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Investigation in stability of eight synthetic piperazines in human whole blood under various storage conditions over time

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INVESTIGATION IN STABILITY OF EIGHT SYNTHETIC PIPERAZINES IN HUMAN WHOLE BLOOD UNDER VARIOUS STORAGE CONDITIONS OVER TIME

by

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ABSTRACT

Over the past decade, synthetic piperazines have been associated with multiple fatalities and was one of the top 25 identified drugs in 2011. (1, 2). While circumventing legislative controls and preventing the detection in standard drug tests, synthetic piperazine derivatives are encountered in forensic casework as "legal" alternatives to ecstasy (3,4-methylenedioxymethamphetamine) (3). These chemically-produced compounds share very similar pharmacological and psychological effects with ecstasy which in turn has led to their popularity as "party pills" (3). The long-lasting duration of synthetic piperazines, especially when 1-benzylpiperazine (BZP) is mixed with 1-(3-trifluoromethylphenyl)-piperazine (TFMPP), has also made them desirable to drug users to receive enhanced hallucinogenic effects (4).

Although most methods are optimized to accurately quantify the amount of drugs in biological specimens submitted for forensic toxicology testing, unforeseeable challenges may arise to complicate the analysis such as postmortem redistribution, enzymatic reactions, the presence of bacterial activities, chemical and matrix interferences as well as the lack of reference materials (5-7). Thus, the purpose of this research was to investigate the stability of synthetic piperazines in human whole blood under various storage

conditions and time ranges. A total of eight synthetic piperazines were assessed on their degrees of degradation using a Shimadzu Ultra-Fast Liquid Chromatography (UFLC) with SCIEX 4000 Q-Trap Electrospray Ionization Tandem Mass Spectrometry in positive ionization mode. These analytes included: 1-benzylpiperazine (BZP), 1-(4-fluorobenzyl)-piperazine (FBZP), 1-(4-methylbenzyl)-piperazine (MBZP), 1-(4-methoxyphenyl)-piperazine (MeOPP), 1-(para-fluorophenyl)-piperazine (pFPP), 1-(3-chlorophenyl)-piperazine (mCPP), 2,3-dichlorophenylpiperazine (DCPP), and 1-(3-trifluoromethylphenyl)-piperazine (TFMPP).

Individual unknown samples were prepared by spiking certified reference standards (Cayman Chemical, Ann Arbor, MI, U.S.A.) of each synthetic piperazine into certified drug-free human whole blood (UTAK Laboratories, Inc., Valencia, CA, U.S.A.) independently at 1000 ng/mL. To closely monitor the stability of each compound and potential drug-drug interactions, mixed samples consisted of all eight piperazines were also stored at room temperature (~20°C), 4°C and -20°C for one, three, six, nine and twelve months in dark sealed containers. Solid phase extraction (SPE) was performed to remove unwanted components prior to the injection into the LC system. Drug of Abuse (DAU) mixed-mode copolymeric columns (Clean Screen®, UCT Inc., Levittown, PA, U.S.A.) were utilized with a positive pressure manifold rack followed by evaporating to dryness with low heat at 65°C. All samples were then reconstituted with 250 µL of 50:50 mixture of methanol and 2mM ammonium formate buffer with 0.2% formic acid (Fisher Scientific, Waltham, MA, U.S.A.).

Analysis was performed in triplicate using a reversed-phase column (Kinetex® F5, Phenomenex®, Torrance, CA, U.S.A.) with a binary gradient of a 2mM ammonium formate buffer with 0.2% formic acid and methanol with 0.1% formic acid. The total run time was 11.5 minutes including equilibration and the flow rate was 0.4 mL/min. Three internal standards including BZP-d7, mCPP-d8 and TFMPP-d4 (Cerilliant, Round Rock, TX, U.S.A) were used to generate calibration curves that were ranged from 20 ng/mL to 2000 ng/mL.

Results revealed that BZP, MBZP and FBZP were more stable than phenyl piperazines over time under all storage conditions, in which MBZP was consistently more stable and still had more than 70% remaining after 12 months. Data showed a smaller degree of degradation when samples were kept frozen or refrigerated; whereas storing at room temperature should be avoided to ensure minimal degradation and detrimental impacts on stability of piperazine compounds. For crime laboratories that are facing backlog situations, case samples with synthetic piperazines should be kept frozen or refrigerated even for time period as short as 30 days or less. However, storing them for too long will clearly affect the quantitation accuracy because phenyl piperazines are more susceptible to degrade completely after six months regardless of storage conditions. Additionally, matrix interference was present due to the outlier of MBZP quantified on Day 270. Drug-drug interaction was also observed in the analyte mixture but the exact stability pattern of phenyl piperazines when mixed together could not be determined from this data set alone due to discrepancies observed on Day 91 and 270.

This research project had shown a solid method to examine how quickly or slowly synthetic piperazines degrade in blood at different storage conditions. To further this study, it would be also important to evaluate the number of freeze-thaw cycles on each specimen in order to minimize the effect of non-metabolic degradation.

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LIST OF ABBREVIATIONS

BZP	1-benzylpiperazine
CA	California
CE	Collision energy
CPS	Counts per second
CSA	Controlled Substances Act
CV	Coefficient of Variation
CXP	Collision cell exit potential
DCPP	2,3-dichlorophenylpiperazine
DEA	Drug Enforcement Administration
DP	Declustering potential
ESI	Electrospray Ionization
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
FBZP	1-(4-fluorobenzyl)-piperazine
FTDTL	Forensic Toxicology Drug Testing Laboratory
GC	Gas Chromatography
HR-MS	High-Resolution Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
MA	Massachusetts

MBZP	1-(4-methylbenzyl)-piperazine
mCPP	1-(3-chlorophenyl)-piperazine
MDMA	3,4-methylenedioxymethamphetamine
MRM	Multiple Reaction Monitoring
MeOPP	1-(4-methoxyphenyl)-piperazine
µL	Microliter
µg	Microgram
min	Minutes
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar
MS/MS	Tandem Mass Spectrometer
MS	Mass Spectrometry
msec	Milliseconds
m/z	Mass-to-charge ratio
ng	Nanogram
NFLIS	National Forensic Laboratory Information System
pFPP	1-(*para*-fluorophenyl)-piperazine
psi	Pounds per Square Inch
®	Registered Trademark
SPE	Solid Phase Extraction

STDEV	Standard Deviation
SWGTOX	Scientific Working Group for Forensic Toxicologists
TFMPP	1-(3-trifluoromethylphenyl)-piperazine
TM	Trademark
TX	Texas
UFLC	Ultra-Fast Liquid Chromatography
U.S.A.	United States of America
UNODC	United Nations Office on Drugs and Crime
UFLC	Ultra-fast liquid chromatography
UHPLC	Ultra-high-performance Liquid Chromatography
V	Volts
°C	Degree Celsius
%	Percent

1. INTRODUCTION

1.1 New Psychoactive Substances

1.1.1 History of Designer Drugs

In the 1970s, "designer drugs" referred to multiple heroin-like synthetic substances derived mostly from the fentanyl molecule (8). Later in the mid-1980s, this term gained widespread popularity when 3,4-methylenedioxymethamphetamine (MDMA) underwent a popularity growth (8). Although the term "designer drugs" has been described informally as "legal highs" in the past ten years, the preferred term is "new psychoactive substances" (NPS) (9). NPS cover a large number of recreational drugs that mimic the effects of existing controlled substances and are often sold as "legal highs" to circumvent legal authorities (2). For example, piperazines derivatives, which belong to a class of amphetamine-like-compounds, are making a resurgence as "legal Ecstasy" (3). Studies showed that NPS may pose a threat to the public health comparable to scheduled substances, but these compounds can be distinguished from classical drugs of misuse such as amphetamine, cocaine, cannabis and heroin because of little or no history of medical use (9).

1.1.2 Wide Spread Internet Availability

The rapid and continuous emergence of NPS that have not been scheduled poses serious safety issues. Due to the growing business of head shops and the internet in the late 1990s and early 2000s, there was a huge explosion in designer drugs being sold in or over these sites because many synthetic or naturally derived psychoactives were legal to possess

(8). As a result, these derivatives appeared in a regular manner on the black market worldwide. Many of these substances are often sold, perceived as safe by the public and mislabeled as “research chemicals” which are not suitable for human consumption (2). During the time that the euphemism “research chemicals” appeared, the internet provided drug sellers new channels for their businesses and even for online “chat-rooms” where the efficacy of drugs could be discussed without censor (9). Selling designer drugs on the internet is still a growing problem that is dangerous to the public.

1.2 Synthetic Piperazines

1.2.1 Original Use

Piperazine-based compounds are completely synthetic rather than “herbal” as suggested by some suppliers. They can be divided into two classes, the benzylpiperazines such as 1-benzylpiperazine (BZP) and the phenylpiperazines such as 1-(3-trifluoromethylphenyl)-piperazine (TFMPP) (10). Piperazines are primarily used as anthelmintic agents in veterinary and clinical practices (1). Although BZP was originally synthesized by researchers in 1944 from a pepper plant to serve as a potential de-worming agent for farm animals in particularly cattle, scientific data was not published in regards to the use of BZP as a treatment for intestinal parasites in human (10). According to the literature in the 1970s, several studies were conducted on BZP being a potential antidepressant medication but was rejected due to its amphetamine-like effects with high potential for abuse (4). Moreover, the piperazine ring and its derivatives are often seen in

the industrial field and used as important raw materials for the hardrning of epoxy resins, corrosion inhibitors, rubber accelerators, urethane catalysts and antioxidants (4).

1.2.2 Arise in Synthetic Piperazines Abuse

The first documented abuse of a piperazine derived drug with BZP was reported in the United States of America (U.S.A.) in 1996 (10). In 2000, piperazine-related abuse has begun to spread in New Zealand and Australia (11). In September of 2004, 1-(3-chlorophenyl)-piperazine (mCPP), which was "marketed" as the new ecstasy-like substance during that time, was detected in street drugs in Sweden as well as the Netherlands by the Drug Information and Monitoring System (10). According to the U.S. National Forensic Laboratory Information System (NFLIS), about 38,230 piperazines were seized from 2006 to 2010 and this class of drugs was one of the top 25 identified drugs in 2011 (1). The accessibility of obtaining piperazine derivatives at a party-type environment and their recovery rates during synthesis stand out among other recreational drugs. This is largely because piperazines can be adulterated with many external binders such as caffeine and vitamins that are usually inexpensive (4). Another reason that piperazines have been promoted to the youth population is their long-lasting duration, which is typically four to six hours. If BZP is mixed with TFMPP in order to enhance its spectrum effects, this specific combination can last up to eight hours (4). According to the United Nations Office on Drugs and Crime (UNODC), there were at least twelve substituted synthetic piperazines drugs on the clandestine drug market as of 2013 (12) in which only few are currently regulated.

Not only are synthetic piperazines used recreationally, but they are also more prevalent in the military (1). In 2008, the Forensic Toxicology Drug Testing Laboratory (FTDTL) within the military system located in Florida had seven unconfirmed urine specimens that were preliminarily detected as amphetamine positive, in which all specimens were subsequently quantitatively confirmed by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) of containing different levels of BZP, TFMPP and mCPP (1). Thus, the result had raised concerns over abuse of piperazines in the military.

1.2.3 Mechanism and Effect

Since an addition or removal of merely one functional group on each chemical structure may entirely alter the effect of each drug (13), it is important for analysts to understand the mechanism behind each class of drugs. Previous animal studies have emphasized that some synthetic piperazines can both stimulate the release and inhibit the reuptake of dopamine, serotonin (5-HT) and noradrenaline, in which serotonergic and dopaminergic effects predominate in most cases (11). Nonetheless, the majority of NPS have not been extensively studied as they are relatively new to the scientific community. As a result, many of the pharmacological properties remain unclear, such as absorption rate, distribution, metabolism, excretion, major psychological effects and even potency of the drug molecule (14). As of today, TFMPP, BZP and mCPP are the only synthetic piperazines that have been studied in detail in terms of their pharmacodynamic and pharmacokinetic properties.

Among the eight synthetic piperazines that were studied in this project including 1-benzylpiperazine (BZP), 1-(4-fluorobenzyl)-piperazine (FBZP), 4-methyl-1-benzylpiperazine (MBZP), 1-(4-methoxyphenyl)-piperazine (MeOPP), 1-(para-fluorophenyl)-piperazine (pFPP), 1-(3-chlorophenyl)-piperazine (mCPP), 2,3-dichlorophenylpiperazine (DCPP), and 1-(3-trifluoromethylphenyl)-piperazine (TFMPP), the pharmacological effect of BZP is proven to be similar to MDMA and shares highly similar dopamine and serotonin agonist mechanism of action (4). Although people who consumed BZP were reported to experience stimulant effects such as elevated blood pressure and increased heart rate, increased euphoria, dysphoria, sociability and drug-liking, it was found to be about 10-fold less potent than MDMA, methamphetamine or amphetamine (11). No death record has been reported following a sole ingestion of BZP, but some of the severe toxic effects include psychosis, renal toxicity and seizure (4).

mCPP, a metabolite of commonly prescribed anti-depressants such as trazodone, nefazodone, enziprazole and etoperidone, has also been found to interact with specific serotonin receptors and thus lead to serotonin release (11, 15). A number of human clinical studies have indicated that the major metabolite of mCPP is believed to be para-hydroxy-mCPP (p-OH-mCPP) via CYP2D6-mediated hydroxylation (11). In order not to overlook further toxic compounds in poisoning cases, confirming the presence of other metabolites is therefore relevant for toxicological risk assessment and for drug screening purposes in forensic toxicology laboratories. Negative effects of mCPP are very similar to those as a result of serotonin syndrome (15). Some of these effects include anxiety, dizziness, confusion, depressive symptoms, panic attacks as well as sensitivity to light and noise (4).

TFMPP acts as a non-selective serotonin receptor agonist in addition to boosting synaptic serotonin levels by blocking serotonin reuptake and increasing its release (11) . Although information is lacking on this compound, TFMPP is rarely administered by itself but often mixed with BZP as a party pill instead (1). As a result, TFMPP has amphetamine-like effects as well.

Besides BZP, TFMPP and mCPP, the following piperazines have been synthesized and introduced into the illicit market and may pose dangerous threat to the public health system: they are MeOPP, pFPP and MBZP. The average duration of MeOPP is shorter than that of BZP and does not have strong stimulant effects, whereas pFPP is a piperazine derivative with mild hallucinogenic and euphoric effects (4, 9). Both MeOPP and pFPP have been found in vitro to play an important role as a serotonin receptor agonist (4). Moreover, MBZP is a stimulant drug which is a derivative of BZP. Despite its stimulant effect is comparatively weaker and it seems to create less problems such as nausea and headaches, the overall effect of MBZP is more or less the same to those of BZP (4).

1.2.4 Legislative Control and Statistics

From the effective “internet marketing” of designer drugs to the huge demand in recreational use, the rapid growth of synthetic piperazines significantly outpace the ability of government to regulate, legislate and to enforce new policies. The UNODC and European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) had categorized piperazines into their standardization list to emphasize the risk of consuming this particular class of drugs (16).

In the United States, some synthetic piperazine compounds are placed under the Controlled Substances Act (CSA), which was amended by the Controlled Substance Analogue Enforcement of 1986 in order to prohibit the illegal manufacturing, selling or possessing of drugs (8). Although the majority of synthetic piperazines seized from 2006 to 2010 were identified as BZP or TFMPP which had 4,180 and 1,084 cases respectively, only BZP has been permanently placed under the CSA and listed as a Schedule I substance (17, 18). The scheduling was determined after several thorough reviews of the drug's abuse pattern, distribution and other relevant scientific data (19). The Drug Enforcement Administration (DEA) ruled that BZP has no legitimate medical use in the United States. Also, it does not seem to be safe to use under medical supervision and has a high potential for abuse which could result in a physical dependence (19). On the other hand, although the DEA temporarily listed TFMPP in 2002 as a Schedule I hallucinogen (17), the Food and Drug Administration (FDA) did not recommend placing regulations on this particular synthetic piperazine and had removed TFMPP from the CSA after March 2014 based on scientific data and multiple medical evaluations (20). Despite TFMPP's lack of placement on the CSA at the federal level, it is still a scheduled drug in Florida according to its State statute (21).

Reports and statistic data reveal that the presence of illicit drugs in whole blood samples originating from motor vehicle drivers might be a contributing factor in causing traffic accidents and in initiating violent behaviors (22). Therefore, it is imperative to have regulations governing the use of these psychoactive substances in order to help lower the rate of criminal cases as well as fatal vehicle accidents.

1.3 Detection of New Psychoactive Substances

1.3.1 Traditional Methods

The successful detection of illicit drug consumption or nonmedical intake of prescription medications is essential in different arenas including criminal investigations, cases involving driving under the influence, performance enhancement tests as well as military drug testing. In spite of the increasing number of designer drugs available, only few comprehensive screening techniques are currently available for NPS detection and quantification in biological specimens (23). While GC-MS and LC-MS are traditionally the analytical method of choice, many of the ever-changing designer drugs entities do not exist within the mass spectral library (22, 23). Although the mass spectrometric theory of the three-dimensional quadrupole ion trap can be traced back to about thirty years ago when the first commercially available instrument got released to the market (24), limitations still exist on these instruments in terms of sensitivity and specificity . For example, the GC-MS method is still limited only to non-polar, volatile and thermally stable compounds that generally requires time-consuming sample preparation and derivatization (22).

In the case of seized drugs, multiple preliminary tests for the presumptive identification of NPS are currently available but false positives with these tests have been known to occur especially if the amphetamine class of compounds are involved in the testing method (10). Although synthetic piperazines have been tested with various color tests and immunoassays, no color test has been accepted in the forensic community and no immunoassay kit has been found to be specific to piperazines (14).

1.3.2 Potential Alternatives

In order to analyze a large number of routine case samples within a given time in the field of forensic science, a user-friendly software coupled with an economically-efficient hardware are highly desirable. For instance, high performance liquid chromatography (HPLC) and ultra-fast liquid chromatography (UFLC) are both considered as a robust alternative with minimal sample preparation and high sample throughput (22).

Moreover, high-resolution mass spectrometry (HR-MS) has been used as a screening tool involving untargeted and general unknowns within the field of forensic toxicology (24). Nevertheless, the cost of HR-MS instrumentation such as a quadrupole-time-of-flight is often a drawback that prohibits its use in toxicology laboratories even though it is an ideal tool to identify designer drugs by exact mass analysis (23). In summary, LC-MS has gained popularity in the field of forensic toxicology because of its ability to perform reliable qualitative and quantitative analysis.

1.4 Multiple Challenges

1.4.1 Chemical Interference

At most medical examiner offices, autopsy samples are usually collected for drug analysis on suspicious cases prior to an embalming process (25). Occasionally, the embalmed tissue will be later analyzed if there is a false negative result from an initial drug screen or no initial suspicion of drug involvement in the death (26). The latter scenario becomes a problem because the presence of formalin and formaldehyde in biological samples which might contribute different degrees of interaction with the drug present in

the decedents' body (26). Since both formalin and formaldehyde are two highly reactive preservative fluid that are commonly used in the embalming process, previous studies have shown that formaldehyde-embalming solution can be reactive with various controlled substances such as barbiturates, diazepam, phenytoin, fenfluramine and tricycli antidepressants (27). With that being said, drug stability is one of the major concerns when body undergoes numerous postmortem changes in forensic casework regardless of if the sample is obtained either ante- or post-mortem.

1.4.2 Limited Toxicological References / Structural Similarity

A lot of NPS are considered as emerging drugs which are not easy to detect in traditional toxicological analysis (28). Most data are obtained mainly from reported intoxication cases or drug users forums. As a result, limited data is available about the pharmacological effects and metabolism of these designer drugs, such as BZP and TFMPP (29). Additionally, due to a high structural similarity of piperazine-based drugs, current analytical methods are often not sensitive or specific enough to distinguish closely-related analytes while cross-reaction with other amphetamines have indeed been reported (28, 29). Therefore, the metabolism pathway and psychological effects of synthetic piperazines remain unclear for the most parts while their popularity as recreational drugs have grown (29).

In some substance abuse cases, drugs can be difficult to identify from a forensic analytical point of view due to the large number of potential structures. However, the constant introduction of novel compounds, the lack of literature references, inadequate

accessibility to standards and the lack of comprehensive analytical methods which all negatively contribute to the issue (23).

1.4.3 Logistic Nature & Storage Conditions

Knowledge of the pharmacological information of a drug is of importance for forensic toxicologists, but backlog situations in laboratories cannot be overlooked. The limitations of a logistic nature often prevent analysts from obtaining reliable quantitative data due to variable time intervals between obtaining a sample and analysis (5).

Furthermore, storage conditions of specific drugs or compounds in biological samples may affect the analytical results due to ongoing enzymatic metabolism and post-mortem redistribution (5, 7). In terms of drugs instability, chemical degradation or even bacterial activities may potentially become an issue that presents challenging interpretations on analytical results (5, 6). For example in 2013, a study on the investigation of stability of mephedrone in blood demonstrated significant degradation after storing it for just seven days at 4°C (7).

1.5 Research Objectives / Hypothesis

Synthetic piperazines, like other emerging designer drugs currently available on the market, have been constantly growing in terms of rate of drug abuse and death rate related to drug overdose. If federal agencies or local legislatures regulate and list them as controlled substances, a well-developed method is necessary to detect and accurately quantify synthetic piperazines in biological matrices such as blood and urine. However, the

forensic community is short of confirmatory and quantitative methods on piperazines and they tend to be limited to the analysis of BZP and TFMPP (29). More importantly, stability of synthetic piperaines in whole blood samples remains unknown due to the fast-changing structure of novel drugs as well as the lack of references. Therefore, in partial to validate a reliable method according to the Scientific Working Group for Forensic Toxicologists (SWGTOX) guidelines for quantitative methods, an investigation on stability of synthetic piperazines is required in order to examine possible degradation of seized drugs in biological samples. This will not only be impactful in terms of quantitative data analysis on routine case samples, but also in maintaining public health and safety.

The ultimate goal of this research was to examine the degree of degradation and the amount loss of each tested synthetic piperazine in blood over time. Since storage condition is one of the key components of analyte stability, all synthetic piperazines including BZP, MBZP, FBZP, MeOPP, pFPP, mCPP, DCPP, and TFMPP were stored separately at room temperature (~20° C), 4° C and -20° C for different periods of time. Quantitation on each analyte was performed with the use of three deuterated internal standards of BZP-d7, mCPP-d8 and TFMPP-d4. Drug-drug interaction and analyte interference were also investigated by storing samples with all piperazines mixed together. Quantitative analysis was performed using an Ultra-Fast Liquid Chromatography – Electrospray Ionization-Tandem Mass Spectrometry (UFLC-ESI-MS/MS) in order to obtain accurate and precise readings. The results were assessed in full accordance with the SWGTOX guidelines including evaluation of accepted range of accuracy and coefficient of determination which is also known as the R^2 value.

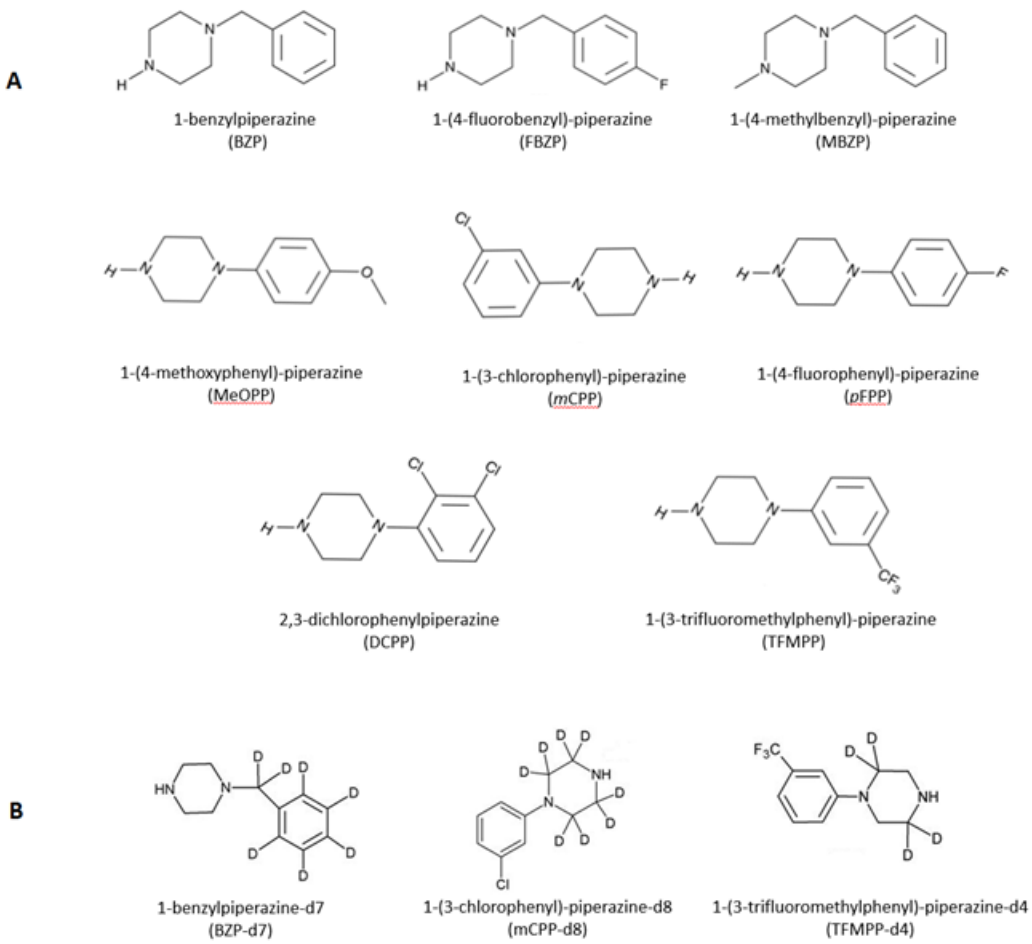


Figure 1: Chemical structures. (A) Synthetic piperazines BZP, FBZP, MBZP, TFMPP, mCPP, MeOPP, DCPP and pFPP (30). (B) Chemical structures of internal standards BZP-d7, mCPP-d8, and TFMPP-d4 (30).

2. MATERIALS AND METHODS

2.1 Theory of Instrumentation

2.1.1 Liquid Chromatography

To optimize the effectiveness of each analytical run, the analyte of interest must be separated from other components in the sample matrix. Liquid chromatography (LC) is widely used as a separation technique based on an adsorption mechanism where molecules and ions adhere to the surface of the absorbent, which is commonly known as the stationary phase (31). In general, stationary and mobile phases are two media in which sample distributes based on levels of affinity or polarity. Samples are dissolved in mobile phase and forced through the column that houses the stationary phase. Molecules with a weak affinity for the stationary phase move rather quickly so that sample components are separated into discrete bands for analysis based on different migration rates. For example, a nonpolar molecule tends to stick to a nonpolar stationary phase via weak intermolecular forces. However, this nonpolar molecule will only have little affinity for a polar stationary phase.

In addition to stationary phase, it is also important to manipulate and optimize each set of parameter independently in order to identify and quantitate complex mixtures. For instance, column dimensions, chemical compositions of column, initial and final mobile phase compositions as well as gradient time are all influential in terms of method optimization (32). Some examples of mobile phases used in a LC system include acetonitrile, methanol, water, formic acid and buffer. A typical analytical column is coated

with silica and may have modified surface depending on the chemistry or interaction between the analyte of interest and the coating material.

In addition to regular LC, high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) are both powerful techniques for identification and quantification. Although HPLC is more cost-effective, UHPLC offers enhanced separation power and reduced run times that make it more desirable in the forensic context (31).

2.1.2 Mass Spectrometry

Mass Spectrometry (MS) is an analytical technique in which sample molecules are ionized in the gas phase and fragmented into different patterns that can be used to obtain structural information (33). It is often used as a definitive detector to ionize, separate and identify generated ions of a wide variety of evidence including controlled substances and fire debris. A typical MS consists of five major components: a vacuum system, an ion source, a mass analyzer, an ion detector and a data recording system. Normally, MS is coupled with either a gas chromatography (GC) or a LC system to accelerate the separate process.

The vacuum system in MS can prevent unwanted collisions between generated gas-phase ions and neutral molecules. In addition, operating under vacuum can increase the mean free path of fragment ions. Once all sample molecules are ionized by the ion source, the produced ions can then be transported to the mass analyzer where they can be separated based on individual mass-to-charge (m/z) ratio. Subsequently, the intensity of each m/z is

recorded so ion signal can be amplified and digitized in the data recording system. Data is then formulated as a spectrum, or a plot of intensity versus m/z (31).

In spite that multiple ion sources exist, electron ionization is a type of ion source where high-energy electrons emitted from a resistively heated filament travel in spiral pathways toward the anode in order to increase the probability of bombarding sample molecules (31). In other words, a high-energy collision occurs which breaks down molecules into smaller pieces of fragment ions, which is also known as fragmentation (31). After the molecule is ionized, fragment ions are charged so they can be accelerated through the mass analyzer (34).

2.1.3 Electrospray Ionization

While atmospheric pressure chemical ionization and electron ionization are two popular choices to volatize and ionize compounds for mass spectrometric analysis, electrospray ionization (ESI) has been gaining recognition and acceptance within the forensic community (31, 33). Unlike chemical ionization, ESI is a soft ionization technique in which pseudo molecular ions can be easily observed due to milder fragmentation (31). The electrospray source is comprised of a capillary tube and a counter electrode that creates electric field. As the sample molecules moving along the capillary tube, there is an accumulation of charge on the surface of the liquid. A pure, hot nebulizing gas, such as nitrogen, is then applied for turning the liquid sample into fine mist. As the solvent evaporates, the droplet size decreases whereas the electrical charge density increases on its

surface (31). Eventually, smaller droplets will be formed due to repulsion between like charges that can overcome the surface tension (31).

2.1.4 Tandem Mass Spectrometry

A tandem mass spectrometer (MS/MS) is two mass spectrometers linked together and consists of two single quadrupole instruments. In theory, tandem mass spectrometers have three quadrupole in series: the first and third quadrupole are mass analyzers whereas the second quadrupoles serves as a collision cell (31). The first quadrupole (Q1) acts as a mass filter that will mainly transmit a selected ion, or precursor ions, towards the collision cell (Q2). In Q2, ion molecules interact and collide with neutral gas under high pressure. The fragment ions are then moved into the third quadrupole (Q3) for another round of scanning mass filtering before entering the detector (35). Thus, tandem mass spectrometry is also known as triple quadrupole mass spectrometry despite the collision cell is not a mass analyzer and does not function as a separator.

The quadrupole mass analyzer is a four-parallel-conducting-rod compartment in square shape. Direct current and radio frequency are applied on assigned rods so only ions of a specific m/z can resonate with the current at a time (31, 35). Although quadrupoles yield a lower resolution, they are comparatively inexpensive and user-friendly (35).

2.2 Materials

2.2.1 Standards / Reagents

The following reagents were purchased from Fisher Scientific (Waltham, MA, U.S.A): LC grade methanol, LC grade 2-propanol (same as isopropanol), optima grade

acetonitrile, optima grade formic acid, methylene chloride, ammonium formate, concentrated ammonium hydroxide, concentrated hydrochloric acid, anhydrous disodium phosphate and monohydrate sodium dihydrogen phosphate. BZP, FBZP, MBZP, MeOPP, pFPP, mCPP, TFMPP and DCPP were purchased through Cayman Chemical Company (Ann Arbor, MI, U.S.A.) All piperazines were received in the form of 10 mg powder except for BZP, which was received as a 1 mg/mL standard in methanol. Three deuterated internal standards, BZP-d7, mCPP-d8 and TFMPP-d4, were received as 100 µg/mL standards in methanol from Cerilliant Corporation (Round Rock, TX, U.S.A.). Fresh millipore water was obtained daily from the Synergy UV water filtration system from EMD Millipore/Merck (Darmstadt, Germany). Certified drug-free human whole blood was purchased from UTAK Laboratories, Inc. (Valencia, CA). During mobile phases and reagents preparation, an Oakton pH meter from Fisher Scientific was used to confirm the pH level. During sample preparation, solid phase extraction (SPE) was conducted on a positive pressure manifold made by UCT, Inc. (Bristol, PA, U.S.A). Additionally, Clean-Screen Drug of Abuse (DAU) 200 mg/ 6 mL solid phase extraction cartridges were also purchased from UCT, Inc.

Table 1: Lot numbers of certified reference materials. All certified reference standards were stored at -20℃

Product	**Company**	**Lot Numbers**
BZP	Cayman Chemical	0470646-1
BZP-d7	Cerilliant	FE06221504
FBZP	Cayman Chemical	0435859-14, 0435859-19
MBZP	Cayman Chemical	0465260-5, 0465260-6
MeOPP	Cayman Chemical	0446981-12, 0446981-13
pFPP	Cayman Chemical	0435857-30

mCPP	Cayman Chemical	0446996-11, 0446996-18
mCPP-d8	Cerilliant	FN071111-01
TFMPP	Cayman Chemical	0435854-36
TFMPP-d4	Cerilliant	FN08011407
DCPP	Cayman Chemical	0446497-12, 0446497-15
Whole Blood	UTAK	B1027, B1086

2.2.2 UFLC-ESI-MS/MS

Quantification of synthetic piperazines was performed using a Shimadzu Prominence Ultra-Fast Liquid Chromatography System with two LC-20AD model pumps and a SIL-20AC model auto-sampler (Kyoto, Japan). The separation was achieved using a Kinetex® F5 2.6µm, 100 Å, 150 mm x 3.0 mm ID column from Phenomenex, Inc. (Torrance, CA, U.S.A). All analytes were detected on a SCIEX 4000 QTRAP tandem mass spectrometer consisting of a Turbo V electrospray ionization source (Framingham, MA, U.S.A). All data were collected using Analyst™ (version 1.6.2) software and quantitation was conducted with MultiQuant™ 3.0 (version 3.05373.0) software (SCIEX).

2.3 Methods

2.3.1 LC-MS/MS Method Parameters

The method utilized in this project was adapted from the compound and source optimization processes conducted by Leblanc, whose thesis focused on method development and validation for the quantification of different synthetic piperazines in blood and urine (30). After evaluating each analyte and internal standard, the two most intense product ions and the single most intense ion were selected respectively. Analytes

that were labelled “1” were the most intense fragment ions that were used for quantitation purposes of that particular analyte; whereas the second most intense fragment ion was labelled “2” as a confirmatory ion of that specific analyte. Table 2 shows the dwell time, declustering potential (DP), collision energy (CE), cell exit potential (CXP) and entrance potential (EP) that were optimized for each analyte and internal standard. The duration of the MRM scan was set to 6.5 minutes and all analyses were conducted in positive ionization mode. In addition, Table 3 shows the optimized gas and ion source parameters used in this experiment.

Table 2: Multiple Reaction Monitoring (MRM) Table

Name of Analyte	Q1 Mass (Da)	Q3 Mass (Da)	Dwell Time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
BZP-d7 IS 1	184.3	98.2	50	70.0	10	30.0	15.0
BZP 1	177.1	91.1	50	66.0	10	32.0	14.0
BZP 2	177.1	65.1	50	66.0	10	63.0	9.0
FBZP 1	195.2	109.1	50	72.0	10	29.0	18.0
FBZP 2	195.2	83.2	50	72.0	10	65.0	12.0
MBZP 1	191.2	91.1	50	75.0	10	31.0	15.0
MBZP 2	191.2	65.2	50	75.0	10	67.0	9.0
MeOPP 1	193.2	150.2	50	70.0	10	28.0	24.0
MeOPP 2	193.2	119.3	50	70.0	10	34.0	19.0
pFPP 1	181.2	138.2	50	75.0	10	29.0	23.0
pFPP 2	181.2	75.2	50	75.0	10	77.0	11.0
mCPP 1	197.1	154.2	50	75.0	10	28.0	26.0
mCPP 2	197.1	118.2	50	75.0	10	48.0	19.0
mCPP-d8 IS 1	205.4	158.2	50	86.0	10	31.0	26.0
DCPP 1	233.1	190.2	50	85.0	10	30.0	32.0
DCPP 2	233.1	117.2	50	85.0	10	67.0	19.0
TFMPP 1	231.1	188.1	50	80.0	10	32.0	33.0
TFMPP 2	231.1	118.3	50	80.0	10	54.0	19.0
TFMPP-d4 IS 1	235.4	190.2	50	84.0	10	32.0	32.0

Table 3: Ion source and gas parameters.

Curtain Gas (psi)	Collision Gas	IonSpray Voltage (V)	Temperature (℃)	Ion Source Gas 1 (psi)	Ion Source Gas 2 (psi)
30	Medium	2500	600	50	80

The two mobile phases used in this method were 2mM ammonium formate buffer with 0.2% formic acid (mobile phase A) and HPLC-grade methanol with 0.1% formic acid (mobile phase B). In terms of LC settings, the flow rate was set to 0.400 mL/min in which the starting condition was 5% mobile phase B. The injection volume was set to 5 µL. The maximum pressure for both LC pumps was set to 5000 psi and a binary flow was selected as the pumping mode. In order to prevent contamination, parameters of the auto sampler were optimized as listed in Table 4.

Table 4: Auto sampler settings

Rinsing Volume	Needle Stroke	Rinsing Speed	Sampling Speed	Purge Time	Rinse Dip Time	Cooler Temperature
1000 µL	52 mm	35 µL/sec	2.0 µL/sec	25 min	0 sec	15°C

This method has a 1.50 minutes of pre-equilibration to ensure the starting conditions such as oven temperature and pressure of the LC system are met. As shown in Table 5, each analysis is designed to run with a gradient flow where the percentage of mobile phase B changes over time. Upon completion of the run, re-equilibration takes place from 6.51 to 10.00 minutes.

Table 5: LC time program.

Time (min)	Module	Event	Parameter (%)
0.01	Pumps	Pump B Concentration	5
0.30	Pumps	Pump B Concentration	5
3.50	Pumps	Pump B Concentration	80
6.50	Pumps	Pump B Concentration	80
6.51	Pumps	Pump B Concentration	5
10.00	Controller	Stop	

2.3.2 Preparation of Calibrators and Stored Samples

Besides BZP which was prepared with methanol by the manufacturer, all piperazine standards purchased from Cayman Chemical were individually prepared at 1 mg/mL by weighting 1 mg of powder and dissolving it in 1 mL of LC grade methanol followed by vortexing it for 30 seconds. Each solution was then serially diluted to make a 10 µg/mL stock from the 1 mg/mL solution. To further prepare a 1 µg/mL stock solution (Stock 1), the above 10 µg/mL stock solution from each analyte were combined and diluted with 50:50 of millipore water and methanol. Stock 1 solution was further diluted to make a 100 ng/mL stock solution (Stock 2) with the same solvent. An internal standard stock solution (Stock 3) was prepared at a concentration of 1 µg/mL by combining BZP-d7, mCPP-d8 and TFMPP-d4 obtained from Cerilliant. Calibrators ranged from 20 ng/mL to 2000 ng/mL were prepared in 15 mL glass tubes that contained different volume of stock 1 or stock 2 along with 100 µL of certified drug-free whole blood. As shown in Table 6, every sample, except for double blanks, were spiked accordingly and fortified with 30 µL of stock 3.

Additionally, a high quality control (QC) sample and a low QC sample were prepared at 1500 ng/mL and 30 ng/mL respectively.

Due to an interference of a stable isotope that has the same m/z in both DCPP and TFMPP, a separate calibration curve was required to accurately quantify the analyte of interest in order to avoid any possible underestimation of TFMPP. To prepare this special calibration curve, stock 1 and stock 2 solutions were prepared with TFMPP only where all other parameters as shown in Table 6 remained the same.

For unknown samples preparation, eight different tubes of 10 mL certified drug-free whole blood containing each synthetic piperazine were prepared independently at a concentration of 1000 ng/mL from the previously diluted 10µg/mL stock. In addition to the eight synthetic piperazines samples, a tube of 10 mL whole blood containing all synthetic piperazines standards as well as another tube of 10 mL whole blood served as control were also prepared. Subsequently, mixture analyte(s) or whole blood from those test tubes were transferred into two separate amber glass vials in which each vial contained 3.3mL. They were then capped and placed at room temperature and 4˚C for 1, 3, 6, 9 and 12 month(s). The remaining 3.4 mL of mixture solution in the tube was also stored and wrapped in opaque tape to prevent degradation by light, and placed in the freezer at -20˚C for 1, 3, 6, 9, 12 month(s). Temperatures for the freezer, refrigerator and the room where samples were stored were monitored and recorded every day except for the weekends. No internal standards were added into any samples on Day 0.

Table 6: Preparation of calibrators for generation of calibration curve. The table shows the amount of stock solution required to generate a calibration curve prior to solid phase extraction. Stock 1 was a 1 µg/mL solution and Stock 2 was a 100 ng/mL solution. Both stocks contained all synthetic piperazines. The internal standard solution (Stock 3) was prepared at 1 µg/mL and contained all three deuterated internal standards.

Calibrator/ Sample	**Stock Solution (µL)**	**Whole Blood (µL)**	**Internal Standard Solution (µL)**
	Stock 1		*Stock 3*
2000 ng/mL	200	100	30
High QC (1500 ng/mL)	150	100	30
1000 ng/mL	100	100	30
500 ng/mL	50	100	30
200 ng/mL	20	100	30
	Stock 2		
100 ng/mL	100	100	30
50 ng/mL	50	100	30
Low QC (30 ng/mL)	30	100	30
20 ng/mL	20	100	30
Negative Control	N/A	100	30
Double Blank	N/A	100	N/A

2.3.3 Solid Phase Extraction

Solid phase extraction (SPE) was performed on all samples to purify and remove unwanted components from whole blood prior to the injection into the LC system. 1 mL of phosphate buffer (100 mM, pH 6) was added to all samples and was vortexed for 10 seconds. Clean Screen Drug of Abuse (DAU) columns were loaded onto a positive pressure manifold rack and were conditioned with 1 mL of methanol followed by another 1 mL of phosphate buffer (100 mM, pH 6). No external artificial pressure was applied and only gravity flow was allowed during cartridges conditioning. Subsequently, samples were poured into each designated pre-conditioned column and were allowed to drip with gravity flow only. A series of wash steps were performed on each column in the order of 1 mL of

millipore water, 1 mL of 0.1 N hydrochloric acid and 1 mL of LC grade methanol. To dry sorbent on SPE columns, a full flow of compressed nitrogen gas was applied for 5 minutes at 40 psi.

Samples were then transferred onto an elution rack followed by adding 2 mL of base elution solvent in which piperazines on the sorbent bed were eluted from the columns into new 15 mL glass test tubes. The base elution solvent was made fresh for each experiment. To avoid misty mixture solution, the base elution solvent was prepared in the order of adding 20% isopropanol (2-propanol), 3% of concentrated ammonium hydroxide and 77% of methylene chloride. Once all samples were eluted via gravity flow, test tubes were placed on a heating block at 65°C until the solvent was completely evaporated.

After drying down all samples, they were reconstituted with 250 µL of 50:50 mixture of methanol and 2mM ammonium formate buffer with 0.2% formic acid. All samples were transferred from the test tube to an LC-MS vial with a flat bottom liner and stored at the auto sampler chamber at 15°C. Using the LC-MS/MS method described previously, all samples were then run under the pre-set conditions.

2.3.4 Quantification of Analytes

The stability of synthetic piperazines was examined by monitoring the amount loss of analytes over time at each storage condition. Quantification of synthetic piperazines in all samples were performed using LC-MS/MS and were compared to the calibration curves generated on both Day 0 and the end of each trial. Each sample was run three times and the average was obtained to serve as the initial concentration on Day 0 or the remaining concentration at the end of each trial. Data analysis was performed with MultiQuant™

software and the following weighting factors were applied to the calibration curve: “area” was selected as the regression parameter while “1/x” was the weighting type to the linear regression line. Among the three deuterated internal standards, BZP-d7 was used to quantify BZP, FBZP and MBZP; mCPP-d8 was used to quantify MeOPP, pFPP, mCPP, and DCPP; TFMPP-d4 was used to quantify TFMPP only.

3. RESULTS AND DISCUSSION

3.1 Analysis of 1-Month Samples

The total length of this method was 11.5 minutes but all synthetic piperazines were expected to elute within the first 6.5 minutes. All benzyl piperazines were separated first with a lower retention time in which BZP was eluted at about 3.82 min, FBZP was eluted at about 4.48 min and MBZP was eluted at about 4.69 min. All phenyl piperazines were also eluted out at a different retention time: MeOPP at 5.09 min, pFPP at 5.33 min, mCPP at 5.80 min, DCPP at 6.11 min and TFMPP at 6.14 min. Although deuterated internal standards might co-elute with the target analytes at a similar retention time, they were all detected distinguishably at 3.71 min, 5.80 min and 6.15 min as BZP-d7, mCPP-d8 and TFMPP-d4 respectively.

Since analyses were performed in triplicate, the quantitated data were recorded and the average of those three runs was calculated. The average value represented the concentration of each analyte stored alone or in mixed-mode on either Day 0 or Day 30. The standard deviation and the percent coefficient of variation (% CV) were calculated as shown in Table 7 by using the following formulas:

$$\text{Standard Deviation} = \sqrt{\frac{\sum \ (Concentration\ from\ each\ replicate - Mean)^2}{2}}$$

$$Coefficient\ of\ Variation\ (\%CV) = \frac{Standard\ Deviation}{Mean}$$

Table 7: Average, standard deviation and percent coefficient of variation for analyte concentration in ng/mL on Day 0. All certified drug-free whole blood samples were fortified with the analytes at a concentration of 1000 ng/mL and run in triplicate. Analytes were prepared separately and in mixed-mode. The average concentration was calculated across these three runs.

Analyte	Average (ng/mL)	Standard Deviation	% CV	Range (ng/mL)
BZP	999.11	14.33	1.43	982.66 – 1008.87
FBZP	1168.10	16.83	1.44	1156.63 – 1187.42
MBZP	1140.45	20.65	1.81	1126.45 – 1164.16
MeOPP	906.95	17.49	1.93	889.99 – 924.93
pFPP	1142.07	18.77	1.64	1124.76 – 1162.02
mCPP	980.95	0.90	0.09	1183.44 – 1224.04
DCPP	795.72	18.31	2.30	778.21 – 814.74
TFMPP	502.83	8.36	1.66	493.29 – 508.86
MIX (BZP)	1195.24	1.70	0.14	1193.62 – 1197.01
MIX (FBZP)	1148.76	21.62	1.88	1130.07 – 1172.44
MIX (MBZP)	993.47	72.67	7.31	917.11 – 1061.78
MIX (MeOPP)	1251.28	26.25	2.10	1223.26 – 1275.30
MIX (pFPP)	1204.51	40.86	3.39	1177.36 – 1251.50
MIX (mCPP)	1175.59	12.05	1.03	1161.72 – 1183.46
MIX (DCPP)	882.04	17.04	1.93	866.54 – 900.28
MIX (TFMPP)	461.74	2.92	0.63	458.48 – 464.12

Table 8: Average, standard deviation and percent coefficient of variation for analyte concentration in ng/mL on Day 30 at different storage conditions. All samples were run in triplicate. The average concentration was calculated across these three runs. RT refers to room temperature.

Analyte / Storage Condition	Average (ng/mL)	Standard Deviation	% CV	Range (ng/mL)
BZP RT	1105.43	62.60	5.66	1054.4 – 1175.28
BZP 4°C	949.15	53.16	5.60	887.76 – 980.12
BZP -20°C	1166.81	51.16	4.38	1107.74 – 1196.79
FBZP RT	787.14	10.27	1.30	776.82 – 797.36
FBZP 4°C	902.95	124.78	13.82	795.24 – 1039.68
FBZP -20°C	1295.06	38.63	2.98	1263.94 – 1338.29

MBZP RT	503.33	12.55	2.49	495.44 – 517.80
MBZP 4°C	606.33	26.30	4.34	582.41 – 634.49
MBZP -20°C	1144.13	60.45	5.28	1082.81 – 1203.68
MeOPP RT	1238.79	50.78	4.10	1189.35 – 1290.82
MeOPP 4°C	767.46	10.33	1.35	759.41 – 779.11
MeOPP -20°C	961.05	3.48	0.36	957.78 – 964.72
pFPP RT	1207.86	14.97	1.24	1192.02 – 1221.77
pFPP 4°C	917.57	37.17	4.05	878.09 – 951.89
pFPP -20°C	1073.07	34.24	3.19	1053.01 – 1112.60
mCPP RT	1010.19	37.80	3.74	966.73 – 1035.41
mCPP 4°C	973.45	13.62	1.40	964.31 – 989.11
mCPP -20°C	978.83	10.23	1.04	971.99 – 990.58
DCPP RT	1060.75	41.01	3.87	1018.16 – 1099.97
DCPP 4°C	705.49	59.09	8.38	641.63 – 758.22
DCPP -20°C	876.19	10.69	1.22	866.81 – 887.83
TFMPP RT	499.42	22.57	4.52	481.18 – 524.66
TFMPP 4°C	385.70	8.71	2.26	375.65 – 390.98
TFMPP -20°C	686.05	54.35	7.92	641.83 – 746.74

Table 9: Average, standard deviation and percent coefficient of variation for analyte concentration (in mixed-mode) in ng/mL on Day 30 at different storage conditions. All samples were run in triplicate. The average concentration was calculated across these three runs. RT refers to room temperature.

Storage Condition	**Analyte**	**Average (ng/mL)**	**Standard Deviation**	**% CV**	**Range (ng/mL)**
MIX RT	BZP	1173.39	87.50	7.46	1091.47 – 1265.56
MIX 4°C	BZP	1077.83	17.19	1.59	1066.53 – 1097.62
MIX -20°C	BZP	1027.91	53.34	5.19	989.22 – 1088.75
MIX RT	FBZP	1239.28	28.59	2.31	1206.85 – 1260.86
MIX 4°C	FBZP	1147.70	14.76	1.29	1132.77 – 1162.28
MIX -20°C	FBZP	1024.82	80.63	7.87	944.53 – 1105.79
MIX RT	MBZP	788.82	18.66	2.37	767.27 – 799.94

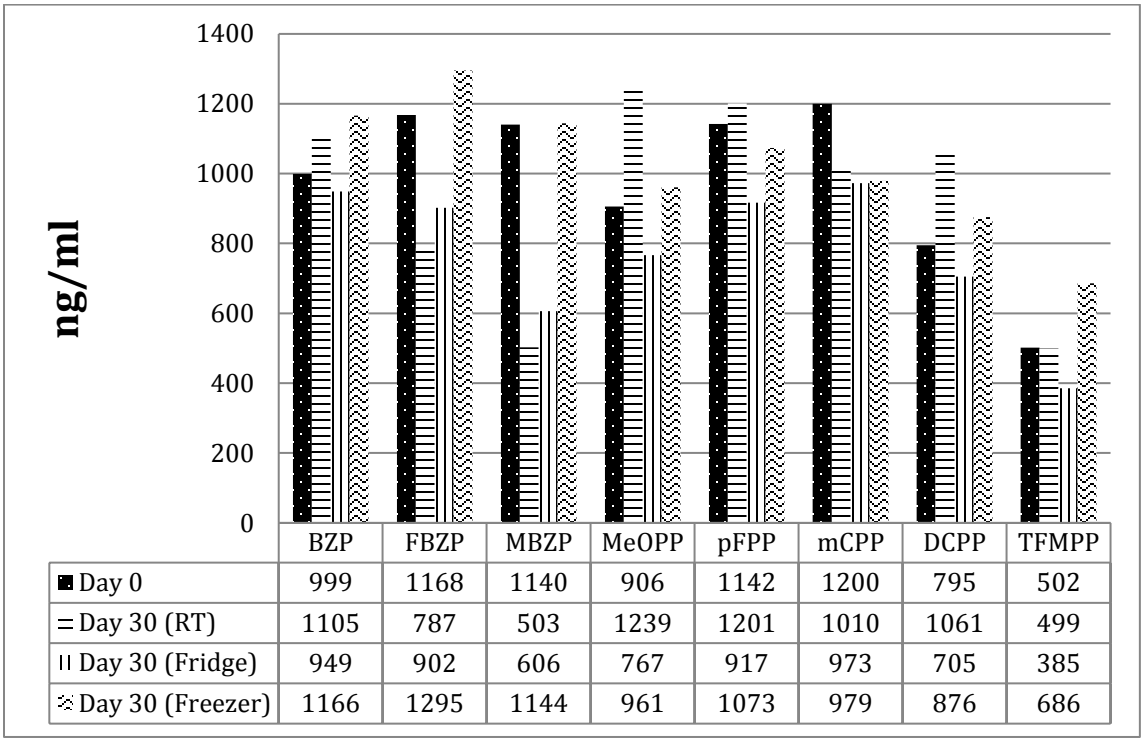
MIX 4°C	MBZP	978.30	18.61	1.90	957.23 – 992.49
MIX -20°C	MBZP	1033.54	52.18	5.05	973.80 – 1070.16
MIX RT	MeOPP	554.17	12.72	2.29	539.51 – 562.24
MIX 4°C	MeOPP	528.09	7.93	1.50	522.66 – 537.20
MIX -20°C	MeOPP	263.30	9.53	3.62	254.35 – 273.32
MIX RT	pFPP	769.77	3.75	0.49	766.96 – 774.03
MIX 4°C	pFPP	728.55	17.42	2.39	709.31 – 743.28
MIX -20°C	pFPP	747.14	29.21	3.91	715.18 – 772.47
MIX RT	mCPP	639.97	26.23	4.10	620.56 – 669.81
MIX 4°C	mCPP	370.44	9.19	2.48	360.06 – 377.53
MIX -20°C	mCPP	409.71	23.30	5.69	386.05 – 432.63
MIX RT	DCPP	654.77	12.40	1.89	641.5 – 666.05
MIX 4°C	DCPP	322.97	8.04	2.49	315.66 – 331.58
MIX -20°C	DCPP	358.43	9.60	2.68	347.74 – 366.31
MIX RT	TFMPP	385.36	5.34	1.39	380.04 – 390.72
MIX 4°C	TFMPP	154.75	0.19	0.12	154.53 – 154.88
MIX -20°C	TFMPP	244.87	11.30	4.61	232.21 – 253.94

A fresh calibration curve was generated for each analysis on both Day 0 and Day 30. The y-axis of the calibration curve indicated the ratio between the peak area of the analyte and peak area of internal standard, which is also known as the peak area ratio. The x-axis of the curve represented the concentration of analytes. As shown in Table 10, all coefficient of determination (R^2 values) were above the minimum accepted value of 0.98.

Table 10: R^2 values of each analyte ran on calibration curve on Day 0 and Day 30.

	BZP	FBZP	MBZP	MeOPP	pFPP	mCPP	DCPP	TFMPP
R^2 value (Day 0)	0.9984	0.9978	0.9876	0.9950	0.9968	0.9990	0.9964	0.9978
R^2 value (Day 30)	0.9938	0.9998	0.9930	0.9936	0.9938	0.9998	0.9904	0.9974

On Day 30, concentrations of synthetic piperazines that were kept in freezer showed little to no degradation of the parent compound as supposed to Day 0. Although similar results from the freezer samples should have been expected for all analytes that were stored in refrigerator, MBZP lost approximately 50% of its parent compound under refrigerator conditions. Likewise, MBZP was the least stable at room temperature where a 55% loss had occurred.

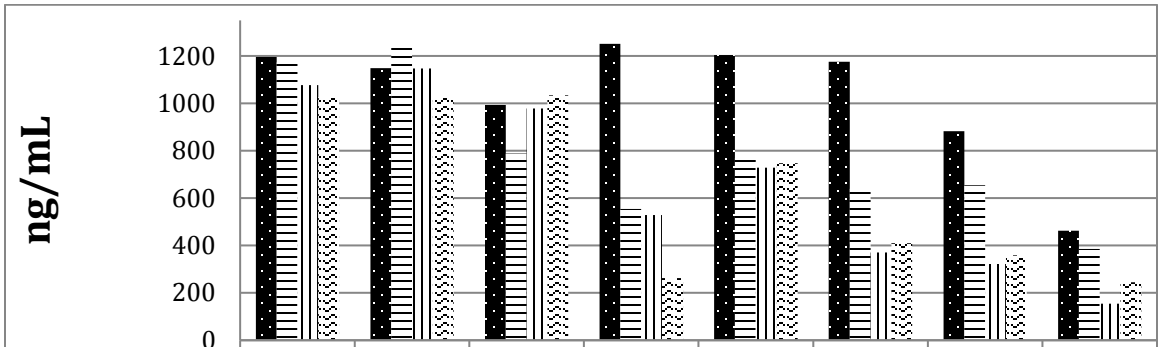


	BZP	FBZP	MBZP	MeOPP	pFPP	mCPP	DCPP	TFMPP
Day 0	999	1168	1140	906	1142	1200	795	502
Day 30 (RT)	1105	787	503	1239	1201	1010	1061	499
Day 30 (Fridge)	949	902	606	767	917	973	705	385
Day 30 (Freezer)	1166	1295	1144	961	1073	979	876	686

Figure 2: Effect of different storage conditions on analyte stability over a 30-day trial. RT refers to room temperature. Temperature in freezer and fridge were at -20˚C and 4˚C respectively.

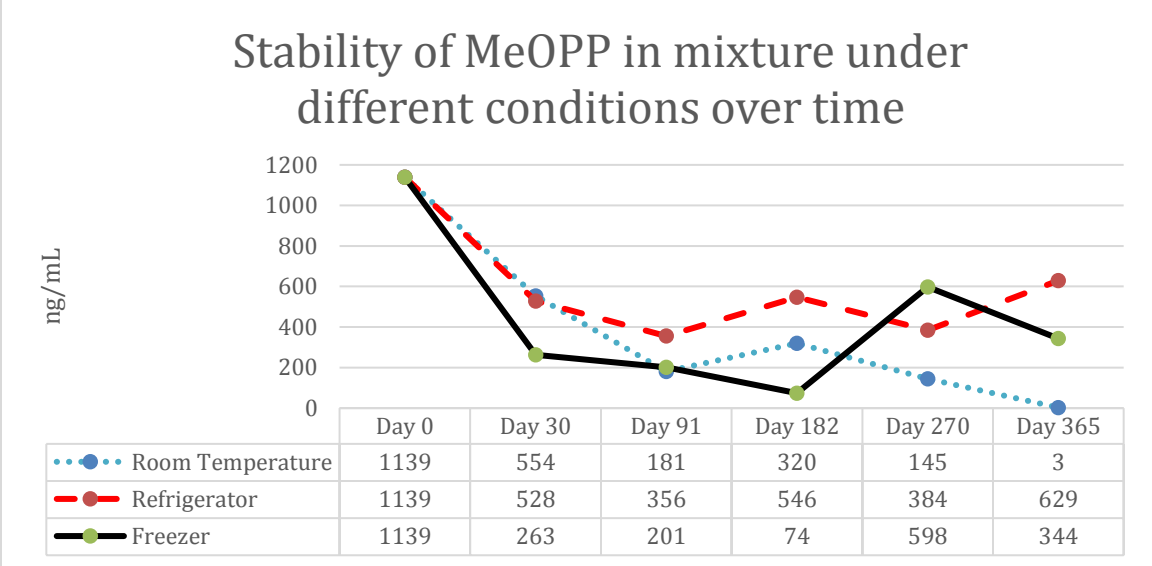
In order to investigate possible analyte inferences, samples containing all synthetic piperazines at a concentration of 1000 ng/mL were also prepared and stored at multiple storage conditions for one month. Data on Figure 3 indicated that benzyl piperazines in

mixture were quite stable over time, but most phenyl piperazines showed moderate to severe degradation after only one month as shown on Figure 4-6. Additionally, the degree of loss for MeOPP, mCPP, DCPP and TFMPP in mixture was relatively smaller at room temperature than those in freezer. Also, the amount of pFPP remained in mixture after 30 days was more or less the same among three storage conditions.



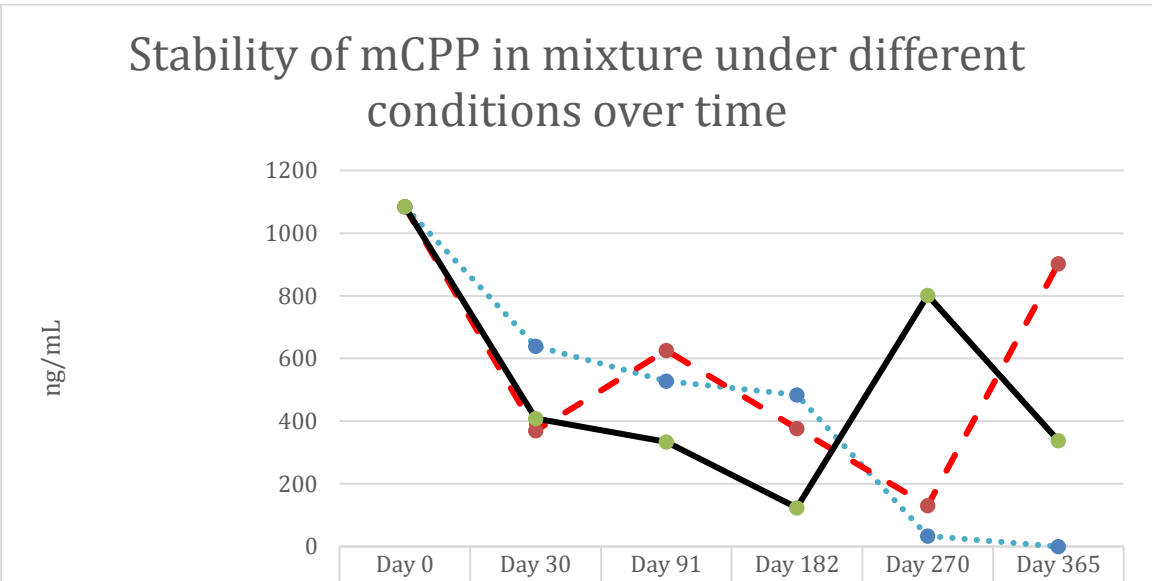
	MIX (BZP)	MIX (FBZP)	MIX (MBZP)	MIX (MeOPP)	MIX (PFPP)	MIX (mCPP)	MIX (DCPP)	MIX (TFMPP)
Day 0	1195	1148	993	1251	1204	1175	882	462
Day30-RT	1173	1239	788	554	769	639	654	385
Day30 Fridge	1077	1147	978	528	728	370	322	155
Day30 Freezer	1027	1024	1033	263	747	409	358	245

Figure 3: Effect of different storage conditions on analyte interference and stability over a 30-day trial. RT refers to room temperature. Temperature in freezer and fridge were at -20˚C and 4˚C respectively.



	Day 0	Day 30	Day 91	Day 182	Day 270	Day 365
Room Temperature	1139	554	181	320	145	3
Refrigerator	1139	528	356	546	384	629
Freezer	1139	263	201	74	598	344

Figure 4: Effect of different storage conditions on MeOPP in mixture overtime.



	Day 0	Day 30	Day 91	Day 182	Day 270	Day 365
Room Temperature	1084	639	528	484	34	0
Refrigerator	1084	370	626	377	131	903
Freezer	1084	409	334	124	802	338

Figure 5: Effect of different storage conditions on mCPP in mixture overtime.

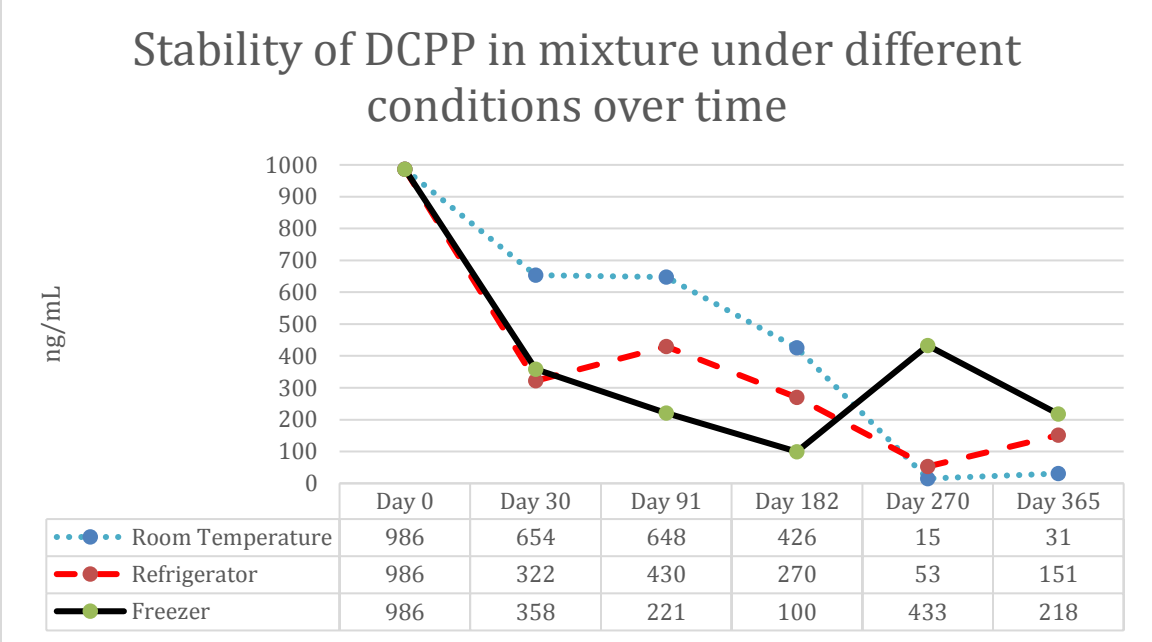


Figure 6: Effect of different storage conditions on DCPP in mixture overtime.

3.1.1 Isotopic Influence of DCPP on TFMPP

In the presence of two principle isotopes of chlorine-35 and chlorine-37, DCPP has a stable isotope with the same mass-to-charge ratio as TFMPP. Although TFMPP and this stable isotope of DCPP both generated a high intensity fragment ion at this specific mass-to-charge ratio of 231, result of quantification of each analyte was still accurate even though both analytes might have coeluted. The crucial key justifying this interference issue is to keep the ratios between the internal standard and the calibrators as well as the ratios between the internal standard and unknown constant. In other words, the ratio of the DCPP isotope influence should always be kept constant.

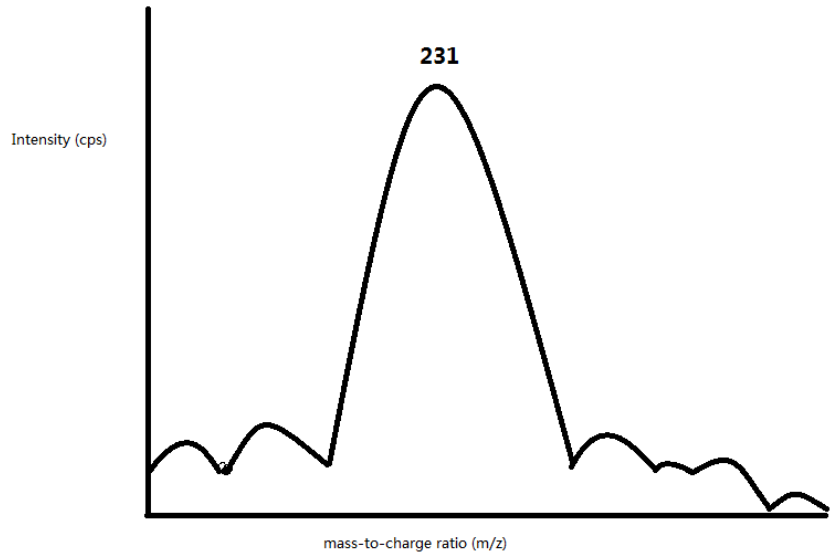


Figure 7: Virtual Q1 MS scan of TFMPP. This scan shows mass-to-charge ratios in Da on the x-axis and intensity in counts per second on the y-axis. Intensity level of the peak should be at about e^5 or above.

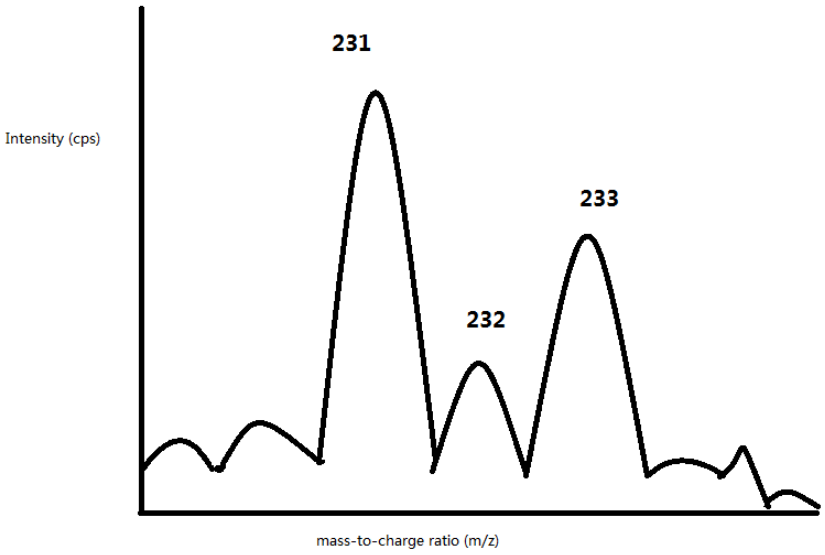


Figure 8: Virtual Q1 MS scan of DCPP. This scan shows mass-to-charge ratios in Da on the x-axis and intensity in counts per second on the y-axis. Intensity level peak "231" should be at about e^5 or above; peak "232" should be at about e^3; peak "233" should be at about e^4 or above.

For example, if TFMPP and DCPP are both present in the calibrators and the mixed-mode samples that consist of eight different piperazines used in this experiment, the result of quantification of each analyte present in mixture should be accurate. However, if the same set of calibrators (including TFMPP and DCPP) are used, the concentration of TFMPP alone in unknown sample will be underestimated (as shown on Figure 9 and 10) due to the absence of the "231" portion from DCPP. In this case, the ratio of the DCPP isotope was not kept constant. This explains the inconsistency of concentration of TFMPP on Day 0 of the 1-month and 6-month trials.

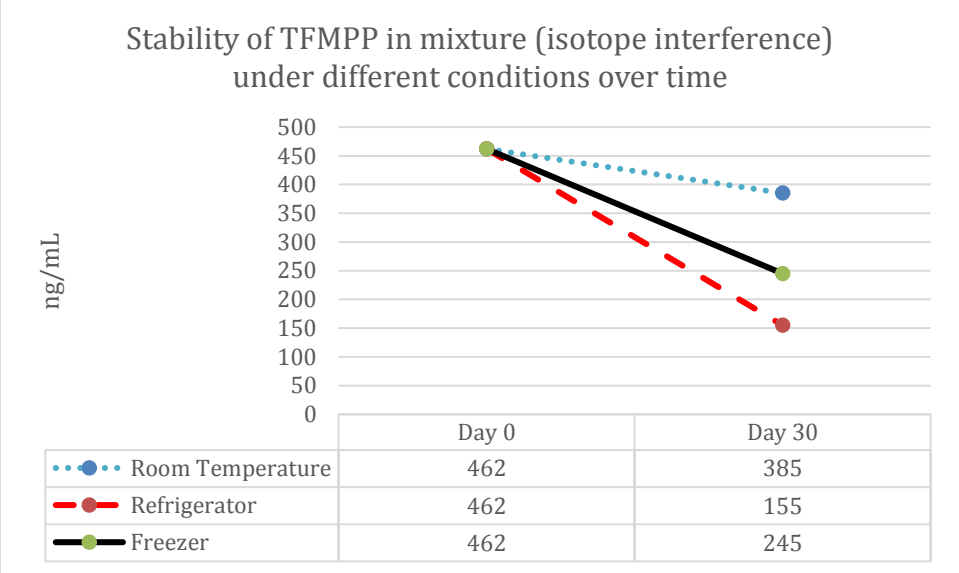


Figure 9: Effect of different storage conditions on TFMPP in mixture on a 30-day trial under isotope interference.

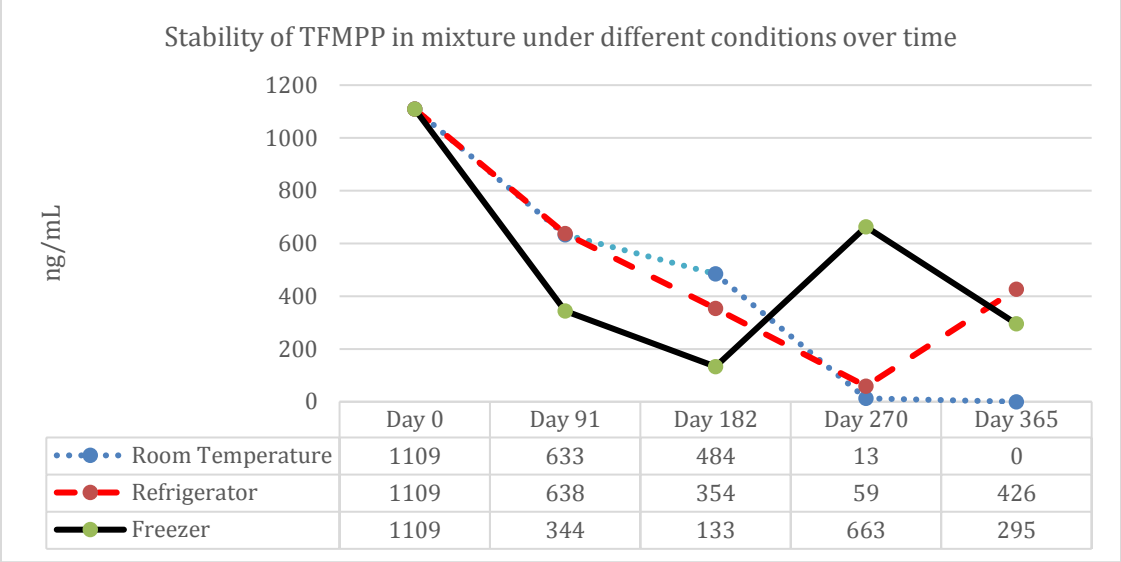


Figure 10: Effect of different storage conditions on TFMPP in mixture overtime.

3.2 Analysis of 3-Month Samples

Quantification of all samples were performed on both Day 0 and Day 91 using the same method. New calibration curves were generated on both Day 0 and Day 91. All % CV were within the standard range of 20%. According to Table 14, all R^2 values were above the acceptable limit of 0.98. In this 91-day trial, two calibration curves were generated where the second one contained TFMPP only.

Table 11: Average, standard deviation and percent coefficient of variation for analyte concentration in ng/mL on Day 0. All certified drug-free whole blood samples were fortified with the analytes at a concentration of 1000 ng/mL and run in triplicate. Analytes were prepared separately and in mixed-mode. The average concentration was calculated across these three runs.

Analyte	Average (ng/mL)	Standard Deviation	% CV	Range (ng/mL)
BZP	1017.21	51.29	5.04	973.11 – 1073.50
FBZP	1092.87	132.69	12.14	1004.95 – 1245.50
MBZP	1063.21	35.87	3.37	1035.76 – 1103.79

MeOPP	1041.88	7.39	0.71	1035.76 – 1050.09
pFPP	1027.77	34.75	3.38	988.38 – 1054.09
mCPP	1025.55	7.00	0.68	1017.52 – 1030.39
DCPP	1160.62	19.31	1.66	1138.36 – 1172.72
TFMPP	1115.92	44.24	3.96	1066.81 – 1152.66
MIX (BZP)	982.18	149.03	15.17	838.84 – 1136.32
MIX (FBZP)	1023.27	132.43	12.94	894.67 – 1159.23
MIX (MBZP)	1021.59	125.52	12.29	890.27 – 1140.38
MIX (MeOPP)	922.58	29.67	3.22	892.99 – 952.33
MIX (pFPP)	1003.93	26.51	2.64	980.11 – 1032.49
MIX (mCPP)	1013.92	31.31	3.09	981.96 – 1044.54
MIX (DCPP)	1036.60	12.44	1.20	1028.09 – 1050.87
MIX (TFMPP)	1038.21	3.38	0.33	1035.19 – 1041.86

Table 12: Average, standard deviation and percent coefficient of variation for analyte concentration in ng/mL on Day 91 at different storage conditions. All samples were run in triplicate. The average concentration was calculated across these three runs. RT refers to room temperature.

Analyte / Storage Condition	**Averag (ng/mL)**	**Standard Deviation**	**% CV**	**Range (ng/mL)**
BZP RT	934.90	110.27	11.79	807.60 – 1000.85
BZP 4°C	1059.16	35.21	3.32	1025.17 – 1095.48
BZP -20°C	1068.37	19.53	1.83	1046.83 – 1084.94
FBZP RT	851.75	21.09	2.48	827.62 – 866.66
FBZP 4°C	1017.92	13.31	1.31	1003.01 – 1028.62
FBZP -20°C	908.15	5.87	0.65	901.63 – 913.01
MBZP RT	1391.77	12.28	0.88	1380.41 – 1404.81
MBZP 4°C	1130.29	36.20	3.20	1105.81 – 1171.88
MBZP -20°C	1191.49	65.11	5.46	1118.61 – 1244.02
MeOPP RT	542.09	19.94	3.68	520.16 – 559.15
MeOPP 4°C	733.51	31.26	4.26	706.73 – 767.86
MeOPP -20°C	645.49	18.74	2.90	633.43 – 667.08
pFPP RT	513.79	10.09	1.96	502.27 – 521.03
pFPP 4°C	744.71	22.15	2.97	719.13 – 758.15
pFPP -20°C	910.21	41.96	4.61	864.18 – 946.32

mCPP RT	788.11	2.90	0.37	785.13 – 790.93
mCPP 4°C	755.23	21.67	2.87	730.42 – 770.42
mCPP -20°C	738.95	14.50	1.96	724.18 – 753.18
DCPP RT	497.08	13.49	2.71	485.29 – 511.79
DCPP 4°C	648.46	16.45	2.54	629.75 – 660.64
DCPP -20°C	483.55	8.96	1.85	477.78 – 493.87
TFMPP RT	686.02	12.80	1.87	675.00 – 700.06
TFMPP 4°C	668.36	13.56	2.03	657.23 – 683.47
TFMPP -20°C	580.24	13.60	2.34	565.76 – 592.75

Table 13: Average, standard deviation and percent coefficient of variation for analyte concentration (in mixed-mode) in ng/mL on Day 91 at different storage conditions. All samples were run in triplicate. The average concentration was calculated across these three runs. RT refers to room temperature.

Storage Condition	Analyte	Average (ng/mL)	Standard Deviation	% CV	Range (ng/mL)
MIX RT	BZP	818.49	21.34	2.61	797.51 – 840.21
MIX 4°C	BZP	1036.19	124.88	12.05	909.06 – 1158.69
MIX -20°C	BZP	947.34	39.78	4.20	907.59 – 987.16
MIX RT	FBZP	859.85	9.42	1.10	848.98 – 865.71
MIX 4°C	FBZP	1028.58	51.75	5.03	969.61 – 1066.42
MIX -20°C	FBZP	937.74	31.75	3.39	915.32 – 974.07
MIX RT	MBZP	567.92	5.35	0.94	563.31 – 573.78
MIX 4°C	MBZP	1207.99	76.06	6.30	1123.56 – 1271.15
MIX -20°C	MBZP	1153.68	33.47	2.90	1121.30 – 1188.14
MIX RT	MeOPP	180.51	2.89	1.60	177.18 – 182.37
MIX 4°C	MeOPP	355.57	11.71	3.29	346.14 – 368.68
MIX -20°C	MeOPP	201.36	5.42	2.69	195.56 – 206.31
MIX RT	pFPP	401.66	7.98	1.99	392.49 – 407.04
MIX 4°C	pFPP	802.10	36.86	4.60	765.67 – 839.37
MIX -20°C	pFPP	638.09	20.32	3.19	614.66 – 650.99

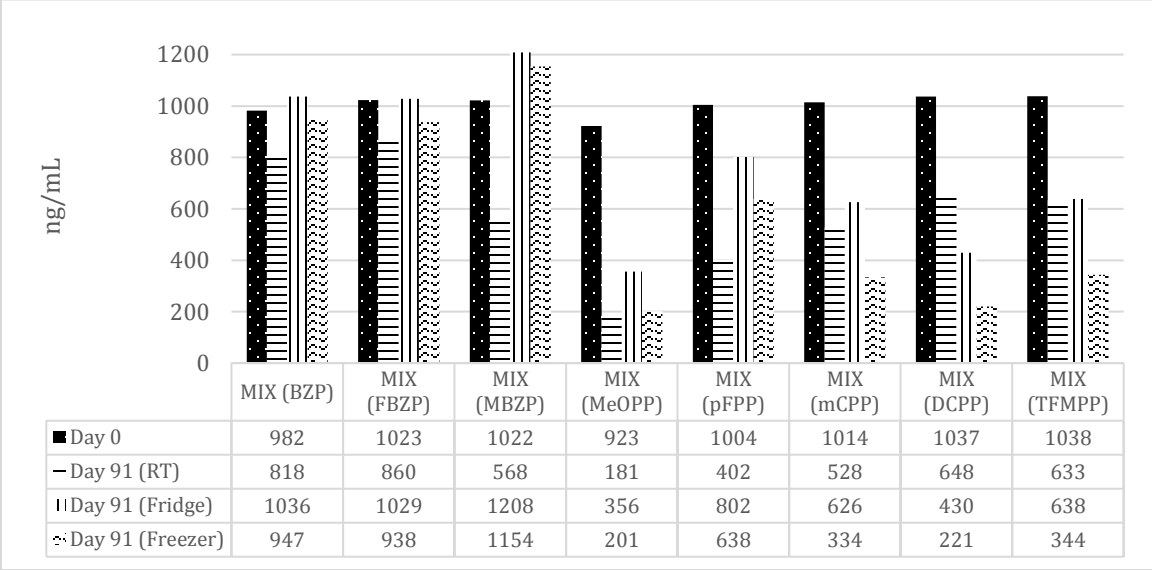
MIX RT	mCPP	528.15	5.70	1.08	521.61 – 532.03
MIX 4°C	mCPP	625.50	16.71	2.67	610.57 – 643.55
MIX -20°C	mCPP	334.02	9.31	2.79	327.15 – 344.62
MIX RT	DCPP	647.73	8.53	1.32	637.90 – 653.10
MIX 4°C	DCPP	429.72	11.27	2.62	416.76 – 437.24
MIX -20°C	DCPP	220.75	10.43	4.72	209.27 – 229.62
MIX RT	TFMPP	632.86	3.26	0.51	629.11 – 635.01
MIX 4°C	TFMPP	638.22	20.21	3.15	614.99 – 650.23
MIX -20°C	TFMPP	343.67	3.32	0.97	339.88 – 346.08

Table 14: R^2 values of each analyte ran on calibration curve on Day 0 and Day 91.

	BZP	FBZP	MBZP	MeOPP	pFPP	mCPP	DCPP	TFMPP
R^2 value (Day 0)	0.9963	0.9983	0.9933	0.9952	0.9970	0.9990	0.9985	0.9989
R^2 value (Day 91)	0.9985	0.9978	0.9872	0.9975	0.9937	0.9973	0.9865	0.9966

The concentration level on Day 91 among three benzyl piperazines that were kept frozen demonstrated minimal variation of less than 15% loss as compared to Day 0. In contrast, phenyl piperazines that were stored in freezer had moderate degradations especially DCPP and TFMPP, which were only left with 42% and 52% of its parent compound, respectively. Furthermore, all phenyl samples kept at 4˚C experienced an approximately 30 to 40% loss since Day 0 while BZP, FBZP and MBZP seemed to be relatively stable. Unlike the result obtained from the 1-month trial, MBZP did not degrade much under both room temperature and 4˚C. As expected, the degree of loss of all synthetic piperazines stored at room temperature was larger than those which were kept at 4˚C or -20˚C except for MBZP, mCPP and TFMPP.

While BZP and FBZP showed very little degradation when mixed with other analytes, MBZP had a 45% loss when kept at room temperature in mixed-mode. Although pFPP in mixed-mode experienced the greatest loss of analyte at room temperature which was contradictory to the pattern observed from the 1-month trial, result of mCPP, DCPP and TFMPP in mixed-mode quantitated on Day 91 were still consistent with it. This was because degree of loss of these three particular piperazines stored in freezer was significantly larger than those kept in ambient temperature. Additionally, there was a dramatic loss of MeOPP in mixed-mode under all storage conditions: less than 20% of MeOPP was recovered in whole blood after 91 days at room temperature while the sample also lost 60 to 80% of its parent analyte at the other two conditions.



	MIX (BZP)	MIX (FBZP)	MIX (MBZP)	MIX (MeOPP)	MIX (pFPP)	MIX (mCPP)	MIX (DCPP)	MIX (TFMPP)
Day 0	982	1023	1022	923	1004	1014	1037	1038
Day 91 (RT)	818	860	568	181	402	528	648	633
Day 91 (Fridge)	1036	1029	1208	356	802	626	430	638
Day 91 (Freezer)	947	938	1154	201	638	334	221	344

Figure 11: Effect of different storage conditions on analyte interference and stability over a 91-day trial. RT refers to room temperature. Temperature in freezer and fridge were at -20˚C and 4˚C respectively. Noted that severe degradation was observed in MeOPP after 91 days.

To overcome the isotope interference in this trial, a separate calibration curve was prepared with only TFMPP as the calibrators on Day 0. This would eliminate the influence of all mass-to-charge ratios of DCPP so that a reliable quantification of TFMPP can be made.

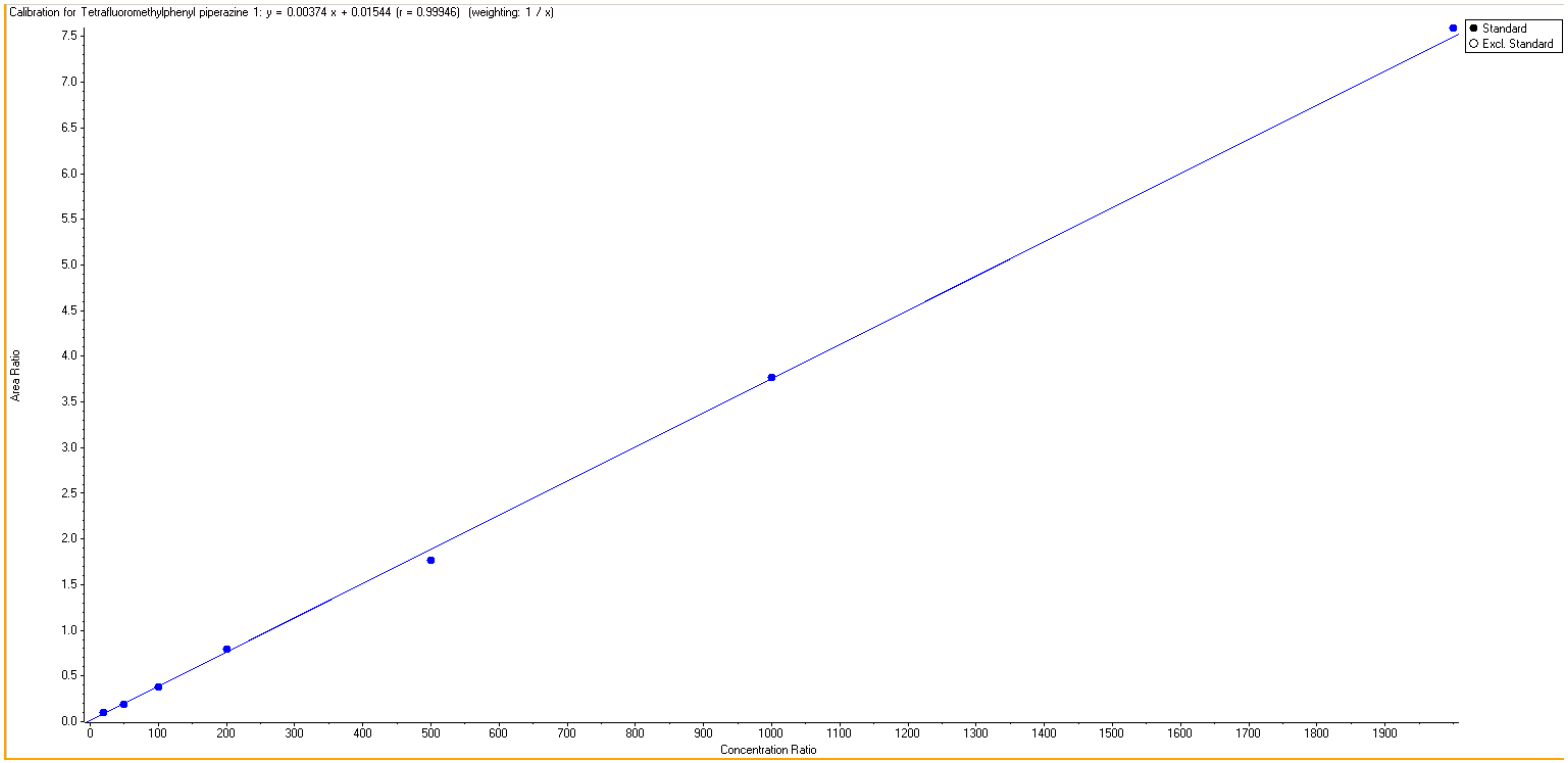


Figure 12: Calibration curve of TFMPP only when prepared and analyzed in blood on Day 0 of the 3-month trial. The R^2 value for this analyte was at 0.99, which was above the minimum accepted value of 0.98.

3.3 Analysis of 6-Month Samples

New calibration curves were generated for quantification analyses on both Day 0 and Day 182. According to Table 18, all R^2 values were above the acceptable limit of 0.98. Although almost every analyte fell within the % CV acceptable range of 20%, MeOPP that was stored under -20˚C, mCPP that was kept at 4˚C, DCPP stored at both room temperature and -20˚C as well as DCPP in mixed-mode that was stored at -20˚C had all exceeded the 20% limit.

Table 15: Average, standard deviation and percent coefficient of variation for analyte concentration in ng/mL on Day 0. All certified drug-free whole blood samples were fortified with the analytes at a concentration of 1000 ng/mL and run in triplicate. Analytes were prepared separately and in mixed-mode. The average concentration was calculated across these three runs.

Analyte	Average (ng/mL)	Standard Deviation	% CV	Range (ng/mL)
BZP	1000.99	40.51	4.05	970.45 – 1046.94
FBZP	1037.91	36.95	3.56	1010.24 – 1079.87
MBZP	1044.95	36.52	3.50	1006.74 – 1079.51
MeOPP	727.61	11.19	1.54	720.68 – 740.52
pFPP	806.31	2.36	0.29	804.06 – 808.78
mCPP	1012.62	6.59	0.65	1005.10 – 1017.41
DCPP	996.67	12.24	1.23	991.74 – 1010.60
TFMPP	548.59	4.22	0.77	544.65 – 553.05
MIX (BZP)	1107.67	53.24	4.81	1046.2 – 1138.49
MIX (FBZP)	1169.57	27.35	2.34	1141.98 – 1196.68
MIX (MBZP)	1066.12	46.43	4.35	1014.12 – 1103.43
MIX (MeOPP)	1242.55	7.64	0.61	1237.53 – 1251.34
MIX (pFPP)	1204.68	6.67	0.55	1197.52 – 1210.73
MIX (mCPP)	1061.60	5.87	0.55	1055.56 – 1067.29
MIX (DCPP)	1038.57	20.43	1.97	1018.89 – 1059.67
MIX (TFMPP)	1178.65	7.84	0.66	1173.84 – 1187.69

Table 16: Average, standard deviation and percent coefficient of variation for analyte concentration in ng/mL on Day 182 at different storage conditions. All samples were run in triplicate. The average concentration was calculated across these three runs. RT refers to room temperature.

Analyte / Storage Condition	Average (ng/mL)	Standard Deviation	% CV	Range (ng/mL)
BZP RT	253.42	0.94	0.37	252.53 – 254.40
BZP 4°C	240.88	3.01	1.25	237.45 – 243.07
BZP -20°C	443.01	4.11	0.93	439.13 – 447.33
FBZP RT	67.76	1.20	1.77	66.63 – 69.02
FBZP 4°C	81.39	1.49	1.83	80.26 – 83.08
FBZP -20°C	317.75	7.57	2.38	311.63 – 326.21
MBZP RT	1144.30	13.53	1.18	1128.98 – 1154.64
MBZP 4°C	959.84	32.84	3.42	922.76 – 985.26
MBZP -20°C	1189.62	77.19	6.49	1100.91 – 1241.49
MeOPP RT	0.00	0.00	0.00	0.00
MeOPP 4°C	0.00	0.00	0.00	0.00
MeOPP -20°C	3.42	5.93	173.21	0.00 – 10.27
pFPP RT	76.25	7.13	9.36	70.12 – 84.09
pFPP 4°C	17.33	0.97	5.62	16.61 – 18.44
pFPP -20°C	105.61	10.33	9.78	98.69 – 117.49
mCPP RT	9.84	0.12	1.25	9.75 – 9.98
mCPP 4°C	15.59	3.15	20.20	13.24 – 19.17
mCPP -20°C	56.39	1.16	2.06	55.15 – 57.44
DCPP RT	13.19	2.92	22.13	9.84 – 15.15
DCPP 4°C	16.64	1.54	9.27	15.45 – 18.38
DCPP -20°C	78.14	17.14	21.94	65.71 – 97.70
TFMPP RT	13.33	0.91	6.82	12.56 – 14.33
TFMPP 4°C	15.72	1.40	8.88	14.77 – 17.32
TFMPP -20°C	63.57	4.22	6.63	58.72 – 66.39

Table 17: Average, standard deviation and percent coefficient of variation for analyte concentration (in mixed-mode) in ng/mL on Day 182 at different storage conditions. All samples were run in triplicate. The average concentration was calculated across these three runs. RT refers to room temperature.

Storage Condition	Analyte	Average (ng/mL)	Standard Deviation	% CV	Range (ng/mL)
MIX RT	BZP	1017.58	23.11	2.27	990.92 – 1032.01
MIX 4°C	BZP	960.53	11.52	1.20	948.21 – 971.04
MIX -20°C	BZP	912.41	6.47	0.71	905.21 – 917.75
MIX RT	FBZP	870.14	28.87	3.32	842.83 – 900.35
MIX 4°C	FBZP	748.80	20.44	2.73	728.83 – 769.67
MIX -20°C	FBZP	690.55	16.60	2.40	674.57 – 707.71
MIX RT	MBZP	948.06	5.71	0.60	943.09 – 954.30
MIX 4°C	MBZP	922.26	41.09	4.45	893.39 – 969.29
MIX -20°C	MBZP	992.61	42.67	4.30	959.61 – 1040.80
MIX RT	MeOPP	320.15	4.26	1.33	315.49 – 323.85
MIX 4°C	MeOPP	546.49	9.44	1.73	536.27 – 554.89
MIX -20°C	MeOPP	74.01	5.79	7.83	70.36 – 80.69
MIX RT	pFPP	901.84	26.14	2.90	877.31 – 929.34
MIX 4°C	pFPP	1106.89	45.09	4.07	1066.69 – 1155.65
MIX -20°C	pFPP	625.25	36.56	5.85	583.14 – 648.80
MIX RT	mCPP	484.10	7.67	1.58	476.78 – 492.08
MIX 4°C	mCPP	376.94	5.41	1.44	370.73 – 380.62
MIX -20°C	mCPP	124.02	6.59	5.31	116.79 – 129.68
MIX RT	DCPP	425.92	7.27	1.71	419.56 – 433.85
MIX 4°C	DCPP	270.05	9.74	3.61	258.85 – 276.54
MIX -20°C	DCPP	99.53	22.56	22.67	73.82 – 116.03
MIX RT	TFMPP	484.28	6.29	1.30	477.08 – 488.69
MIX 4°C	TFMPP	353.65	0.62	0.18	353.30 – 354.37
MIX -20°C	TFMPP	133.15	1.03	0.78	131.99 – 133.99

Table 18: R^2 values of each analyte ran on calibration curve on Day 0 and Day 182.

	BZP	FBZP	MBZP	MeOPP	pFPP	mCPP	DCPP	TFMPP
R^2 value (Day 0)	0.9963	0.9944	0.9977	0.9964	0.9878	0.9936	0.9951	0.9932
R^2 value (Day 182)	0.9926	0.9915	0.9848	0.9914	0.9847	0.9983	0.9940	0.9995

Figure 13-16 suggested that most piperazines in blood experienced the highest rate of degradation between Day 91 and 182; the amount loss was significantly larger than those shown on Day 30 and Day 91. When stored at -20°C, all three benzyl piperazines had a smaller degree of loss as opposed to all of the phenyl piperazines; this was consistent with the degradation pattern observed from the 1-month and the 3-month trial. Under both room temperature and 4°C, the degree of degradation of BZP and FBZP revealed a 75% and 90% loss respectively, and showed almost no differences indicating stability at a particular storage condition over another. However, MBZP was comparably stable under all storage conditions where more than 90% of its parent compound was still detected after six months. In general, all phenyl piperazines were slightly more stable at -20°C because only approximately 2% of their parent compounds were still available under room temperature and 4°C. Among all phenyl piperazines, MeOPP experienced the largest degradation in which only approximately 3 ng/mL of its parent compound remained in the blood sample when stored at -20°C. No MeOPP was detected under room temperature or 4°C. In addition to phenyl piperazines, pFPP was more stable when stored in freezer and room temperature instead of refrigerating condition.

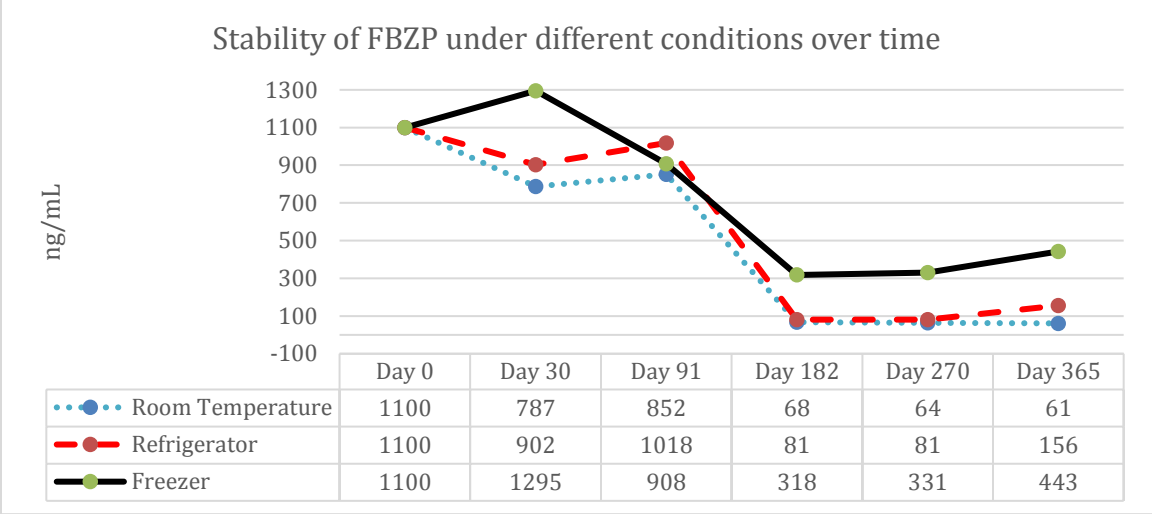


Figure 13: Effect of different storage conditions on FBZP overtime.

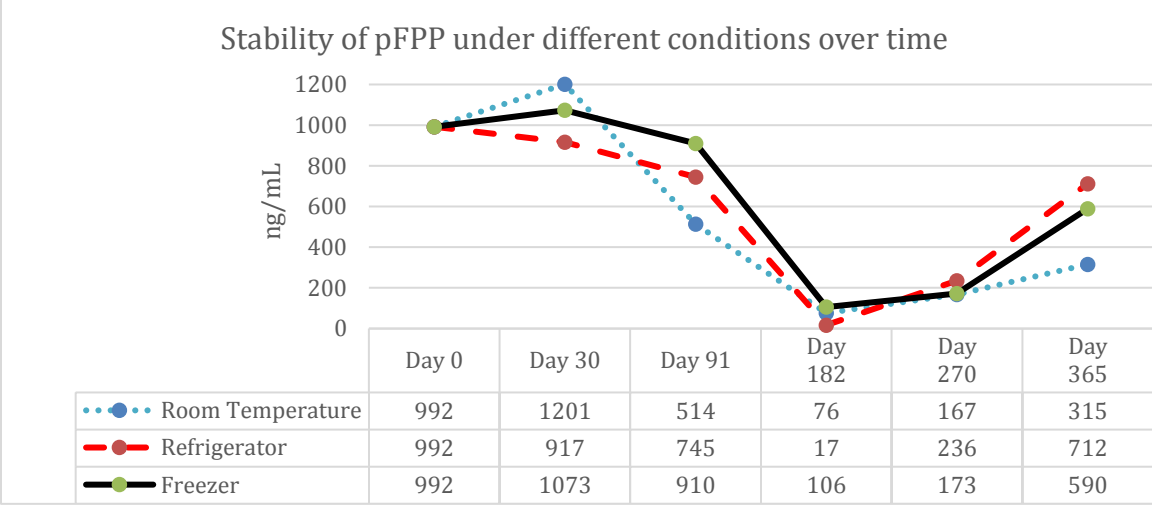


Figure 14: Effect of different storage conditions on pFPP overtime.

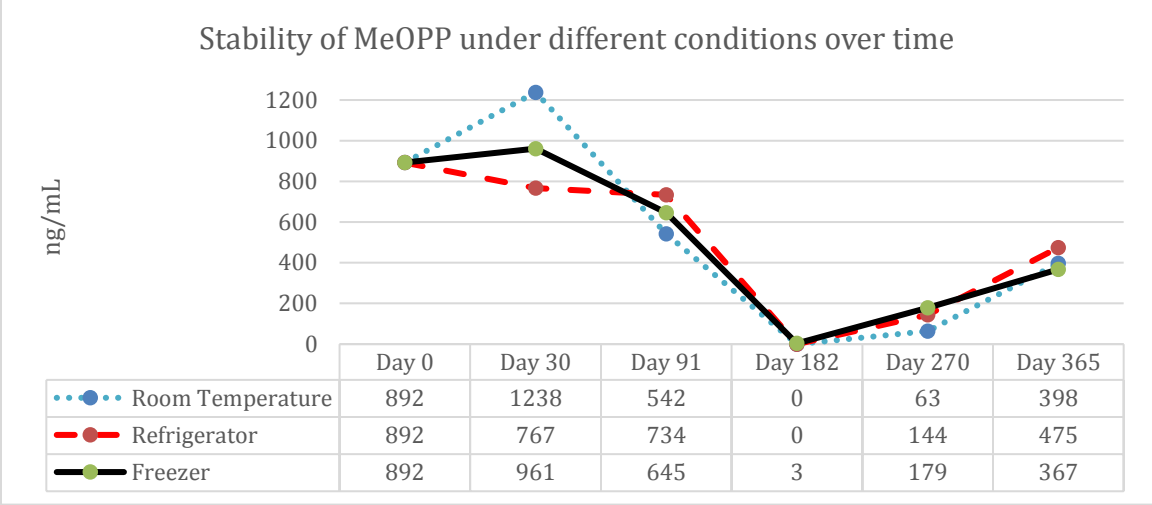


Figure 15: Effect of different storage conditions on MeOPP overtime.

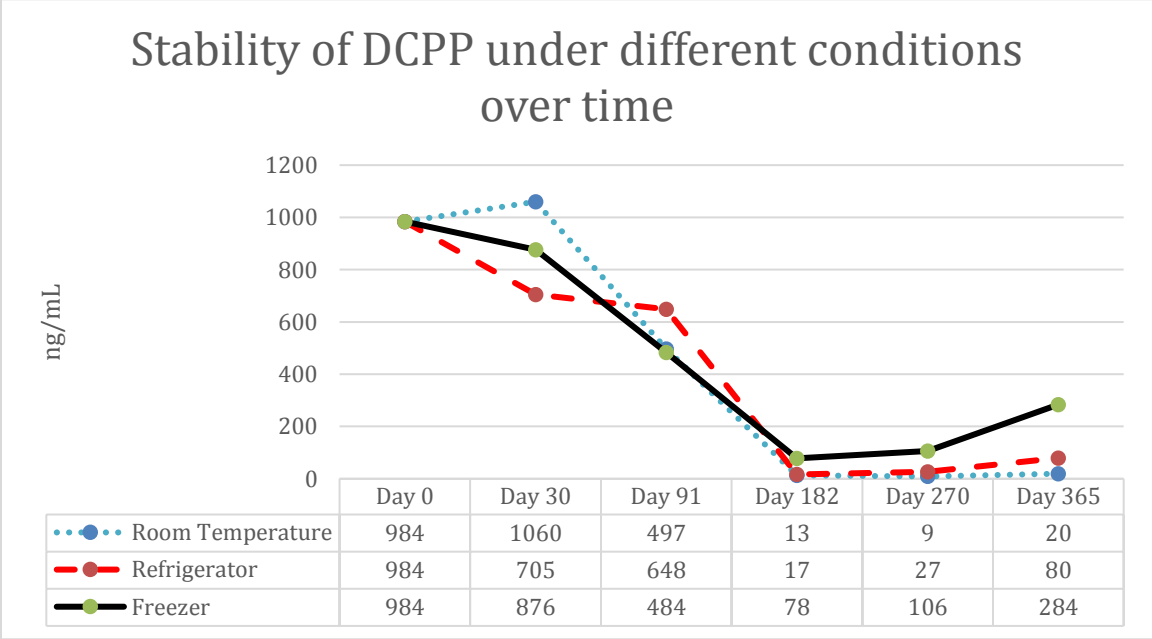


Figure 16: Effect of different storage conditions on DCPP overtime.

After storing all piperzines in mixed-mode for six months under various conditions, all benzyl piperazines exhibited a slight to moderate analyte degradation where BZP and

FBZP were found to be the most stable under room temperature when mixed with other piperzines. Moreover, MBZP had the least amount of loss out of all piperzines under the influence of analyte interferences. In contrast, all phenyl piperazines, except for pFPP, lost more than 50% of their parent analytes at every storage condition. In addition, MeOPP, pFPP, mCPP, DCPP and TFMPP were all clearly experiencing a significant loss over time at -20˚C; and this was consistent with other trials where a shorter period of storage time was implemented in which all of the non-benzyl piperazines were relatively more stable under room temperature. Nonetheless, it should be pointed out that only 74 ng/mL of MeOPP remained in blood when kept under -20˚C in mixed-mode. This particular analyte demonstrated a degree of degradation of more than 95% loss over a 182-day trial under freezing condition in mixed-mode.

To further support illustrating data analysis, a representative total ion chromatogram of a 2000ng/mL calibrator and a representative calibration curve are shown on Figure 17 and Figure 18.

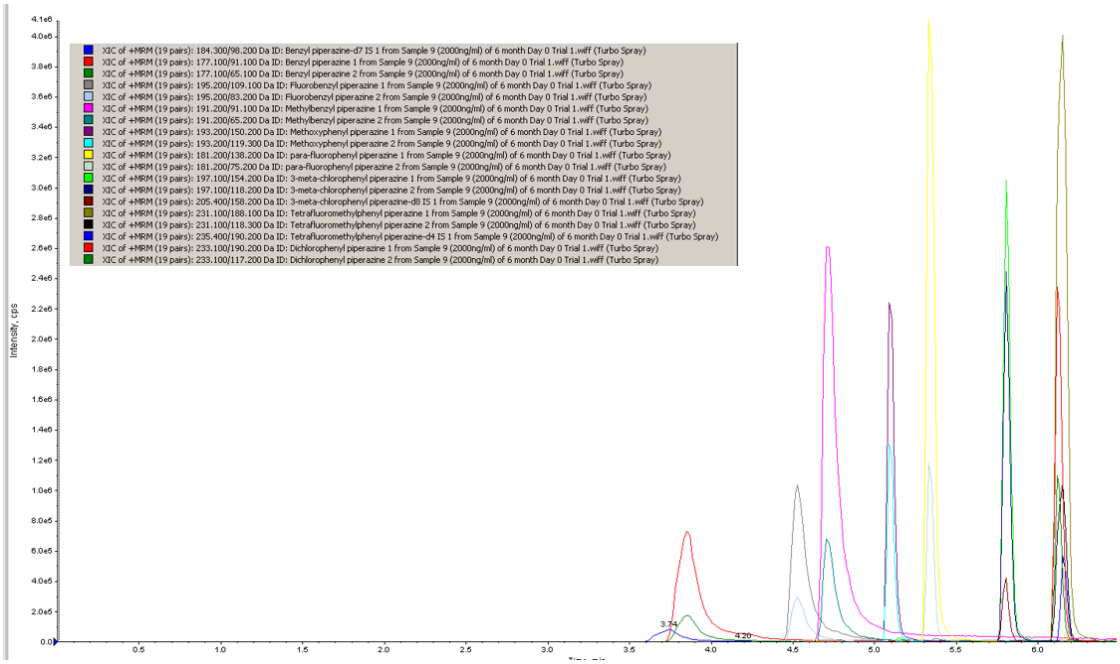


Figure 17: Total ion chromatogram of a 2000 ng/mL calibrator in blood on Day 0 of the 6-month trial. The x-axis is time ranging from 0-6.5 min and the y-axis is intensity measured in counts per second (cps) ranging from 0-4.1e^6. Analytes elute at the following retention times: BZP-d7 at 3.74 min, BZP at 3.85 min, FBZP at 4.53 min, MBZP at 4.71 min, MeOPP at 5.10 min, pFPP at 5.34 min, mCPP-d8 at 5.80 min, mCPP at 5.81 min, DCPP at 6.12 min, TFMPP at 6.15 min, and TFMPP-d4 at 6.16 min.

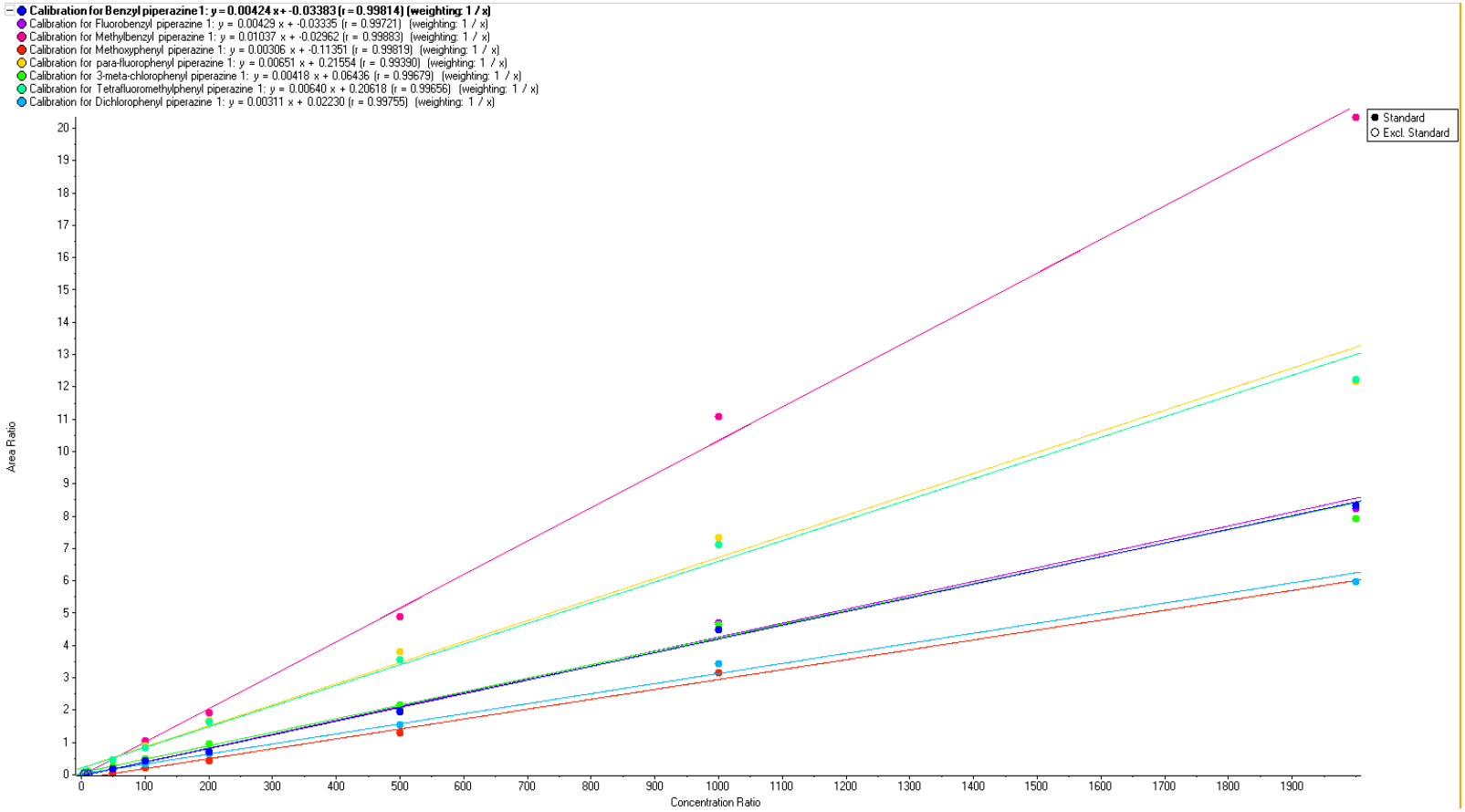


Figure 18: Calibration curve of BZP, FBZP, MBZP, MeOPP, pFPP, mCPP, DCPP and TFMPP and when prepared and analyzed in blood on Day 0 of the 6-month trial. The R^2 value for each analyte was above the minimum accepted value of 0.98.

3.4 Analysis of 9-Month Samples

On Day 270, a set of new calibration curves were prepared to estimate and quantitate the amount of piperazines left in blood. According to Table 21, all R^2 values were above the acceptable limit of 0.98.

Table 19: Average, standard deviation and percent coefficient of variation for analyte concentration in ng/mL on Day 270 at different storage conditions. All samples were run in triplicate. The average concentration was calculated across these three runs. RT refers to room temperature.

Analyte / Storage Condition	Average (ng/mL)	Standard Deviation	% CV	Range (ng/mL)
BZP RT	600.41	10.58	1.76	590.36 – 611.45
BZP 4°C	671.34	40.60	6.05	630.82 – 712.01
BZP -20°C	576.30	31.97	5.55	545.85 – 609.60
FBZP RT	63.75	1.03	1.61	63.08 – 64.93
FBZP 4°C	81.48	5.87	7.20	75.38 – 87.09
FBZP -20°C	331.61	9.39	2.83	320.98 – 338.80
MBZP RT	1223.00	11.17	0.91	1210.14 – 1230.32
MBZP 4°C	1530.80	50.88	3.32	1486.61 – 1586.42
MBZP -20°C	1262.52	16.33	1.29	1244.10 – 1275.24
MeOPP RT	63.06	17.85	28.31	51.15 – 83.58
MeOPP 4°C	143.96	40.22	27.94	117.15 – 190.21
MeOPP -20°C	179.06	40.41	22.57	132.70 – 206.77
pFPP RT	167.07	46.91	28.08	139.24 – 221.23
pFPP 4°C	236.47	62.92	26.61	199.48 – 309.12
pFPP -20°C	172.96	42.75	24.72	124.09 – 203.45
mCPP RT	26.12	0.45	1.73	25.60 – 26.43
mCPP 4°C	69.02	3.07	4.45	66.47 – 72.43
mCPP -20°C	151.02	5.46	3.61	147.58 – 157.31
DCPP RT	9.10	1.50	16.49	8.23 – 10.83

DCPP 4°C	26.52	9.26	34.92	19.71 – 37.07
DCPP -20°C	106.07	22.58	21.28	80.10 – 120.98
TFMPP RT	0.71	1.22	173.21	0.00 – 2.12
TFMPP 4°C	22.46	30.63	136.38	4.63 – 57.83
TFMPP -20°C	88.80	34.47	38.81	60.68 – 127.25

Table 20: Average, standard deviation and percent coefficient of variation for analyte concentration (in mixed-mode) in ng/mL on Day 270 at different storage conditions. All samples were run in triplicate. The average concentration was calculated across these three runs. RT refers to room temperature.

Storage Condition	**Analyte**	**Average (ng/mL)**	**Standard Deviation**	**% CV**	**Range (ng/mL)**
MIX RT	BZP	666.34	24.07	3.61	643.32 – 691.35
MIX 4°C	BZP	1056.03	21.99	2.08	1041.38 – 1081.31
MIX -20°C	BZP	1150.42	43.97	3.82	1117.84 – 1200.44
MIX RT	FBZP	248.16	17.36	7.00	229.11 – 263.09
MIX 4°C	FBZP	591.88	10.65	1.80	579.59 – 598.29
MIX -20°C	FBZP	755.16	24.23	3.21	728.98 – 776.78
MIX RT	MBZP	1015.99	38.53	3.79	973.52 – 1048.72
MIX 4°C	MBZP	1420.35	23.77	1.67	1393.60 – 1439.06
MIX -20°C	MBZP	1213.90	37.52	3.09	1170.61 – 1237.14
MIX RT	MeOPP	144.78	2.34	1.62	143.19 – 147.47
MIX 4°C	MeOPP	383.79	104.29	27.17	312.57 – 502.50
MIX -20°C	MeOPP	597.88	103.62	17.33	510.90 – 712.52
MIX RT	pFPP	131.94	32.37	24.53	111.28 – 169.25
MIX 4°C	pFPP	362.36	90.71	25.03	296.89 – 465.90
MIX -20°C	pFPP	659.38	132.56	20.10	552.67 – 808.77
MIX RT	mCPP	34.36	1.49	4.35	32.94 – 35.92
MIX 4°C	mCPP	131.46	6.54	4.97	124.89 – 137.96
MIX -20°C	mCPP	801.51	21.91	2.73	781.27 – 824.77
MIX RT	DCPP	15.00	3.58	23.88	12.82 – 19.13
MIX 4°C	DCPP	53.35	11.72	21.97	44.00 – 66.50

MIX -20°C	DCPP	433.09	4.88	1.13	427.52 – 436.57
MIX RT	TFMPP	12.83	11.52	89.78	5.82 – 26.12
MIX 4°C	TFMPP	59.17	6.26	10.58	55.34 – 66.39
MIX -20°C	TFMPP	663.43	8.02	1.21	657.98 – 672.64

Table 21: R^2 values of each analyte ran on calibration curve on Day 270.

	BZP	FBZP	MBZP	MeOPP	pFPP	mCPP	DCPP	TFMPP
R^2 value (Day 270)	0.9993	0.9993	0.9814	0.9973	0.9966	0.9986	0.9952	0.9977

Similar to the degradation pattern observed on Day 91 and Day 182, all benzyl piperazines were comparatively more stable than phenyl piperazines in blood. Although data on Day 270 showed that MBZP perhaps was the most stable compound among all piperazines, the amount of MBZP remained in blood after storing in a refrigerator for nine months was detected at an exceptionally high concentration of 1530 ng/mL. Since all samples were spiked at a designated initial concentration of 1000 ng/mL, the presence of MBZP as a metabolite from other piperazines or compounds that are structurally-related to MBZP in the same matrix could not be ruled out as the source of this outlier. Overall, MeOPP, mCPP, DCPP and TFMPP were determined to be the most stable in blood at -20˚C, followed by 4˚C and room temperature.

While the degree of degradation of BZP and MBZP were smaller in mixed-mode samples, there was a moderate loss of FBZP at -20˚C and 4˚C. Table 20 also indicated that FBZP in blood was very unstable at room temperature under mixed-mode. Unlike the findings obtained from Day 30, 91 and 182, all phenyl piperazine derivatives were the most

stable in freezer. For example, more than 80% of mCPP was still detected on Day 270 when it was stored at -20˚C under mixed-mode condition.

3.4.1 Matrix Interference

As shown on Table 20, MBZP in mixed-mode sample which was originally spiked at 1000 ng/mL had reached 1420 ng/mL after storing at 4˚C for 270 days. Since slight coagulation was observed in some samples after nine months, matrix interference could not be excluded as one of the causes of this exceptionally high level of MBZP. This is because blood coagulation could be a result of alternation of physiochemical properties such as viscosity and surface tension. Although limited information is currently available on synthetic piperazines' metabolic pathways, the excess amount of MBZP quantified in this trial could be a result of the presence of active metabolites that share the same mass-to-charge ratio with MBZP. Also, the possibility of having MBZP as the metabolite derived from other piperazines under mixed-mode could confound the validity of this result as well.

3.5 Analysis of 12-Month Samples

To quantitate the amount of synthetic piperazines left in blood after storing for 365 days, a set of new calibration curves were prepared. All R^2 values were above the acceptable limit of 0.98 (Table 24). All % CV were within the 20% limit range.

Table 22: Average, standard deviation and percent coefficient of variation for analyte concentration in ng/mL on Day 365 at different storage conditions. All samples were run in triplicate. The average concentration was calculated across these three runs. RT refers to room temperature.

Analyte / Storage Condition	Average (ng/mL)	Standard Deviation	% CV	Range (ng/mL)
BZP RT	284.83	13.00	4.56	269.94 – 293.85
BZP 4°C	585.98	6.80	1.16	581.91 – 593.83
BZP -20°C	662.86	22.46	3.39	640.48 – 685.41
FBZP RT	63.72	3.62	5.68	60.91 – 67.80
FBZP 4°C	166.41	9.44	5.67	155.52 – 172.03
FBZP -20°C	442.59	5.57	1.26	436.68 – 447.73
MBZP RT	874.87	31.06	3.55	843.32 – 905.41
MBZP 4°C	713.65	49.57	6.95	673.20 – 768.96
MBZP -20°C	966.52	69.82	7.22	917.80 – 1046.51
MeOPP RT	398.36	4.84	1.21	394.67 – 403.83
MeOPP 4°C	474.92	5.07	1.07	469.10 – 478.31
MeOPP -20°C	366.65	5.58	1.52	360.49 – 371.34
pFPP RT	314.91	3.86	1.22	311.80 – 319.22
pFPP 4°C	711.98	1.97	0.28	710.04 – 713.99
pFPP -20°C	590.29	21.05	3.57	567.86 – 609.61
mCPP RT	75.59	2.19	2.90	73.14 – 77.36
mCPP 4°C	621.57	4.15	0.67	616.79 – 624.31
mCPP -20°C	408.93	17.93	4.38	398.15 – 429.63
DCPP RT	20.07	1.74	8.67	18.70 – 22.03
DCPP 4°C	79.98	2.95	3.69	77.79 – 83.34
DCPP -20°C	283.65	7.92	2.79	275.08 – 290.70
TFMPP RT	73.32	4.34	5.92	68.89 – 77.57
TFMPP 4°C	420.73	4.87	1.16	416.98 – 426.23
TFMPP -20°C	343.50	13.00	3.78	333.01 – 358.04

Table 23: Average, standard deviation and percent coefficient of variation for analyte concentration (in mixed-mode) in ng/mL on Day 365 at different storage conditions. All samples were run in triplicate. The average concentration was calculated across these three runs. RT refers to room temperature.

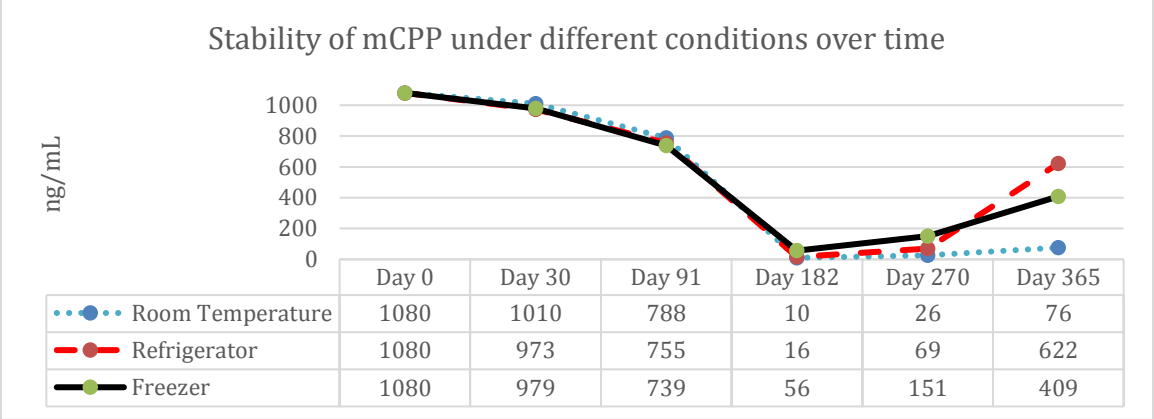
Storage Condition	Analyte	Average (ng/mL)	Standard Deviation	% CV	Range (ng/mL)
MIX RT	BZP	819.04	25.49	3.11	790.08 – 838.10
MIX 4°C	BZP	841.29	34.43	4.09	803.83 – 871.55
MIX -20°C	BZP	851.54	17.90	2.10	832.67 – 868.27
MIX RT	FBZP	428.13	18.51	4.32	407.44 – 443.14
MIX 4°C	FBZP	533.04	40.65	7.63	487.67 – 566.15
MIX -20°C	FBZP	709.08	46.53	6.56	663.66 – 756.64
MIX RT	MBZP	839.11	52.80	6.29	787.67 – 893.16
MIX 4°C	MBZP	955.15	100.15	10.49	849.42 – 1048.59
MIX -20°C	MBZP	1008.18	93.28	9.25	907.27 – 1091.26
MIX RT	MeOPP	2.62	0.12	4.62	2.55 – 2.76
MIX 4°C	MeOPP	628.54	16.34	2.60	610.48 – 642.30
MIX -20°C	MeOPP	343.71	2.10	0.61	341.42 – 345.55
MIX RT	pFPP	0.00	0.00	0.00	0.00-0.00
MIX 4°C	pFPP	1015.68	45.69	4.50	963.61 – 1049.09
MIX -20°C	pFPP	553.10	1.81	0.33	551.46 – 555.03
MIX RT	mCPP	0.00	0.00	0.00	0.00 – 0.00
MIX 4°C	mCPP	903.02	41.98	4.65	859.23 – 942.91
MIX -20°C	mCPP	337.90	2.91	0.86	336.15 – 341.26
MIX RT	DCPP	31.20	2.16	6.93	29.10 – 33.43
MIX 4°C	DCPP	150.89	8.31	5.50	144.24 – 160.20
MIX -20°C	DCPP	217.99	10.70	4.91	205.76 – 225.68
MIX RT	TFMPP	0.00	0.00	0.00	0.00 – 0.00
MIX 4°C	TFMPP	426.00	10.19	2.39	414.71 – 434.51
MIX -20°C	TFMPP790.0	294.50	6.79	2.31	288.45 – 301.84

Table 24: R^2 values of each analyte ran on calibration curve on Day 365.

	BZP	FBZP	MBZP	MeOPP	pFPP	mCPP	DCPP	TFMPP
R^2 value (Day 365)	0.9976	0.9993	0.9865	0.9953	0.9995	0.9993	0.9970	0.9998

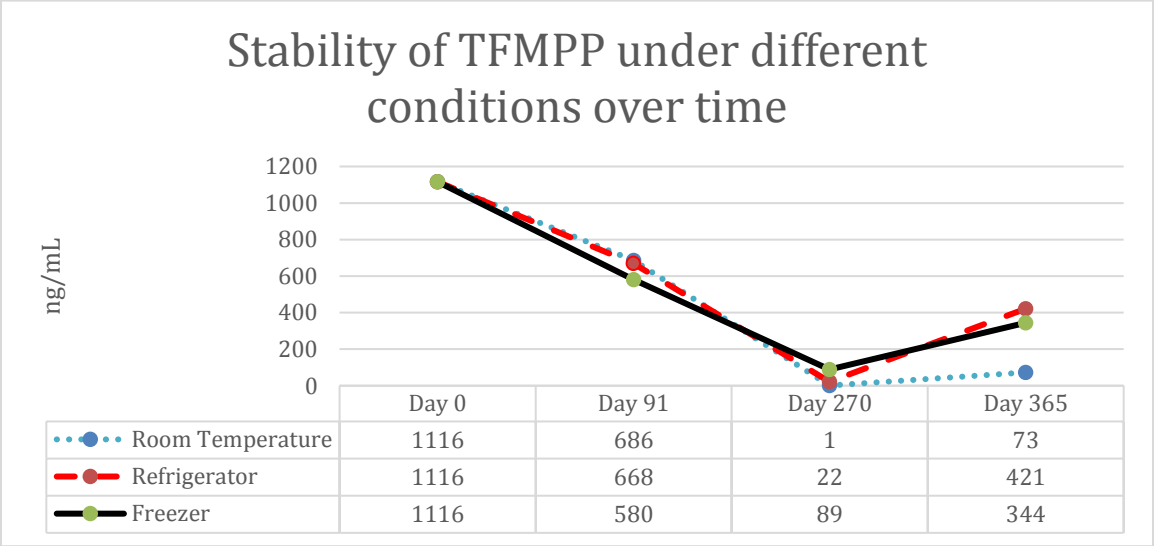
Analytes had the smallest degrees of loss when stored at -20°C, which was consistent with the hypothesis that synthetic piperazines should be more stable in blood under freezing condition. In addition, MBZP was found to be extremely stable regardless of storage conditions; more than 70% of its parent analyte was still detected after 12 months. Overall, each phenyl piperazine, except for DCPP, lost most of its analyte at 4°C instead of -20°C. Furthermore, mCPP, DCPP and TFMPP exhibited the largest degradation in which only ~10% of their parent analytes were left in blood when stored at room temperature.

Similar to all of the other trials, benzyl piperazines in mix-mode were significantly more stable than phenyl piperazines. However, it was clear that all phenyl piperazines had a dramatic decline in concentration level after storing them for 12 months at room temperature under mix-mode. For example, MeOPP, pFPP, mCPP and TFMPP were not detectable after 365 days. As a result, it is highly recommended to store matrices containing synthetic piperazines at an appropriate storage condition rather than at room temperature. This might help to retain more of the compound(s) when samples are retained for extended periods of time.



	Day 0	Day 30	Day 91	Day 182	Day 270	Day 365
Room Temperature	1080	1010	788	10	26	76
Refrigerator	1080	973	755	16	69	622
Freezer	1080	979	739	56	151	409

Figure 19: Effect of different storage conditions on mCPP overtime.



	Day 0	Day 91	Day 270	Day 365
Room Temperature	1116	686	1	73
Refrigerator	1116	668	22	421
Freezer	1116	580	89	344

Figure 20: Effect of different storage conditions on TFMPP overtime.

Moreover, pFPP, MeOPP, mCPP and TFMPP had a resurgence on analyte concentration level after keeping them for 12 months as shown in Figure 14, 15, 19 and 20. The phenomenon could be a result of: the presence of active metabolite sharing the same mass-to-charge ratio with its parent compound; having compounds that owned similar structures; or having the identical molecular weight with the original piperazines spiked on Day 0. This might falsely mislead the way how the MS would detect designated ions and

affect the precision of the whole quantification process where specificity could have been impacted.

3.6 Background Noise

Although a higher background noise at the end of the column separation was expected for this method, multiple double blanks and solvent blanks between 0 and 5.5 minutes had a relatively high background noise intensity after a new roughing pump had been replaced on the instrument as part of the annual maintenance. As shown in Figure 22, a normal baseline should be between 0 and 1000cps. However in Figure 21, the background noise has reached approximately 2000cps for one particular analyte. According to the SWGTOX guidelines, the chromatogram is acceptable as long as the response (peak area) of the analyte's lower LOQ is greater than the background noise. Since all lower LOQ (defined as 25% of the LOQ) of each analyte had a response greater than the highest baseline noise of about 4000cps, the background noise issue was justified and could be ignored. Thus, the concentration of each analyte in unknown samples was accurately quantified.

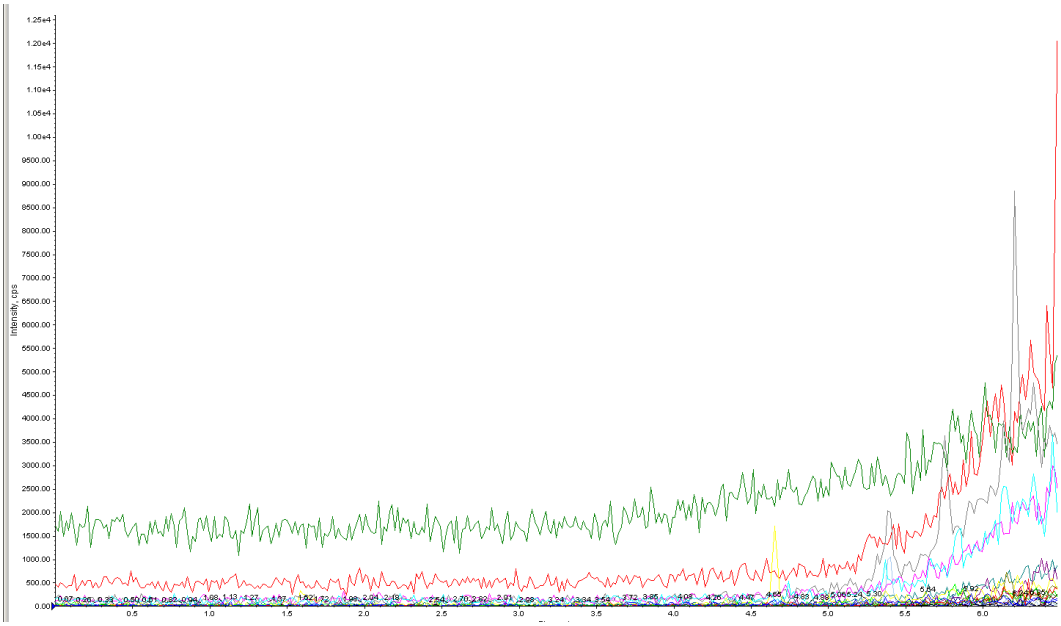


Figure 21: Total ion chromatogram (with high background noise at about 2000cps) of a double blank on Day 0 of the 3-month trial. This scan shows mass-to-charge ratios in Da on the x-axis and intensity in counts per second on the y-axis.

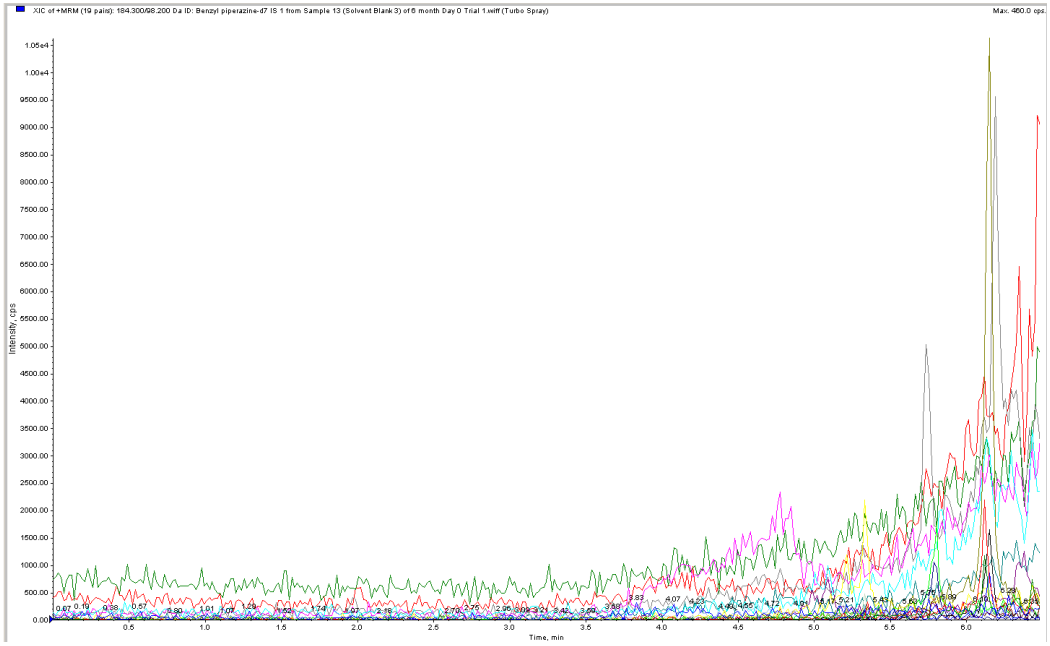


Figure 22: Total ion chromatogram (with normal background noise between 500 and 1000cps) of a solvent blank on Day 0 of the 6-month trial. This scan shows mass-to-charge ratios in Da on the x-axis and intensity in counts per second on the y-axis.

4. CONCLUSION

4.1 Summary of Findings

The use of solid phase extraction as a sample preparation technique was proven to be successful to obtain analytes of interest by removing matrices and unwanted components from the sample. Not only the quantification of synthetic piperazines using a UFLC-MS/MS system was robust and reliable, but also the method utilized in this project including equilibrations had a quick total run time of 11.5 minutes. All calibrations curves exhibited an R^2 value above 0.98 and had met the criteria according to the SWGTOX guideline.

Data on this stability investigation revealed that BZP, MBZP and FBZP were generally more stable than all other phenyl piperazines over time under all storage conditions, in which MBZP was extremely stable and still had at least 70% left after 12 months. Most data, if not all, showed a smaller degree of degradation if proper storage conditions were maintained that is when samples were kept in either a refrigerator or a freezer. In contrast, storing samples at room temperature should be avoided because of detrimental impacts on stability of piperazine compounds. This information is very valuable when analyzing data for postmortem specimens that are collected from a decomposed body or a body that is found in an exceptionally warm environment. For crime laboratories that are facing backlog situations, it is recommended that specimen containing synthetic piperazines should be kept frozen or refrigerated even for time period as short as 30 days or less. However, they should not be stored for more than six months because

phenyl piperazines are more susceptible to lose all parent compounds after six months regardless of storage conditions.

4.2 Analyte and Matrix Interference

Similarly, all benzyl piperazines were comparably more stable than phenyl piperazines in mixed-mode under all storage conditions. For this reason, it was determined that the stability of BZP, MBZP and FBZP were less likely to be affected by analyte interferences in blood over time. However, all phenyl piperazines including TFMPP, DCPP, mCPP, pFPP and MeOPP experienced severe degradation after only 30 days. In addition, they had lost more than 50% of their initial concentration after three months.

Phenyl piperazines when mixed with other derivatives appeared to be more stable at 4˚C or even at room temperature as opposed to -20˚C. Data from Day 91 and 182 indicated that DCPP and TFMPP under mixed-mode were consistently more stable at room temperature. Although this was contradicting the hypothesis that synthetic piperazines in biological specimens are more likely to degrade at a slower rate when stored in a freezer or refrigerator, this piece of information is very useful for laboratories that have limited freezer storage; this is because detectable levels of analytes in whole blood might still be able to be quantified after three to six months without freezing. However, the exact stability pattern of phenyl piperazines when mixed together could not be determined from this data set alone due to discrepancies observed on Day 91 and 270.

The high level of MBZP concentration quantitated on Day 270 could be a result of matrix interference because of slight coagulation observed in some blood samples. In addition, the presence of metabolites derived from other piperazines or the presence of

structurally-related compounds that shared the same mass-to-charge ratio with the target analyte could have contributed to the late peak increase of mCPP, DCPP and MeOPP under mixed-mode. Thus, this result emphasized that further work is needed to explore the most stable storage condition and necessary precautions for specimens containing more than one synthetic piperazines in order to ensure minimal degradation.

4.3 DCPP and TFMPP

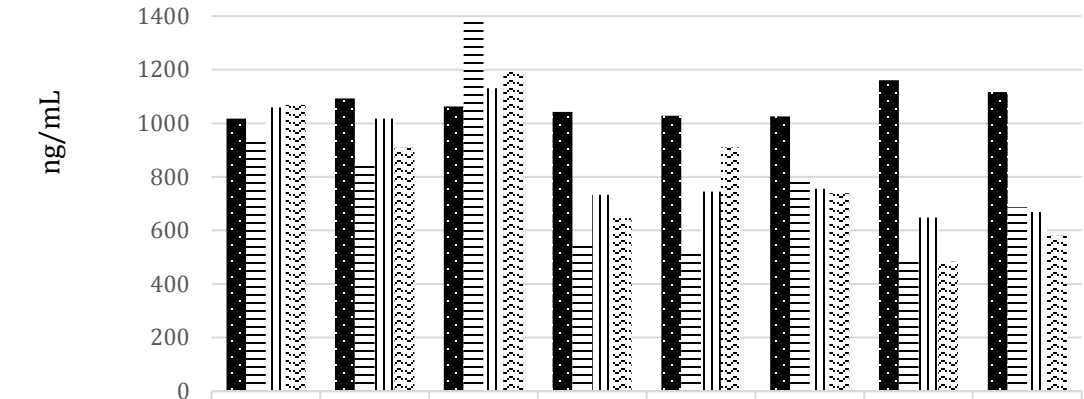
For cases that involve the mixture of BZP and other piperazine drugs such as TFMPP that shares certain pharmacodynamic traits with MDMA, data suggested that both of these piperazines will still be detectable after three months or even a longer period of time that can be encountered in backlog situations. Moreover, if DCPP and TFMPP are both present in whole blood specimen, a separate calibration curve containing only TFMPP is required in order to obtain an accurate quantitative result that reflects the actual concentration of TFMPP in blood. Another possible solution to avoid the DCPP influence on TFMPP is to optimize this current method so that both analytes will have their individual discrete mass-to-charge ratio values.

4.4 Future Direction

As the number, categories and analogs of designer drugs continues to emerge, the stability of synthetic piperazines is crucial in terms of data analyses for forensic casework. This research project has shown a solid method to examine how quickly or slowly synthetic piperazines degrade in blood at each storage condition. For future research, it is also

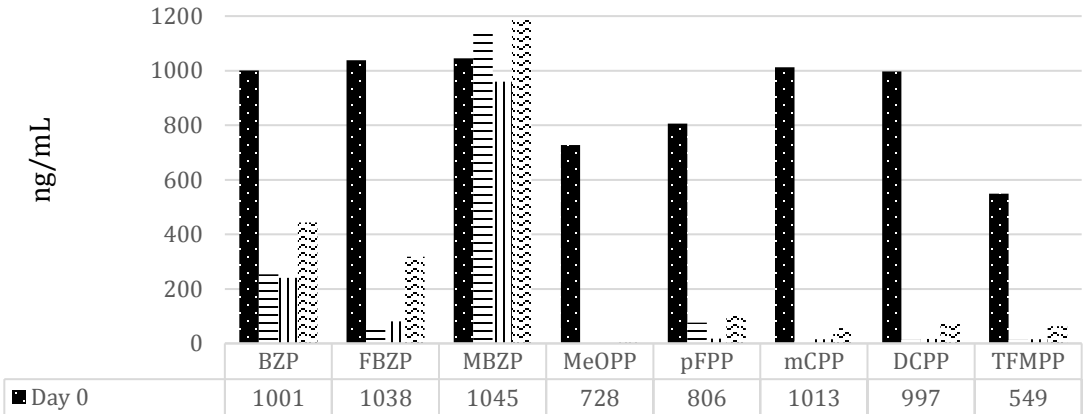
important to evaluate the number of freeze-thaw cycles on each specimen in order to minimize the effect of non-metabolic degradation. At practical levels, the stability of piperazines when stored at -60°C to -80°C should also be investigated to support the hypothesis that lower temperature freezing conditions are believed to be the most stable environment for piperazines in blood.

APPENDIX A: BAR GRAPH / LINE GRAPH DATA



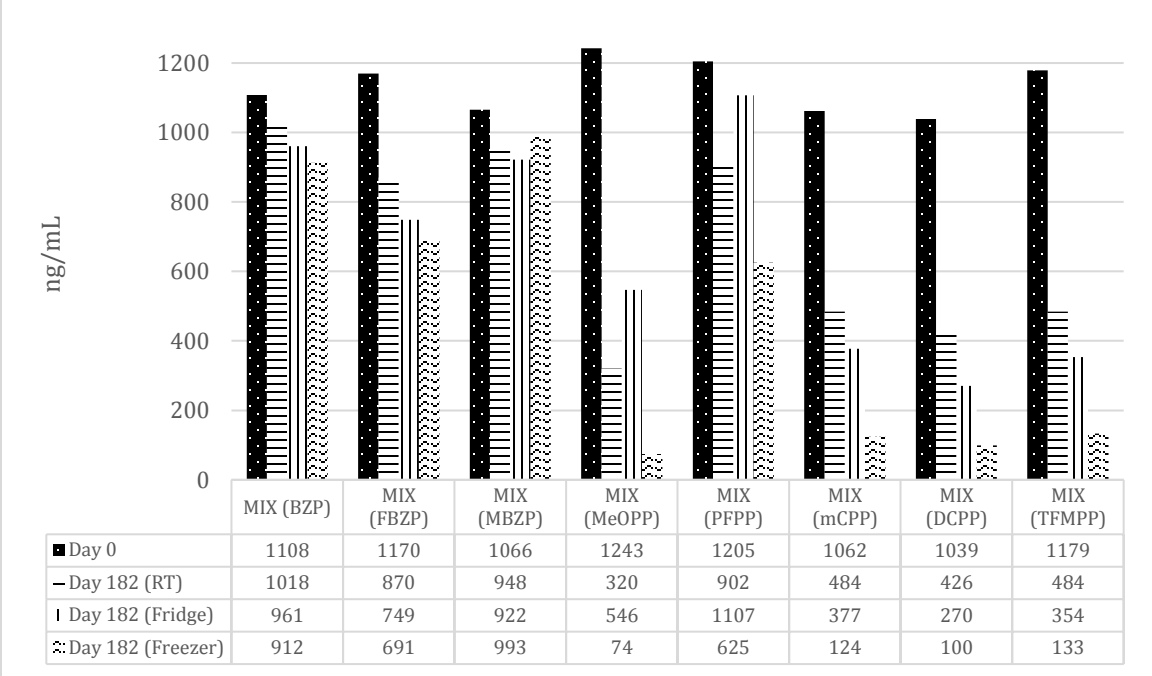
	BZP	FBZP	MBZP	MeOPP	pFPP	mCPP	DCPP	TFMPP
Day 0	1017	1093	1063	1042	1028	1026	1161	1116
Day 91 (RT)	935	852	1392	542	514	788	497	686
Day 91 (Fridge)	1059	1018	1130	734	745	755	648	668
Day 91 (Freezer)	1068	908	1191	645	910	739	484	580

Figure A: Effect of different storage conditions on analyte stability over a 91-day trial. RT refers to room temperature. Temperature in freezer and fridge were at -20˚C and 4˚C respectively.



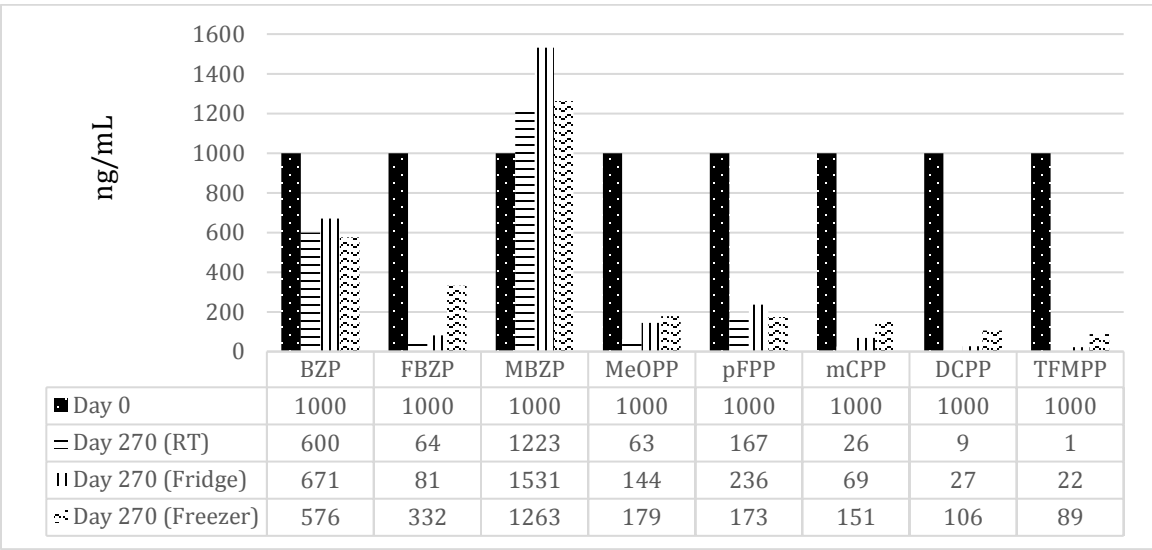
	BZP	FBZP	MBZP	MeOPP	pFPP	mCPP	DCPP	TFMPP
Day 0	1001	1038	1045	728	806	1013	997	549
Day 182 (RT)	253	68	1144	0	76	10	13	13
Day 182 (Fridge)	241	81	960	0	17	16	17	16
Day 182 (Freezer)	443	318	1190	3	106	56	78	64

Figure B: Effect of different storage conditions on analyte stability over a 182-day trial. RT refers to room temperature. Temperature in freezer and fridge were at -20˚C and 4˚C respectively.



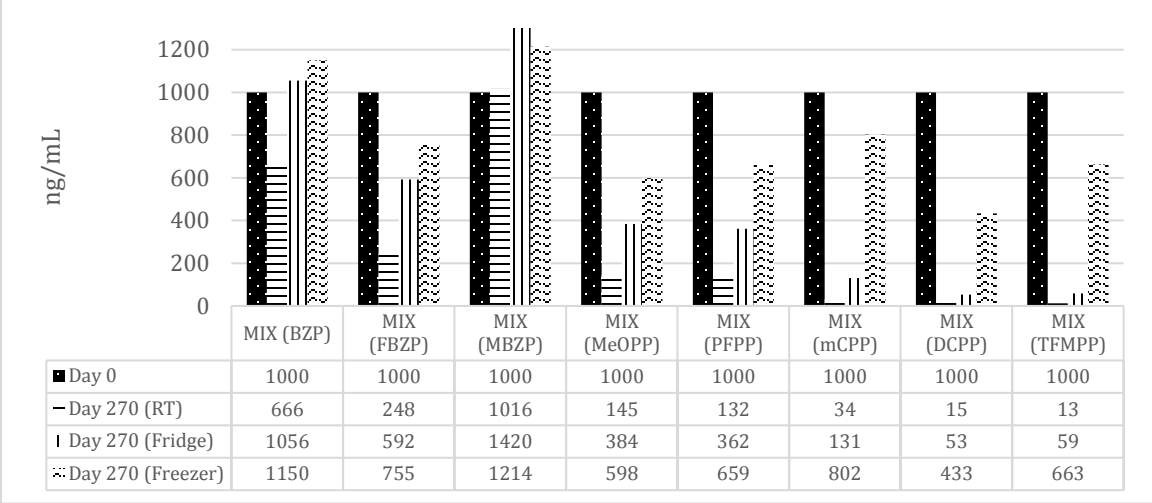
	MIX (BZP)	MIX (FBZP)	MIX (MBZP)	MIX (MeOPP)	MIX (PFPP)	MIX (mCPP)	MIX (DCPP)	MIX (TFMPP)
Day 0	1108	1170	1066	1243	1205	1062	1039	1179
Day 182 (RT)	1018	870	948	320	902	484	426	484
Day 182 (Fridge)	961	749	922	546	1107	377	270	354
Day 182 (Freezer)	912	691	993	74	625	124	100	133

Figure C: Effect of different storage conditions on analyte interference and stability over a 182-day trial. RT refers to room temperature. Temperature in freezer and fridge were at -20˚C and 4˚C respectively.



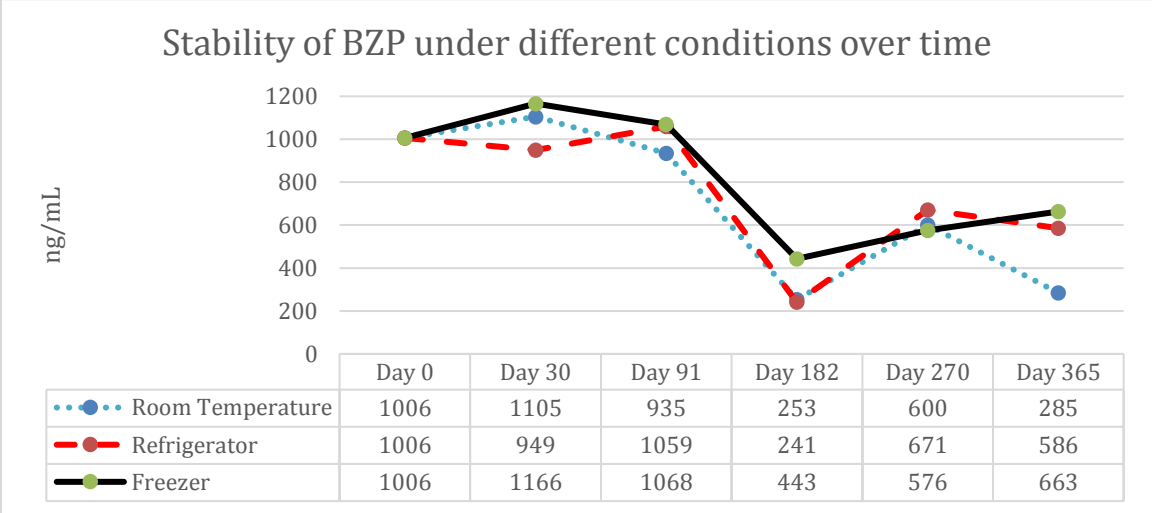
	BZP	FBZP	MBZP	MeOPP	pFPP	mCPP	DCPP	TFMPP
Day 0	1000	1000	1000	1000	1000	1000	1000	1000
Day 270 (RT)	600	64	1223	63	167	26	9	1
Day 270 (Fridge)	671	81	1531	144	236	69	27	22
Day 270 (Freezer)	576	332	1263	179	173	151	106	89

Figure D: Effect of different storage conditions on analyte stability over a 270-day trial. RT refers to room temperature. Temperature in freezer and fridge were at -20˚C and 4˚C respectively.



	MIX (BZP)	MIX (FBZP)	MIX (MBZP)	MIX (MeOPP)	MIX (PFPP)	MIX (mCPP)	MIX (DCPP)	MIX (TFMPP)
Day 0	1000	1000	1000	1000	1000	1000	1000	1000
Day 270 (RT)	666	248	1016	145	132	34	15	13
Day 270 (Fridge)	1056	592	1420	384	362	131	53	59
Day 270 (Freezer)	1150	755	1214	598	659	802	433	663

Figure E: Effect of different storage conditions on analyte interference and stability over a 270-day trial. RT refers to room temperature. Temperature in freezer and fridge were at -20˚C and 4˚C respectively.



	Day 0	Day 30	Day 91	Day 182	Day 270	Day 365
Room Temperature	1006	1105	935	253	600	285
Refrigerator	1006	949	1059	241	671	586
Freezer	1006	1166	1068	443	576	663

Figure F: Effect of different storage conditions on BZP overtime.

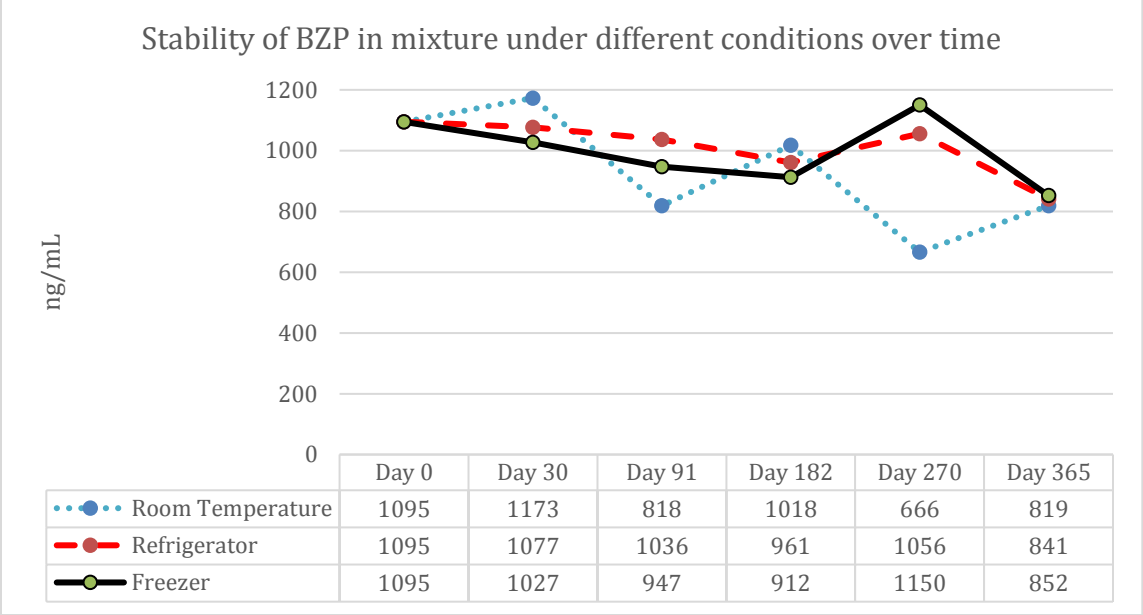


Figure G: Effect of different storage conditions on BZP in mixture overtime.

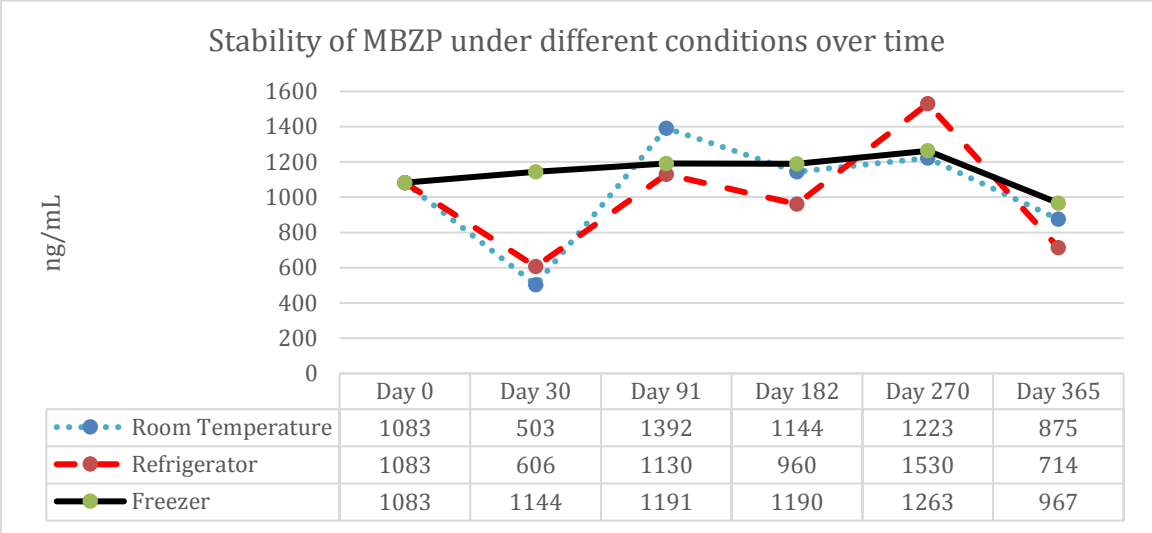
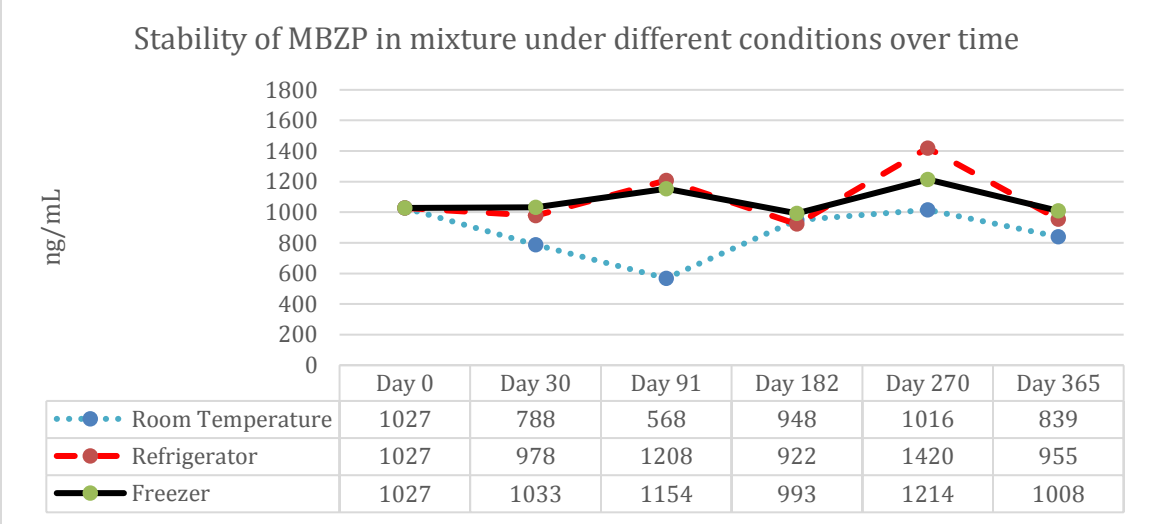
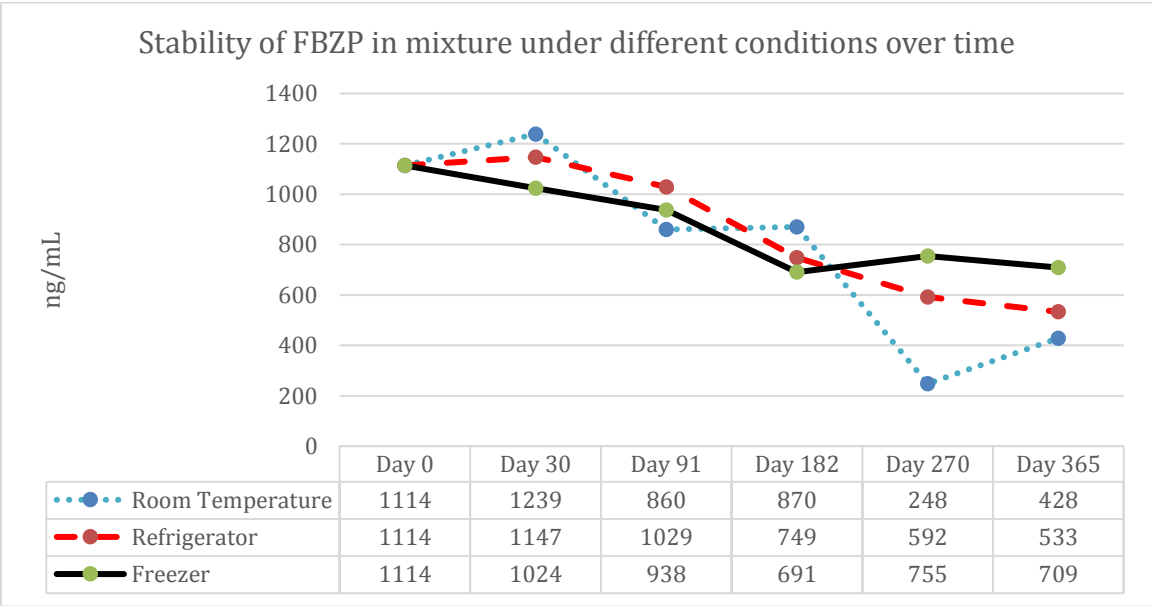


Figure H: Effect of different storage conditions on MBZP overtime.



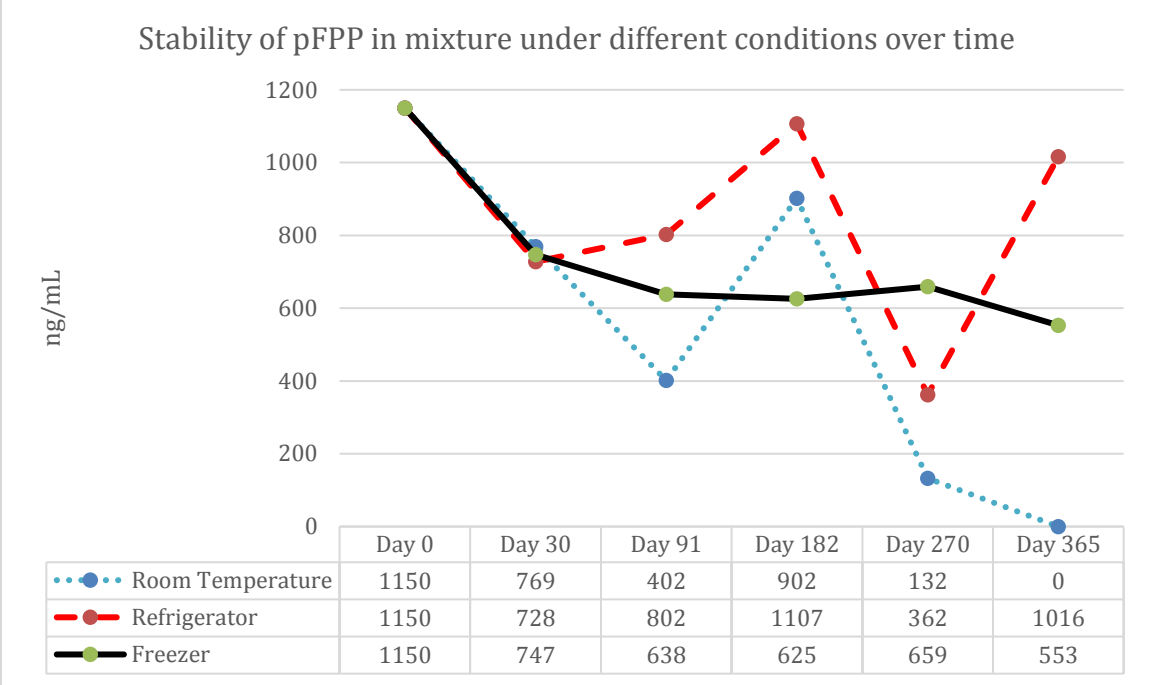
	Day 0	Day 30	Day 91	Day 182	Day 270	Day 365
Room Temperature	1027	788	568	948	1016	839
Refrigerator	1027	978	1208	922	1420	955
Freezer	1027	1033	1154	993	1214	1008

Figure I: Effect of different storage conditions on MBZP in mixture overtime.



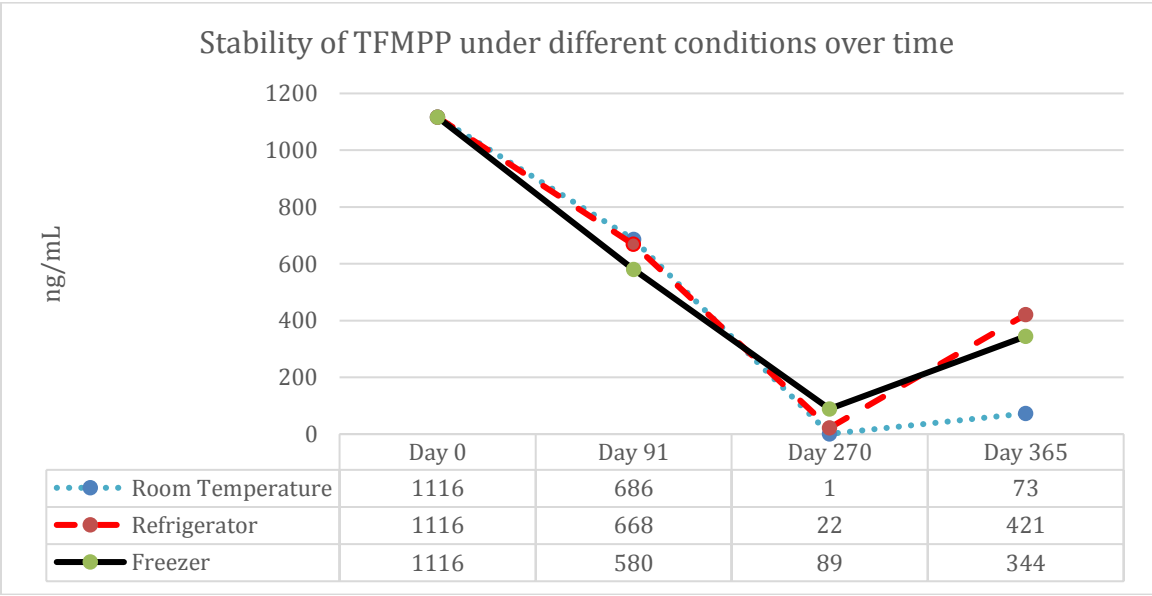
	Day 0	Day 30	Day 91	Day 182	Day 270	Day 365
Room Temperature	1114	1239	860	870	248	428
Refrigerator	1114	1147	1029	749	592	533
Freezer	1114	1024	938	691	755	709

Figure J: Effect of different storage conditions on FBZP in mixture overtime.



	Day 0	Day 30	Day 91	Day 182	Day 270	Day 365
Room Temperature	1150	769	402	902	132	0
Refrigerator	1150	728	802	1107	362	1016
Freezer	1150	747	638	625	659	553

Figure K: Effect of different storage conditions on pFPP in mixture overtime.



	Day 0	Day 91	Day 270	Day 365
Room Temperature	1116	686	1	73
Refrigerator	1116	668	22	421
Freezer	1116	580	89	344

Figure L: Effect of different storage conditions on TFMPP overtime.

APPENDIX B: CHROMATOGRAPHIC DATA

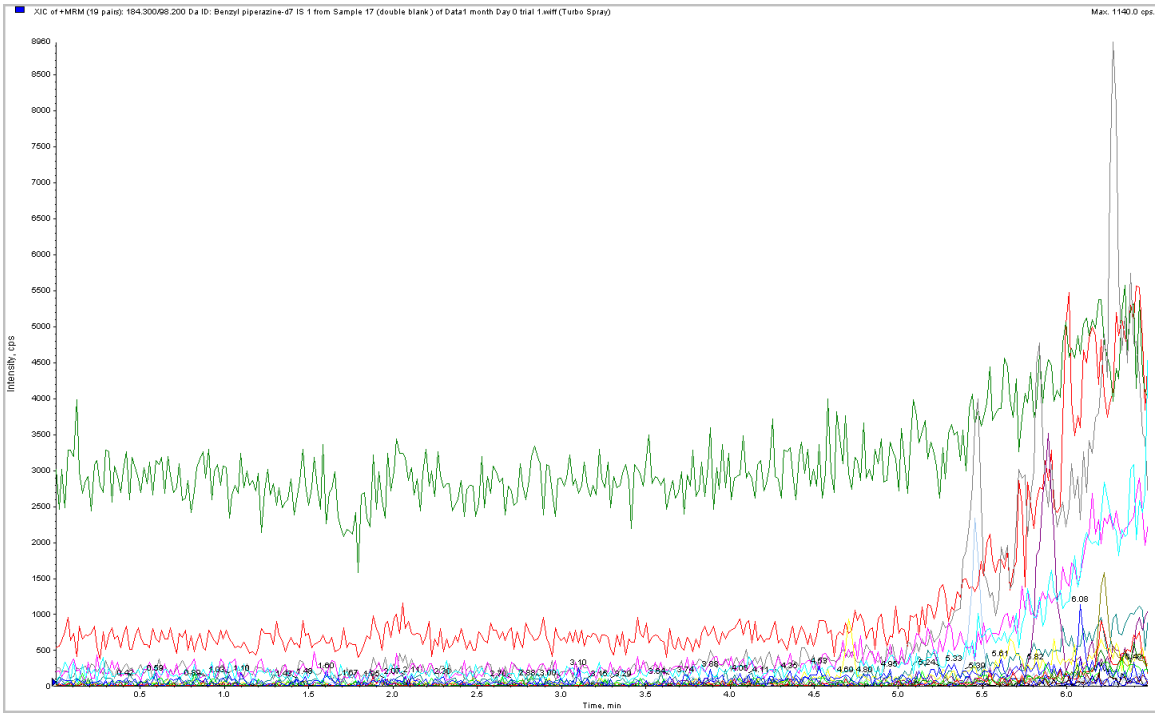


Figure A: Total ion chromatogram of certified drug-free whole blood (Lot#: B1027) on Day 30 of the 1-month trial. This scan shows mass-to-charge ratios in Da on the x-axis and intensity in counts per second on the y-axis.

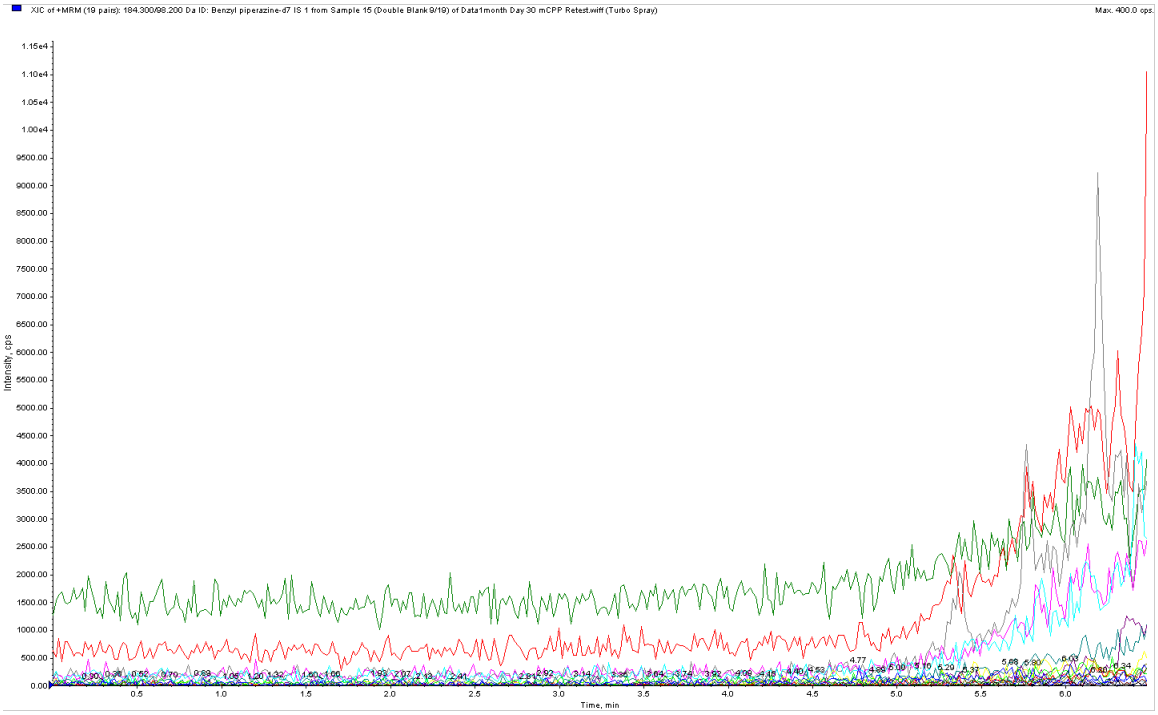


Figure B: Total ion chromatogram of certified drug-free whole blood (Lot#: B1086) on Day 0 of the 1-month trial. This scan shows mass-to-charge ratios in Da on the x-axis and intensity in counts per second on the y-axis.

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