracts with excellent analytical recoveries.

Figure 39. Total ion chromatogram for an authentic urine sample from a cathinone user (Sample 7) containing flephedrone (2.95 mins), internal standard, MDPV-D8 (10.38 mins), nicotine (3.16 mins) and cotinine (6.2 mins).

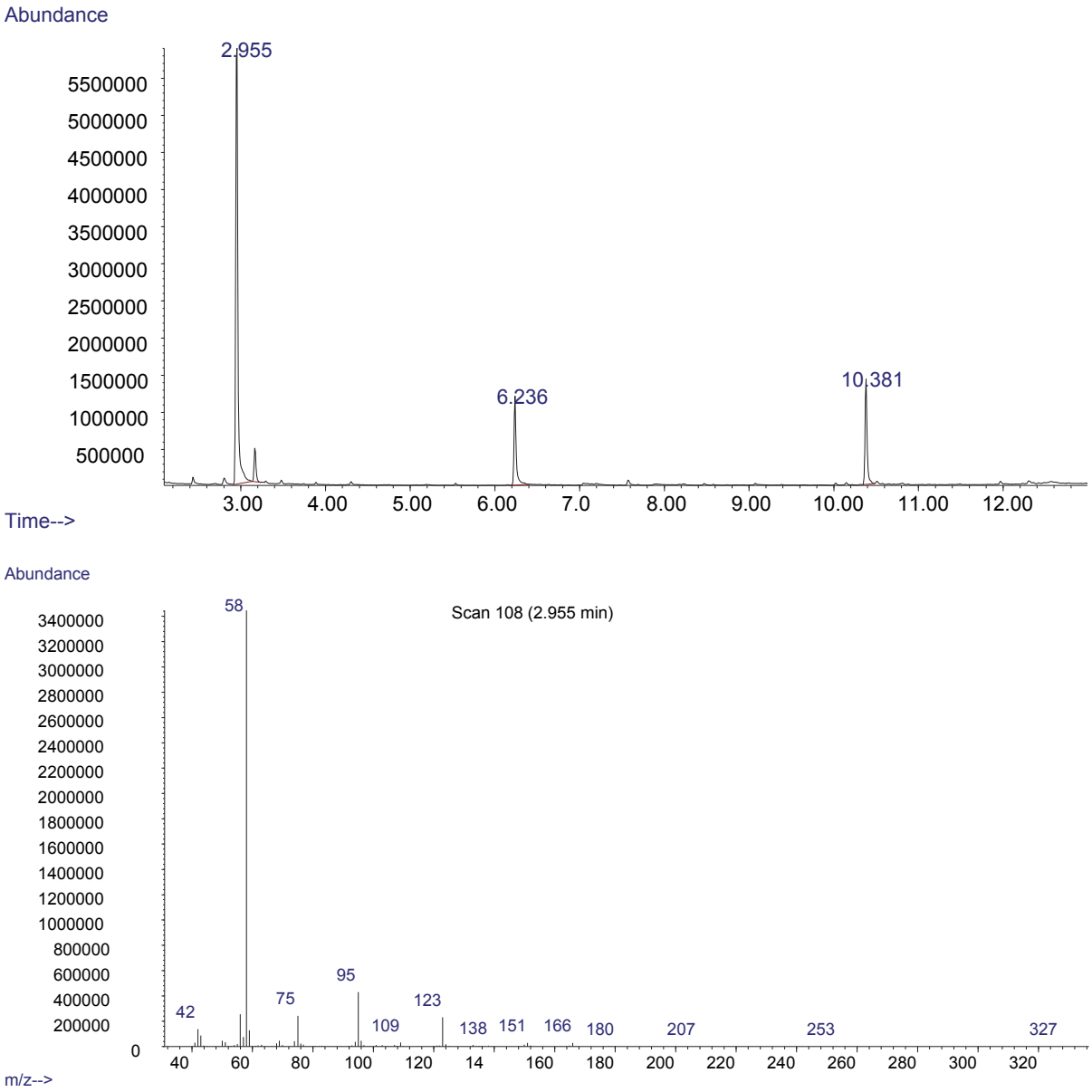


Figure 40. Total ion chromatogram for an authentic urine sample from a cathinone user (Sample 18) containing ethylone (6.83 mins), internal standard, MDPV-D8 (10.38 mins) and dextromethorphan (10.58 mins).

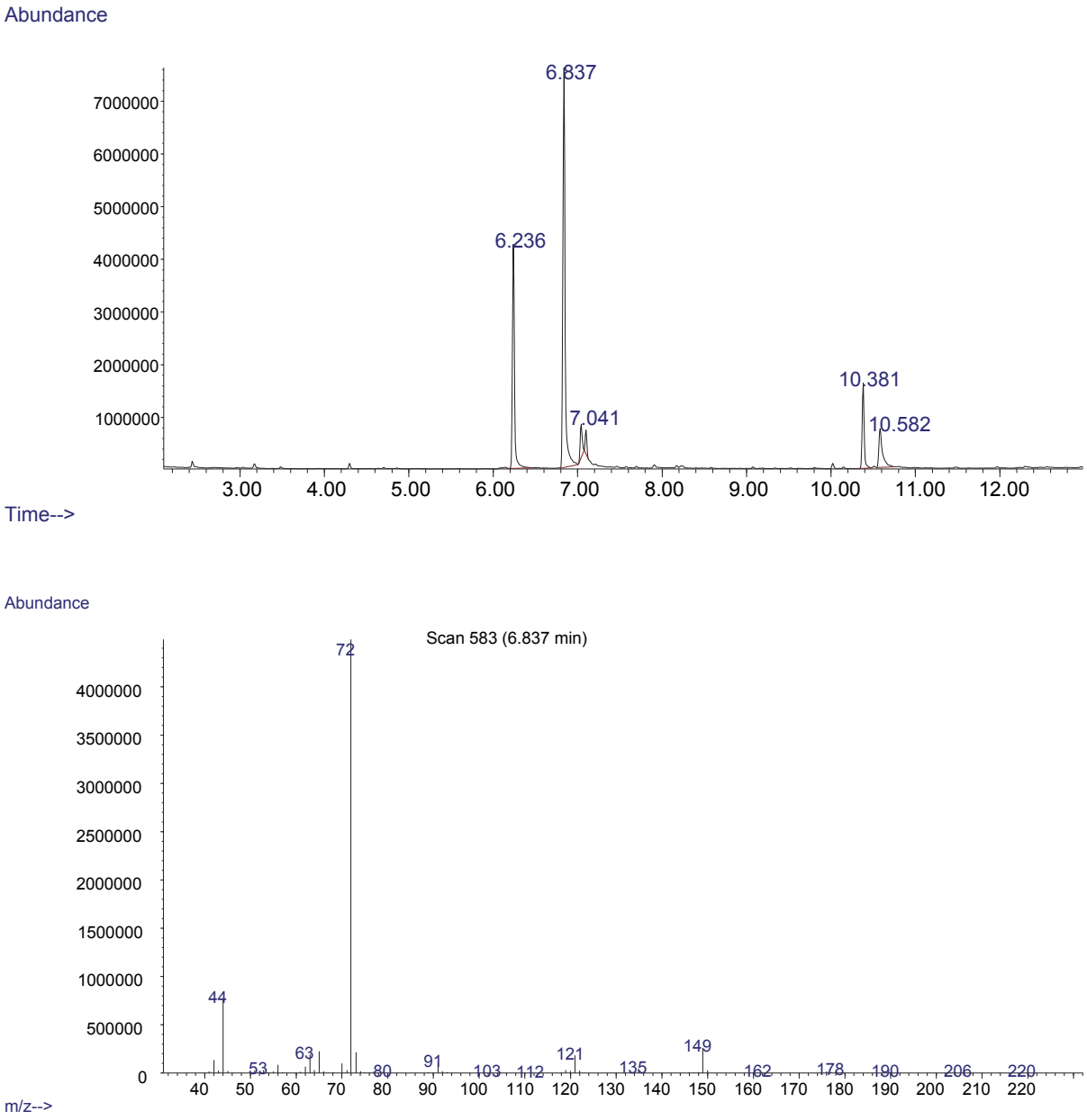
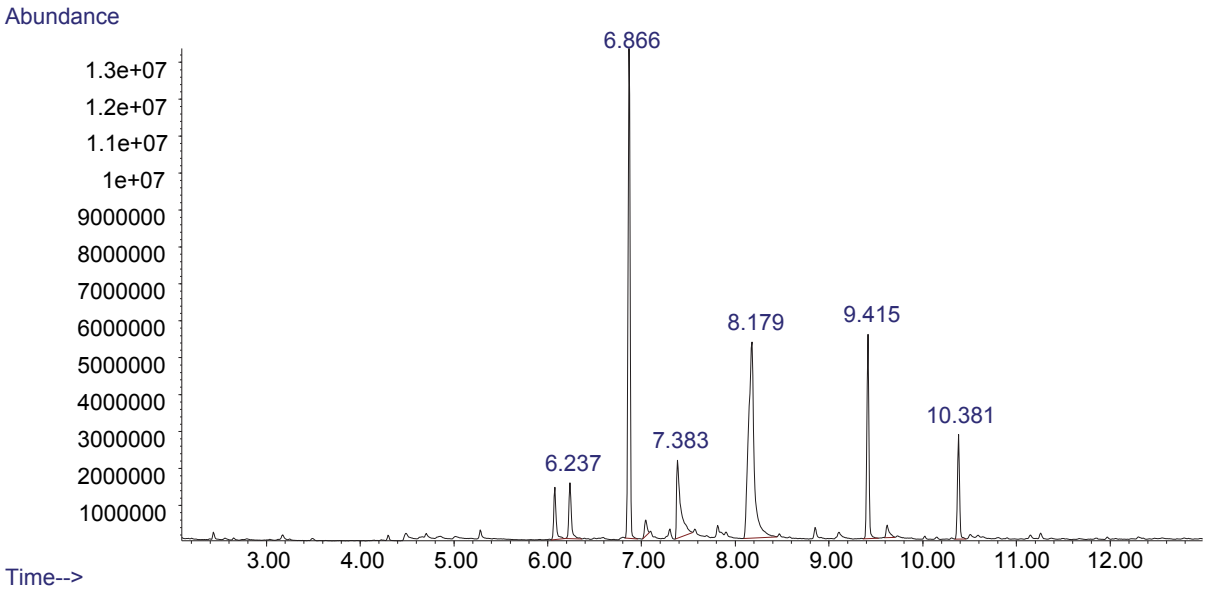


Figure 41. Total ion chromatogram for an authentic urine sample from a cathinone user (Sample 21 containing PVP (6.86 mins), internal standard, MDPV-D8 (10.38 mins) and cotinine (6.2 mins).



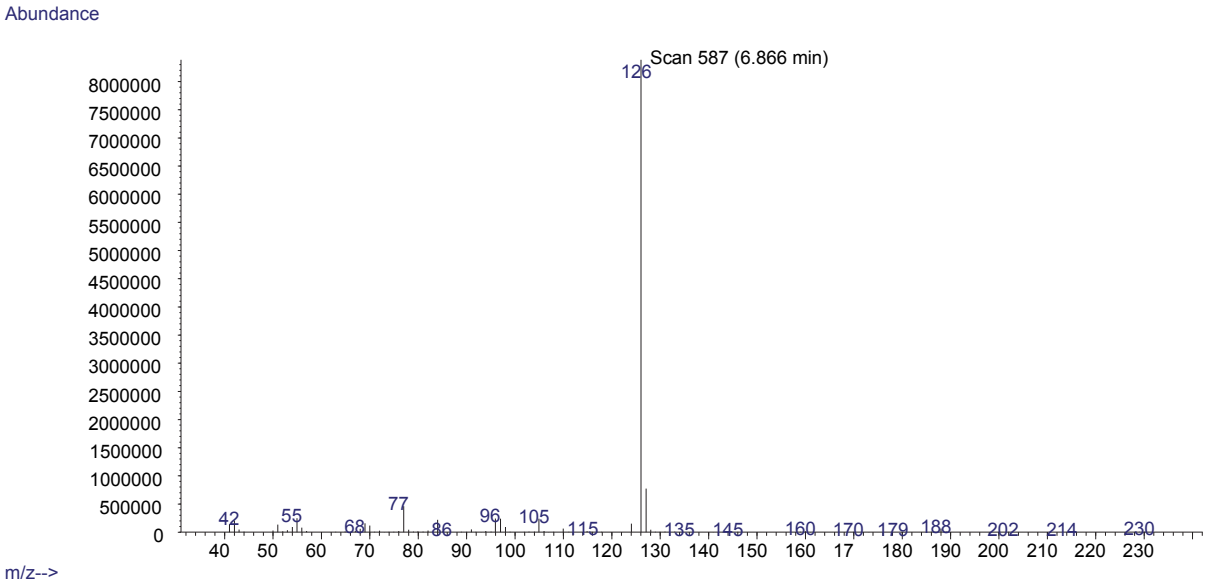
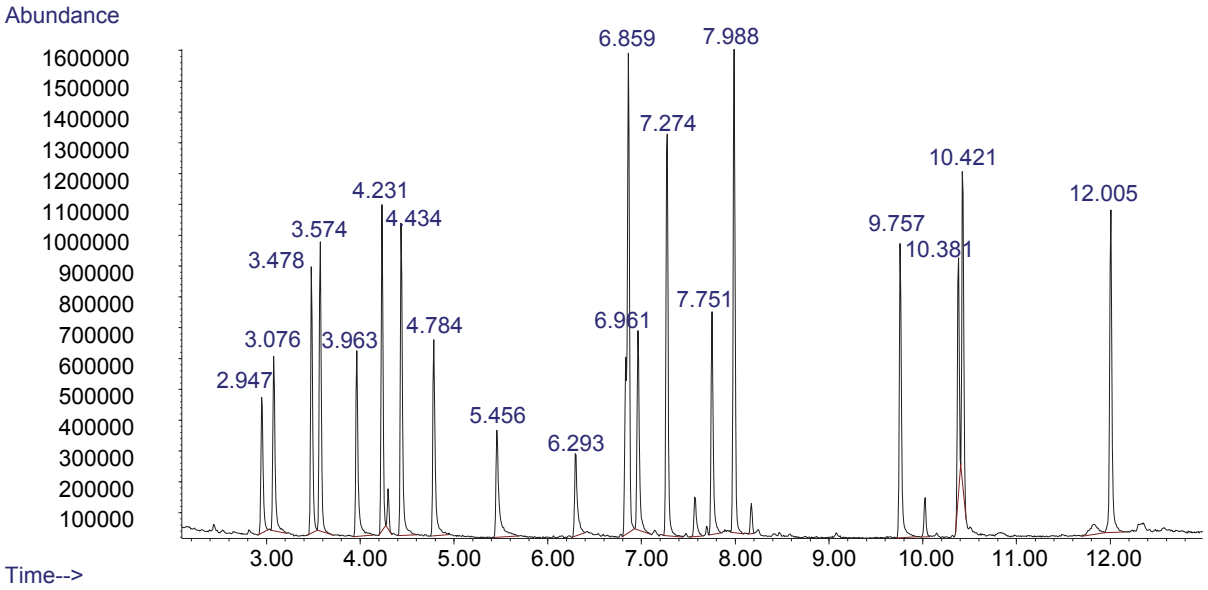


Figure 42. Total ion chromatograms for positive urine control (250 ng/mL) (top) and negative urine control (bottom), shown with internal standard, MDPV-D8 (10.38 min). Flephedrone (2.947 min), methcathinone (3.076 min), ethcathinone (3.478 min), buphedrone (3.574 min), mephedrone (3.963 min), pentedrone (4.231 min), 4-MEC (4.434 min), 4-EMC (4.784 min), methedrone (5.456 min), methylone (6.293 min), α-PVP (6.859 min), butylone (6.961 min), MPBP (7.274 min), pentylone (7.751 min), pyrovalerone (7.988 min), MDPBP (9.757 min), MDPV-D8 (10.381), MDPV (10.421 min), naphyrone (12.005 min).



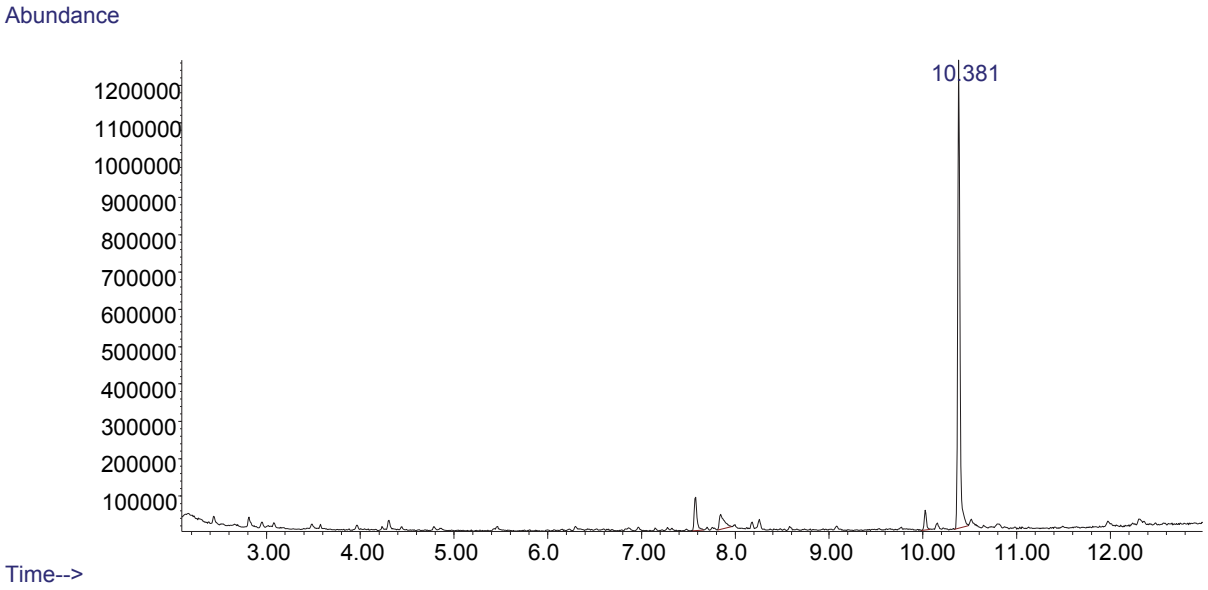


Table 17. Analysis of authentic urine samples from cathinone users. Inconsistent results are shown in bold.

Sample	Reference Laboratory Result	Confirmed Result (GC/MS)	Comment
1	Ethylone, Methylone, **Pentedrone and/or metabolite**	Ethylone, Methylone	Pentedrone confirmed by LC-Q-TOF
2	Ethylone, Methylone, **Pentedrone and/or metabolites**	Ethylone, Methylone	Pentedrone confirmed by LC-Q-TOF
3	4-MEC	4-MEC	
4	PVP, Methylone	PVP, Methylone	
5	Ethylone	Ethylone	
6	Ethylone	Ethylone	
7	Flephedrone and/or metabolite	Flephedrone	
8	Flephedrone and/or metabolite	Flephedrone	
9	PVP, Ethylone	PVP, Ethylone	
10	Pentylone	Pentylone	
11	Ethylone, Methylone	Ethylone, Methylone	
12	Ethylone	Ethylone	
13	Methylone, **Pentedrone and/or metabolite**	Methylone	Pentedrone confirmed by LC-Q-TOF
14	Ethylone	Ethylone	
15	Ethylone, **Methylone**	Ethylone	Methylone confirmed by LC-Q-TOF
16	PVP, Methylone	PVP, Methylone	
17	PVP, Methylone	PVP, Methylone	
18	Ethylone	Ethylone	
19	PVP	PVP	
20	Ethylone	Ethylone	
21	PVP	PVP	
22	Ethylone, Methylone	Ethylone, Methylone	
23	Methylone	Methylone	
24	PVP, **Methylone**	PVP	No methylone by LC-Q-TOF; No methylone on

			retest at reference laboratory
25	MDPV	MDPV	
26	PVP, **Pentedrone and/or metabolite**	PVP	No pentedrone by LC-Q-TOF
27	PVP, Methylone	PVP, Methylone	
28	MDPV	MDPV	
29	MDPV, Butylone	MDPV, Butylone	
30	PVP	PVP	
31	MDPV	MDPV	
32	MDPV	MDPV	
33	PVP, Methylone	PVP, Methylone	
34	PVP, Methylone	PVP, Methylone	
35	PVP, Methylone	PVP, Methylone	
36	PVP	PVP	
37	PVP, Methylone	PVP, Methylone	
38	**Ethylone, Methylone, Pentedrone and/or metabolite**	-	No ethylone, methylone or pentedrone by LC-Q-TOF; No ethylone or methylone on retest at reference laboratory
39	Methylone, Ethylone	Methylone, Ethylone	
40	PVP, Methylone	PVP, Methylone	

Table 18. Summary of positive findings in cathinone users by drug.

Drug	Reference Laboratory Positives	Confirmed Positives	Concentration Range*
4-MEC	1	1	-
Butylone	1	1	177 ng/mL
Ethylone	14	13	26 - 405,129 ng/mL
Flephedrone	2	2	-
MDPV	5	5	257 - 6,261 ng/mL
Methylone	19	16	32 - 6,315 ng/mL
Pentedrone	5	0	-
Pentylone	1	1	-
PVP	16	16	72 - 15,045 ng/mL
	64	55	-

*Reported by the reference laboratory.

IV. Conclusions

Discussion of Findings

Our study using eighteen synthetic cathinones confirms earlier observations regarding the in-situ degradation and thermal instability of the cathinone species. Degradation products were characterized by base peaks 2 Da lower than the parent drug. This phenomenon was first documented by DeRuiter and Noggle two decades ago using methcathinone. In this study, we confirm that all of the cathinones tested, including ring substituted, unsubstituted, methylenedioxy-type and pyrrolidine-containing species are capable of in-situ degradation during GC analysis. Degradation can be minimized by lowering inlet temperatures, decreasing residence time in the inlet and minimizing active sites. Chromatographic and mass spectral characteristics for all eighteen breakdown products were evaluated. Nevertheless, their similarity in terms of chromatographic and mass spectral characteristics should be carefully considered and evaluated during method development, validation and routine analysis.

Although synthetic cathinones bearing a secondary amine are readily derivatized using well established techniques, a universal derivatization for all cathinones with favorable yields, single products and improved mass spectral qualities was not identified. Attempts to derivatize the ketone met with limited success, even using well established methodology which has proven successful for other drug classes including keto opioids and keto steroids. Although pentafluorobenzyloxime derivatives offered the most promise and greatly enhanced mass spectral quality, the formation of *syn*- and *anti*- geometric isomers was not ideal. Reductive silylation, although readily accomplished using a simple two-step derivatization approach, yields mass spectra that are not significantly improved due to extensive alpha cleavage and the predominance of the very stable iminium ion.

Synthetic cathinones were extracted successfully using a mixed-mode polymeric solid phase extraction to yield clean extracts with high recoveries. Underivatized extracts from blood and urine were readily identified by GC/MS using full scan acquisition. Although qualitative assay

performance was within acceptable limits there are many drawbacks to this approach. The dominance of the iminium ion (and in particular the pyrrolidinium ion for cathinones bearing a tertiary amine) is very limiting if sensitivity is to be increased using targeted analysis (*i.e.* selected ion monitoring or SIM). The absence of secondary or tertiary ions, in addition to the target ion makes it difficult to acquire data with acceptable ion ratios, due to their exceptionally low abundance. Identification of a suitable, universal, ketone reactive derivatization might have provided some resolve. However, the arylaminoketones are inherently more complex than their non-carbonyl counterparts (amphetamines). Keto-enol equilibria and multiple products associated with these polyfunctional drugs present a significant challenge.

Given the thermal lability of these compounds, an alternative analytical approach is desirable. Although the GC/MS analysis of non-derivatized cathinones provided sound qualitative data and provided favorable results using authentic specimens from cathinone users, hyphenated LC procedures offer many advantages. Aside from thermal degradation, the stability of the drug in biological evidence must be carefully considered. The reanalysis of authentic samples from cathinone users highlighted the importance of this issue. The stability of the synthetic cathinones in biological matrices has not yet been systematically evaluated and deserves additional study.

Implications for Policy and Practice

Once analytical testing has concluded, forensic toxicologists are often required to interpret those findings in criminal and civil proceedings. Forensic science and the broader criminal justice system relies upon analytical testing and interpretation of the results in criminal, civil and postmortem death investigations. In order for a forensic toxicologists to provide an interpretation of the results it is important to understand how concentrations of these drugs might be influenced during analysis or storage. This report highlights some of the analytical variables associated with GC/MS analysis of the synthetic cathinones.

Implications for Further Research

Although steps can be taken to minimize degradation, the synthetic cathinones are a thermally labile class of compounds, consistent with earlier reports involving methcathinone. Alternative analytical approaches may overcome these issues and should be carefully considered for this class of drug. Aside from their thermal degradation, a much broader question that must be answered relates to their stability in biological evidence. The chemistry of these polyfunctional compounds is inherently more complex than their amphetamine counterparts. Although research in this area is in its infancy, preliminary reports suggest that there are matrix and pH related stability effects that must be investigated. Due to the speed at which these drugs have evolved, a systematic approach is needed. We must understand how the various structural facets of the synthetic cathinones influence their stability. This approach is more likely to lend itself to meaningful interpretation of newer analogs and derivatives that have not yet been developed, or are still emerging as new designer drugs.

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VI. Dissemination of Research Findings

Sarah Kerrigan, Megan Savage, Cassandra Cavazos and Paige Bella. Thermal Degradation of Synthetic Cathinones: Implications for Forensic Toxicology. Journal of Analytical Toxicology, accepted (2015).

Sarah Kerrigan and Rebecca Ponsini. Identification of Synthetic Cathinones from Electron Impact Mass Spectra. Proceedings of the American Academy of Forensic Sciences, Orlando, FL (2015).

Sarah Kerrigan and Paige Bella. Stability of the Synthetic Cathinones: Implications for Forensic Toxicology. Proceedings of the American Academy of Forensic Sciences, Seattle, WA (2014).

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