Universidade de São Paulo
Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto
Departamento de Química
Programa de Pós-Graduação em Química

Novas drogas de abuso e estratégias bioanalíticas em Toxicologia Forense: metabolômica, análise em sangue, urina ou DBS e casos de alteração de desempenho humano e post-mortem

Novel drugs of abuse and bioanalytical strategies in Forensic Toxicology: metabolomics, analysis in blood, urine or DBS and human performance and post mortem cases

Eduardo Geraldo de Campos

Tese apresentada à Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto da Universidade de São Paulo, como parte das exigências para a obtenção do título de Doutor em Ciências, Área de Concentração: **Química**

RIBEIRÃO PRETO - SP

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Orientador: Prof. Dr. Bruno Spinosa De Martinis
Co-orientador: Prof. Dr. Barry Kerr Logan

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“Dreams can come true, but there is a secret. They are realized through the magic of persistence, determination, commitment, passion, practice, focus and hard work. They happen a step at a time, manifested over years, not weeks”

– Elbert Hubbard

RESUMO

De Campos, E. G. **Novas drogas de abuso e estratégias bioanalíticas em Toxicologia Forense: metabolômica, análise em sangue, urina ou DBS e casos de alteração de desempenho humano e post-mortem**. 2020. 204f. Tese (Doutorado). Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto.

Nos últimos anos, o problema do abuso de drogas se tornou mais complexo com o surgimento de novas drogas, incluindo as novas substâncias psicoativas (NSP). As NSP são substâncias desconhecidas, nunca antes reportadas, ou substâncias previamente conhecidas que passaram a ser usadas de modo diferente daquele para o qual foram desenvolvidas. Além das NSP, outras novas drogas de abuso também têm surgido em casos forenses, como o 2,4-dinitrofenol (2,4-DNP), um agente utilizado ilicitamente para perda de peso, que tem causado várias mortes e não possui antídoto para sua intoxicação. Nesse contexto, novos estudos e métodos de análise toxicológica em fluidos biológicos são necessários. Dried Blood Spots (DBS) têm sido propostos como uma alternativa bioanalítica para determinação de NSP utilizando um baixo volume de amostra de sangue depositado em papel, reduzindo os volumes necessários para coleta e extração, facilitando o transporte, minimizando riscos de coleta, sem reduzir a estabilidade de várias drogas. Outra abordagem analítica adotada no estudo de NSP é a combinação de incubações em microssomas hepáticos humanos e espectrometria de massas de alta resolução (HRMS) para elucidação estrutural de potenciais metabólitos de novas drogas. Com isso, os objetivos do presente trabalho foram: (I) o desenvolvimento e validação de métodos para análise de NSP em DBS via cromatografia em fase gasosa acoplada à espectrometria de massas (GC-MS) ou cromatografia líquida de ultra eficiência acoplada à espectrometria de massas sequencial (LC-MS/MS); (II) o desenvolvimento de validação de métodos para análise de 2,4-DNP em sangue e em urina via GC-MS; e (III) o estudo do metabolismo *in vitro* de NSP via incubação com microssomas hepáticos e HRMS. Como resultados, foi desenvolvido um método alternativo para o preparo de DBS, utilizando uma pastilha em substituição ao papel, para análise de cocaína, metanfetamina e MDMA via GC-MS, e derivatização assistida por ultrassom. Contudo, o método é sensível apenas para concentrações elevadas dessas drogas em DBS suportado em sal. O método para triagem de drogas de abuso clássicas e NSP via cromatografia em fase líquida de ultra pressão acoplada à espectrometria de massas com analisador quadrupolo-tempo de voo (UPLC-QTOF-MS) apresentou limites de detecção de 0,5 a 20 ng/mL, com recuperações superiores a 70%, exceto para alfa-PVP, LSD, heroína e mitraginina. As drogas investigadas apresentaram-se estáveis por até 37 dias em DBS a -20ºC, exceto mitraginina. À temperatura ambiente, a maioria dos compostos mostrou-se estável em DBS, exceto 4-fluoro-anfetamina, acrilfentanil, heroína, mefedrona, metilona, N-etil-hexedrona, pentilona, alfa-PVP e N-etil-pentilona. Boa correlação foi observada entre resultados obtidos na análise de amostras autênticas de sangue líquido e de DBS. Bons resultados também foram obtidos com o método de triagem de canabinóides sintéticos em DBS, com limites de detecção entre 0,5 e 2 ng/mL usando UPLC-MS/MS e alta estabilidade após 20 dias à temperatura ambiente ou à – 20ºC. O método confirmatório para análise de catinonas sintéticas em DBS também apresentou bons resultados, com R^2 maiores do que 0,99, exatidão média de 4,43% - 20,0%, precisão intra-ensaio média de 5,44% - 13,3% e precisão inter-ensaio média de 9,44% - 16,1%. Nos estudos de metabolismo *in vitro*, foram identificados e caracterizados oito, três, cinco, cinco e seis metabólitos de Fase I para 4-MDEC, 3,4-DCEC, tertilona, N-etil-hexedrona e N-etil-hexilona, respectivamente. O método de análise da nova droga 2,4-DNP também apresentou resultados satisfatórios de linearidade, imprecisão inter-ensaio (inferior a 10,6%), imprecisão intra-ensaio (inferior a 10,7%) e recuperação de 92,1%. Três casos de intoxicação humana foram positivos, apresentando concentrações na faixa de 61.6 - 220 mg/L em urina e < 3 - 114 mg/L em sangue. Com a realização desse estudo foi possível a utilização de métodos modernos para análise de NSP e/ou drogas ilícitas tradicionais, com implicações e potencial para aplicação em análises forenses de rotina.

Palavras-chave: Novas Substâncias Psicoativas. 2,4-dinitrofenol. Dried Blood Spots. Metabolismo *in vitro*.

ABSTRACT

De Campos, E. G. **Novel drugs of abuse and bioanalytical strategies in Forensic Toxicology: metabolomics, analysis in blood, urine or DBS and human performance and postmortem cases**. 2020. 204p. Thesis (Doctorate). Ribeirão Preto School of Philosophy, Science and Literature – University of São Paulo, Ribeirão Preto.

In recent years, the problem of drug abuse has become more complex with the emergence of new drugs, including new psychoactive substances (NPS). NPS are unknown substances, never reported before, or previously known substances that have been used in a different way. In addition to NPS, other new drugs of abuse have also emerged in forensic casework, such as 2,4-dinitrophenol (2,4-DNP), a weight loss agent illegally used that has caused many deaths and has no antidote. In this context, further studies and methods of toxicological analysis in biological fluids are required. Dried Blood Spots (DBS) have been proposed as a bioanalytical alternative for NPS determination based on application of a low volume of blood onto a paper card, reducing the volumes required for collection and extraction, making shipping easier, minimizing collection risks, and not decreasing the stability of many drugs. Another analytical approach adopted in the study of NPS is the combination of human liver microsome incubations and high resolution mass spectrometry (HRMS) for structural elucidation of potential metabolites of new drugs. Thus, the aims of this work were: (I) the development and validation of methods for NPS analysis in DBS via gas chromatography-mass spectrometry (GC-MS) or ultra-high pressure liquid chromatography tandem mass spectrometry LC-MS/MS; (II) the development of validation methods for the analysis of 2,4-DNP in blood and urine via GC-MS; and (III) in vitro metabolism studies of NPS via incubation with human liver microsomes and HRMS. As results, an alternative method was developed for the preparation of DBS, using a salt tablet for replacing the paper, for the analysis of cocaine, methamphetamine and MDMA via GC-MS, and ultrasound-assisted derivatization. However, the method is sensitive only to high concentrations of these drugs in salt-supported DBS.The ultra-high pressure liquid chromatography quadrupole time of flight mass spectrometry (UPLC-QTOF-MS) screening method for classic abuse drugs and NPS had detection limits of 0.5 to 20 ng/mL, with recoveries greater than 70% (except for alpha-PVP, LSD, heroin and mitragynine). The investigated drugs were stable for up to 37 days in DBS at -20ºC, except mytraginine. At room temperature, most compounds were stable in DBS except 4-fluoro-amphetamine, acrylfentanyl, heroin, mephedrone, methylone, N-ethyl-hexedrone, pentylone, alpha-PVP and N-ethyl-pentylone. Good correlation was observed between results obtained in the analysis of authentic liquid blood samples and DBS. Good results were also obtained with the synthetic cannabinoid screening method in DBS, with detection limits between 0.5 and 2 ng/mL using UPLC-MS/MS and high stability after 20 days at room temperature or at -20ºC. The confirmatory method for analysis of synthetic cathinones in DBS also yielded good results, with R^2 greater than 0.99, accuracy between 4.43% - 20.0%, intra-assay precision between 5.44% - 13.3% and inter-assay precision between 9.44% - 16.1. In *in vitro* metabolism studies, eight, three, five, five and six Phase I metabolites were identified and characterized for 4-methyldiethcathinone (4-MDEC), 3,4-dichloroethcathinone (3,4-DCEC), tertylone, N-ethylhexedrone and N-ethylhexylone, respectively. The method of analysis of 2,4-DNP also showed satisfactory results of linearity, inter-assay inaccuracy (less than 10.6%), intra-assay inaccuracy (less than 10.7%) and 92.1% recovery. Three human intoxication cases were positive, with concentrations in the range of 61.6 - 220 mg/L in urine and <3 - 114 mg / L in blood. By performing this study, it was possible to use modern methods for the analysis/study of NPS and / or traditional illicit drugs, with implications and potential for application in routine forensic analyzes.
Keywords: Novel Psychoactive Substances (NPS). 2,4-dinitrophenol (2,4-DNP). Dried Blood Spots (DBS). *In vitro* metabolism.

LIST OF ABBREVIATIONS

2,4-DNP	2,4-dinitrophenol
2-DPMP	2-desoxypipradrol
3,4-DCEC	3,4-dichloroethcathinone
3-MeO-PCP	3-methoxy-PCP
4-MDEC	4-methyldiethcathinone
4-MeO-PCP	4-methoxy-PCP
6-MAM	6-monoacetylmorphine
Alpha-PVP	Alpha-pyrrolidinopentiophenone
ANOVA	Analysis of Variance
BAC	Blood-alcohol content
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CFSRE	Center for Forensic Science Research and Education
CNS	Central Nervous System
CV	Coefficient of variation
CYP	Cytochrome P450
DBS	Dried blood spots
DEA	Drug Enforcement and Administration
DFC	Drug-facilitated crimes
DLLME	Dispersive liquid-liquid microextraction
DMMC	3,4-dimethylmethcathinone
DMSO	Dimethylsulfoxide
DPX	Disposable pipette tips extraction
DUID	Driving under influence of drugs
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EIA	Enzyme immunoassay
FMO	Flavin-containing monooxygenase
GC	Gas chromatography
GC-FID	Gas chromatography with flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GC-NPD	Gas chromatography with nitrogen-phosphorus detector
GHB	Gamma hydroxybutyrate

LIST OF ABBREVIATIONS

HLM	Human liver microsomes
HQC	High quality control
IDA	Information Dependent Acquisition
ISTD	Internal standards
LC	Liquid chromatography
LC-DAD	Liquid chromatography with diode array detector
LC-MS	Liquid chromatography-mass spectrometry
LC-QTOF	Liquid chromatography-quadrupole-time of flight mass spectrometry
LC-UV	Liquid chromatography-ultraviolet-visible spectrophotometry
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LOD	Limit of detection
LQC	Low quality control
LSD	Lysergic acid dethylamide
MDA	Methylenedioxymethamphetamine
MDMA	3,4-methylenedioxy-methamphetamine
MPA	Methiopropamine
MRM	Multiple Reaction Monitoring Mode
MS	Mass Spectrometry
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAME	National Association of Medical Examiners
NMDA	N-methyl-D-aspartate
NPS	Novel psychoactive substances
PCP	Phencyclidine
p-FiBF	4-fluoroisobutyrylfentanyl
QC	Quality control
QuEChERS	Dispersive solid phase extraction
RSM	Response Surface Methodology
S/N	Signal-to-noise
SIM	Selected Ion Monitoring

LIST OF ABBREVIATIONS

SN_2	Nucleophilic bimolecular substitution reactions
SPE	Solid phase extraction
SPME	Solid phase micro-extraction
SRM	Selective Reaction Monitoring Mode
STA	Systematic toxicological analysis
SWGDRUG	Scientific Working Group for the Analysis of Seized Drugs
TCMS	Trimethylchlorosilane
THC	Δ^9-tetrahydrocannabinol
TMS	Trimethylsilyl
UGT	UDP-glucuronyl transferase
UPLC	Ultra-high pressure liquid chromatography
UPLC-MS/MS	Ultra-high pressure liquid chromatography tandem mass spectrometry
UPLC-QTOF-MS	Ultra-high pressure liquid chromatography quadrupole time of flight mass spectrometry
VH	Vitreous humor
WADA	World Anti-Doping Agency

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CHAPTER 1. INTRODUCTION

1. ANALYSIS IN FORENSIC TOXICOLOGY

Forensic Toxicology is the science related to the application of concepts, methods and practices of Toxicology in the resolution of cases with legal interest (PETERS et al. 2017). The major sub-fields are Postmortem, Human Performance, Doping Control and Workplace Drug Testing. **Postmortem Forensic Toxicology** is dedicated to investigate substances that potentially could be involved in causing death, in biological fluids. In **Human Performance Toxicology**, the interest of forensic toxicologists is to investigate drugs that can alter human behavior involved in impaired driving cases (driving under influence of drugs, DUID) and drug-facilitated crime (DFC), for example (TIAFT, 2001; WYMAN, 2012; SMITH; BLUTH, 2016). **Doping Control** in Forensic Toxicology involves the analysis of the abuse of prohibited substances (as steroids, for example) by professional or non-professional athletes in order to enhance their physical performance during a competition (WYMAN, 2012). **Workplace Drug Testing** is another major area in Forensic Toxicology, where the investigations are conducted as pre-employment policies or random or legally requested monitoring of employees (WYMAN, 2012).

1.1. Systematic Toxicological Analysis (STA)

The role of the forensic toxicologist is the analysis of biological specimens in order to provide a report that can be used in court, followed by the interpretation of the result. The toxicological analysis must follow the scientific method and standard practices to produce a forensically defensible result (LOGAN, 2019). In this context, the main goal is to analyze a sample in order to provide the identification and quantification of compounds of interest, followed by the interpretation of the results in the context of the case (TIAFT, 2001). **Systematic Toxicological Analysis (STA)** is the tool used for general forensic toxicological analysis, by using a well-planned analytical approach to identify compounds with potential toxicity and their metabolites in biological fluids (MUELLER et al. 1994; DE ZEEUW, 1997; TIAFT, 2001). STA can be a complex and challenging investigation, based on the case history, available biological samples, required extraction and analytical technique and the available database (JOYE et al., 2019).

The first step of STA is related to getting all the information available, such as the case history, medical reports and autopsy findings, including which substance might be expected to be involved (SKOPP, 2004; DRUID; HEARN, 2007). Some pieces of evidence can be obtained by the externally examination of the body or from microscopic

analysis/histology at autopsy (WYMAN, 2012). A few examples are pills and tablets at the scene, syringes, or powder around the nasal area of a body. Hemorrhagic gastritis may indicate exposure to Ar or Fe whereas reddish skin or pink coloration of the blood can be a sign of carbon monoxide intoxication (WYMAN, 2012). The information obtained from the scene and/or the pathologists reports are important in determining the type of analysis to be performed in the laboratory (SKOPP, 2004). For example, an arson case requires for an investigation of gases such as carbon monoxide (CO) while a fatality involving a car crash may require analysis of blood-alcohol content (BAC). If no specific requirements for analysis are requested by the medical examiner or by the police, the toxicologist decides which analysis will be performed based on the case history and additional findings (DRUID; HEARN, 2007). If the individual has received medical treatment, all the medications must be included in the scope of analysis (SKOPP, 2004).

Drug screening is the initial analysis conducted in STA, when a comprehensive analysis is performed to detect as many compounds with forensic interest as possible without reducing the sensitivity of the method, searching for compounds that are not expected to be present in that sample based on the subjects history (SKOPP, 2004; DRUID; HEARN, 2007; DRUMMER, 2017). Usually, screening is performed by the analysis of biological fluids in which the levels of compounds tend to be elevated; the sample preparation is performed using a non-selective extraction method (to cover a broad range of compounds) and the analysis is non-targeted, resulting in the use of a generic gradient of separation in chromatographic methods, for example (PETERS et al., 2017). The main goal is to find if there is any substance with forensic relevance to which the subject has been exposed via screening before performing confirmatory analysis (JOYE et al., 2019). In general, to cover a wide range of analytes, more than one technique is used, combining immunoassays and chromatography/mass spectrometry (e.g. GC-MS and LC-MS) (DRUID; HEARN, 2007; GRAPP et al., 2018). Historically, screening was performed by immunoassay only, but recently the availability of high sensitive chromatographic techniques with mass spectrometric detectors, has changed this scenario, making chromatography/mass spectrometry-based screening more common (MERCOLINI; PROTTI, 2016). Immunoassays are used to detect classes of substances, based on the interaction between drugs and the antibodies, while chromatography/mass spectrometry is used to identify compounds with similar polarity and similar behavior with respect to extraction and system response (DRUID; HEARN, 2007). More recently, LC-MS/MS based methods have become increasingly used in multi-targeted screening due to the selectivity and sensitivity of these platforms (GRAPP et al., 2018). However,

in some laboratories, the use of less sensitive/selective techniques such as immunoassays, are still used for screening purposes.

After providing a preliminary identification, STA requires a second analytical stage where all the positive results obtained through screening tests are confirmed (RICHARDSON, 2000). Confirmation is performed using a different technique and testing the same specimen or another specimen (DRUMMER et al., 2013). In analytical terms, a potential cross-reactivity in immunoassays (with other drugs and metabolites) used for screening or potential interferents in chromatographic methods or in biological specimens are source of uncertainties (such as false-negative or false-positive results), which requires confirmation (MAURER, 2020; DRUID; HEARN, 2007). In addition, a complex sample such as postmortem blood or decomposed tissues can generate complex results as well, or a possible contamination from the environment where a body has been found or from errors during the collection makes the identification of some components very difficult, which supports the approach of using a second analysis to confirm the results. However, not all findings in the screening report must be confirmed. Common medications (as acetaminophen), drugs used for resuscitation (such as atropine) and other compounds that are commonly ingested by people (as caffeine) are examples of substances that may not be confirmed (DRUID; HEARN, 2007). However, if the compound could be related to the intoxication, a confirmation may be required. An example is when acetaminophen or a prescribed benzodiazepine is found in samples collected in a suicide case.

Confirmatory analysis is performed using techniques, which are based on a different chemical principle, in order to eliminate the possibility of having two false-positive results in different analyses (DRUID; HEARN, 2007). In contrast to screening methods, it is recommended for confirmation to use a more selective sample preparation method and targeted analysis (PETERS et al., 2017). The method used for confirmation should use a technique different from that used in the screening method, with higher sensitivity and selectivity (RICHARDSON, 2000). To confirm a result obtained by immunoassay, a less sensitive chromatographic technique such as GC-MS can be used (DRUID; HEARN, 2007). The confirmatory analysis of screening results obtained by chromatography can be performed using a chromatographic technique with a different system of detection or based on a different principle (DRUID; HEARN, 2007). If the screening was performed by GC-MS, Liquid Chromatography coupled to Triple Quadrupole or to Quadrupole-Time of Flight Mass Spectrometry (LC-QTOF) are examples of techniques that could be used for the confirmation. It is recommended to use

mass spectrometry (MS) techniques whenever possible in confirmation methods (RICHARDSON, 2000). The confirmatory analysis by MS based techniques is indicated due to its high selectivity, but the selectivity is related to the amount of ions or transitions that are been monitored (MAURER, 2007).

In some cases, in addition to the identification, it is necessary to know the concentration. One of the most widely practiced applications of quantitations in a forensic setting is the determination of ethanol in breath and blood. The presence of ethanol in blood collected from a driver in a DUI case is not enough and quantitation is required, because in most countries' legislations there are different penalties regarding alcohol levels in blood. The quantification of the parent drug only may be not enough and the determination of metabolite levels may be required, since the metabolites can be pharmacologically active and exhibit higher toxicity than the parent (RICHARDSON, 2000). Eventually the parent drug undergoes a rapid metabolism or is unstable in blood, requiring the determination of the metabolite as evidence of parent drug use (RICHARDSON, 2000). In general, carbon monoxide, cyanide, heavy metals (such as Pb) and drugs that induce alteration of human performance should be quantified (DRUID; HEARN, 2007). Drugs and metabolites are important for quantification as this information is useful for pharmacokinetics calculations in relation to the parent drug (DRUID; HEARN, 2007), and also for purposes of comparing drug concentrations in a case under investigation with cases previously reported elsewhere.

The toxicological analysis is focused on biological specimens but seized material analysis can be very useful and can provide valuable additional information. For example, if a powder is found close to a victim of oversode, the analysis of that material can complement the toxicological findings. For seized drugs, a systematical analysis is also recommended. The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) has an official guideline for the analysis of seized materials, recommending the analysis of seized materials by using more than one technique (SWGDRUG, 2019). According to SWGDRUG (2019), the techniques are classified as Category A (based on analysis of structural data), B (based on analysis of physico-chemical properties) or C (based on chemical class information), with more sensitive techniques being in Category A and less sensitive techniques being in Category C. It is recommended to combine at least one analysis by using a technique in Category A (such as Mass Spectrometry) with another analysis using a technique in Categories A, B or C (SWGDRUG, 2019). If no Category A techniques are available, the laboratories should

perform three analyzes, combining Categories B (at least two) and C (one technique) (SWGDRUG, 2019).

1.2. Biological Matrices

STA is conducted by analyzing specimens, to search for drugs and other compounds relevant to the forensic investigation. The biological fluid to be chosen for the analysis depends of the context of the case as well as the goal of the analysis (DRUMMER, 2004; PETERS et al., 2017). The biological fluids traditionally available for collection in vivo are blood, urine, sweat, oral fluid and hair (PETERS et al., 2017). In postmortem investigations, in addition to blood, urine and hair, other specimens can be collected such as vitreous humor, stomach content, bile, nails and tissue samples (brain, lungs, liver and kidney) (PETERS et al., 2017). In some cases, bones can be tested as well.

It is very important to highlight that the quality of the specimen determines the value of the toxicological finding (DINIS-OLIVEIRA, 2010). A well-performed and correct analysis is useless if the sample is completely degraded, which compromise the validity of the results. For this reason, the chain of custody of the sample must be respected.

1.1.1. Blood

Blood is the traditional matrix of choice used in toxicological analysis for identification, quantification and interpretation of drugs and its metabolites levels (SKOPP, 2004). It is a complex matrix due to its composition, which is a combination of water, proteins, fat, salts and cells (BORDIN et al., 2015). For analysis, whole blood, plasma or serum can be selected. Plasma is the portion of blood obtained by centrifugation of whole blood containing anticoagulant whereas serum is collected by centrifugation of coagulated blood (BORDIN et al., 2015). For this reason, if the drug present high affinity for proteins, the analysis should be performed in whole blood, as part of the drug content can be precipitated in serum or plasma (BORDIN et al., 2015). Blood is traditionally collected using gray top tubes with both preservative (sodium fluoride) and anticoagulant (potassium oxalate) added (WYMAN, 2012).

The drug concentration can change according to the body site where blood is collected (YAREMA; BECKER, 2005; SKOPP, 2004). Central and peripheral bloods are sources of postmortem blood, which can be collected for analysis and indicate recent drug use (PETERS et al., 2017). However, the interpretation of postmortem findings in blood

specimens should be done with caution as differences in drug concentrations can occur due to incomplete distribution at the time of death or to postmortem redistribution (MAURER, 2020; PETERS et al., 2017; SKOPP, 2004). Postmortem redistribution consists in changes in drugs concentrations in blood after death, usually from organs such as lungs and livers (YAREMA; BECKER, 2005). Blood from the thoracic and abdominal cavities can be collected in cases of violent deaths but should be avoided if there is other available sources of blood due to the potential for contamination (SKOPP, 2004; HEPLER; ISENSCHIMID, 2007). In general, peripheral blood samples are used for quantitation while other blood sources are indicated for qualitative analysis (SKOPP, 2004). Heart blood is usually more abundant and presents higher drug levels than peripheral blood, being an alternative specimen for screening purposes (SKOPP, 2004; PETERS et al., 2017).

The use of whole blood (plasma and serum as well) in toxicological investigations is well established, especially to assess recent drug use (MERCOLINI; PROTTI, 2016; PETERS et al., 2017). However, it is not recommended to state behavioral effects or impairment based only in drug concentrations (ASB, 2019). Serum and plasma collected at the time of admission in hospitals are very important in the investigation of delayed deaths, since these samples are not subjected to postmortem changes whereas whole blood will be collected a few days later during autopsy (BYARD; BUTZBACH, 2012). The risk of adulteration is minimal (JOYE et al., 2019). However, it is know that the collection of blood is invasive and requires medical supervision (FARRELL; KERRIGAN; LOGAN, 2007; PETERS et al., 2017; JOYE et al., 2019). The detection time is short compared with other matrices and the analysis of whole blood usually requires an extensive sample preparation procedure (PETERS et al., 2017).

1.1.2. Urine

Urine is composed of ions and organic substances such as creatinine, uric acid and urea and it is also the major route of elimination in the body, carrying away drugs, but mostly, their metabolites (BORDIN et al., 2015; PETERS et al., 2017). Based on its composition, urine is also a “cleaner” matrix, with low levels of proteins and lipids, requiring a less complex extraction procedure (SKOPP, 2004; JOYE et al., 2019). In general, drugs and metabolites are accumulated in urine, increasing their concentrations compared to other matrices (SKOPP, 2004; PETERS et al., 2017). The detection time in urine is longer than in blood (up to weeks) (PETERS et al., 2017). Urine collection is easy

and simple, with large volume available (MERCOLINI; PROTTI, 2016; PETERS et al., 2017; JOYE et al., 2019). In addition, the analysis of urine samples is easier to automate.

Urine testing has some important disadvantages. Drug levels in urine cannot be correlated to pharmacological behavior/effect (FARRELL; KERRIGAN; LOGAN, 2007). Adulteration of urine is possible to occur, especially in doping control cases, which requires a supervised collection. A urine sample can adulterated by dilution (by diluting urine with water or by drinking large amounts of water), replacing the urine sample by a "blank sample" or adding a substance to the urine, making the sample invalid for testing (MAURER, 2020; SMITH; BLUTH, 2016). In addition, as some drugs/metabolites can be excreted as conjugates after undergoing a Phase II Metabolism, a step of conjugates cleavage or hydrolysis must be included in the sample preparation, which takes time to perform but is needed in order to avoid a potential false-negative result if the cleavage is not performed (MAURER, 2020; MAURER, 1999).

1.1.3. Oral Fluid

In the last few years, oral fluid has gained more attention due to its application in DUID cases, as oral fluid testing can be performed on site (e.g. at roadside). Oral fluid is a mix of salivary fluids that is released by numerous glands, and also contains mouth cells, microorganisms and food residue (BORDIN et al. 2015; MERCOLINI; PROTTI, 2016). The mechanism of transference of drugs from blood to the oral fluid is by ultrafiltration or by passive diffusion (BORDIN et al. 2015; MERCOLINI; PROTTI, 2016). The transfer of a drug to the oral fluid and, therefore, its concentration, depends on the pH of the fluid and the pKa and protein-binding rate of the drug, with drugs unbound to proteins being transferred to the oral fluid (BORDIN et al. 2015; DRUMMER; 2008). Usually, oral fluid is slightly acid (with pH ranging from 6.2 to 7.4) and free and non-charged basic drugs (such as amphetamines and opiates) diffuse from blood into oral fluid, getting ionized and not transferring back to blood, increasing the concentrations of these drugs in the oral fluid (DESROSIERS; HUESTIS, 2019). On the other hand, acidic drugs (such as benzodiazepines) and neutral drugs as well as their metabolites can be found in oral fluid at lower concentrations (MERCOLINI; PROTTI, 2016; DRUMMER; 2008).

The advantages of using oral fluid in STA are the non-invasive and easy collection, the ability to assess recent drug use and the possibility of correlating free drug levels in plasma with those in oral fluid, according to some studies (PETERS et al., 2017; MERCOLINI; PROTTI, 2016). However, it is not recommended to estimate blood

concentrations based on oral fluid levels due to inter and intra-subject variability (DESROSIERS; HUESTIS, 2019). The low content of proteins in oral fluid makes the fluid more hydrophilic and, by consequence, extracting drugs from oral fluid specimens is less subjected to endogenous interferents (DRUMMER; 2008). More specifically regarding the collection, the procedure is simple and fast, and can be performed under supervision without constraints (LILLSUNDE, 2008). However, the amount of oral fluid for collection is limited and it is subjected to contamination (PETERS et al. 2017). Some contaminations can arise from smoked or ingested drugs as well, resulting in elevated concentrations in oral fluid. In addition, some compounds are unstable in oral fluid and some drugs present short half-life in plasma, making difficult to detect them in oral fluid (MERCOLINI; PROTTI, 2016). Another limitation is that dry mouth can make the collection of oral fluid more difficult, and this condition can be caused either by physiological factors or drugs (such as amphetamines and cannabis) (DRUMMER, 2008).

1.1.4. Other samples for STA

Although blood, urine and oral fluid are traditionally used in STA, hair, sweat and postmortem matrices can be used in STA biological matrices in forensic analysis.

The composition of hair is proteins, including keratin (65% - 95%), water, lipids and inorganic elements (BORDIN et al., 2015; BARROSO et al., 2011). The average rate of hair growing is 1 cm per month and this rate is variable depending on type of hair and body location (COOPER; KRONSTRAND; KINTZ, 2012; BARROSO et al., 2011). Due to this growing rate, drugs and other substances can be gradually incorporated into hair over time, enabling the assessment of history of past or chronic use (from weeks to years), making the length of the hair being collected an important factor for analysis and findings interpretation (COOPER; KRONSTRAND; KINTZ, 2012; BORDIN et al., 2015; MERCOLINI; PROTTI, 2016; KINTZ, 2017). In some cases, the goal of hair testing is to detected if an individual has been exposed to a drug (such as in custody cases of children being exposed to illicit substances); in other cases, such as workplace monitoring, the goal of hair testing is to find if a person has actually used the drug (MONTGOMERY; LEBEAU; MORRIS-KUKOSKI, 2017).

The use of hair in STA allows the assessment of long-term drug use or absence, with an extended detection time (weeks, months and years) (DRUMMER, 2004; COOPER; KRONSTRAND; KINTZ, 2012). The collection procedure is easier, non-invasive and simple, and do not require specialized staff (MERCOLINI; PROTTI, 2016; KINTZ, 2017). Although hair is preferred collected from the head, hair from other areas

such as pubic, underarm and beard regions can also be collected for analysis (COOPER; KRONSTRAND; KINTZ, 2012). Hair specimens do not require refrigeration and can be stored at room temperature (COOPER; KRONSTRAND; KINTZ, 2012). On the other hand, extracting drugs from hair requires an extensive procedure, which also includes a decontamination or wash step. Hair can be readily contaminated by environmental exposure, resulting in a false-positive result for a person who has not used a drug, for example, and by the use of cosmetic products, which may degradate drugs present in the hair (PETERS et al. 2017).

Sweat is another alternative matrix composed by water (99%), lactate, albumin, urea, electrolytes, ions, enzymes and organic compounds, eventually containing bacteria and cellular debris, and exhibiting a pH between 4 and 6.8 (BORDIN et al., 2015; DE MARTINIS et al., 2007; HUSSAIN; MANTRI; COHEN, 2017; MENA-BRAVO; LUQUE DE CASTRO, 2014). Four glands produce sweat: eccrine, apocrine, apoeccrine and sebaceous glands (HUSSAIN; MANTRI; COHEN, 2017). Xenobiotics can be excreted through the sweat and the incorporation into the sweat can occurs through passive diffusion from blood or transdermal migration (BORDIN et al., 2015; DE GIOVANNI; FUCCI, 2013). In general, the collection of sweat is through a patch composed by a cellulose pad attached to an adhesive polyurethane support (DE MARTINIS et al., 2007).

As an alternative biological matrix, sweat presents a number of advantages over other conventional matrices. The collection is non-invasive, detection window is longer in relation to plasma or urine and sweat specimens are not easily adulterated (DE MARTINIS et al., 2007; PETERS et al., 2017; SCHWILKE et al., 2006). The extraction is usually easier than other more complexes matrices. However, to collect sweat a special collector is required (a patch) and the sample volume is low, compared to blood or urine, for example. In addition, the concentrations of drugs in sweat are usually low (DE MARTINIS et al., 2007).

Many postmortem specimens may be available for collection and for STA but it is important to consider they might be altered due to autolysis and bacterial action (STIMPFL; VYCUDILIK, 2004). Vitreous humor (VH), bile, gastric content and organs tissues are some examples. VH is one of the most interesting postmortem specimens due to its protection (except in violent death cases) made by the eye globe, which avoids contact with microorganisms. This protection of VH makes this matrix less affected by postmortem redistribution effects (CHESSER et al., 2019). In addition, VH is clean, with a low number of endogenous components, and it allows the investigation of recent use of

drugs (PETERS et al. 2017). Drug levels in VH reflect the levels in blood between 1 and 2 hours before death and there is no esterase activity in VH, avoiding the hydrolysis of some drugs and metabolites (HEPLER; ISENSCHIMID, 2007).

Gastric contents are useful for the investigation of drug overdoses, as the ingestion through oral route is one of the most important routes of drug consumption and high levels of the drug can be found in the stomach (HEPLER; ISENSCHIMID, 2007). In addition, basic drugs can be trapped in gastric contents, showing high levels in these fluids. Bile and tissues from liver, brain, kidney, lung and spleen can provide important data, as drugs can accumulate in these fluids, generating results that complement blood findings (HEPLER; ISENSCHIMID, 2007; PETERS et al. 2017). All these matrices are complex, with a high number of endogenous components, requiring extensive sample preparation, including homogenization.

1.3. Sample Preparation methods used in forensic STA

Sample preparation is a critical stage performed in STA to isolate and to concentrate the compounds of interest (DE ZEEUW, 1997; HE; CONCHEIRO-GUISAN, 2019). Some specimens require steps prior the extraction for cleavage of conjugates or decontamination. Many techniques are used for extracting organic compounds from biological fluids in forensic STA, with liquid-liquid extraction (LLE), solid phase extraction (SPE) and headspace being the most common used in forensic laboratories.

1.3.1. Protein Precipitation

Protein Precipitation consists in an easy-to-perform method, used to remove as much as possible the proteic content of a biological fluid, to minimize possible matrix effects, resulting in satisfactory extraction efficiency of polar compounds (PETERS et al., 2017). The technique consists in promoting the precipitation of proteins followed by the analysis of the supernatant after centrifugation (FLANAGAN et al., 2006). For methods using liquid chromatography-mass spectrometry (LC-MS) methods, endogenous proteins can induce ion suppression or enhancement, especially considering dilute-and-shoot procedures. However, the use of protein precipitation in sample pretreatment can also lead to matrix effects, especially ion suppression (DAMS et al., 2003). Methods for protein precipitation include solvent addition, heat, acids and centrifugation.

1.3.2. Liquid-Liquid Extraction

LLE is one of the techniques used for preparing biological fluids samples for STA. The general mechanism consists in generating a biphasic system composed by an aqueous phase, containing the biological fluid, and an organic phase, containing the extractor solvent (PETERS et al., 2017). Sample has the pH adjusted by adding an acid, base or buffer, to guarantee that the drug will be in the non-ionized form, in order to be transferred to the organic phase. LLE is a versatile technique as there are many solvents available and different buffer systems can be used for adjusting the pH of the medium. In addition, a conventional LLE method is easy to perform and it involves a few additional steps (centrifugation and agitation) (FLANAGAN et al., 2006). This is a technique commonly adopted for screening procedures due to its non-selective characteristics, when a broad range of compounds are searched in a sample. However, for some complex matrices (as blood), a more selective method may be required for confirmatory or quantitative analysis.

1.3.3. Solid Phase Extraction

In SPE, the sample is simultaneously submitted to a cleanup and extracted using a solvent or combination of solvents. The process is performed through a cartridge containing a solid phase, normally with a hydrophobic phase (such as C8 or C18) (PETERS et al., 2017). In the first step, the solid phase is activated using water, reagents and solvents, which is the conditioning process (ANDRADE-EIROA et al., 2016). The following step consists in passing the sample through the cartridge, retaining the target analytes as well as interferents. A wash step is performed to remove all these interferents, without eluting the analyte. Finally, the target compounds are eluted using a solvent or solvent mix. The process of passing the sample and solutions through the cartridges are usually promoted by the application of pressure (positive or negative). Methods using SPE are more selective than LLE, with selectivity depending on the sorbent used as solid phase. There are several different types commercially available, most of them based on alkyl-bonded silica or copolymers phases, enabling the extraction to be used for many compounds and in many applications (ANDRADE-EIROA et al., 2016). The extracts obtained by SPE are cleaner than using LLE (DE ZEEUW, 1997). One of the main disadvantages of SPE is that the entire process of SPE is time-consuming and increases the costs of analysis. However, SPE can be automated and used on-line with analytical instrumentation (ANDRADE-EIROA et al., 2016).

1.3.4. Headspace

Headspace technique is both an injection system for GC and an extraction method. The sample is incubated under high temperature sometimes with agitation, to reach an equilibrium state between the liquid or solid phase containing the sample and the corresponding vapor phase, and later the vapor phase is collected for GC analysis (SNOW; BULLOCK, 2010). The target compounds present in the sample are partitioned between both phases, migrating to the gas phase formed inside the vial, until equilibrium is reached. Once equilibrium is reached, the concentration of the analyte in the gas phase is proportional to the concentration in the sample, at a given temperature. It is very important to run quality controls and calibrators simultaneously with authentic specimens in testing since the temperature in the incubation is not monitored. An aliquot of the gas phase is collected and injected in the GC system.

Extraction using headspace minimizes the matrix effects because most of the endogenous components from the sample are not evaporated and remains in the liquid or solid phase. However, the extraction is more effective for volatile compounds, such as alcohols and aldehydes.

1.3.5. Other techniques

Although LLE, SPE and headspace are the techniques that are traditionally used in STA, there are several techniques of sample preparation available, which can also be used for forensic analysis. Microextraction techniques as Solid Phase Microextraction (SPME) and Dispersive Liquid-Liquid Microextraction (DLLME) were developed based in the traditional LLE and SPE. SPME is performed using a procedure similar to headspace, with incubation and agitation, and a syringe coated with a solid adsorbent phase (fiber) is inserted inside the vial, allowing the compounds that evaporate from the sample to adsorb onto the fiber. DLLME involves the extraction by a fiber containing an acceptor solution, which extracts that compounds that are present in the sample. Other modern techniques as Dispersive Solid Phase Extraction (such as QuEChERS or DSPE) and Disposable Pipette Tips Extraction (DPX) are based in using a free solid phase that interacts with the sample.

1.3.6. Derivatization

Derivatization consists of a chemical reaction of the compound of interest with a derivatizing agent to increase the volatility of the compound as well as to reduce its polarity, and change its relative retention time, prior to Gas Chromatographic analyzes

(POLETTINI et al., 1998; WELLS, 1999). Although not all analytes require derivatization to obtain a good chromatographic profile, it is known that this step can improve the analysis in different ways, for example, by reducing the possibility of compound degradation and lowering the injector and column temperatures necessary to conduct the chromatographic separation.

Silylation is traditionally the most commonly reaction used in derivatizations and consists of nucleophilic bimolecular substitution reactions (SN_2) in which active hydrogens of the molecule are replaced by trimethylsilyl groups (TMS) and the reaction is favored by presence of strong leaving group in the target molecule (SEGURA; VENTURA; JURADO, 1998; BOWDEN et al., 2009; SCHUMMER et al., 2009; PRATA; EMÍDIO; DOREA, 2012; MOLNÁR, MOLNÁR-PERL, 2015; HSU et al., 2016).

According to Green Analytical Chemistry theory, derivatization should be avoided as possible in a method (LAVILLA et al., 2014). However, in many analyzes, derivatization is a fundamental step for the detection of an analyte and directly influences the sensitivity of the method. In this sense, some analytical strategies have been studied and adopted for green derivatization procedures, considering different aspects such as, for example, reduction of energy consumption and use of less toxic reagents (LAVILLA et al., 2014). These analytical strategies include the use of microwave or ultrasound radiation.

Ultrasound-assisted derivatization is promoted and accelerated using soft reaction conditions and requires less energy to be performed, as the ultrasound is a more efficient source of energy (LAVILLA et al., 2014). For example, thermal energy consists of a form of energy propagation in which losses occur to the surrounding environment, requiring an additional energy expenditure to compensate for these (LAVILLA et al., 2014). Microwave is also a good alternative for derivatization, reducing the time for reaction. However, derivatization under microwave requires specific and more expensive instruments. Ultrasound reactions are easily executable and safe, do not require expensive instrumentation (a simple ultrasonic bath can be used) and does not induce degradation of the analytes (PRATA; EMÍDIO; DOREA, 2012).

1.4. Analytical Instrumentation used in STA

Several analytical platforms are available in Forensic Toxicology. The technology of analytical instrumentation has been improved over the years, with the development of new and more sensitive instruments, which is important and helpful for STA. There are

several factors that need to be considered for the selection of an analytical method, such as chemical properties of target compounds, the number of target compounds and their metabolites, the analyte levels in the sample and the nature of the sample.

In STA, a broad range of compounds is screened and identified, which requires a powerful separation technique (MAURER, 1999). Chromatography is the most used technique applied in the separation of compounds in STA. Gas Chromatography (GC) and Liquid Chromatography (LC) are both used in screening, confirmation or quantitative analysis and the selection of the technique depends of the analyte and the purpose of the analysis.

GC is used in the analysis of volatile compounds, with low molecular mass, and is more efficient with compounds that have greater lipophilicity and less polarity (unless derivatized). The detection is usually performed by Mass Spectrometry (MS), Flame Ionization Detector (GC-FID) and GC-NPD. GC-FID is still the preferred technique for BAC analysis. GC-NPD has a large application in pesticides determination. GC-MS is used for drugs of abuse testing and it can be performed by using Single Quadrupole, Triple Quadrupole or QTOF. The three instrumentations have different sensitivities, with Single Quadrupole GC-MS being the least sensitive. However, the cost of GC-Triple Quadrupole-MS and GC-QTOF-MS instruments is higher than for a Single Quadrupole GC-MS. For STA in forensic laboratories, although the most sensitive technique is ideal, budget constraints are a limitation for acquiring such a high technology instrumentation and not all laboratories have the capability of using them.

LC has no limitation regarding volatility and it is used for analyzing compounds in a large range of molecular masses, and polarities. Detection systems for LC used more often in forensic routine testing are MS, Ultraviolet-Visible Spectrophotometer (LC-UV) and LC-DAD. Triple Quadrupole-Time of Flight and Ion Trap are common systems of detection in MS used for coupling to LC. Nowadays, LC-MS has been used in confirmatory and quantitation techniques in routine STA due to its sensitivity in addition to its accuracy and precision.

1.5. Relevant target compounds in STA

Many compounds have relevance in Forensic Toxicology and could be included in the scope of screening and confirmatory methods for STA. As described earlier, there is a wide field of interest in Forensic Toxicology and the consequence is that a large number of substances can be investigated in STA.

Drugs of abuse are one of the most common classes of substances of forensic interest. This class covers compounds that induce a psychoactive effect, such as alcohol, cocaine, methamphetamine and fentanyl, and other drugs that have potential for abuse but do not induce a psychoactive effect, as acetaminophen or sibutramine. Some of these compounds are not prohibited for human consumption but they have potential for abuse, such as fentanyl or benzodiazepines (alprazolam and diazepam, for example). In addition to these drugs, new designer drugs are also of growing concern and are discussed later. All these compounds can be found in postmortem, doping and human performance cases.

Other classes of substances that are relevant in Forensic Toxicology include heavy metals. Lead (Pb), cadmium (Cd), arsenium (As) and mercury (Hg) may appear in routine analysis of homicide or accidental exposure cases. Similar cases are found for pesticides, another class of compounds. Pesticides can be the cause of accidental intoxication or intentional intoxications, as in homicide or suicide cases.

For doping control, the World Anti-Doping Agency (WADA) regulates the substances that are prohibited in and out-competition and only during the competition. Non-approved substances, anabolic agents, peptide hormones, growth factors, beta-2 agonists, hormone and metabolic modulators, diuretics and masking agents are forbidden in and out-competition (WADA, 2019). Cannabinoids, stimulants, opioids and opiates, glucocorticoids and beta-blockers are regulated by WADA only during competition (WADA, 2019).

As mentioned before, the list of compounds with potential relevance in forensic investigations is very long. However, this is a “constantly updated list”, once new compounds have been emerging in the drug market and in forensic casework. When performing a STA, it is not possible to search for every single substance at once. Metals are not extracted and analyzed in the same way as steroids, for example. For this reason, the target compounds must be defined according to the case history and autopsy findings, if available. In addition, the right technique and the adequate specimen should be chosen, considering the toxicokinetics of the substance (where it is more absorbed, distributed, biotransformed and excreted in the body) and that not all the target compounds have the same chemical response to analytical instrumentation.

2. CURRENT CHALLENGES IN FORENSIC TOXICOLOGY

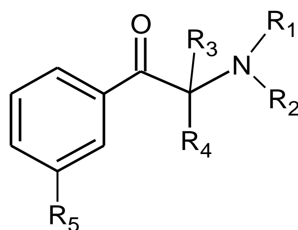
2.1. Novel Psychoactive Substances

Modern Forensic Toxicology has several well established guidelines and standards for practice of STA in routine casework. However, in the last 10 to 15 years, the phenomenon of Novel Psychoactive Substances (NPS) has introduced an additional level of complexity in toxicological analysis. NPS are natural, synthetic or semisynthetic substances that have been (I) discovered; or (II) emerged in the drug market or appeared in toxicological casework samples; or (III) used in a different way (in terms of dosage forms and amounts) from the original intended use (LOGAN, 2018). These compounds have emerged as alternatives of the traditional drugs of abuse, to mimic the effects induced by known drugs, as well as an attempt to avoid legal prosecution, if these substances are non-regulated. However, over the last few years, public health agencies in many countries have adopted an approach of scheduling the core structure of NPS, prohibiting analogs of controlled substances, in order to control the dissemination of these new drugs. There are several classes of NPS: cathinones, phenethylamines, synthetic cannabinoids, opioids analogs of fentanyl, designer benzodiazepines and novel hallucinogens.

2.1.1. Synthetic Cathinones

Synthetic cathinones are beta-keto amphetamines (a group of phenethylamines) and act as stimulants in the Central Nervous System (CNS) and mediate the action of catecholamines dopamine, norepinephrine and serotonin, through the inhibition of reuptake or release (KRAEMER et al., 2019; ANVISA, 2018; UNODC, 2015). The chemical structure of cathinones is similar to methamphetamine and 3,4-methylenedioxy-methamphetamine (MDMA) and they have been designed based on the structure of cathinone (a natural compound obtained from the plant *Catha edulis*) **(Figure 1)** (KRAEMER et al., 2019; ANVISA, 2018; UNODC, 2015). Usually, synthetic cathinones are ingested in powder, tablets or capsules, by ingestion or insufflation, but smoking or injection can also occur (DEA, 2018a; ANVISA, 2018; UNODC, 2015). Some examples are methylone, alpha-pyrrolidinopentiophenone (Alpha-PVP), butylone, dibutylone, pentylone, N-ethyl-hexedrone and N-ethyl-pentylone.

Figure 1. General structure for synthetic cathinones showing substitution patterns



The psychoactive effects induced by cathinones are similar to those evoked by cocaine, MDMA and methamphetamine (ANVISA, 2018). In general, cathinones induce psychostimulant and hallucinogenic effects (KRAEMER et al., 2019). The effects that are desirable for users include mood improvement and alert state, libido and energy increase (KRAEMER et al., 2019). However, users also report tolerance, dependence and abstinence syndrome involving cathinone use (UNODC, 2015). Undesirable effects can occur in gastrointestinal, cardiovascular and the central nervous systems (KRAEMER et al., 2019). Paranoia, agitation, psychosis, hypertension, tachycardia, hyperthermia and hallucinations are described in cathinone intoxications, including in overdose cases (KRAEMER et al., 2019).

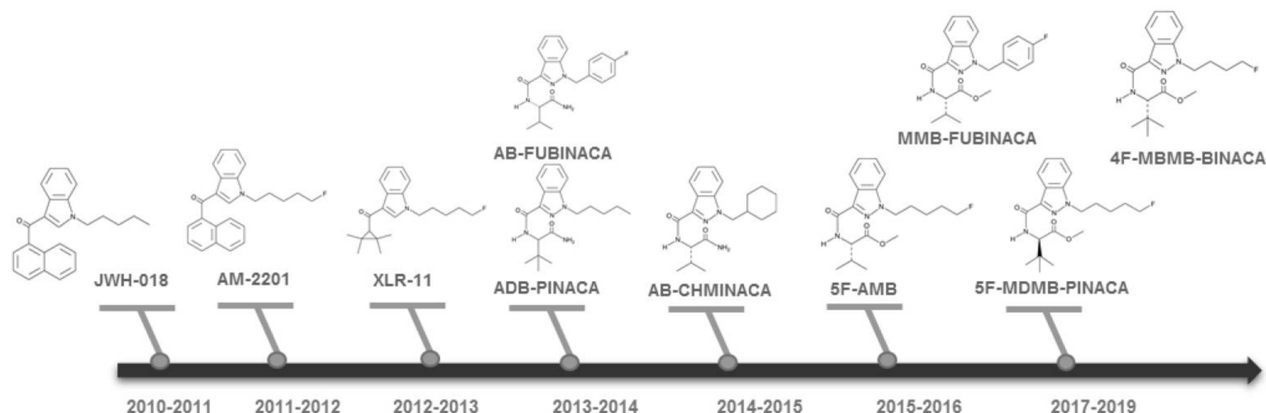
This group of NPS are one of the most prevalent class of NPS in the drug market around the world. In Europe, synthetic cathinones and synthetic cannabinoids are the most commonly seized NPS (EMCDDA, 2019; DEA, 2018a). Synthetic cathinones have been reported as commonly found NPS in United States and Brazil as well (DEA, 2018a; ANVISA, 2018). In 2017, cathinones were the most commonly found NPS class in seized drugs by Federal Police in Brazil and N-ethyl-pentylone was the most detected cathinone in 2017 (ANVISA, 2018). In 2018, in United States, cathinones were the third most detected class of NPS in seized drug samples, behind opioids and synthetic cannabinoids only (DEA, 2018b). N-ethyl-pentylone was still the most reported cathinone in United States in 2018 (DEA, 2018b).

2.1.2. Synthetic Cannabinoids

Synthetic cannabinoids are another class of common NPS, which are available in the drug market labeled as “herbal incenses”, “research chemicals” or “legal highs” and not for human use (HASSAN et al. 2017). They are chemical compounds sprayed on plant or herbal material for smoking, or for making an oil to be used in electronic cigarettes (YEAKEL; LOGAN, 2013; DEA, 2018a). They can also be available as powders for smoking or injecting. The structure of these chemicals is variable, and has evolved over the years (YEAKEL; LOGAN, 2013; LOGAN et al., 2017). Examples are ADB-

FUBINACA, AB-FUBINACA, FUB-AMB, 5F-MDMB-BINACA and JWH series **(Figure 2)**.

Figure 2. Chemical structures of synthetic cannabinoids and their emergence over time, between 2010 and 2019.



Sources: Cayman Chemical Website and based on Adams et al. (2017).

The pharmacology of these compounds is mainly based on their agonism at CB_1 and CB_2 cannabinoid receptors, inducing effects similar to those caused by Δ^9-tetrahydrocannabinol (THC), such as relaxation, euphoria and mood elevation (YEAKEL; LOGAN, 2013; KRAEMER et al., 2019). Due to an elevated affinity and potency at the CB_1 receptor, several synthetic cannabinoids mediate psychotropic effects, causing undesirable side effects such as paranoia, psychosis, altered perception, seizure and more (KRAEMER et al., 2019).

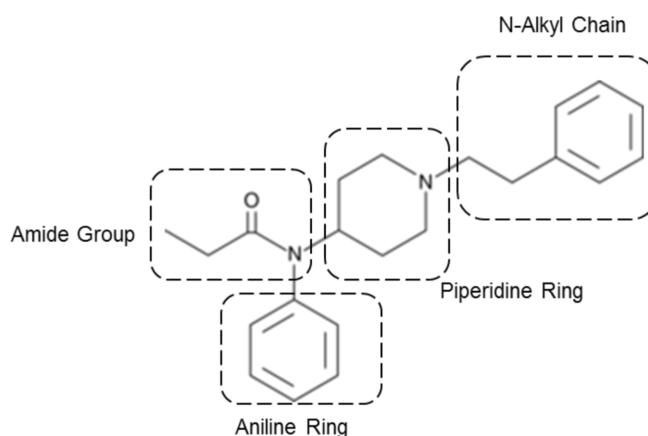
The prevalence of synthetic cannabinoids has changed over the years as the drug dealers adapt to drug laws and regulations (DEA, 2018a). In Brazil, synthetic cannabinoids were detected by Federal Police in 20% of the drug samples in 2016 and only in 6% in 2017 (ANVISA, 2018).According to DEA, in United States, in 2018, synthetic cannabinoids were the second most prevalent NPS class detected (after opioids), with 5F-MDMB-PINACA and FUB-AMB being the most reported compounds (DEA, 2018b).

2.1.3. Fentanyl and Designer Opioids

The ongoing opioid epidemics in United States are currently attributed to the abuse of fentanyl and synthetic opioids, causing a high number of hospitalizations and deaths. Fentanyl is still the most common opioid available in United States and it has been sold in combination with heroin (DEA, 2018a). Analogs of fentanyl have been produced

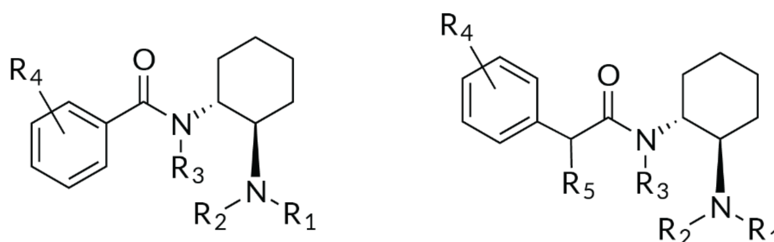
by substitution of fentanyl structure with alkyl groups or halogen substituents on the aromatic ring (LOGAN et al., 2017). Some examples of novel opioids are fentanyl analogs as furanylfentanyl, cyclopropylfentanyl, methoxyacetylfentanyl, butyrylfentany and carfentanil. A generic structure of fentanyl analogs is shown in **Figure 3**. Other opioids from the "utopioids" class such as U-47700 and U-48800 have seen periods of popularity in forensic casework **(Figure 4).** In Europe, although synthetic opioids have a small contribution to the drug market, the risk of fentanyl derivatives exists in Europe as these drugs have been reported causing deaths (EMCDDA, 2019). In 2018, three countries have reported death cases involving cyclopropylfentanyl and four countries reported death cases linked to methoxyacetylfentanyl (EMCDDA, 2019). In 2017, Brazil has identified furanylfentanyl and U-47700 (ANVISA, 2018).

Figure 3. A generic structure of fentanyl analogs



Source: Based on Cayman Standardized Naming of Substituted Fentanyls Guide (2019)

Figure 4. Generic molecular structures of utopioids based on U-47700 (left) and U-51754 (right)



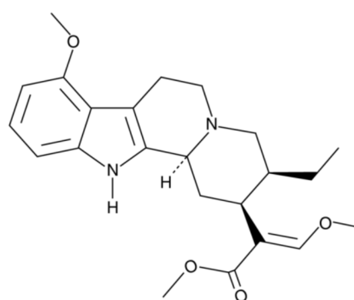
Source: Cayman Chemical (2018)

The opioids have CNS depressant activity in addition to their other μ, κ and δ opioid agonist mediated effects, with the potencies at this receptor depending of the compound (KRAEMER et al., 2019; LOGAN et al., 2017). The effects caused by opioids such as fentanyl and its analogs include bradycardia, hypotension, analgesia, alteration of mental status, hypothermia, loss of consciousness and respiratory depression

(KRAEMER et al., 2019; ZAWILSKA, 2017). Side effects include nausea, fatigue, dizziness and constipation and tolerance and dependence have been reported (ZAWILSKA, 2017). Some effects induced by activation of μ receptors are analgesia, sedation, decreased breathing and reduction of gastrointestinal motility (LOGAN et al., 2017). The action of opioids on κ receptors include analgesia, sedation, alteration of mental status and hallucinogenic/dissociative effects (LOGAN et al., 2017). Binding to δ receptors, novel opioids also cause analgesia and contribute to physical dependence (LOGAN et al., 2017).

Mitragynine (also known as Kraton) **(Figure 5)** is a natural opioid that is the major active component in the plant *Mitragyna speciosa* (LOGAN et al., 2017). The compound acts as μ-receptor agonist, producing stimulant-like effects (similar to cocaine) at low doses, and opioid-like effects at higher doses (DOMINGO et al., 2017; LOGAN et al., 2017). This pharmacological profile has made mitragynine very popular among people suffering of chronic pain or with mental health issues (MATSON; SCHENK, 2019). Mitragynine also has agonist properties on both the δ- and κ-opioid-receptors (DOMINGO et al., 2017). However, in general, the drug is purchased online, as herbal mixtures, and it is abused for stimulant-euphoric effects (DOMINGOS et al., 2017). Some effects induced by mitragynine include agitated behavior, hypertension, tachycardia, hallucinations and abdominal pain (LOGAN et al., 2017).

Figure 5. Molecular structure of mitragynine



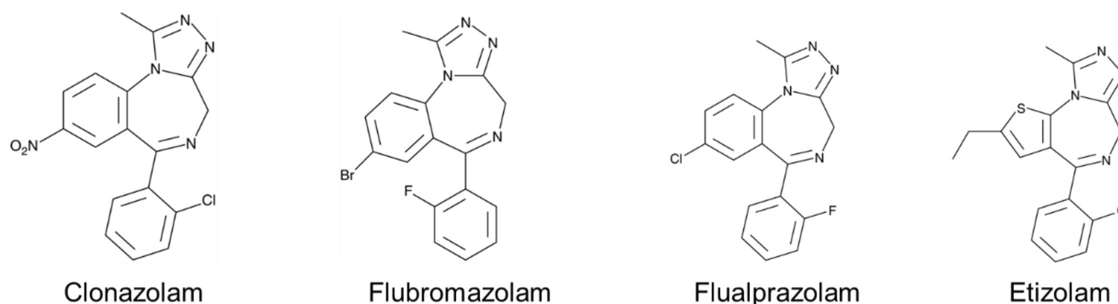
Source: Cayman Chemical Website

2.1.4. Designer Benzodiazepines

Designer benzodiazepines are compounds related to traditional benzodiazepines, which are prescribed for treatment of anxiety and depression. Some of them are sold as counterfeit alprazolam or diazepam (EMCDDA, 2019). In the last 10 years, the number of designer benzodiazepines has increased. Some examples are clonazolam, flubromazolam, flualprazolam and etizolam **(Figure 6)**. Etizolam is one of the most

frequently reported designer benzodiazepine, with cases in Europe and United States (EMCDDA, 2019; DEA, 2018b). In Europe, etizolam, diclazepam, flubromazolam and phenazepam combined account for 80% of tablets samples containing designer benzodiazepines seized since 2005 (EMCDDA, 2015). In United States, etizolam, flubromazolam and flualprazolam have been reported (DEA, 2018b). However, as observed for other NPS classes, prevalences and trends of designer benzodiazepines change following the scheduling of these drugs under drug legislations (EMCDDA, 2015).

Figure 6. Example of designer benzodiazepines



Source: Cayman Chemical

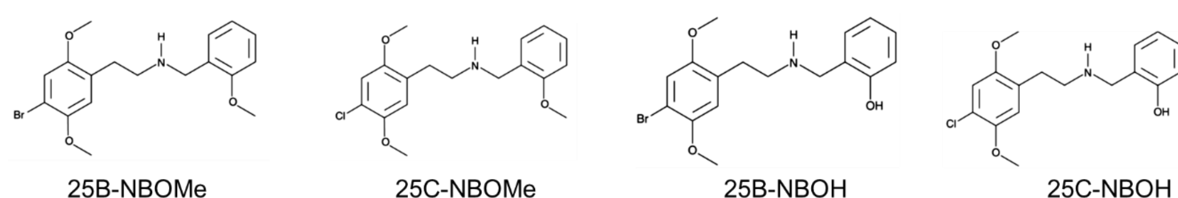
Benzodiazepines act through the allosteric modulation of $GABA_A$ receptors, binding to α subunits (ZAWILSKA; WOJCIESZAK, 2019; LOGAN et al., 2017). Many effects are induced by benzodiazepines and their depend of the subunit that they bind: addiction, sedation and anticonvulsivant effects (subunit α1), anxiolytic effects (subunit α2) and myorelaxant effects (subunits α2, α3 and α5) (ZAWILSKA; WOJCIESZAK, 2019). The recreational use of benzodiazepines cause relaxation and increase of empathy, sociability and mood (ZAWILSKA; WOJCIESZAK, 2019). However, low doses of these compounds can induce potent effects, as sedation and amnesia (LOGAN et al., 2017). The use of novel benzodiazepines may cause dizziness, loss of coordination, drowsiness, somnolence, impaired speech and thinking and, at elevated doses, they may cause hallucinations, delirium, seizures and coma (ZAWILSKA; WOJCIESZAK, 2019; LOGAN et al., 2017).

2.1.5. Novel hallucinogens and dissociative anesthetics

Novel hallucinogens are a class of NPS that induce hallucinations, dissociated behavior and “out-of-body” experiences, and they are derived from traditional hallucinogenic drugs as lysergic acid dethylamide (LSD) (LOGAN et al., 2017).

NBOMe and NBOH series are a group of compounds derived from the 2C compounds (as 2C-C, 2C-B and 2C-I), which have emerged back in 1990s (LOGAN et al. 2017). Some examples are shown in **Figure 7**. In general, these compounds are available as crystalline powders and they are abused in a similar manner as LSD (LOGAN et al. 2017). These compounds act as full or partial agonists of $5HT_{2A}$ serotonergic receptors, showing a high potency at these receptors (LOGAN et al., 2017). *In vitro*, 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOH and 25N-NBOMe showed high affinity and potency at $5HT_{2A}$ and $5HT_{2C}$ receptors (ESHLEMAN et al., 2018). The abuse of NBOMe drugs may cause undesirable effects such as agitation and increased aggressive behavior (LOGAN et al., 2017).

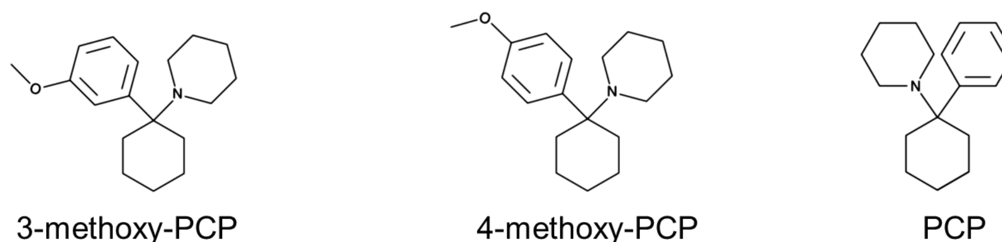
Figure 7. Examples of novel hallucinogens of NBOMe and NBOH series



Source: Cayman Chemical Website

3-methoxy-PCP (3-MeO-PCP) and 4-methoxy-PCP (4-MeO-PCP) are novel dissociative compounds that are similar to ketamine and PCP (LOGAN et al., 2017) **(Figure 8)**. They are available as powder or tablets (KINTZ et al. 2019). 3-MeO-PCP act as an antagonist of N-methyl-D-aspartate (NMDA) receptor, more potent than PCP (DE JONG et al. 2019; LOGAN et al. 2017). The MeO-PCP compounds produce dissociative effects, inducing hallucinations, alterations in the mind state and “out-of-body” sensation (DE JONG et al. 2019; KINTZ et al. 2019). Severe side effects include psychosis, aggressive behavior, tachycardia, hypertension and suicidal thoughts (KINTZ et al. 2019).

Figure 8. Molecular structures of isomers 3-MeO-PCP and 4-MeO-PCP, in comparison with PCP



Source: Cayman Chemical Website

2.2. Other drugs of abuse: 2,4-dinitrophenol (2,4-DNP)

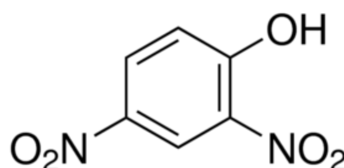
In addition to the NPS common classes, other drugs of abuse, psychoactive or not, have emerged or re-emerged in the last few years, adding some complexity in the world drug scene. In general, these substances were developed for medical or other purposes and have been emerged or re-emerged in the drug market due to their recreational or performance enhancement properties.

There are a few popular substances in this group of new drugs. Loperamide is an antidiarrheal agent that induces euphoria and relief of opioid withdrawal, with abuse potential (POWELL; PRESNELL, 2019). Tianeptine is an atypical tricyclic antidepressant approved in Europe, Asia and Latin America that acts on μ opioid receptor, inducing euphoria (BAKOTA et al., 2018). More recently, another drug, 2,4-DNP, has re-emerged in the drug market as a non-centrally acting weight loss drug, gaining forensic interest because of several deaths reports caused by this drug around the world.

2.2.1. History and use of 2,4-DNP

2,4-DNP **(Figure 9)** was first-synthetized in the early 1900s. One of the first uses of 2,4-DNP was in the production of ammunition for armaments in France during the First World War (1914-1918) (ZACK et al., 2016). Later, the drug became widely used as a weight-loss drug, in the treatment of obesity (MIRANDA et al., 2006). In the 1930s, in the United States, the drug was used at low doses for losing weight (BARTLETT et al., 2010). However, the severe adverse effects induced by 2,4-DNP in humans led to the prohibition of this drug by the Federal Food, Drug and Cosmetic Act of 1938 (MIRANDA et al., 2006; BARTLETT et al., 2010; GRUNDLINGH et al., 2011; YEN; EWALD, 2012). Nowadays, more countries have prohibited 2,4-DNP for human consumption.

Figure 9. Molecular structure of 2,4-dinitrophenol



Source: Sigma-Aldrich

2,4-DNP is still used in industry for manufacturing dyes, explosives and insecticides (POLITI et al., 2007). However, in 1980s, 2,4-DNP re-emerged in United States (BARTLETT et al., 2010). It is a crystalline powder with a characteristic yellow color, soluble in water and musty odor (GRUNDLINGH et al., 2011). The drug is sold

mostly online, being sold as nutritional supplement for bodybuilders (POLITI et al., 2007; BARTLETT et al., 2010; GRUNDLINGH et al., 2011).

2.2.2. Pharmacology and Toxicity of 2,4-DNP

The pharmacology of 2,4-DNP is based on the uncoupling of oxidative phosphorylation (MIRANDA et al., 2006; POLITI et al., 2007). The uncoupling consists in inhibiting ATP synthesis by preventing that electrons produced through respiratory chain be used for phosphorylation; activity of ATPase and the electron flow are not inhibited but are uncoupled from ATP synthesis (TERADA, 1990).

As 2,4-DNP is lipophilic and a weak acid, it crosses mitochondrial membrane in a protonated form, releases the H^+ ion into the matrix and returns to the extramitochondrial space in anionic form (VAN SCHOOR; KHANDERIA; THORNILEY, 2018). The rate of electron transfer is increased, with energy produced not being stored as adenosine triphosphate (ATP) but released as heat (GRUNDLINGH et al., 2011; YEN; EWALD, 2012; VAN SCHOOR; KHANDERIA; THORNILEY, 2018). At higher levels of 2,4-DNP, the production of energy is uncoupled, leading to unbalance of ATP, increased heat and cell death (VAN SCHOOR; KHANDERIA; THORNILEY, 2018). The uptake of inorganic phosphate into the mitochondria is blocked also by 2,4-DNP, with accumulation of inorganic phosphate in the space extra-mitochondrial and loss of electrochemical gradient (GRUNDLINGH et al., 2011; VAN SCHOOR; KHANDERIA; THORNILEY, 2018).

The consequences of exposure to 2,4-DNP are an accelerated metabolism and elevated body temperature, heart rate and oxygen consumption (POLITI et al., 2007). The weight loss is associated with an increase by 50% of the basal metabolic rate in humans (ZACK et al., 2016). The pathological thermogenesis induced by 2,4-DNP causes enzyme denaturation, failure of metabolic pathways, cell necrosis and organ failure (HOLBOROW; PURNELL; WONG, 2016). Adverse effects induced by 2,4-DNP include besides hyperthermia (equal or higher than 40ºC), shortness of breath, excessive sweating, fatigue, reduction of urine volume, renal failure, cardiac arrest and death (MIRANDA et al., 2006; POLITI et al., 2007; YEN; EWALD, 2012). A characteristic pathological finding in a 2,4-DNP intoxication is the yellow color of skin and/or body fluids, which is an important finding for diagnosis (KOPEC et al., 2018).

There are no antidotes for 2,4-DNP intoxication and the treatment is only supportive, by providing cooling procedures and administration of benzodiazepines (TEWARI et al., 2009; GRUNDLINGH et al., 2011; YEN; EWALD, 2012). 2,4-DNP

has a large volume of distribution, making dialysis or haemoperfusion ineffective (TEWARI et al., 2009). Dantrolene has been proposed as an effective agent for 2,4-DNP intoxications. Due to uncoupling of oxidative phosphorylation, intracellular calcium is increased, leading to muscle contraction and hyperthermia (TEWARI et al., 2009). Dantrolene inhibits the release of calcium from the sarcoplasmic reticulum in the cell, reducing the concentration of calcium and helping to manage the hyperthermia (TEWARI et al., 2009). However, Van Schoor, Khanderia and Thorniley (2018) reported an acute DNP intoxication case that dantrolene was ineffective; suggesting this compound may have limitations as an “antidote” for DNP poisoning.

2.2.3. Fatalities related to 2,4-DNP intoxications

In the literature, several cases of 2,4-DNP intoxications have been reported. Deaths can be due to unintentional overdose, occupational exposure or suicide. One of the first death related to 2,4-DNP was reported in 1918, due to occupational exposure (WARTHIN, 1918; GRUNDLINGH et al., 2011). In 1919, a report of occupational exposure to 2,4-DNP in France was reported (PERKINS, 1919). In the 1930s, deaths related to 2,4-DNP were associated to weigh loss use (GRUNDLINGH et al., 2011). **Table 1** shows a summary of reports available in the literature.

Table 1. Review of some literature reports about 2,4-DNP poisonings

Country	Case details	Reference
United States	**Case 1.** Female, 17-years-old, presented at the hospital, showing rapid breathing and fatigue. Two pills containing yellow powder were found in deceased's bedroom. 2,4-DNP was found at 36.14 mg/L in admission blood and 29.68 mg/L in admission serum. **Case 2.** Male, 28-years-old, was found unconscious, in a bathtub with ice. A friend stated that the deceased had been using "bodybuilding supplements". 2,4-DNP levels were as following: admission blood: 28 mg/L; peripheral blood: 31 mg/L; peripheral serum: 29 mg/L; urine: 53 mg/L; gastric contents: 850 mg; and vitreous humor: 3.4 mg/L.	MIRANDA et al., 2006
	Female, 17-years-old, ingested between 12 and 15 "diet pills" with intent to commit suicide. She went to pediatric emergency presenting vomiting and diaphoresis. A bag containing yellow pills labeled as "60 DNP" was found at her home. Her condition worsened in a few hours, with body temperature increase, tachycardia, reduced mental status, dying around 5h 46 min after arriving at the hospital and 10h after ingesting the pills.	HSIAO et al., 2005
Italy	Male, 30-years-old, died and had ingested 2,4-DNP before. 2,4-DNP was found at 48.4 mg/L in admission blood.	POLITI et al., 2007

Table 1. Review of some literature reports about 2,4-DNP poisonings (continuing)

Country	Case details	Reference
United Kingdom	Male, 46-years-old, ingested 14 pills of 2,4-DNP (with 200 mg each) to commit suicide. He exhibited the following symptoms: short breathing, tachycardia, excessive sweating and agitation. He died 7h after arriving at the hospital and 21h after ingesting 2,4-DNP.	BARTLETT; BRUNNER; GOUGH, 2010
	Male, 21-years-old, presented to the hospital 2h after ingesting 17 tablets with 4250 mg of 2,4-DNP, in combination with alcohol, with suicidal intent. The patient reported fatigue and presented shortness of breath and the condition later worsened, with him presenting tachycardia, diaphoresis, excessive sweating with yellow coloration and cardiac arrest. 2,4-DNP was found in blood (23 mg/dL) and in urine.	HOLBOROW; PURNELL; WONG, 2016
China	**Case 1.** Male, 30-years-old, presented with shortness breath and reported a recent ingestion of “bodybuilding pill” over 10 days, which caused excessive sweating. The content of 2,4-DNP in the pill was 72 mg. After monitoring, the patient was discharged on day 2. **Case 2.** Female, 25-years-old, presented tachycardia and rapid breathing, after ingesting a slimming pill for over a month. The pill was found positive for 2,4-DNP. The patient was discharged on day 4 and showed no symptoms 1 month later.	LEE et al., 2014
Denmark	Male, 39-years-old, died after the ingestion of 4 g of 2,4-DNP. He presented a severe condition of muscular rigidity and went to cardiac arrest. Resuscitation was ineffective.	LARSEN; HALBERG; BAKKE, 2015

Table 1. Review of some literature reports about 2,4-DNP poisonings (conclusion)

Country	Case details	Reference
Germany	Male, 50-years-old, died after ingesting 2,4-DNP over 43 days. He presented excessive sweat, nausea, dizziness and had vomited a yellow liquid. Police found notes of 2,4-DNP consumption over 43 days, with a daily dose between 200 and 600 mg. 2,4-DNP was quantitated as follows: femoral blood: 21.6 µg/mL; urine: 95.3 µg/mL; cerebrospinal fluid: 4.61 µg/mL; heart blood: 4.24 µg/g; lung: 6.02 µg/g; brain: 2.00 µg/g; liver: 1.11 µg/g and kidney: 4.38 µg/g.	ZACK et al., 2016

The characteristics of 2,4-DNP poisonings occurred in the last 10 to 15 years are very typical. The reports have shown that the major intentions of using 2,4-DNP is to lose weight or to commit suicide. In general, 2,4-DNP users are adults and presented the classical symptoms of 2,4-DNP poisoning: increased body temperature, excessive sweating, tachypnea, tachycardia and agitation. In published reports, the majority of reports are fatal intoxications, especially considering that there is no effective antidote for 2,4-DNP intoxication. Another observation is that the selected cases have been reported by different countries in North America, Europe and Asia, showing that 2,4-DNP is a drug easily available in any part of the world.

2.2.4. 2,4-DNP analytical challenges

2,4-DNP is an analytically challenging compound. Nitrophenols, as 2,4-DNP, present a high polarity and a strong interaction with active hydroxyl groups present in the stationary phase when analyzed by GC (KIM et al., 1993). On the other hand, LC-MS analysis of 2,4-DNP was proved to exhibit ionization enhancement, which can be caused by the higher content of organic modifier in the mobile phase (POLITTI et al., 2007). In imunoassays, Kopec et al. (2018) recently reported that 2,4-DNP shows cross-reactivity in salicylate assays, producing “false positive” results, which can generate a misinterpretation. In addition, as both substances have the same toxicity mechanism, the need for a confirmatory method may occur, considering these substances are very common in forensic casework but not necessarily in combination. In **Chapter 3**, the development, validation and application of an analytical method for 2,4-DNP is described.

2.3. Polydrug use in the context of NPS

The scenario of drug abuse in the world has an additional complexity when **polydrug use** is considered. The definition of polydrug use is the combined use illegal drugs with other legal or illegal drugs (EMCDDA, 2002). Drug users are, in general, polydrug users, both occasionally and regularly (UNODCE, 2016). According to UNODC (2019), polydrug use is a current threat in the NPS scenario. Only in 2018, more than half of the postmortem cases reported to the United Nations Office on Drugs and Crime (UNODC) Early Warning Advisory (EWA) Tox-Portal have presented more than four substances involved (UNODC, 2019).

In DUID cases, it is common to find suspects of impaired driving using multiple drugs (FARRELL; KERRIGAN; LOGAN, 2007). In post-mortem cases, the presence of multiple drugs increases the complexity in the assessment of how a particular drug contributed to the death (UNODC, 2019). This is a major issue, especially because some of the combinations that might have caused the death are common drug use patterns, which means that they could be occurring with other drug users as well. Serious interactions can occur by using fentanyl in combination with cocaine, heroin, ethanol and benzodiazepines (ZAWILSKA, 2017). Combinations of designer benzodiazepines with other drugs, as synthetic cannabinoids, are also reported (EMCDDA, 2015). Benzodiazepines are often used by opioid users, to potentiate the opioid high and to induce sedation in the descent phase; by stimulant users, to reach a calm state, and by ethanol users, in order to increase the effects by synergy (ZAWILSKA; WOJCIESZAK, 2019). This scenario is always challenging for a forensic toxicologist, in terms of selecting the right analytical workflow to follow as well as interpreting the results.

2.4. Analytical challenges to study novel drugs of abuse

One of the biggest challenges of modern STA practice in Forensic Toxicology is how to incorporate a rapid and effective analytical workflow in order to study a new drug, whether an NPS or a re-emergent drug.

In some cases, if a drug is found for the first time and it is similar or an analog of a "known" NPS, the routine method should be redesigned in order to extend its scope and to include the new drug within the list of target substances, not requiring a new method. Some drugs are not chemically similar to any other NPS, as mitragynine, requiring a different approach and fully method development by testing extraction, separation and detection.

It is not possible to analyze every single possible drug using a single method or technique at once. For this reason, there are some drugs that were included in the scope of routine analytical methods in the past but, due to the reduction in the prevalence or even disappearance, the laboratories updated the scope, by removing them. With re-emergence, drugs must be re-tested and a method development is needed. Some substances, such as 2,4-DNP, were tested in the past using different methods and techniques, which also require an update in analytical workflow for improved performance and robustness.

As discussed in the previous sections, polydrug use is a key factor in forensic toxicological analyses. In this context, for STA, one of the best approaches is to develop a comprehensive screening method that would extract and detect a large number of drugs as possible. Then the forensic toxicologist would be able to have a large panel of compounds in the sample to decide which confirmatory or quantitative tests would be required. Another challenge is the emergence of new drugs simultaneously with the re-emergence of “old” drugs. Some drugs may not be tested simultaneously anymore or might never been, requiring the constant discussion, monitoring and update of routine methods.

As the NPS are unknown substances or know substances that have been used in different doses, the effects induced by them in the body is unknown. For this reason, the study of toxicokinetics and toxicodynamics of these compounds is important for STA. Information regarding the absorption, distribution, biotransformation and excretion of these compounds can help forensic toxicologists to identify the best specimens to analyze in order to search for a specific compound and to develop the most appropriate analytical method for screening, confirmation and quantitation. In addition, knowing possible metabolic profiles of the NPS helps to include major metabolites in the scope of analytical methods, in the case of the parent drug is not excreted unchanged.

Another source of complexity for modern forensic STA practice is to perform tests and studies in a timely manner. Some novel drugs are identified as potential new threats based in the growing number of cases over time. However, other novel drugs may emerge in outbreak events. One example is the “Zombie” outbreak occurred in New York, United States, in 2016 (ADAMS et al., 2017). A mass intoxication of 33 persons occurred July 12, 2016, due to the potent synthetic cannabinoid AMB-FUBINACA (ADAMS et al., 2017). In July, 2018, another outbreak occurred in Philadelphia, United States, with over 160 persons (estimated) admitted to hospitals in the area of Philadelphia, showing symptoms of overdose (CFSRE, 2018). Although no new drugs were found, a new and dangerous combination of heroin, fentanyl, and 5F-ADB was found in the drug packaged for illicit sale under the brand “Santa Muerte”, which was consumed by the users (CFSRE, 2018). In these cases, if the subjects are hospitalized, by knowing the identity of the possible toxic compound involved, the diagnosis and the treatment can be optimized to save the life of the users. For this reason, it is important to adopt an effective analytical strategy to test samples for the identification of known and unknown drugs and their metabolites. However, if the laboratory has no facilities that allow the test in house,

alternative strategies should be adopted to send sample to other laboratories for testing. In addition, it is also very important to consider how the data from these cases are communicated to the stakeholders in public health. After the preliminary analyses, the dissemination of data from outbreak cases must follow a timely approach, since new cases can arise at any time from other parts of the country. As soon this information is released to the community, hospitals and medical teams can be more prepared to face potential intoxications in their regions.

Biological fluids analysis of NPS can present some challenges as well. Specimens are not always readily available for collection and analysis. Although the ideal situation is to analyze different sources of sample (as blood and urine or blood and oral fluid), this may be difficult in some situations. Collecting large volume of blood samples for some populations, as drug user and psychiatric patients, may be difficult (MERCOLINI; PROTTI, 2016; JOYE et al., 2019). Victims of violent death also may have a small amount of blood available for collection after an accident, suicide, or a homicide, depending of the gravity of the injuries (SKOPP, 2004). For this reason, any alternative approach that can combine both easy sampling and possibility of analysis is desired.

3. MODERN BIOANALYTICAL STRATEGIES

3.1. Dried Blood Spots (DBS)

Dried matrix drugs testing has been used in the last few years and DBS is one the most reported application (JOYE et al., 2019). The use of dried blood for analysis date back from 1900s, by Ivar Bang (ZAKARIA et al., 2016; BANG, 1913). One of the first reports of DBS use in research was in the early 1960s, with the development of an assay for the diagnostic of phenylketonuria in newborns (GUTHRIE; SUSI, 1963).

DBS consists of an alternative technique for obtaining, shipping and storing blood samples by depositing a low volume of blood on paper cards followed by drying under room temperature (**Figure 10**). The blood can be capillary, obtained by finger prick, or venous, obtained by conventional procedure (in the latter collecting a reduced blood volume) (AMBACH et al., 2019). Endogenous components and drugs present in the blood are adsorbed onto the surface of a cellulose-based matrix, such as a paper card (WAGNER et al., 2016). The drying process should fully occur before storage or transport and it is recommended to let DBS samples drying for 1.5h to 4h, in low humidity

conditions, protected from light, on a non-absorbent surface (KEEVIL, 2011; ZAKARIA et al., 2016).

Figure 10. DBS on card

Source: Provided by the author of this work

3.1.1. Advantages of DBS

DBS approach has several advantages over liquid blood collection. A reduced amount of whole blood is used for DBS preparation (WILHELM et al., 2014). A few microliters of blood sample is needed for DBS whereas for whole blood testing, usually 0.5 mL to 1 mL is required. The collection is also easier and less invasive, especially when considering *in vivo* procedures (STOVE et al., 2012; WILHELM et al., 2014; MERCOLINI; PROTTI, 2016; WAGNER et al., 2016).

Manipulation of DBS offers less biohazard risks, as many pathogens such as HIV-1, are deactivated, by drying the blood (MERCOLINI et al. 2010; STOVE et al., 2012; ODOARDI et al., 2014; MERCOLINI; PROTTI, 2016; WAGNER et al., 2016; JOYE et al., 2019). Both transportation and storage of DBS specimens are less complex, without requiring any special procedures such as refrigeration (MERCOLINI et al. 2010; ODOARDI et al., 2014; WAGNER et al. 2016; ZAKARIA et al., 2016). In the literature, there are several reports of good stability of different compounds in DBS (WAGNER et al. 2016; WILHELM et al. 2014).

3.1.2. Limitations of DBS

The use of DBS in routine analysis has some limitations. As DBS combine a small volume of sample and complex matrix (blood), and some compounds are found in the sub ng/mL range, a sensitive and selective analytical technique is required (MERCOLINI; PROTTI, 2016; WAGNER et al., 2016). Although chromatographic and mass

spectrometric instrumentation has been improved in the last 10-15 years, high resolution instruments present elevated costs and are not accessible to all laboratories, which is also a major limitation. However, DBS is readily prepared and can be sent through regular mail to other laboratories, which is a way to overcome this limitation.

Regarding the interpretation of results from DBS toxicological analysis, some points should be considered. Method development requires additional tests, resulting in more time needed to achieve a new method. The type of paper used in the card, volume of blood, homogeneity and other DBS-specific procedures should be evaluated (WILHELM et al., 2014). Some blood and paper properties need to be taken into account as the hematocrit levels, the type of paper used and the homogeneity of the sample distribution, which may influence the results (HERNANDES et al., 2017). The paper composition (including its thickness and density) and source can influence the rate of adsorption and the dispersion of drugs within blood, onto the surface of the material (ZAKARIA et al., 2016). The use of DBS introduce the paper as a new component in the extracting mixture, generating another residue in addition to the biological sample.

When a quantitative analysis is performed, difference between drug levels in capillary blood and in venous blood should be considered (KEEVIL, 2011; WILHELM et al., 2014). Both arterial and venous circulation occur through capillaries and capillary blood has more similarity with arterial blood (KEEVIL, 2011). For example, levels of total protein and bilirubin were lower in capillary than in venous serum (KUPKE et al., 1981). However, some studies have shown that for drug testing, the differences between capillary and venous blood are not significant. *In vivo*, a good correlation between capillary and venous GHB levels was observed (SADONES et al., 2015). A similar result was observed for benzodiazepines in capillary and cardiac blood collected from postmortem cases (MORETTI et al., 2019).

Variations in hematocrit content in DBS affect the plasma amount in the same sample, which will affect the relative plasma percentage in each spot (ZAKARIA et al., 2016). At high hematocrit content, the blood distributes less homogeneously in the paper, and drugs present in the blood will diffuse in a shorter distance onto the paper (ZAKARIA et al., 2016). For example, for cocaine and metabolites, hematocrit produced effect in the results, especially at 30%, but in the range of 37% to 47%, the analytes can be quantified without adjusting the results (AMBACH et al., 2019). Due to the inhomogeneity of blood, when a spot is selected, the quantification results may overestimate or underestimate the concentration, which depends the position were the spot is taken (ZAKARIA et al., 2016).

3.1.3. Analytical aspects of DBS testing

The analytical workflow for DBS testing is simple but requires a standardized protocol, in order to avoid contamination or bias. The analysis starts by collecting the spot from the paper card for extraction, selecting a spot with size between 3 mm and the whole spot (ZAKARIA et al., 2016). In general, considering the hematocrit effect and possible contaminations between samples, the most common approaches to avoid carry over, inaccuracy or bias are (ZAKARIA et al., 2016):

(a) to pre-punch the whole spot in the clean paper card (without blood) and to analyze the whole spot;

(b) to punch a smaller portion of the whole spot, applying less blood onto this spot and;

(c) to apply the blood in the whole spot and, after dried, to punch several small spots from the whole spot, from different positions, using all them for analysis.

The spotting should be performed carefully and precisely to avoid double spotting, mixing of different spots or contaminations (TIMMERMAN et al., 2011). When collecting capillary blood through finger prick, the collection site should not be pressed against the paper card and only used in one single circle/spot (KEEVIL, 2011). Blood (venous or capillary) should be uniformly absorbed, saturating the paper (KEEVIL, 2011). In addition, the inappropriate manipulation of DBS sample must avoid the contact with hands of the subject or collector (TIMMERMAN et al., 2011).

After the spot is properly removed from the paper, the compounds need to be extracted to a liquid medium in order to perform a chromatographic separation. The more efficient the extraction, the lower is probability of losing drugs during the processing (ZAKARIA et al., 2016). In general, methanol is one of the most successful elution solvents for DBS extractions (ZAKARIA et al., 2016). Additional procedures can also be evaluated as agitation, sonication, heating, and centrifugation, if a low recovery is found.

Analysis of DBS is possible through GC-MS or LC-MS. GC-MS provide good resolution but it is limited to the analysis of volatile, thermally stable and mildly polar or apolar substances (ZAKARIA et al., 2016). LC-MS is a more sensitive technique, without restrictions regarding volatility or polarity of the substances, and with improved sensitivity, showing potential to improve DBS analysis (KEEVIL, 2011; ZAKARIA et al., 2016). In addition, the introduction of modern instrumentations have provided good improvement in DBS analysis. UPLC instrumentations enabled better chromatographic resolution and Selective and Multiple Reaction Monitoring Modes (SRM and MRM,

respectively) in Mass Spectrometry have improved the sensitivity, lowering the limits of detection (LOD) (ZAKARIA et al., 2016). Two dimensional chromatography systems also have improved DBS analysis, by reducing matrix and carry over effects (ZAKARIA et al., 2016).

3.1.4. Novel DBS approaches

DBS have a potential for technological innovation and modifications to the conventional DBS on paper card have been developed over the years. Automation of the analysis represented a large advantage in DBS testing. Automated systems make both extraction and analysis easier, avoiding the punching step, reducing the generation of biohazard waste and improving the efficiency of the process (OLIVEIRA; HENION; WICKREMSINHE, 2014). In a study by Oliveira, Henion and Wickremsinhe (2014), an on-line extraction and analysis by two dimensional LC-QTOF was used for the analysis of midazolam and desipramine in rats. The method was satisfactory, presenting acceptable linearity, precision and accuracy, showing the validity of the automation in DBS analysis (OLIVEIRA; HENION; WICKREMSINHE, 2014). A similar study by Verplaetse and Henion (2016) used an online extraction system coupled to UPLC-MS/MS for the analysis of opioids (fentanyl, morphine, codeine, oxycodone and hydrocodone), resulting in a satisfactory quantitative method (VERPLAETSE; HENION, 2016).

As mentioned before the hematocrit content can introduce bias and imprecision in the results, which is a limitation of DBS, and a potential solution is to use plasma rather than whole blood (RYONA; HENION, 2016). Ryona and Henion (2016) have proposed an alternative technology creating DBS as a “book”, with several layers that separate the whole blood and serum, collecting only a dried plasma spot. This method can be automated and analysis by on-line SPE-LC-MS/MS is used for analysis (RYONA; HENION, 2016). However, the authors state that this dried plasma spots approach is not to replace DBS, since some drugs may have more affinity to red blood cells and analysis in DBS will be preferred (RYONA; HENION, 2016). In **Chapter 2**, an alternative approach to paper DBS, using a salt tablet, is presented.

3.1.5. DBS in the context of drugs of abuse and NPS

Although DBS approaches have some limitations, the logistic and analytical gains of using them in Forensic Toxicology are very promising. One of the key factors of using

DBS is to use a sensitive technique able to detect concentrations in ng/mL range, in a very complex matrix (blood) and using a low volume of sample. As discussed before, these techniques are available in the industry but not all laboratories have the budget for purchasing such a high technology instrumentation or have facilities to host these instruments. However, the implementation of DBS by forensic agencies and laboratories is a tool for sending these samples to other laboratories, at low cost, for testing.

The easy, fast and possibility of *in loco* sampling of DBS can be applied to the analysis of drugs with short half-life as heroin and gamma hydroxybutyrate (GHB) (STOVE et al., 2012). GHB is frequently used in DFC cases and the use of DBS is very relevant considering the dynamics of these cases, when the victim goes to the police a few hours after crime, while the drug is still undergoing biotransformation in the body (STOVE et al., 2012).

Another application of DBS is to monitor current drug users under treatment (STOVE et al., 2012). Although urine is traditionally used in these cases, DBS provide a minimally invasive and lower likelihood of adulterated collection, and without the need of medical experts to perform the sampling (STOVE et al., 2012). In addition, due to the use of syringes for drug use, the risk of contamination with viral or bacterial pathogens is minimal, providing more safety to the analyst (STOVE et al., 2012).

DBS also show potential for DUID cases investigation. Drug testing in DBS can be done on site, like roadside, and the results can be complementary to oral fluid testing, which is adopted by many countries in DUID investigations. The possibility of sample contamination is minimal and, again, no medical team is required to perform the collection (STOVE et al., 2012).

Another potential benefit of DBS use in forensic testing is regarding to the stability of drugs. Cocaine and metabolites, MDMA, 6-monoacetylmorphine (6-MAM) and NBOMe compounds are some of the drugs that presented good stability in DBS for long periods of storage. Enzymatic activity is minimal or zero in dried samples, as DBS, which acts as a stabilizing factor for some drugs in DBS, as for cocaine; ester chemical groups are readily hydrolyzed in whole blood and this do not occur in DBS (SIMÕES et al., 2018). Cocaine and some metabolites were stable up to 262 days in DBS under -20ºC and -80ºC (AMBACH et al., 2019). Good stability was also found for cocaine, methadone, benzoylecgonine, morphine, codeine, 6-MAM, amphetamine, methamphetamine, MDMA and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (SIMÕES et al., 2018). In DBS, NBOMe series compounds (25B-, 25C-, 25H-,

25I-, 25G-, 25D- and 25E) were stable at 4ºC and -20ºC for 6 months and at room temperature (except for 25B- and 25I- compounds that presented a degradation of 20%) (CUNHA; EBERLIN; COSTA, 2018a). In general, positive reports regarding drugs stability in DBS have been published, showing the versatility and relevance of DBS in Forensic Toxicology.

DBS methods are also applicable and relevant for retrospective sample-mining, in order to re-test samples searching for recently discovered drugs. As discussed before, NPS have a cycle of prevalence, which depends of geographical region and scheduling under regulatory acts. However, when some drugs are detected in seized samples or biological fluids, the retrospective analysis of "old" samples may reveal trends and patterns, as when or from where this drug was first seen by a laboratory. This helps laboratories and law enforcement to build and to release a database of NPS trends. By using DBS, laboratories can store a large number of samples in a reduced physical space, for a future re-analysis, instead keeping whole blood samples. In **Chapter 2**, a set of screening, confirmatory and quantitative methods using DBS for drugs of abuse and NPS using GC-MS and UPLC-MS/MS is described.

3.2. *In vitro* metabolism studies

3.2.1. Metabolomics

Metabolomics is the investigation of the **metabolome**, which is the set of substances produced in biochemical reactions that occur in cellular pathways of a biological system of interest (HOUNOUM et al., 2016; VUCKOVIC, 2012). The metabolites are molecules with low molecular weight, involved in the maintenance, growth and normal function of a cell (DUNN; ELLIS, 2005).

When xenobiotics are introduced in a biological system, they undergo a process of **biotransformation** (or **metabolization**), consisting in biochemical processes, usually enzymatic, of converting non-polar and lipophilic substances into polar and hydrophilic metabolites, facilitating the elimination of the substance, especially through the urine (VENKATAKRISHNAN et al., 2001; KARCH, 2006; DIAO; HUESTIS, 2019). By consequence, metabolites are generated, constituting the metabolome of a particular xenobiotic in that biological system.

Biotransformation takes place mainly in the liver, occurring to a lesser extent in other organs such as kidneys, lungs, intestine and heart (BRANDON et al., 2003;

FASINU et al., 2012; DIAO; HUESTIS, 2019). The set of biotransformation reactions is divided into **Phase I** and **Phase II** reactions. **Phase I** metabolism involves reactions of introduction or exposure of functional groups of higher polarity into the parent substance structure and comprise hydrolysis, oxidation and reduction reactions, including N-dealkylation, O-dealkylation, aromatic and aliphatic hydroxylations, deamination, N-oxidation and S-oxidation (BRANDON et al., 2003; KARCH, 2006; VENKATAKRISHNAN et al., 2001). Phase I reactions are mainly performed by the cytochrome P450 (CYP) enzymes present in the liver and also in the kidneys, intestines and other organs (BRANDON et al., 2003; FASINU et al., 2012). **Phase II** metabolism is the set of conjugation reactions, which produces covalent bonding to glucuronic acid, sulfate, glutathione, acetate or amino acids, and it is conducted by different enzymes such as UDP-glucuronyl transferase (UGT) and N-acetyl transferase, among others (FASINU et al., 2012; KARCH, 2006).

3.2.2. Metabolism study models

The study of metabolism of a particular xenobiotic is a powerful tool to assess how a biological system responds after the introduction of an exogenous substance, including the biomarkers produced due to biotransformation. To have this data, studies can be performed using *in vivo* or *in vitro* models. *In vivo* models include humans and animals and consist in the controlled administration of the compound of interest to the subjects, followed by monitoring their responses over time. Although these models can be very informative by providing data from real and living subjects, research with humans and animals have some limitations as high cost and ethical issues.

The use of *in vitro* models has an important application in Forensic Toxicology. These models can reproduce human biotransformations, providing useful data in the study of drugs (DIAO; HUESTIS, 2019). Using *in vitro* systems, it is possible the biosynthesis of metabolites for metabolite identification and analytical method development (WRIGHTON et al., 1995). *In vitro* models include cellular systems as liver tissue, hepatocytes in suspension or in monolayer cultures and cell lines derived from hepatocytes; and preparations with subcellular fractions (microsomes and cytosolic) and purified or cDNA heterologously expressed enzymes (BRANDON et al., 2003; VENKATAKRISHNAN et al., 2001; WRIGHTON et al., 1995). HLM are some of the most commonly used model in Forensic Toxicology and will be discussed in detail.

3.2.3. Human Liver Microsomes

Human liver microsomes (HLM) are hepatocyte endoplasmic reticulum vesicles, obtained by differential centrifugation of liver homogenates and cell fractions (VENKATAKRISHNAN et al., 2001; DIAO; HUESTIS, 2019). Some of the characteristics of this model are: (I) high availability, (II) reduced cost compared with other models, (III) easy handling, (IV) presence of enzymes responsible for drug metabolism (as CYP, flavin-containing monooxygenases (FMO) and UDP-glucuronyltransferases) and (V) stability for long periods if stored at -80ºC (VENKATAKRISHNAN et al., 2001; BRANDON et al., 2003; ZHANG et al., 2012; TEMPORAL et al., 2017; DIAO; HUESTIS, 2019). However, HLM are not useful for quantitative analysis as CYPs and UGTs enzymes are enriched without the competition of other enzymes, drugs are exposed to the medium without the need of crossing membrane barriers and some enzymes that might metabolize some drugs are not present, such as N-acetyltransferase and Glutathione S-transferases (DIAO; HUESTIS, 2019).

The HLM incubation conditions should be assessed carefully as they may affect the *in vitro* metabolism profile as well as the integrity of the HLM. It is recommended that the incubation medium present ionic strength and pH similar to those of hepatic system *in vivo* (VENKATAKRISHNAN et al., 2001). However, the medium pH depends also of the enzyme activity, which might be optimal at a pH different from the physiological pH; an example is flavin-containing monooxygenase (FMO) that has an optimum pH of 9.0 and the physiological pH is around 7.4 (VENKATAKRISHNAN et al., 2001). Some commonly used buffers in metabolism assays are Tris, sodium phosphate and potassium phosphate (VENKATAKRISHNAN et al., 2001). Solubilizing solvents can be used with target compounds or inhibitors, to prepare the incubations. Hydrophobic drugs may require an organic solvent for solubilization and the effect on the HLM incubation should be assessed (VENKATAKRISHNAN et al., 2001).

3.2.4. In vitro metabolism assays in the context of NPS

In vitro metabolism studies are able to generate metabolites for a specific drug, which is useful for both metabolite identification and method development. As most of NPS are unknown substances, it is important to comprehend how this drug is biotransformed by the organism and which compounds are the biomarkers of exposure to this drug (i.e. the metabolites). Once potential metabolites are identified and the analytical

method is developed, STA can be performed in authentic specimens to search for parent NPS and their metabolites.

According to Dunn and Ellis (2005), **metabolic profiling** consist in the qualitative and quantitative analysis of selected metabolites produced by a metabolic pathway. The metabolic profiling for a new drug can be performed based on Phase I and/or Phase II mechanisms, according to the purpose of the analysis. For example, if after identifying the metabolites of a particular drug, the toxicologist needs to compare with the metabolites found in authentic samples, it is important to check which samples are available. Phase II metabolites are more likely to be detected in urine specimens so it is required an *in vitro* study of conjugated metabolites.

The method for preparation and analysis of *in vitro* assays samples is developed to extract the metabolites from the medium, with minimal matrix effects (DUNN; ELLIS, 2005). Extraction starts with the stop of metabolic reaction by freezing or adding organic solvents. Samples are extracted using organic solvents (polar or apolar), which also involves the disruption of cells (to release metabolites in the medium) and centrifugation to remove cell pellet and other residues (DUNN; ELLIS, 2005). Analyses can be performed by chromatography/mass spectrometry techniques (GC-MS or LC-MS), commonly used in metabolomics because their sensitivity and selectivity (DUNN; ELLIS, 2005).

There are several reports in the literature regarding NPS metabolism. HLM and human hepatocytes have been used for studying the metabolism of novel drugs (KROTULSKI et al., 2018b). Some of these studies with NPS are summarized in **Table 2.** In **Chapter 4**, the *in vitro* metabolism study of novel synthetic cathinones, recently found in seized samples in the ports of entry in the United States, is described.

Table 2. Review of literature on *in vitro* metabolism studies for novel drugs of abuse

	Drug	*In vitro* model	Reference
Designer Opioids (fentanyl analogs and utopioids)	AH-7921	Human hepatocytes	WOHLFARTH et al., 2016
	U-47700 and U-49900	HLM	KROTULSKI et al., 2018b
	MT-45 and 2-fluoro-MT-45	HLM and human and mouse hepatocytes	McKENZIE et al., 2018
	Cyclopropylfentanyl	HLM	CUTLER; HUDSON, 2019
Synthetic Cannabinoids	JWH-015, JWH-098, JWH-251 and JWH-307	Rat liver slices	STRANO-ROSSI et al., 2014
	ADB-FUBINACA	HLM and human hepatocytes	CARLIER et al., 2017
	CUMYL-PeGACLONE	HLM	MOGLER et al., 2018
	MN-18 and 5-fluoro-MN-18	Human hepatocytes	DIAO et al., 2017
	MDMB-CHMINACA	HLM	PRESLEY et al., 2019
Synthetic Cathinones	2-desoxypipradrol (2-DPMP), 3,4-dimethylmethcathinone (3,4-DMMC), alpha-PVP, and methiopropamine (MPA)	HLM	TYRKKÖ et al., 2013
	Mephedrone	DNA-expressed CYP enzymes and HLM	PEDERSEN et al., 2013

Table 2. Review of literature on *in vitro* metabolism studies for novel drugs of abuse (continuing)

	Drug	***In vitro* model**	**Reference**
Synthetic Cathinones	4-methoxy-alpha-PVP	HLM and human hepatocytes	ELLEFSEN et al., 2016
	PV-8	HLM and human hepatocytes	SWORTWOOD et al., 2016a
	Alpha-PVT	Human hepatocytes	SWORTWOOD et al., 2016b
	N-ethyl-pentylone	HLM	KROTULSKI et al., 2018c
	Dibutylone	HLM	KROTULSKI et al., 2018a
Novel hallucinogens	25B-NBOMe, 25C-NBOMe, 25H-NBOMe and 25I-NBOMe	HLM	TEMPORAL et al., 2017
	5-fluoro-DALT, 7-methyl-DALT, and 5,6-methylenedioxy-DALT	HLM	MICHELY et al., 2017
	25D-NBOMe, 25E-NBOMe and 25N-NBOMe	HLM and Cunninghamella elegans (C. elegans)	GRAFINGER et al., 2018a
	5-MeO-MiPT	HLM	GRAFINGER et al. 2018b
	5-MeO-2-Me-DALT, 5-MeO-2-Me-ALCHT, and 5-MeO-2-Me-DIPT	HLM and human liver cytosols	CASPAR et al., 2018
	25N-NBOMe	HLM	SEO et al., 2019

4. GENERAL GOALS OF THIS WORK

Based on the current scenario of Forensic Toxicology, there are many opportunities for research, which is recommended or required in the field. Considering the challenges of novel drugs of abuse and novel bioanalytical approaches for the identification, confirmation or quantitation of these new drugs, it is recommended to explore and to develop as much as possible methods for the analysis of these new drugs, along with other traditional illicit drugs, in biological specimens. In addition, it is also recommended to study other aspects of these drugs, including the metabolism of these compounds, in order to obtain important data regarding how these new compounds will be biotransformed.

The general goal of this research is the analysis of illicit drugs (such as cocaine and methamphetamine), novel psychoactive substances (NPS) and non-psychoactive substances (in this work, 2,4-DNP) drugs of abuse in biological fluids or in *in vitro* models, using traditional and/or novel analytical methods (such as DBS and high sensitivity/resolution techniques). The methods and studies performed with these novel drugs of abuse are described in **Chapters 2, 3** and **4**.

CHAPTER 2. Analysis of illicit drugs and NPS in dried blood spots (DBS) using GC-MS and LC-MS/MS

1. INTRODUCTION

The use of DBS in forensic STA has increased in the last few years. Low volume of blood, easy handling and possibility of long-term storage in reduced spaces and shipping to other laboratories, without special requirements (such as refrigeration) are some of the positive aspects of using DBS in routine analysis. Nowadays with the polydrug use and emergence of novel drugs, a methodology that provides fast processing or transport is important in forensic investigations.

The number of publications on analytical methods using DBS for screening, confirmatory and quantitative analysis has increased. However, the number of new drugs has increased as well, and there are no methods using DBS for some NPS or no methods with expanded scope including traditional drugs and newer psychoactive substances. In addition, DBS uses a reduced amount of blood, which requires a very sensitive analytical technique as LC-MS/MS. However, as some laboratories have no capability for high-resolution techniques, the use of less sensitive techniques such as GC-MS, which is more commonly found in forensic laboratories due to low cost, for DBS analysis may not be possible, requiring different approaches.

2. OBJECTIVES

General objective

Development and validation of analytical methods using LC-MS/MS and GC-MS for screening, confirmatory or quantitative analysis of traditional and novel drugs in DBS.

Specific objectives

I. Development of a novel salt-supported DBS approach for screening of cocaine, MDMA and methamphetamine using GC-MS.

II. Development and validation of a screening method for 35 psychoactive substances (traditional drugs, synthetic cathinones, designer benzodiazepines, NBOMe series compounds, fentanyl analogs and utopioids) in paper DBS using UPLC-QTOF-MS/MS and analysis of postmortem and DUID specimens.

III. Development and validation of a screening method for 10 synthetic cannabinoids in paper DBS using UPLC-Triple Quadrupole-MS/MS.

IV. Development and validation of a confirmatory and quantitative method for six synthetic cathinones in paper DBS using UPLC-Triple Quadrupole-MS/MS and analysis of postmortem specimens.

3. MATERIALS AND METHODS

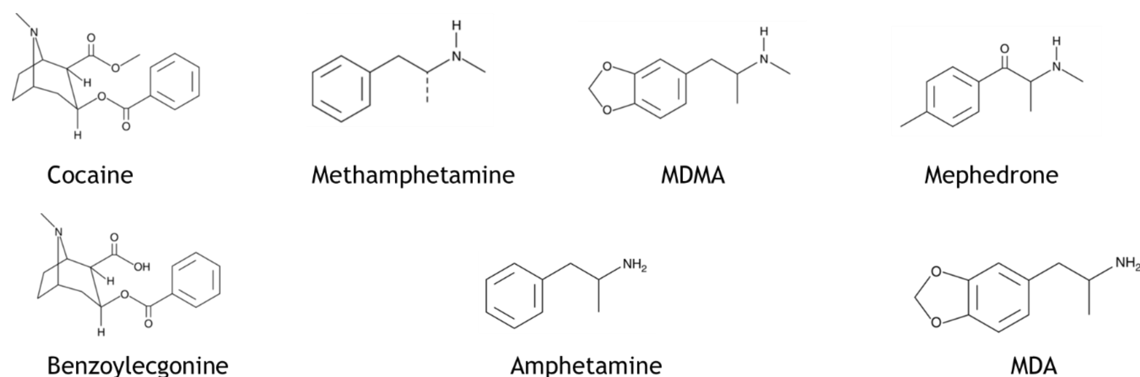
3.1. Development of a novel salt-supported DBS approach for screening of classic drugs of abuse using GC-MS

3.1.1. Reagents, materials and solutions

Methanol, ethyl acetate, N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and sodium carbonate were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Cocaine, MDMA, methylenedioxymethamphetamine (MDA), methamphetamine, mephedrone, amphetamine, benzoylecgonine, amphetamine-d_{11}, MDMA-d_5 and cocaine-d_3 were purchased as 1 mg/mL solutions in methanol or acetonitrile from Cerilliant (Round Rock, TX, USA). Defibrinated sheep's blood used in method development was purchased from Newprov (Pinhais, PR, Brazil).

Standard (**Figure 11**) and ISTD working solutions were prepared in methanol by serial dilution from the reference materials at the concentrations of 10 and 1 µg/mL and stored under refrigeration.

Figure 11. Molecular structures of target compounds



Source: Cayman Chemical Website

3.1.2. Salt-DBS preparation

For salt-DBS preparation, a manual press and a cylinder holder were used **(Figure 12)**. 400 mg of sodium carbonate were weighted out and inserted inside the holder. 130 µL of defibrinated sheep's blood, spiked with the analytes, was added on the top of the solid, inside the holder. The holder was closed and manually compressed using the press.

The bottom part of the holder was removed and a second round of compression was performed to remove the tablet from the holder. The tablet was stored for later analysis.

Figure 12. Manual press (on the left) and cylinder holder (on the right) used for salt-supported DBS preparation

Source: Provided by the author of this work

3.1.3. Salt-DBS extraction

The salt-supported DBS was inserted in a glass tube followed by the addition of 1 mL of deionized water and 1 mL ethyl acetate followed by vortex homogenization for 90 s, orbital agitation for 15 min and centrifugation. The organic phase was collected and dried under air compressed flow at 45ºC (Caliper TurboVap® LV). The derivatization was performed using ultrasound assisted derivatization with 20 µL of MSTFA at 30°C for 20 minutes. Derivatization was performed in sonicator with potency of 100 W and ultrasound frequency of 40 kHz.

3.1.4. Ultrasound assisted derivatization method development

First, a derivatization method using ultrasound was developed. The reaction of silylation using MSTFA was optimized in ultrasound for target (cocaine, MDMA and methamphetamine) and other possible target drugs of abuse/metabolites (amphetamine, benzoylecgonine and mephedrone).

The study was performed using the Box-Behnken method of Experimental Design (BOX; BEHNKEN, 1960; FERREIRA et al., 2007), for three variables within 3-levels each, with 3 central points. The selected parameters to study in silylation reaction catalyzed by ultrasound were the temperature of the medium (in this case, the ultrasonic

bath), the volume of MSTFA and the time of reaction, at three levels each (low, medium and high). The values selected for each parameter are as follows: for temperature, 25°C, 30°C and 40°C; for volume of MSTFA, 10, 20 and 30 µL; and for time of reaction, 5, 10 and 20 min. All 15 experiments were performed in random order and each run was performed in triplicate.

Aliquots of 10 µL of solutions of analytes and internal standards (ISTD) in methanol or acetonitrile (10 µg/mL) were added to a glass tube and the mixture was dried at 45°C in an evaporator TurboVap LV (Caliper®) to remove the solvent. The residue was redissolved in 70 µL of ethyl acetate and an aliquot of 50 µL of this solution were transferred to an ambar glass vial. To perform the derivatization reaction, MSTFA was added to vials, according to the volume of interest (10, 20 or 30 µL). All reaction mixtures were prepared to a final volume of 80 µL. Ultrasound-assisted derivatization experiments were performed a 1440 D ultrasonic bath, with power of 100 W and frequency of 40 kHz (Odontobras®), at 25, 30 or 40°C and for 5, 10 or 20 min. Any temperature variations in ultrasonic bath along the reaction (2 to 3 °C) were not relevant in the analysis. For comparison, conventional thermal derivatization reactions were performed in a TE-021 dry-block (Tecnal®) by heating at 90°C and for 20 minutes.

The results were analyzed according to the relative chromatographic response (ratio analyte peak area/ISTD peak area) for each of the analytes (amphetamine, methamphetamine, mephedrone, MDA, MDMA and benzoylecgonine). All statistical analyses were performed using the software Minitab® 18 (Minitab Inc.). Analysis of Variance (ANOVA) was used in the analysis of the effects of each variable (reaction parameters) on the analytes chromatographic responses and of how well the model fits the analytical responses data. ANOVA followed by Tukey Post-hoc Test (if necessary) were used for multiple comparisons between derivatization methods. Statistical significance data was considered for $p < 0.05$ at a 95% of confidence level. The optimization of the conditions of reaction were performed using the Response Surface Methodology (RSM) and desirability function

3.1.5. GC-MS analysis

Analyses were performed using an Agilent 7890A GC coupled to Agilent 5975C MS (Santa Clara, CA, USA) with electron impact ionization mode. Chromatographic separation was performed using a HP-5MS capillary column (30 m × 250 µm × 0.25 µm). The temperature gradient used for separation is described as follows: started at 90ºC with

holding time of 2 min; increased to 220ºC, at 10ºC/min rate; and increased to 290ºC, at 20ºC/min, with holding time of 4 min. Total run time was 22.5 min. Helium was used as carrier gas at 1 mL/min flow rate. Temperatures of injection port, MS interface, source and quadrupole were 280ºC, 300ºC, 230ºC and 150ºC, respectively. MS operated in Selected Ion Monitoring (SIM) Mode and the monitored ions are described in **Table 3**.

Table 3. Selected ions monitored for target compounds and ISTD

Analyte/ISTD	Qualifier and quantifier ions (m/z)
Amphetamine	91, 116, 192
Amphetamine-d11	98, 120
Methamphetamine	130, 91, 206
Mephedrone	91, 130, 219, 234
MDA	116, 135, 236
MDMA	130, 135, 250
MDMA-d5	134, 255
Benzoylecgonine	82, 240, 361
Cocaine	82, 182, 303
Cocaine-d3	85, 185

3.2. Development and validation of a screening method for 35 psychoactive substances in paper DBS using UPLC-QTOF-MS/MS

3.2.1. Reagents and materials

Methanol and acetonitrile were obtained from Honeywell (Morris Plains, NJ, USA). Hydrochloric acid was obtained from VWR Chemicals (Radnor, PA, USA). Formic Acid was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Sodium borate decahydrate was acquired from Millipore Corporation (Darmstadt, Germany). Whatman® 903 cards were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Human blank blood for method development and validation was purchased from Bio IVT (Hicksville, NY, USA).

Reference materials of 25B-NBOMe, 25C-NBOMe, 25I-NBOMe, 4-fluoroamphetamine, acetylfentanyl, alpha-PVP, alprazolam, butylone, clonazepam, cocaine, diazepam, fentanyl, heroin, ketamine, LSD, MDMA, mephedrone,

methamphetamine, methylone, phencyclidine (PCP) and pentylone were acquired as 1 mg/mL solutions in methanol or acetonitrile from Cerilliant (Round Rock, TX, USA). Reference materials of 3-MeO-PCP, acrylfentanyl, butyrylfentanyl, carfentanil, dibutylone, etizolam, 4-fluoroisobutyrylfentanyl (FiBF), flubromazolam, furanylfentanyl, methoxyacetylfentanyl, N-ethyl-hexedrone, N-ethyl-pentylone, U-47700, U-48800 and valerylfentanyl were purchased from Cayman Chemical (Ann Arbor, MI, USA) as 1 mg powders and dissolved in methanol to the concentration of 1 mg/mL (**Figure 13**). Alprazolam-d_5, fentanyl-d_5, methylone-d_3 and MDMA-d_5 were also purchased from Cerilliant as 100 µg/mL solutions in methanol.

3.2.2. Standards solutions and controls

Standard working solutions containing all target compounds were combined and diluted from the reference materials to the concentrations of 10, 1 and 0.1 µg/mL in methanol. ISTD working solution with all ISTD was prepared at the concentration of 1 µg/mL in methanol. All working solutions were prepared in amber vials and stored at -20°C.

Positive controls were prepared at concentrations of 500, 50, 20, 10, 5, 2, 1 and 0.5 ng/mL, in a volume of 0.5 mL of blank human blood. The highest control (500 ng/mL) was prepared by spiking 25 µL of 10 µg/mL standards mix solution in 0.5 mL blood and the other controls were prepared by serial dilution, using blank human blood. For recovery, matrix effects and stability assays, positive control at 50 ng/mL was prepared by adding 25 µL of 1 µg/mL standards solution to 0.5 mL of blank blood.

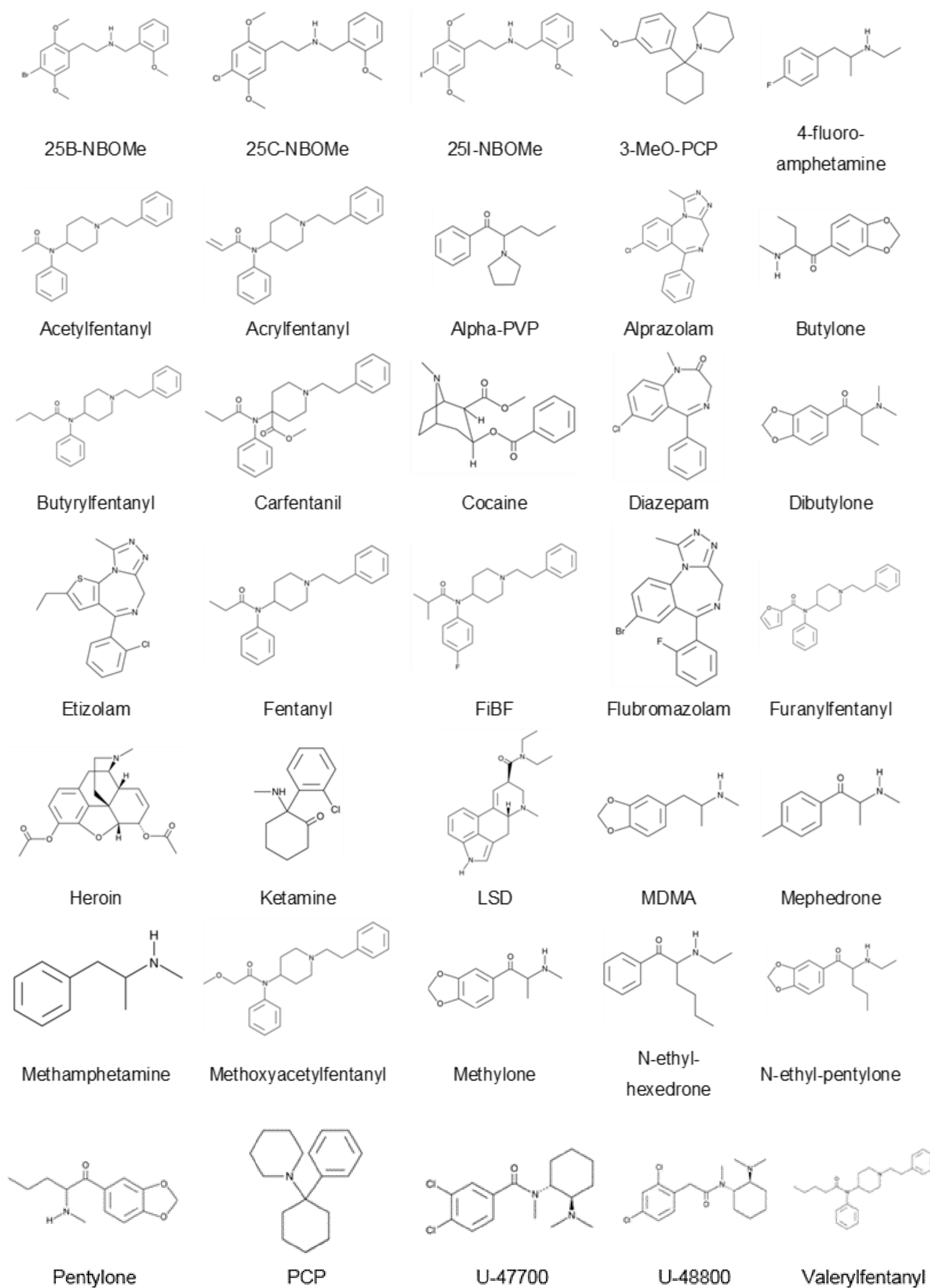
3.2.3. DBS preparation and extraction

For DBS preparation, 50 µL of blood were applied onto 1-cm diameter spots, pre-punched from Whatman® cards. The spots were dried at room temperature, protected from light, for 2h. After dried, the whole spots were collected, to avoid hematocrit variation effects, and analyzed or stored.

Extraction of DBS were performed by adding two spots per sample into a microcentrifuge plastic tube containing 1 mL of methanol, 50 µL of borate buffer pH 10.4 and 25 µL of ISTD working solution. DBS samples were incubated under agitation (1000 rpm) at 25°C for 10 min using an Eppendorf ThermoMixer F2.0. Extracts were transferred to clean glass tubes containing 100 µL of 10% HCl in methanol and

evaporated to dryness at 35ºC (Caliper TurboVap® LV). For reconstitution of dried extracts, 100 µL of mobile phase at initial conditions (90:10 0.1% 5 mM ammonium formate in water (pH 3)/0.1% formic acid in acetonitrile) were added. The extracts were transferred to microcentrifuge tubes and centrifuged for 5 min under 10,000 rpm and 4°C (Eppendorf Centrifuge 5415R). Finally, supernatant was collected and transferred to glass insert vials. 10 µL was injected into the UPLC-QTOF system.

Figure 13. Chemical structures of target compounds



Source: Cayman Chemical Website

3.2.4. UPLC-QTOF-MS/MS analysis

Liquid chromatography analyses was performed using a Waters Acquity I-class UPLC® (Milford, MA, USA). Separation was performed using an Acquity UPLC® BEH C18 (2.1 mm x 150 mm, 1.8 µm) column, at 50°C. Flow rate was set at 0.4 mL/min. The mobile phases were 5 mM ammonium formate in water pH 3 (MPA) and 0.1% formic acid in acetonitrile (MPB). Gradient conditions are detailed in **Table 4**. The total run time was 15 min. Weak and strong wash solvents were 5mM ammonium formate (pH 3.0) and 5:95 H_2O:ACN with 0.1% formic acid, respectively (MOHR; FRISCIA; LOGAN, 2016).

Table 4. Gradient conditions of chromatographic method

Time (min)	MPA (%)	MPB (%)
Initial	87	13
0.5	87	13
10	50	50
10.75	5	95
12.25	5	95
12.5	87	13
15	87	13

High-resolution mass spectrometry analyses were performed using Waters Xevo® G2-S QTOF (Milford, MA, USA). Electrospray ionization in positive mode was used, with cone voltage of 25 V, capillary energy of 0.8 kV, source temperature at 150°C and desolvation temperature at 400°C. Masses were scanned in the range 50 m/z - 1000 m/z with scan time of 0.1 s. Collision energy ramp started at 10 eV and ended at 40 eV (MOHR; FRISCIA; LOGAN, 2016). Leucine enkephalin ($C_{28}H_{37}N_5O_7$) was used for tuning and calibration by monitoring the mass m/z 556.2766. All analyzes were performed using Waters UNIFI Scientific Information System software.

3.2.5. Method Validation

A fit-for-purpose validation was performed, according to ASB guidelines (ASB, 2017). LOD, matrix effects, recovery, interferences, carryover and stability were evaluated.

Sensitivity was assessed using DBS samples prepared from whole blood fortified in decreasing concentrations (20, 10, 5, 2, 1 and 0.5 ng/mL), in triplicate, over different runs (days). The criteria for a positive identification were as follows: retention time within ±0.25 min of target in the library, mass error within ±5 ppm for the molecular ion and ±2 mDa for the fragment ion and peak area of 800 or higher. The LOD was considered the lower concentration that the drug was detected in all samples and following the criteria. Blank DBS as well as blank paper were also analyzed along with positive controls in every run, to assess potential false-negative results.

Recovery and matrix effects were determined at 50 ng/mL, in triplicate, using three set of samples: (1) neat standard solutions dried and reconstituted in mobile phase at initial conditions; (2) extracted DBS samples, following the procedure described before and (3) blank DBS samples spiked with the target compounds after extraction at 50 ng/mL. Recovery and matrix effects were calculated as follows:

$$\text{Recovery (\%)} = \left(\frac{\text{Mean peak area of extracted DBS samples}}{\text{Mean peak area of post spiked blank DBS samples}}\right) \times 100$$

$$\text{Matrix Effects (\%)} = \left[\left(\frac{\text{Mean peak area of post spiked blank DBS samples}}{\text{Mean peak area of neat standards in MP}}\right) - 1\right] \times 100$$

Matrix interferences were tested using 10 different blank blood samples spiked on Whatman® 903 paper cards and extracted as described previously. Interferences from stable isotope ISTD on target compounds responses were tested, in triplicate, using blank DBS extracted within the ISTD at 25 ng/mL. In a similar fashion, interferences from all target compounds on ISTD responses were tested in triplicate, by extracting a high concentration DBS sample (500 ng/mL), without adding ISTD. Interferences from other substances that can be found in forensic casework as medical drugs, steroids or pesticides were assessed by analyzing DBS samples spiked at 100 ng/mL with these drugs. Drugs tested as potential interferents were acetaminophen, caffeine, cannabidiol, Δ^9-THC, diphenhydramine, fluoxetine, methadone, naloxone, nicotine, oxycodone, pentobarbital, salicylic acid, sertraline, tramadol, androstenedione, testosterone 17O-acetate, phentermine, strychnine, carbofuran, 3-hydroxy-carbofuran, monocrotophos, aldicarb, aldicarb sulfoxide, aldicarb sulfone, carbaryl and methomyl. Carryover effects were evaluated by injecting a 90:10 MPA/MPB mobile phase three times after running a high concentration (500 ng/mL) DBS extracted sample.

Stabilities of drugs in DBS samples were studied after processing and for long-term storage. Post-processing stability was determined by re-analysis of extracted DBS samples after kept at room temperature in the auto sampler, for 24h and 48h. Stability was calculated by comparing average peak areas at time zero, after 24h and after 48h.

Long-term stability was evaluated with the preparation of DBS samples spiked with all drugs at 50 ng/mL, stored at room temperature and frozen at -20°C, for 24h, 14 days, 22 days and 37 days. Samples were stored without any desiccant agent, to assess stability at worst case scenario, similarly to Moretti et al. (2019). The studies were performed in triplicate for each condition of temperature and time. The drug was considered stable in DBS if it was detected (following the positive identification criteria) in all samples, after being stored at room temperature or at -20°C for 24h, 14 days, 22 days and 37 days.

3.2.6. Authentic specimens

Authentic whole blood specimens previously analyzed and discarded by NMS Labs (Willow Grove, PA, USA) were gently donated to CFSRE for drug testing. 68 whole blood specimens (66 postmortem cases and 2 DUID cases) were collected from USA and Canada between March 2016 and October 2017 and re-tested using DBS method. Specimens had tested positive by GC-MS, LC-Triple Quadrupole-MS/MS or LC-QTOF-MS/MS, for at least one drug from the scope of the method developed in this work.

3.3. Development and validation of a screening method for synthetic cannabinoids in paper DBS using UPLC-Triple Quadrupole-MS/MS.

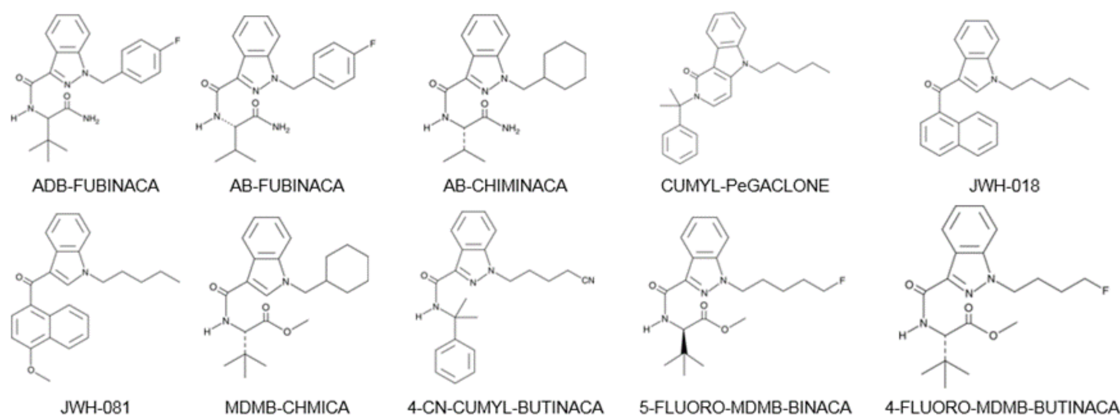
3.3.1. Reagents and materials

Methanol and acetonitrile were purchased from Honeywell (Morris Plains, NJ, USA). Formic Acid was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Whatman® 903 cards were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Human blank blood used in development and validation of the method was purchased from Bio IVT (Hicksville, NY, USA).

Reference materials of ADB-FUBINACA, AB-FUBINACA, AB-CHMINACA, CUMYL-PEGACLONE, JWH-081, JWH-018 and MDMB-CHMICA were acquired as 10 mM solutions in dimethylsulfoxide (DMSO), in the Synthetic Cannabinoid Screening Library from Cayman Chemical (Ann Arbor, MI, USA). 4-CN-CUMYL-BUTINACA

and 5F-MDMB-PINACA were purchased from Cayman Chemical (Ann Arbor, MI, USA), as 1 mg solids and dissolved in methanol, to a concentration of 1 mg/mL solution. 4F-MDMB-BINACA, AB-FUBINACA-d_4 and JWH-018-d_9 were purchased as 1 mg/mL solution in methanol from Cayman Chemical (Ann Arbor, MI, USA) (**Figure 14**).

Figure 14. Molecular structures of target synthetic cannabinoids



Source: Cayman Chemical Website

3.3.2. Standards solutions and controls

Standard working solutions of all synthetic cannabinoids were prepared by diluting the reference materials to the concentrations of 1, 0.1 and 0.01 µg/mL in methanol. ISTD working solution with AB-FUBINACA-d_4 and JWH-018-d_9 was made at 1 µg/mL in methanol. Working solutions were stocked in amber vials at -20ºC.

Positive controls were prepared at 2, 1 and 0.5 ng/mL. 0.5 mL of blank human blood were spiked with 10 µL of 0.1 µg/mL standards mix solution, to reach the final concentration of 2 ng/mL. Controls at 1 and 0.5 ng/mL were also prepared to a final volume of 0.5 mL of blood, by serial dilution. For recovery, matrix effects and stability assays, additional controls at 10 ng/mL (spiking 5 µL of 1 µg/mL solution in 0.5 mL of blood) and 4 ng/mL (spiking 20 µL of 0.1 µg/mL solution in 0.5 mL of blood).

3.3.3. DBS preparation and extraction

Spots of 1-cm diameter were pre-punched from Whatman® cards and 50 µL of whole blood spiked with synthetic cannabinoids standards was spotted. Blood on paper was kept at room temperature and protected from light, for drying over 2h. DBS were then analyzed or stored. Extraction of synthetic cannabinoids from DBS was performed by adding a spot into a clean, plastic microcentrifuge tube containing 1 mL of methanol

and 25 µL of AB-FUBINACA-d_4 and JWH-018-d_9 working solution. DBS samples were incubated for 10 min at 25°C, at under 1000 rpm agitation, using the Eppendorf® ThermoMixer F2.0. Extracted samples were collected to a clean glass tube and evaporated to dryness at 35ºC (Caliper TurboVap® LV). Dried extracts were redissolved in 200 µL of 60:40 MPA/MPB and centrifuged for 5 min under 10,000 rpm at 4ºC (Eppendorf Centrifuge 5415R). 190 µL were collected, added to a glass insert vial and 10 µL was injected into the UPLC-MS/MS system.

3.3.4. UPLC-Triple Quadrupole-MS/MS analysis

Analysis was performed using a Waters Acquity I-class UPLC® coupled to Waters Xevo TQ-S Micro (Milford, MA, USA). Column used for separation was an Agilent Poroshell EC C18 (3 mm × 150 mm × 2.7 µm) (Santa Clara, CA, USA), kept at 40ºC. The mobile phases were 0.1% formic acid in water (MPA) and 0.1% formic acid in methanol (MPB). The gradient is described in the **Table 5**, and was developed based on Ambroziak and Adamowicz (2018).

MS operated with positive electrospray ionization, in resolution mode. The capillary voltage and cone voltage were 3.0 kV and 40 V, respectively. Source temperature was 150ºC. Dessolvation temperature and gas flow were 500ºC and 1000 L/h, respectively. The instrument also operated under multiple reaction monitoring (MRM). The specific transitions and settings for each synthetic cannabinoid are listed in **Table 6**. The dwell time for all cannabinoids was set 10 ms. For the determination of MRM transitions, solutions of each synthetic cannabinoid at 1 µg/mL in 50:50 methanol/acetonitrile were prepared. These solutions were directly infused in the MS and the two most intense transitions for each compound were selected. For infusions, cone voltage range was 2 – 100 V and collision energy range was 2 – 80 eV. The lowest fragment mass monitored was 70 Da and excluded losses were 18 Da and 44 Da. All analyzes were performed using Waters MassLynx V. 4.1 software.

Table 5. Gradient conditions of chromatographic method

Time (min)	Flow rate (mL/min)	MPA (%)	MPB (%)
Initial	0.6	60	40
1.0	0.6	60	40
2.0	0.6	40	60
3.0	0.6	10	90
5.0	0.6	10	90
6.0	0.6	60	40

3.3.5. Method Validation

The validation of this method was carried out based on ASB guidelines (ASB, 2017). A fit-for-purpose approach was adopted evaluating LOD, recovery, matrix effects, interferences, carryover and stability.

LOD was tested by running DBS samples spiked with synthetic cannabinoids at concentrations of 2, 1 and 0.5 ng/mL, in triplicate, over three different runs. Criteria for a positive identification were ion ratio tolerance of 30%, retention time within ±0.25 min of target and signal-to-noise (S/N) higher than 3:1. The lowest concentration that the drug was consistently detected in all three replicates, following the criteria for positive identification, was accepted as the LOD.

Recovery and matrix effects were determined at two levels, 4 and 10 ng/mL, in triplicate. Three set of samples were used for recovery and matrix effects calculations: (1) standard solutions dried and reconstituted in 60:40 MPA/MPB; (2) extracted DBS samples, following the procedure described before and (3) extracted blank DBS samples, spiked with synthetic cannabinoids after extraction at 4 and 10 ng/mL. Both recovery and matrix effects were calculated as described in the section 3.2.5.

Table 6. MRM transitions and settings for selected synthetic cannabinoids

Compound	Precursor ion (m/z)	Product ions (m/z)	Cone (V)	Collision energy (eV)
4-CN-CUMYL-BUTINACA	361.1	226.2	38	20
		243.2	38	10
4F-MDMB-BINACA	364.1	145.1	38	40
		219.1	38	24
5F-EDMB-PINACA	392.1	233.1	38	24
		318.2	38	16
AB-CHMINACA	357.1	145.1	14	38
		241.2	14	24
AB-FUBINACA	369.1	253.1	4	22
		352.2	4	8
ADB-FUBINACA	383.1	109.0	40	48
		253.1	40	24
CUMYL-PEGACLONE	373.1	91.1	4	54
		255.2	4	10
MDMB-CHMICA	385.1	144.1	42	36
		240.1	42	14
JWH-018	342.1	127.0	80	64
		155.1	80	32
JWH-081	372.1	114.1	70	70
		185.1	70	22
AB-FUBINACA-d_4	373.1	91.1	36	78
		109.0	36	34
JWH-018-d_9	351.1	127.0	66	72
		155.0	66	40

Matrix interferences were assessed by preparing 10 blank DBS samples using different sources of blank blood and extracting them following the method described previously. Interferences from synthetic cannabinoids on ISTD responses were tested by extracting a high concentration DBS sample (100 ng/mL), without adding the ISTD. Interferences from ISTD, AB-FUBINACA-d_4 and JWH-018-d_9, on target synthetic

cannabinoids responses were analyzed by extracting blank DBS samples within the ISTD at 25 ng/mL, in triplicate. Interferences from substances commonly found in forensic routine were analyzed at a high concentration, 200 ng/mL. The compounds tested as potential interferents were: acetaminophen, alprazolam, amphetamine, caffeine, cannabidiol, citalopram, clonazepam, cocaine, codeine, Δ^9-THC, cotinine, diazepam, diphenhydramine, ephedrine, fentanyl, fluoxetine, lidocaine, lorazepam, MDMA, methadone, morphine, naloxone, nicotine, oxycodone, oxymorphone, pseudoephedrine, salicylic acid, sertraline, tramadol, zolpiclone and zolpidem.

Potential carryover effect was tested after analyzing a high concentration on synthetic cannabinoids DBS sample (100 ng/mL), by running mobile phase three times and investigating potential residual peaks.

Post-processing stability of synthetic cannabinoids was evaluated at two levels, 4 and 10 ng/mL. Samples were analyzed after extraction and again, 24h and 48h later, after kept in the auto sampler at 10ºC. Initial average peak areas for each synthetic cannabinoid were compared with average peak areas after 24h and after 48h. Long-term stability of synthetic cannabinoids at 10 ng/mL was studied in DBS samples stored at room temperature and frozen at -20°C, after 24h and 20 days (without desiccants). The experiments were conducted in triplicate. Synthetic cannabinoids were considered stable in DBS if they were still detected in all samples, according to the positive identification criteria.

3.4. Development and validation of a confirmatory and quantitative method for six synthetic cathinones in paper DBS using UPLC-Triple Quadrupole-MS/MS

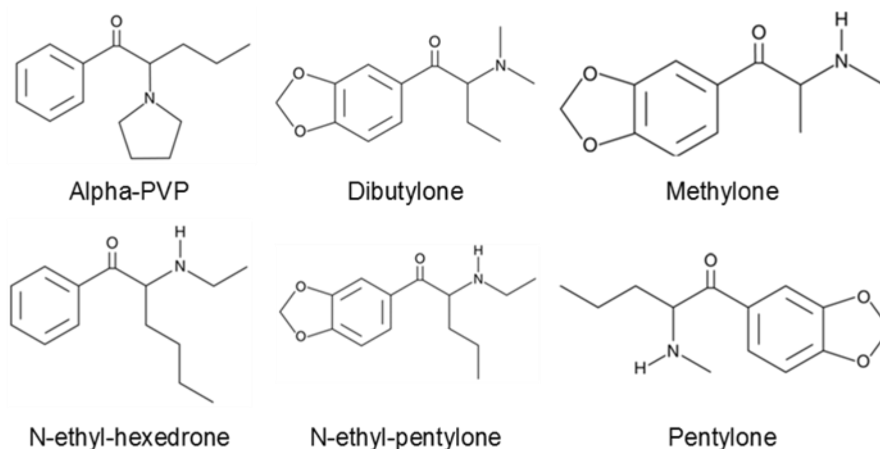
3.4.1. Reagents and materials

Whatman® 903 cards were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Hydrochloric acid was purchased from VWR Chemicals (Radnor, PA, USA). Methanol and acetonitrile were obtained from Honeywell (Morris Plains, NJ, USA). Formic Acid was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Human blank blood used for method development and validation was purchased from Bio IVT (Hicksville, NY, USA).

Reference materials of alpha-PVP, methylone, pentylone, alpha-PVP-d_8, butylone-d_3 and pentylone-d_3 were obtained as 1 mg/mL or 100 µg/mL solutions in

methanol from Cerilliant (Round Rock, TX, USA). Reference materials of dibutylone, N-ethyl-hexedrone and N-ethyl-pentylone were obtained from Cayman Chemical (Ann Arbor, MI, USA) as 1 mg powders and dissolved in methanol to the concentration of 1 mg/mL (**Figure 15**).

Figure 15. Chemical structures of target synthetic cathinones



Source: Cayman Chemical Website

3.4.2. Standard solutions, controls and calibrators

Working solutions of all synthetic cathinones were prepared from reference materials to the concentrations of 10, 1 and 0.1 µg/mL, in methanol. Working solution of alpha-PVP-d_8, butylone-d_3 and pentylone-d_3 was made at 1 µg/mL in methanol. All solutions were prepared in amber vials and kept at -20ºC.

Calibrators were prepared at 500, 200, 100, 50, 25 and 10 ng/mL in blank human blood. 500 ng/mL calibrator was prepared by adding 25 µL of 10 µg/mL standards mix solution into 0.5 mL of blank blood and serial diluted to make the other calibrators. Quality controls (QC) were made at 400 ng/mL (high QC, HQC) and 20 ng/mL (low QC, LQC). HQC was prepared by adding 20 µL of 10 µg/mL standard solution into 0.5 mL of blood and LQC was prepared by dilution of HQC to a final volume of 0.5 mL of blood. Calibrators and QC were prepared by different analysts daily.

3.4.3. DBS preparation and extraction

50 µL of whole blood was applied to 1-cm diameter pre-punched spots from Whatman® cards. Whole blood was previously spiked with target cathinones at concentration of interest. DBS were allowed to dry for 2 h at room temperature and protected from light.

Synthetic cathinones were extracted from DBS in microcentrifuge plastic tubes with 1 mL of methanol and 25 µL of ISTD solution. Samples were incubated under agitation (1000 rpm) at 25ºC, for 10 min (Eppendorf ThermoMixer F2.0), followed by centrifugation at 12000 rpm and 4ºC, for 2 min (Eppendorf Centrifuge 5415R). Extracted samples were transferred to a glass tube with 100 µL of 10% HCl in methanol and evaporated to dryness at 35ºC (Caliper TurboVap® LV). Reconsitution was performed using 200 µL of 90:10 MPA/MPB and an additional centrifugation under 12,000 rpm at 4ºC, for 2 min, was performed. 150 µL were collected, inserted into a glass vials and 10 µL was injected into the UPLC-MS/MS system.

3.4.4. UPLC-Triple Quadrupole-MS/MS analysis

Analysis was performed using a Waters Acquity I-class UPLC® coupled to Waters Xevo TQ-S Micro (Milford, MA, USA). Chromatographic separations was carried out using an Agilent Poroshell EC C18 (3 mm × 150 mm × 2.7 µm) (Santa Clara, CA, USA). The temperature of the column was set at 40ºC. Mobile phases used were 0.1% formic acid in water (MPA) and 0.1% formic acid in acetonitrile (MPB). The gradient is described in the **Table 8**. Run time was 4.3 min.

Table 7. Gradient conditions of chromatographic method

Time (min)	Flow rate (mL/min)	MPA (%)	MPB (%)
Initial	0.3	90	10
0.5	0.3	70	30
1.5	0.3	60	40
4.2	0.3	60	40
4.3	0.3	90	10

MS operated in positive electrospray ionization mode, under MRM. Capillary voltage and cone voltage were 3.0 kV and 40 V, respectively. The temperature of source was 150ºC. Dessolvation temperature was 500ºC and the gas flow was set at 1,000 L/h. Transitions and parameters of cone voltage and collision energy for each cathinone are listed in **Table 9**. The dwell time for all compounds was 9 ms. For the determination of MRM transitions, individual solutions of each synthetic cathinone at 1 µg/mL in 50:50 methanol/acetonitrile were prepared. These solutions were infused into the MS and the

two most intense transitions for each compound were selected. For infusions, the cone voltage and collision energy ramps were 2 – 100 V and 2 – 80 eV, respectively. The lowest fragment mass and excluded losses were 70 Da and 18 Da and 44 Da, respectively. Waters MassLynx V. 4.1 software was used for data analysis and processing.

Table 8. MRM transitions and settings for selected synthetic cannabinoids

Compound	Precursor ion (m/z)	Product ions (m/z)	Cone (V)	Collision energy (eV)
Alpha-PVP	232.1	77.0	60	46
		91.0	60	22
Methylone	208.1	132.0	26	28
		160.1	26	16
Dibutylone	236.1	86.0	48	22
		161.0	48	18
N-ethyl-hexedrone	220.1	91.0	22	50
		118.0	20	50
N-ethyl-pentylone	250.1	174.0	38	12
		202.2	46	36
Pentylone	236.1	175.1	28	20
		188.2	28	18
Alpha-PVP-d$_8$	240.1	90.8	32	24
		104.9	32	28
Butylone-d$_3$	225.1	75.0	50	22
		177.3	50	28
Pentylone-d$_3$	239.1	89.0	32	24
		191.2	32	28

3.4.5. Method Validation

The validation of this method was carried out based on ASB guidelines (ASB, 2017). Calibration model, lower limit of quantitation (LLOQ), accuracy and precision, LOD, recovery, matrix effects, interferences, carryover and stability were assessed.

LOD was investigated using three different sources of whole blood spiked on card and extracted in duplicate, over three days, at concentrations of 1 or 5 ng/mL. Criteria for

a positive identification were ion ratio tolerance of 30%, retention time within ±0.25 min of target and S/N higher than 3:1. LLOQ was calculated in a similar approach, in duplicate and over three days, using three different sources of whole blood spiked at 10 ng/mL and spotted on card. LLOQ was calculated against a calibration curve prepared at the same day. Calibration model was evaluated using a 6-point calibration curve, with quadratic fitting, over five different days. Calibrators were prepared at concentrations of 10, 25, 50, 100, 200 and 500 ng/mL. Accuracy and precision were tested at three levels, LLOQ, low quality control (LQC) of 20 ng/mL and high quality control (HQC) of 400 ng/mL, in triplicate, over five days. Acceptable imprecision, expressed as coefficient of variation (CV), were within ± 20%. Acceptable bias were within 20% of nominal concentration.

As described for the methods in previous sections, matrix interferences were analyzed using whole blood samples (N = 10) spotted on card and extracted as described before, searching for potential peaks at the same retention time of those presented by target compounds. Analysis of interferences from target cathinones on ISTD responses were performed running an extracted DBS sample at 400 ng/mL, with no ISTD added. Analysis of interferences from ISTD were searched by running blank DBS samples, extracted within ISTD at 25 ng/mL. These analyses were conducted in triplicate.

The following compounds were analyzed for interferences on target cathinones responses: acetaminophen, alprazolam, amphetamine, caffeine, cannabidiol, citalopram, clonazepam, cocaine, codeine, Δ^9-THC, cotinine, diazepam, diphenhydramine, ephedrine, fentanyl, fluoxetine, lidocaine, lorazepam, MDMA, methadone, morphine, naloxone, nicotine, oxycodone, oxymorphone, pseudoephedrine, salicylic acid, sertraline, tramadol, zolpiclone and zolpidem. The analysis was performed in triplicate.

Analysis of recovery and matrix effects were performed using LQC and HQC, in triplicate. The assays were conducted as described in Section 3.2.5. Carryover effects were evaluated along with calibration curve, running mobile phase sample after running the highest calibrator (500 ng/mL), over five days.

As all target cathinones in the scope of this confirmatory/quantitative method were previously studied regarding post-processing and long-term stability during the screening method development, here, post-processing and 24h-stability studies were performed. Samples were re-analyzed after 24h and 4 days after first analysis. During this time, samples were kept in the auto sampler at 10ºC. A comparison between average peak areas at first analysis and 24h and 4 days later was performed. For 24h-stability, a comparison

between average peak areas of freshly prepared DBS samples and DBS samples prepared 24h earlier was performed.

3.4.6. Authentic Specimens

Whole blood specimens from cases of intoxication with cathinones previously analyzed and discarded by NMS Labs (Willow Grove, PA, USA) were gently donated to CFSRE for drug testing. The specimens were collected in 2016 and 2017 in United States (Florida, Vermont, New York, Pennsylvania and Lousiana) and re-analyzed through DBS in 2019. Some of these samples were already screened positive for cathinones using the screening method described in the section 3.2.

4. RESULTS AND DISCUSSION

4.1. Development of a novel salt-supported DBS approach for screening of cocaine, MDMA and methamphetamine using GC-MS

4.1.1. Ultrasound derivatization method development and optimization

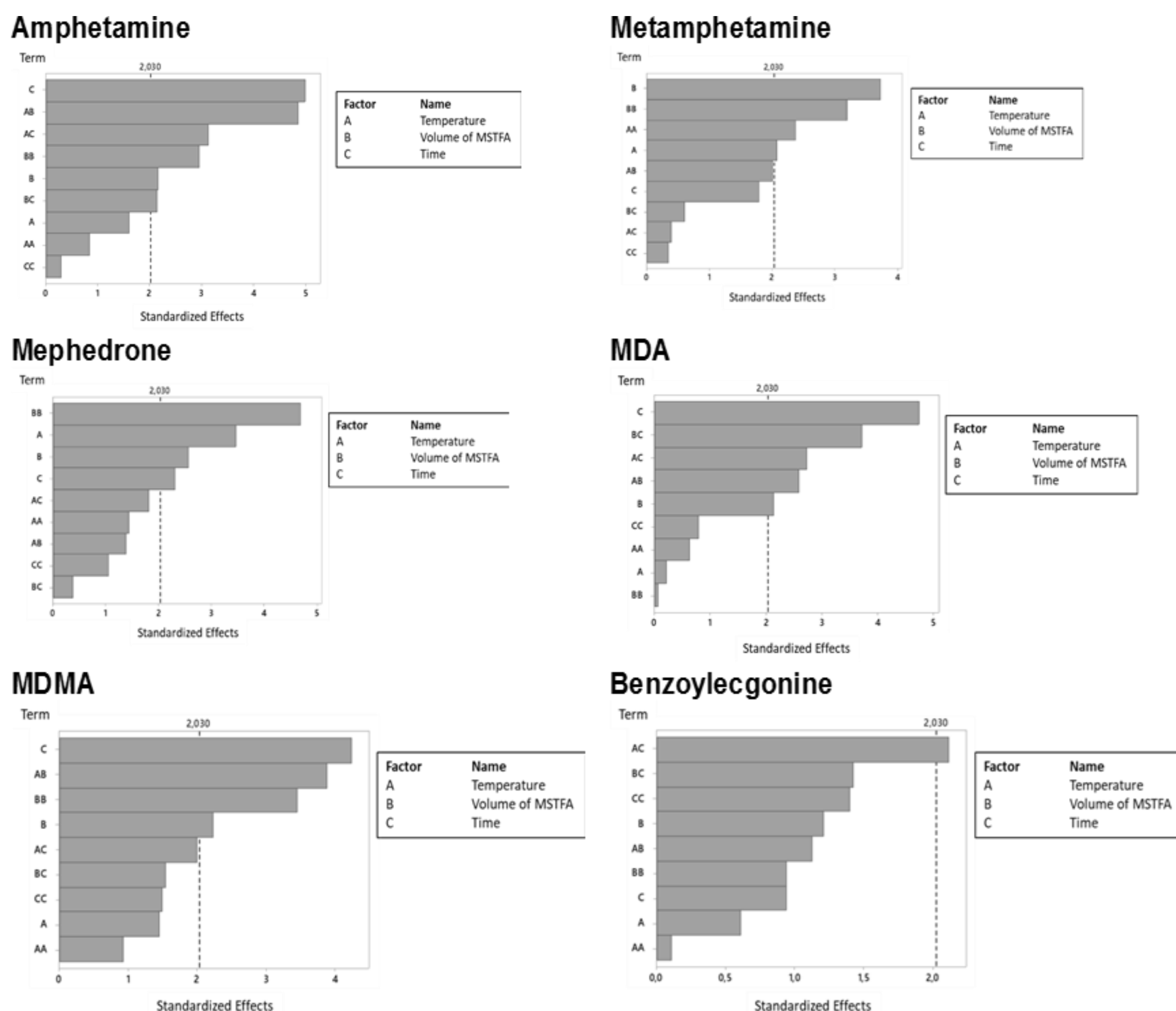
The development of a novel approach for silylation of target drugs using ultrasound was developed and optimized. The values selected for each variable (temperature, time and volume of MSTFA) were defined according to previous pilot studies. The values were in line with analytical and experimental aspects in order to: (I) adopt soft temperature conditions, to reduce energy consumption and to prevent analyte degradation, (II) reduce the total time of analysis, (III) minimize reagent use and generation of waste and (IV) develop an easy implementation experimental protocol.

The first analysis that was conducted was to detect which effects present greater statistical significance for the model, among the main effects, second-order effects and the interactions effects, using Pareto charts. In this graph, the most important effects and interactions are presented at the top of the graph, with a longer bar (NIST, 2019). Statistical significance is identified according to the effects whose bar on the graph exceeds a vertical line (NIST, 2019).

Figure 16 shows Pareto charts for all analytes, at a significance level of 0.05. The most significant first-order effects for each analyte were as follows: time of reaction and volume of MSTFA, for amphetamine, MDA and MDMA; volume of MSTFA and temperature, for methamphetamine; time of reaction, volume of MSTFA and

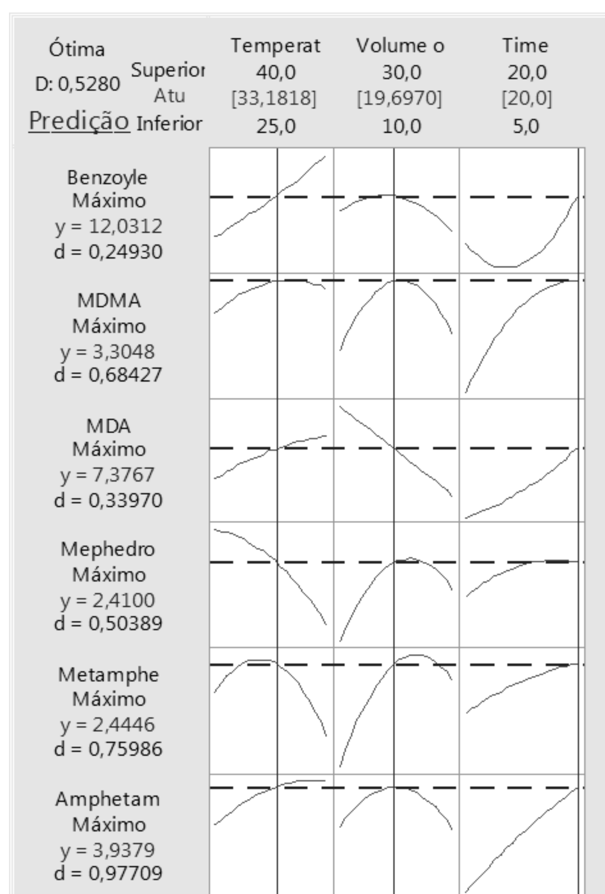
temperature, for mephedrone. For benzoylecgonine, none of the main effects was found to be statistically significant. The quadratic terms of each variable can also indicate some important model information, such as function curvature (BRERETON, 2003). In our results, the second-order effects of volume of MSTFA were relevant in amphetamine, methamphetamine, mephedrone and MDMA responses. Second-order effects of temperature were found to be statistically significant only in methamphetamine responses. A more detailed analysis of the interactions between the effects of the variables made it possible to obtain important information. In general, combined-dependent effects of two factors affect the responses (BRERETON, 2003). The interactions that were statistically significant are as follows: between temperature and time for amphetamine, MDA and benzoylecgonine; between temperature and volume for amphetamine, MDA and MDMA; and between time and volume for amphetamine and MDA.

Figure 16. Pareto Charts of main standardized effects



The final step of the analysis was the determination of the optimal reaction conditions, using Response Surface Methodology and desirability function method. The desirability profiles were plotted (**Figure 17**). The values of desirability of each response are defined in the range between 0.0 and 1.0: a function equal to 0.0 means an undesirable response, a function equal to 0.5 means intermediary response and a function of 1.0 is very desirable (DERRINGER, SUICH, 1980; CANDIOTI et al., 2014). The overall desirability (D) was equal to 0.5280. The values of desirability were: 0.24930 for benzoylecgonine; 0.68427 for MDMA; 0.33970 for MDA; 0.50389 for mephedrone; 0.75986 for methamphetamine and 0.97709 for amphetamine.

Figure 17. Desirability profiles for chromatographic responses of all analytes.



The increase in the ultrasonic bath temperature increases the response of benzoylecgonine, MDMA, MDA and amphetamine. On other hand, increasing temperature exerted a negative effect on chromatographic responses for mephedrone and methamphetamine. More specifically for methamphetamine responses, a hyperbolic curve was found for temperature increase effects. Based on desirability criteria, the effects of increasing the volume of MSTFA were negative for all analytes. The chromatographic

response of MDA was inversely proportional to volume of MSTFA increases. For all other analytes, a desirability hyperbolic curve was resulted from increasing volume of MSTFA. The time of reaction exerted a positive effect in all analytes responses: as longer the reaction time was, better analytical responses were reached. According to the observations, the selected optimum values were 30°C for temperature, 20 µL of MSTFA and 20 minutes of reaction.

Using the optimized method for ultrasound-assisted silylation of the compounds, a comparison with conventional protocol of derivatization by heating was performed. For some analytes as methamphetamine and mephedrone, the mean response was lower for ultrasound-assisted derivatization; for MDA, MDMA and benzoylecgonine, an increased response was found by ultrasonic method. However, according to one-way ANOVA, no statistically significant difference was found between the chromatographic responses by ultrasonic assisted derivatization and those by derivatization by heating, with a confidence level of 95% and a p-value of 0.05 (**Table 10**). Although the ultrasound-assisted method results in higher chromatographic responses without statistical significance in relation to heating derivatization method, the proposed method is at least equivalent to the conventional method by heating.

Table 9. One-way ANOVA for multiple comparisons between the derivatization methods.

	Mean response for thermal derivatization (± SD)	Mean response for ultrasound-assisted derivatization (± SD)	F	p-value
Amphetamine	0.16405 ± 0.01359	0.1915 ± 0.0446	1.38	0.285
Methamphetamine	0.2798 ± 0.1047	0.957 ± 1.364	0.98	0.360
Mephedrone	0.04843 ± 0.00710	1.03 ± 2.02	0.96	0.366
MDA	0.01199 ± 0.00446	0.219 ± 0.423	0.96	0.366
MDMA	0.1099 ± 0.0230	0.1326 ± 0.0276	1.60	0.253
Benzoylecgonine	0.04813 ± 0.00346	0.0670 ± 0.0429	0.77	0.414

4.1.2. Salt-supported DBS method development

The salt-supported DBS consist in mixing a salt with blood followed by compressing the mixture, leading to a tablet of salt and blood. This is a variation of conventional DBS, using the same concept of spotting blood onto a solid support.

At first, solubility tests were performed for salt-supported DBS. 20 µL of blood were mixed with 200 mg of sodium carbonate and compressed to make the salt-supported DBS. Solubility was evaluated in 2 mL of water or carbonate buffer (pH 9) for 5, 10, 15 and 20 min using agitation at 200 rpm. Best results were found using water to dissolve the material blood + salt.

Different salts were tested for salt-DBS preparation. Initially, sodium carbonate and sodium sulfate were tested without blood. During the processing, a higher loss of solid is observed for sodium carbonate but tablets with sodium sulfate were not resistant enough, disintegrating immediately after being produced. Other salt, NaCl, was not resistant to make DBS. Sodium carbonate (Na_2CO_3) was found to be the best salt for the method. Other salts were tested with and without blood but they were found to be mechanically unstable. **Figure 18** shows the results of preparing DBS with some specific salts.

Figure 18. Comparison between tablets made of sodium sulfate (left), sodium carbonate (right) and sodium chloride (down) showing the mechanical instability of sodium sulfate and NaCl tablets.

Different masses of Na_2CO_3 as well as the proportion of salt and blood were tested. Initially, 200 mg of sodium carbonate and 20 to 30 µL were used. Extraction methods were tested, using liquid-liquid extraction approaches. Ethyl acetate, 1-chlorobutane/ethyl acetate 70:30 (v/v) (TEMPORAL et al., 2014) and ethyl ether / isopropanol 9:1 (v/v) (FARINA; YONAMINE; SILVA, 2002) were tested as solvent extractors. Another method tested was the use of a desiccant agent Na_2SO_4/NaCl 2:1 (m/m) added to the extracting solvent (MATSUTA, 2013). The use of activated carbon was also tested to promote a clean-up of the sample at 10 and 100 mg mass. However, the GC-MS did not present a good sensitivity using only 20-30 µL of blood.

Based on the first results, the amount of blood to make a salt-supported DBS was then increased, in order to increase the sensitivity of the instrument; amount of salt was also increased, as more salt is needed to load an increased volume of blood. The volume of blood was increased to 80 μL of blood and the amount of sodium carbonate was increased to 400 mg. Based on previous results, a LLE with ethyl acetate and SPE procedure were tested. The salt-supported DBS were added to 3 mL of carbonate buffer (pH 9) to perform SPE. However, results with SPE were not good and using LLE the response of analytes was still low. Other tests were conducted in order to optimize the sensitivity of the method, by increasing the amount of sample. Finally, the best combination was found for 400 mg of sodium carbonate and 130 μL of blood. Variations of salt-supported DBS were also tested as the blood inside and on top of the tablet and the tablet immersed in liquid blood. However, based on the purpose of DBS, the procedure of mixing blood and salt was adopted. Examples of salt-DBS are presented in **Figure 19** and **Figure 20**.

Figure 19. An example of salt-supported DBS with 400 mg of sodium carbonate containing blood inside the tablet.

Figure 20. An example of an alternative salt-supported DBS containing blood spotted onto the sodium carbonate tablet.

Based in all previous findings, ethyl acetate was the best option regarding the lowest number of interferents, and for this reason, this solvent was used for extraction of drugs from salt-supported DBS prepared by 400 mg of salt and 130 μL of blood.

Extraction of the analytes from the salt-supported DBS was performed by mixing the tablets with deionized water and ethyl acetate, followed by vortexing, agitation and centrifugation for 5 min at 2000 rpm. The supernatant containing the organic phase was collected and the solvent was evaporated under a stream of compressed air at 45ºC. The dried extract was reconstituted in 70 µL of ethyl acetate and 50 µL were destined for ultrasonic assisted derivatization with MSTFA described earlier. However, the extraction works better for cocaine, methamphetamine and MDMA and, for this reason, the other compounds were not included in the scope of the method (mephedrone, MDA and benzoylecgonine).

The optimization of the method described above was performed using the Box-Behnken method, with 4 factors at 3 levels each (low, medium and high), with 3 central points. Different volumes of water and ethyl acetate, time for vortex homogenization and for extraction, and the addition of Na_2SO_4/NaCl to the mixture were tested. The method parameters and the evaluated levels are described in **Table 10**. The analyzed responses were the relative chromatographic response of each of the analytes (cocaine, methamphetamine, and MDMA).

Table 10. Extraction Method Parameters for Optimization

Parameter	Levels
Volume of deionized H_2O and ethyl acetate	0.5 mL, 0.75 mL and 1.0 mL
Homogenization (vortex) time	30, 90 and 120 s
Extraction time	5, 15 and 25 min
Salt addition (Na_2SO_4/NaCl, 2:1, m/m)	0, 150 and 300 mg

According to one-way ANOVA and Response Surface Modelling, the optimal conditions for extraction were as follows: addition of 1 mL of water and 1 mL of ethyl acetate; homogenization with vortex for 90 s; extraction over 15 min; and no addition of desiccant agents.

No interferences from blank salt-supported DBS or from ISTD were observed for any of the target compounds.

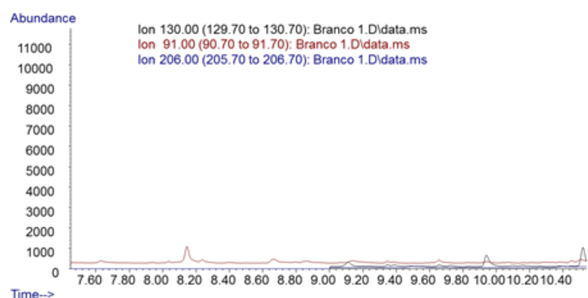
Different concentrations of target drugs of abuse in salt-supported DBS were tested using the extraction and GC-MS methods described earlier. Initially, concentrations of 20, 120 and 200 ng/mL for target compounds were tested. However, the method was unable to provide good extraction and good chromatography of the drugs.

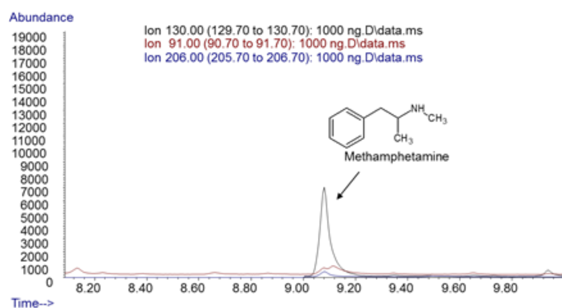
For this reason, a higher range was investigated, at 1 µg/mL and 2 µg/mL, as some drugs can be found in forensic casework at these concentrations. **Figure 20** shows SIM chromatograms for each drug (cocaine, MDMA and methamphetamine).

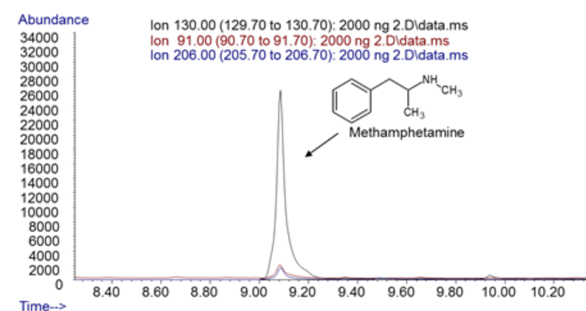
Figure 21. SIM chromatograms of: **(A)** blank salt-supported DBS sample; **(B)** salt-supported DBS sample at 1 µg/mL and **(C)** salt-supported DBS sample at 2 µg/mL

A **B** **C**

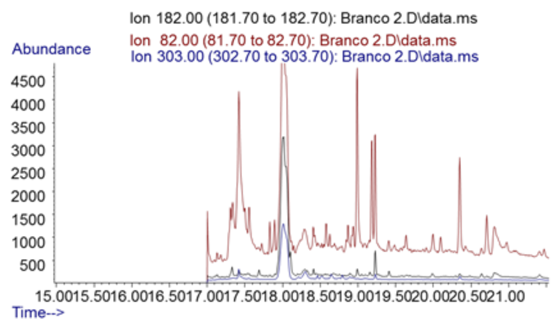
Methamphetamine

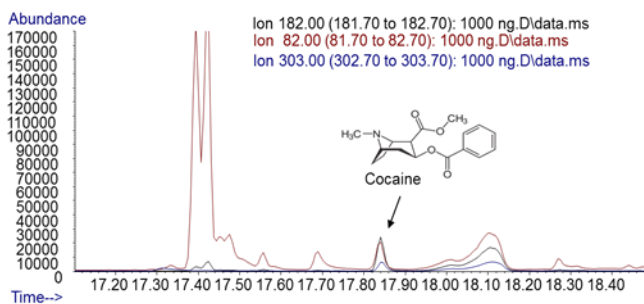






Cocaine





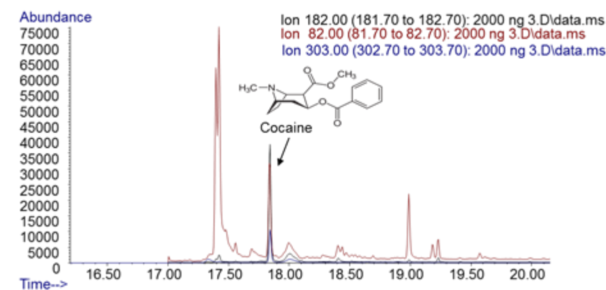
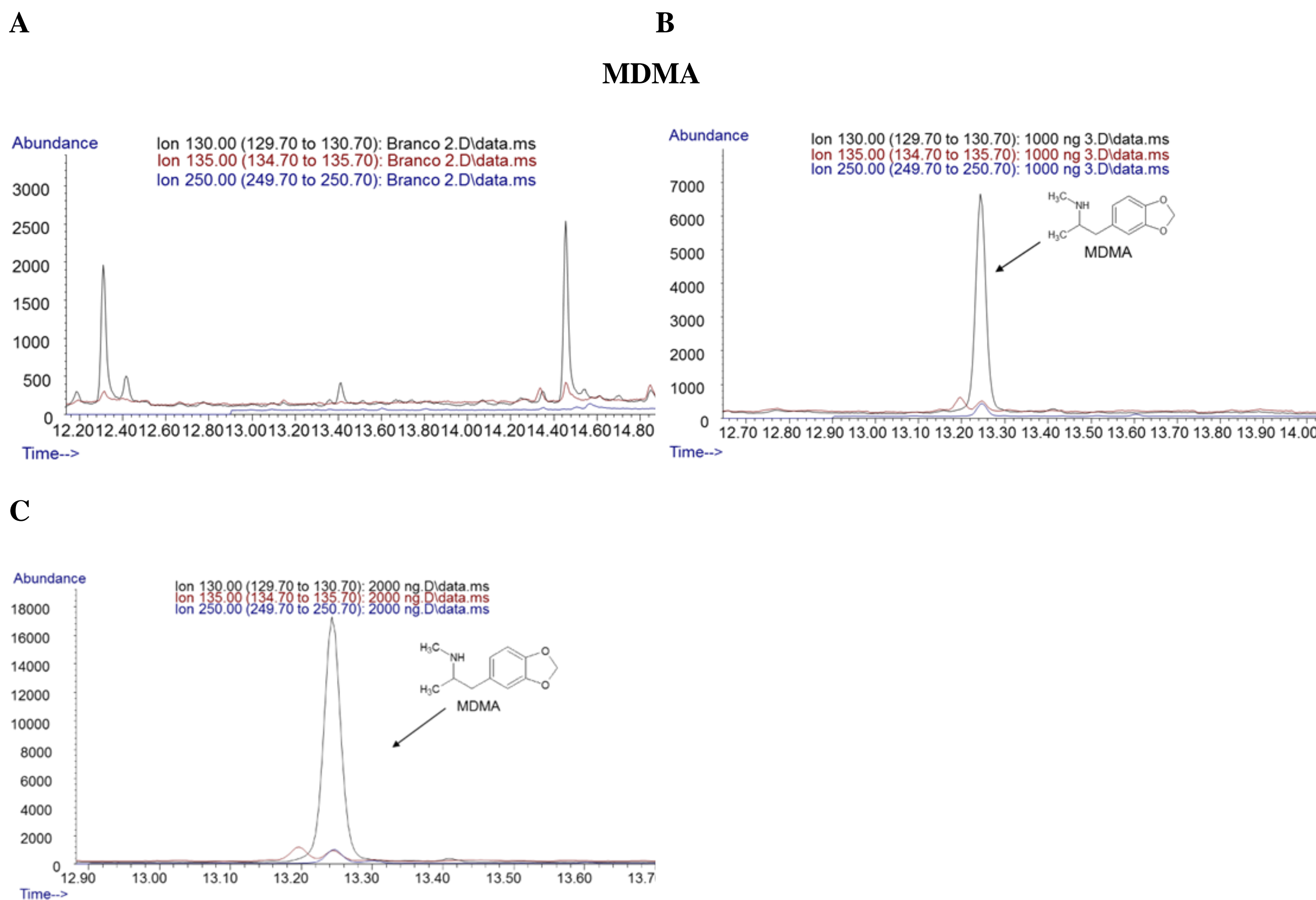


Figure 21. SIM chromatograms of: (**A**) blank salt-supported DBS sample; (**B**) salt-supported DBS sample at 1 µg/mL and (**C**) salt-supported DBS sample at 2 µg/mL (conclusion)



The method developed in this work is the first report of a salt DBS-GC-MS method for cocaine, methamphetamine and MDMA, to best of our knowledge. However, Single Quadrupole GC-MS has provided good detectability/chromatography only at concentrations of 1 µg/mL and 2 µg/mL, after using a one-step liquid-liquid extraction. Although the range of concentrations of these compounds in forensic casework is broad, including concentrations higher than 1 µg/mL, reduced concentrations (below 1000 ng/mL) are also commonly reported.

Ultimately, the method did not display clear advantages over the more traditional paper DBS approach, based on ease of preparation, sensitivity and recovery/signal strength. It is very important to consider and to assure the reproducibility of this process. As an alternative method, the salt-supported DBS provides an alternative approach for DBS by using a salt tablet as support for the blood, instead of paper. By using this, the paper is removed from the analytical process and the solid can be dissolved into the solution, minimizing the residues. In addition, more blood can be spotted on the solid (130 µL), which is beneficial for analysis, as more sample is available for extraction.

The salt-supported DBS has a major limitation, which is still regarding the sensitivity of the analytical technique. Here, salt-supported DBS approach enabled the loading of more blood onto the solid for preparing the DBS, which was thought to be useful for low sensitivity techniques, such as GC-MS. However, even reaching a higher capacity for blood spotting, the use of high sensitivity techniques is required to detect trace concentrations in salt-supported DBS and techniques as GC-MS are not always applicable, only at elevated concentrations (> 1 µg/mL), as demonstrated here.

The sensitivity limitation is not only observed for salt-supported DBS but it is also a limitation exhibited by paper-based DBS. The use of DBS in the GC-MS analysis of drugs of abuse is very limited and applicable only for high concentrations drugs. For example, in the literature, the use of conventional DBS (with paper) for GC-MS analysis of drugs has been reported only for GHB, a drug found at elevated concentrations, especially in DFC cases. Ingels, Lambert and Stove (2010) developed a method for detection of GHB in DBS using an "on spot" derivatization and GC-MS, reporting a LOD of 1 µg/mL and a calibration range of 2 to 100 µg/mL. Sadones et al. (2016) used a microwave derivatization for GC-MS analysis of GHB, reporting a calibration range of 10 - 100 µg/mL. For cocaine, methamphetamine and MDMA, DBS-GC-MS methods in the literature have not been reported yet.

Another important aspect of discussion is the instrumental sensitivity limitation of gas chromatograph-mass spectrometers. In general, even for liquid samples, as whole blood, GC-MS analysis requires a high volume of sample. In the literature, several methods for GC-MS analysis of drugs in blood have been published, reaching low LOD but only using volumes of blood much higher than 130 µL (used in the salt-supported DBS method). Pelição et al. (2014) developed a GC-MS method for cocaine, amphetamines and cannabinoids in 1-mL of whole blood and obtained LOD of 5 or 10 ng/mL. Margalho et al. (2016) described a method using 250 µL of blood for analysis of cathinones and 2C-compounds, reaching LOD of 5 ng/mL. Ferrari Junior and Caldas (2018) measured drugs and pesticides in 1 mL of blood using dispersive solid phase extraction and large volume injection-programmed temperature vaporization GC-MS, obtaining LOQ of 20 or 30 ng/mL. Mercieca et al. (2018) described DLLME method using 2 mL of blood for GC-MS analysis of stimulants, reaching LOD of 1 to 10 ng/mL. Nisbet et al. (2019) developed a GC-MS method for some cathinones and NBOMes, reaching LOD between 0.3 and 1 ng/mL but using 1 mL of blood and SPE.

For this reason, the use of salt-supported DBS in GC-Single Quadrupole-MS methods are limited; however, the combination of salt-supported DBS with more sensitive techniques such as Triple Quadrupole or QTOF UPLC-MS/MS would be interesting and further research is needed.

4.2. Development and validation of a screening method for 36 psychoactive substances in paper DBS using UPLC-QTOF-MS/MS

4.2.1. Method development

Initially, a full screening method was designed for a Triple Quadrupole UPLC-MS/MS. The platform presented a good sensitivity for all drugs. Different extractions were performed using methanol, 0.1% formic acid in methanol and methanol/acetonitrile, with or without sonication and agitation. However, even exploring different approaches, the reproducibility for all drugs was not acceptable. The ion ratio criteria for all compounds was not reproducible over different runs. For this reason, in order to have a sensible and more reproducible method for a large range of different compounds, the UPLC-QTOF-MS/MS was selected.

Using UPLC-QTOF, a preliminary test using one, two or three spots per sample was performed. A one-step extraction with methanol and borate buffer (pH 10.4) was

adopted due to less complexity of the procedure. The best results were achieved extracting two or three spots, reaching lower LOD. In order to have a less complex sample preparation process, two spots were used for extraction. The whole spots were used for extraction, to avoid variations due to hematocrit content between different samples.

4.2.2. Method validation

The qualitative method produced acceptable results for all validation parameters assessed. The DBS-based method presented good sensitivity for all compounds, with LOD ranging from 0.5 ng/mL to 20 ng/mL. Negative controls (blank DBS and blank paper) produced no false negative results. **Table 12** summarizes the LOD for each compound as well as a concentration range consistent with that reported in fatal intoxications, showing the method's ability to detect the compounds at appropriate concentrations.

The method showed good specificity without interference from blood components or paper (**Figures 21 and 22**). The ISTD were also appropriate producing no interferences in the detection of target compounds. In a similar way, the target compounds were not a source of interference for ISTD responses. Chemicals that may appear in forensic casework such as medical drugs and pesticides were tested as interferents but produced no interference with target drugs detection. The only limitation of this method is regarding the differentiation of isomers, with this method not being able to discriminate the following isomers: 3-MeO-PCP and 4-MeO-PCP; butyryl fentanyl and *m*- or *p*-methyl-fentanyl; FiBF and *o*-,*m*- or *p*-fluorobutyrylfentanyl; U-48800 and U-51754.

No carryover effects were found at concentration of 500 ng/mL.

Table 11. LOD of the DBS method and review of reported range of postmortem blood concentrations in the literature.

	LOD (ng/mL)	Reported postmortem blood concentrations in the literature
25B-NBOMe	2	66.5 and 661 ng/mL (WIERGOWSKI et al., 2017)
25C-NBOMe	0.5	0.7 ng/mL (KUEPPERS AND COOKE, 2015) 2.8 ng/mL (peripheral blood) and 1.43 ng/mL (central blood) (MORINI et al., 2017) 1.69, 2.37 and 4.04 ng/mL (NISBET et al., 2019)
25I-NBOMe	0.5	0.405 ng/mL (peripheral blood) and 0.410 ng/mL (heart blood) (POKLIS et al., 2014) 28 ng/mL (KUEPPERS AND COOKE, 2015) 1.32 and 1.77 ng/mL (NISBET et al., 2019)
3-MeO-PCP	20	139 ng/mL (BAKOTA et al., 2016) 0.63 ng/mL (peripheral blood) and 3.2 mg/L (central blood) (MITCHELL-MATA et al., 2017) 63 ng/mL (KINTZ, et al., 2019) 152 ng/mL (DE JONG; OLYSLAGER; DUIJST, 2019)
4-fluoroamphetamine	20	n.a.
Acetylfentanyl	0.5	0.14 - 0.16 ng/mL (SOFALVI et al., 2017) 17 - 945 ng/mL (FINKELSTEIN et al., 2019) 0.1 - 5.3 ng/mL (AVEDSCHMIDT et al., 2019)
Acrylfentanyl	0.5	0.1 – 29 ng/mL (MOODY et al., 2018)
Alpha-PVP	1	100, 500 and 290 ng/mL (RICHARDS-WAUGH et al., 2013)

Table 11. LOD of the DBS method and review of reported range of postmortem blood concentrations in the literature (continuing)

	LOD (ng/mL)	Reported postmortem blood concentrations in the literature
Alprazolam	20	13 – 1200 ng/mL (WOLF et al., 2005) 20 – 1600 ng/mL (JONES AND HOLMGREN, 2013) 5 – 2100 ng/mL (DARKE; TOROK; DUFLOU, 2014)
Butylone	5	<10 – 600 ng/mL (KROTULSKI et al., 2018a)
Butyrylfentanyl	2	0.10 – 760 ng/mL (MOODY et al., 2018)
Carfentanil	0.5	0.11 – 0.88 ng/mL (SOFALVI et al., 2017) <0.2 – 0.73 ng/mL (KAHL et al., 2018) 0.1–120 ng/mL (MOODY et al., 2018) 0.03 – 1.66 ng/mL (RAB; FLANAGAN; HUDSON, 2019)
Cocaine	0.5	10 – 3000 ng/mL (PILGRIM; WOODFORD; DRUMMER, 2013)
Diazepam	2	n.a.
Dibutylone	5	10 – 1400 ng/mL (KROTULSKI et al., 2018a)
Etizolam	5	19 – 17000 ng/mL (HØISETH; TUV; KARINEN, 2016)
Fentanyl	5	1.1 – 15 ng/mL (SOFALVI et al., 2017) 0.21 - 107 ng/mL (RAB; FLANAGAN; HUDSON, 2019)
FiBF	0.5	30.6 - 91.7 ng/mL (KAHL et al., 2018) 0.10 – 760 ng/mL (MOODY et al., 2018)

Table 11. LOD of the DBS method and review of reported range of postmortem blood concentrations in the literature (continuing)

	LOD (ng/mL)	Reported postmortem blood concentrations in the literature
Flubromazolam	20	0.48 – 100 ng/mL (HØISETH; TUV; KARINEN, 2016)
Furanylfentanyl	0.5	2.0 – 42.9 ng/mL (PAPSUN et al., 2017) 1.9 ng/mL (peripheral blood) and 2.8 ng/mL (cardiac blood) (MARTUCCI et al., 2018)
Heroin	20	**
Ketamine	5	6900 ng/mL (heart blood) and 1800 ng/mL (peripheral blood); 1600 ng/mL (heart blood) and 600 ng/mL (peripheral blood) (LALONDE AND WALLAGE, 2004) 1300 ng/mL (DINIS-OLIVEIRA et al., 2010)
LSD	20	3.2 ng/mL (FAVRETTO et al., 2007)
MDMA	5	100 – 3700 ng/mL (GILL et al., 2002) 470 – 28390 ng/mL (ELLIOT, 2005) 410 – 84000 ng/mL (VERSCHRAAGEN et al., 2007)
Mephedrone	5	51 – 22000 ng/mL (PAPASEIT et al., 2017)
Methamphetamine	20	> 60000 ng/mL (LOGAN; WEISS; HARRUFF, 1996) 20 – 22000 ng/mL (methamphetamine toxicity only) (DARKE et al., 2017)
Methoxyacetylfentanyl	0.5	0.21 – 39.9 ng/mL (FOGARTY; PAPSUN; LOGAN, 2018)
Methylone	5	3130 ng/mL (peripheral blood) and 6640 ng/mL (central blood) (BARRIOS et al., 2016) 80, 110 and 10600 ng/mL (NISBET et al., 2019)

Table 11. LOD of the DBS method and review of reported range of postmortem blood concentrations in the literature (conclusion)

	LOD (ng/mL)	**Reported postmortem blood concentrations in the literature**
Mitragynine	20	20 - 1060 ng/mL (WANG AND WALKER, 2018)
N-ethyl-hexedrone	0.5	285 ng/mL (KOVÁCS et al., 2019)
N-ethyl-pentylone	0.5	12 – 1200 ng/mL and one case at 50000 ng/mL (KROTULSKI et al., 2018c)
Pentylone	1	200 ng/mL and 96 ng/mL (KROTULSKI et al., 2018a)
PCP	20	10 – 900 ng/mL (BUDD; LIU, 1982) <1 ng/mL to 598 ng/mL (DEROUX; SGARLATO; MARKER, 2011)
U-47700	2	189 and 547 ng/mL (PAPSUN et al., 2017) 0.2 – 3800 ng/mL (MOODY et al., 2018)
U-48800	1	n. a.
Valerylfentanyl	0.5	0.44 ng/mL (MOODY et al., 2018)

n.a.: non-available in the literature at the time of this study.

Figure 22. Total chromatogram of blank card (without blood), showing no interferences for any of target compounds (the compounds identified in the chromatogram are based on the attempted software identification and are not present in the sample; only a not significant background level of cocaine was observed).

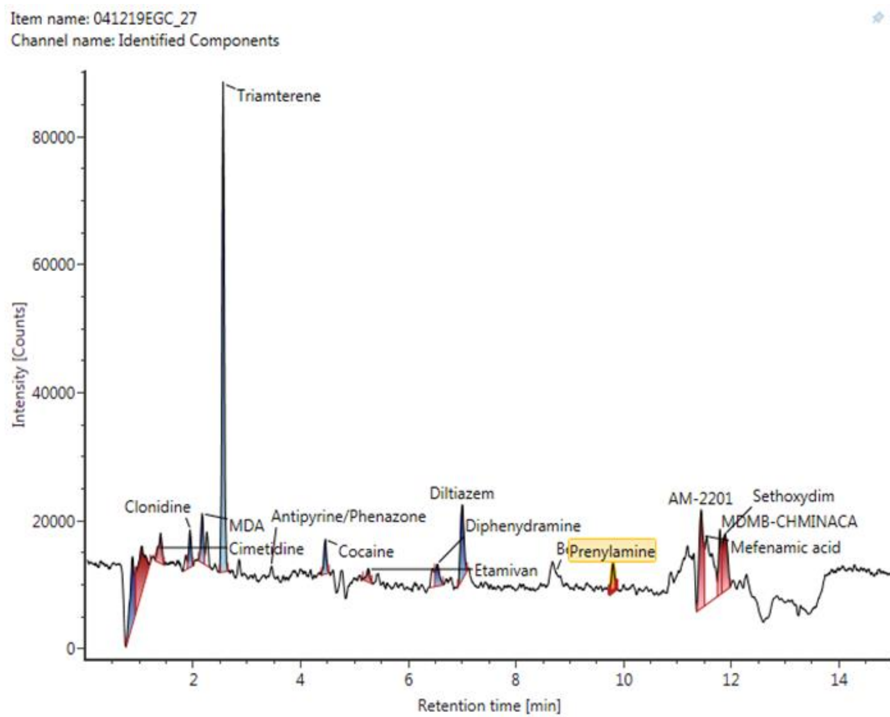
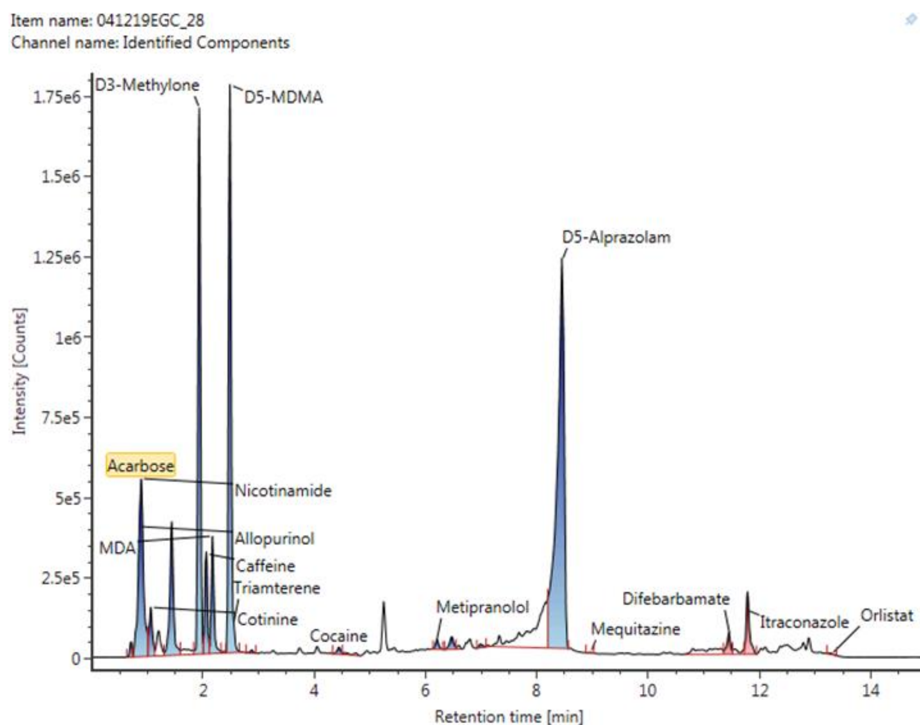


Figure 23. Total chromatogram of blank DBS sample, with ISTD. No endogenous compounds from blood generated peaks for the target analytes.



The DBS extraction was found to be very satisfactory, resulting in recoveries greater than 80% for most of the target drugs and using a simple and fast one-step procedure with methanol and borate buffer pH 10.4. Reduced recoveries (lower than (80%) were found only for three drugs, alpha-PVP (78.4%), heroin (69.9%) and LSD (69.7%). Significant matrix effects (exceeding ±20%) were noted only for 11 compounds (25C-NBOMe, 25I-NBOMe, 3-MeO-PCP, alprazolam, etizolam, FiBF, furanylfentanyl, heroin, MDMA, LSD and valerylfentanyl). These effects may explained due to the use of borate buffer during the extraction, which introduces aqueous solution into the medium that can increase the content of non-organic components in the extract, suppressing or enhancing the responses. Mitragynine presented a very low recovery, of 28.5%, and a different behavior in DBS, and for this reason, it is discussed separately from the other compounds. **Table 12** presents the values of recovery and matrix effect for each compound.

Table 12. Qualitative method validation data: recovery and matrix effects

	Retention Time (min)	Matrix Effects	Recovery
25B-NBOMe	8.09	-10.5	92.7
25C-NBOMe	7.81	33.4	96.4
25I-NBOMe	8.59	-30.4	96.1
3-MeO-PCP	6.33	28.9	93.1
4-fluoroamphetamine	2.60	33.0	85.9
Acetylfentanyl	5.11	6.79	94.6
Acrylfentanyl	5.99	2.77	94.0
Alpha-PVP	4.24	9.09	78.4
Alprazolam	8.48	132	91.3
Butylone	2.78	9.09	99.4
Butyrylfentanyl	7.20	0.001	98.1
Carfentanil	7.04	5.06	97.5
Cocaine	4.50	15.3	93.5
Diazepam	10.5	2.83	110

Table 12. Qualitative method validation data: recovery and matrix effects (conclusion)

	Retention Time (min)	Matrix Effects	Recovery
Dibutylone	2.77	13.1	93.6
Etizolam	9.28	-54.9	98.9
Fentanyl	6.27	6.65	94.9
FiBF	7.43	52.6	99.9
Flubromazolam	8.41	22.7	87.6
Furanylfentanyl	6.45	31.1	98.2
Heroin	4.11	-28.3	69.9
Ketamine	3.15	9.14	96.2
LSD	4.97	149	69.7
MDMA	2.51	91.9	104
Mephedrone	3.08	6.19	85.4
Methamphetamine	2.47	-0.311	89.2
Methoxyacetylfentanyl	4.92	-1.56	99.5
Methylone	2.04	-10.6	94.8
N-ethyl-hexedrone	5.21	6.27	83.9
N-ethyl-pentylone	4.08	13.6	96.2
Pentylone	3.68	16.2	95.4
PCP	5.81	0.333	92.8
U-47700	6.21	5.82	89.3
U-48800	6.97	11.7	85.9
Valerylfentanyl	8.26	-46.8	90.8

4.2.3. Stability of drugs in DBS

All target drugs presented a post-processing stability higher than 80% after 24h at room temperature in auto sampler, except etizolam (78.1%). After 48h, an expected decrease in the response was observed and the majority of the drugs presented a post-processing stability of 70% or higher, except alprazolam (69%), diazepam (67.5%), fentanyl (48%) and flubromazolam (59.6%). In a specific case, diazepam showed a high imprecision of peak area after 48h **(Table 13).**

Table 13. Post-processing stability data

	After 24h	CV (%) of Peak Area	After 48h	CV (%) of Peak Area
25B-NBOMe	94.4	7.48	76.2	3.65
25C-NBOMe	95.1	10.3	79.6	3.03
25I-NBOMe	92.3	9.46	75.9	5.32
3-MeO-PCP	81.3	11.3	75.2	8.06
4-fluoroamphetamine	103	7.74	78.9	2.59
Acetylfentanyl	105	0.425	85.2	0.712
Acrylfentanyl	96.7	7.90	88.0	1.81
Alpha-PVP	102	1.81	88.5	7.41
Alprazolam	83.1	16.0	69.0	8.25
Butylone	103	2.22	87.9	2.46
Butyrylfentanyl	95.7	8.58	88.2	6.53
Carfentanil	95.7	7.25	86.1	8.03
Cocaine	104	3.72	92.6	5.41
Diazepam	99.3	12.0	65.3	42.9
Dibutylone	101	3.80	86.7	5.09
Etizolam	78.1	8.13	67.5	2.32
Fentanyl	90.6	10.1	48.3	9.19
FiBF	98.7	4.24	92.3	8.84
Flubromazolam	86.3	13.8	59.6	4.83
Furanylfentanyl	89.1	6.68	86.6	6.27
Heroin	114	2.56	73.7	19.3
Ketamine	103	1.95	85.4	4.03
LSD	96.3	6.04	73.4	3.18
MDMA	94.8	2.79	80.3	3.11
Mephedrone	101	2.31	82.5	8.23
Methamphetamine	90.8	1.49	75.7	7.56

Table 13. Post-processing stability data (conclusion)

	After 24h	CV (%) of Peak Area	After 48h	CV (%) of Peak Area
Methoxyacetylfentanyl	103	1.25	85.4	3.87
Methylone	96.6	11.8	80.9	9.40
N-ethyl-hexedrone	102	2.18	89.4	1.30
N-ethyl-pentylone	101	3.81	90.6	3.13
Pentylone	95.7	4.17	87.9	4.04
PCP	92.4	3.17	72.4	6.71
U-47700	83.0	7.54	79.8	6.92
U-48800	114	0.810	99.3	6.00
Valerylfentanyl	94.3	7.63	86.2	4.49

Mitragynine presented a stability of 131% (with a CV of 10.7%) after 24h and 67.1% (with a peak area CV of 30.7%) after 48h, in post-processing analysis. This can be explained due to the low recovery presented by mitragynine.

For long-term stability, an intermediate concentration of 50 ng/mL for all drugs was used in order to qualitatively investigate the chemical behavior of each drugs in a dry matrix, in a multidrug sample. A summary is presented in the **Table 14.** At room temperature, the majority of target drugs were stable up to 37 days. Alpha-PVP and N-ethyl-pentylone were detected in all DBS sample stored at room temperature only up to 22 days. 4-fluoro-amphetamine, acrylfentanyl, heroin, mephedrone, methylone, N-ethyl-hexedrone and pentylone were detected in DBS only up to 14 days at room temperature. Mitragynine was unstable at room temperature in DBS, not reaching the positive identification criteria. In DBS samples stored at -20ºC, all target drugs remained detectable after 37 days. Although the method was developed for qualitative investigations, it was found that less variability in the responses of drugs in DBS samples kept at -20ºC, supporting an increased stability for all compounds in these conditions. Mitragynine showed reduced stability in relation the other compounds (24h). In particular for mitragynine, the instability in other biological fluids, such as liquid blood, has been observed (PAPSUN et al., 2019).

Table 14. Stability of analytes in DBS at room temperature and at -20ºC

	Sets of DBS samples fully screened positive for each analyte after storage (# is time of storage)	
	Room Temperature	**-20ºC**
25B-NBOMe	37 days	37 days
25C-NBOMe	37 days	37 days
25I-NBOMe	37 days	37 days
3-MeO-PCP	37 days	37 days
4-fluoroamphetamine	14 days	37 days
Acetylfentanyl	37 days	37 days
Acrylfentanyl	14 days	37 days
Alpha-PVP	22 days	37 days
Alprazolam	37 days	37 days
Butylone	37 days	37 days
Butyrylfentanyl	37 days	37 days
Carfentanil	37 days	37 days
Cocaine	37 days	37 days
Diazepam	37 days	37 days
Dibutylone	37 days	37 days
Etizolam	37 days	37 days
Fentanyl	37 days	37 days
FiBF	37 days	37 days
Flubromazolam	37 days	37 days
Furanylfentanyl	37 days	37 days
Heroin	14 days	37 days
Ketamine	37 days	37 days
LSD	37 days	37 days
MDMA	37 days	37 days
Mephedrone	14 days	37 days
Methamphetamine	37 days	37 days
Methoxyacetylfentanyl	37 days	37 days
Methylone	14 days	37 days

Table 14. Stability of analytes in DBS at room temperature and at -20ºC (conclusion)

	Sets of DBS samples fully screened positive for each analyte after storage (# is time of storage)	
	Room Temperature	**-20ºC**
N-ethyl-hexedrone	14 days	37 days
N-ethyl-pentylone	22 days	37 days
Pentylone	14 days	37 days
Phencyclidine	37 days	37 days
U-47700	37 days	37 days
U-48800	37 days	37 days
Valerylfentanyl	37 days	37 days

Our findings are coherent with reports in the literature. Cocaine was detected in DBS samples after 37 days kept at both room temperature and -20ºC. For cocaine, a study performed by Simões et al. (2018) found a variation of -5% to 12% in relation to positive controls in DBS samples at 2-8ºC and -10ºC after 8 months, with high variations of -44% and -25% observed at room temperature (SIMÕES; AJENJO; DIAS, 2018). In another study, cocaine exhibited reduced stability at room temperature and high stability (more than 85%) at -80ºC after 1 or 6 months (CHEPYALA et al. 2017).

MDMA was detected in all samples in both temperatures up to 37 days. Ambach et al. (2014) reported stabilities higher than 93.8% for MDMA after one or two weeks in DBS stored at room temperature or 4ºC. Simões et al. (2018) showed that MDMA showed a variation between -15% and -7% in relation to controls, after 8 months at room temperature, 2-8ºC or -10ºC. Chepyala et al. (2017) found that MDMA presented high stability (more than 85%) after 1 month (room temperature and -80ºC) or 6 months (room temperature).

Our results suggest methamphetamine is stable in DBS up to 37 days, in both conditions (room temperature and -20ºC). In a different study, methamphetamine was less than 50% stable at room temperature after 1 or 6 months or at -80ºC after 6 months, showing high stability after 1 month at -80ºC (CHEPYALA et al. 2017). Ambach et al. (2014) reported high stability, more than 95%, for methamphetamine, after one or two weeks at room temperature or 4ºC. Simões et al. (2018) also found variations of -18% to

-7% in relation to controls for methamphetamine in three different conditions (ambient temperature, 2-8ºC and -10ºC), after 8 months.

Fentanyl presented acceptable stability in all samples after 1, 14, 22 and 37 days, in both temperatures. Chepyala et al. (2017) found fentanyl stable at room temperature and -80ºC up to 6 months. Another study by Verplaetse and Henion (2016) showed that fentanyl was stable for 30 days at room temperature, for 55 days at 4ºC and for 95 days at -20ºC. For acrylfentanyl, similarly to observed in this study, a report by Kahl, Cunha and Costa (2019) found that this compound exhibits a degradation of 21.7% after 15 days at room temperature.

Ketamine exhibited a stability profile similar to fentanyl, MDMA and methamphetamine. In the literature, Chepyala et al. (2017) reported ketamine was more than 85% stable after one month (room temperature and -80ºC), more than 50% after 6 months (room temperature) and less than 50% after six months (at -80ºC). Stability of ketamine in DBS was also evaluated by Ambach et al. (2014), resulting between 97.5% and 103.2% after one or two weeks.

LSD and PCP were identified in DBS samples stored for 1, 14, 22 or 37 days, at room temperature and at -20ºC. PCP stability was reported in the literature between 50% and 85% after 1 month (at room temperature and -80ºC) and after 6 months at room temperature, with poor stability after 6 months at -80ºC for PCP (CHEPYALA et al. 2017). Another work showed stability between 97.7% and 100% for PCP after one or two weeks, at room temperature or 4ºC (AMBACH et al. 2014). Chepyala et al. (2017) reported high stability for LSD up to 6 months in DBS.

Alprazolam and diazepam were stable in DBS sample in all tested conditions (up to 37 days). In the study conducted by Déglon et al. (2012), alprazolam and diazepam presented good stability at room temperature and at -20°C up to 30 days.

In this work, mephedrone showed stability in all samples up to 14 days at room temperature and up to 37 days at -20ºC. A report stated that mephedrone was not detected after 7 days at room temperature and, at reduced temperatures, mephedrone presented an increased stability up to 90 days (CUNHA; EBERLIN; COSTA, 2018b). Ambach et al. (2014) observed a reduced stability of mephedrone at room temperature (78.5% after 1 week and 61.9% after 2 weeks).

In our study, we observed that pentylone was stable in DBS at -20ºC up to 37 days, whereas at room temperature, all samples remained positive for pentylone only up to 14 days. In a different study, pentylone was detected after 90 days when stored at -

20ºC and at ambient temperature at 75 and 750 ng/mL (CUNHA; EBERLIN; COSTA, 2018b). Other report observed a good stability for pentylone after one week in DBS kept at room temperature and 4ºC, with reduced stability after two weeks at room temperature (78.2%) and 4ºC (86.1%) (AMBACH et al. 2014).

Methylone showed a stability similar to pentylone. All samples had positive identification for methylone up to 14 days at room temperature and up to 37 days, at -20ºC. In other work, methylone was more than 92.7% stable after 1 week at room temperature and at 4ºC and after 2 weeks at 4ºC, with reduced stability (75.1%) at room temperature after 2 weeks (AMBACH et al. 2014).

Butylone was detected up to 37 days at room temperature and at -20ºC in DBS. Other works report a stability of 90 days (CUNHA; EBERLIN; COSTA, 2018b) and higher than 83.7% after 1 or 2 weeks at room temperature and 4ºC (AMBACH et al. 2014).

We found that NBOMe compounds (25C-, 25I- and 25B-) were detected in all DBS samples stored at -20ºC or room temperature after 37 days. A study in the literature showed that NBOMe series compounds had good stability when stored at room temperature, 4ºC and -20ºC, except for 25I-NBOMe (at 8 ng/mL) and 25B-NBOMe (at 0.3 ng/mL) showing a degradation higher than 20% after 180 days (CUNHA; EBERLIN; COSTA, 2018a)

4.2.4. Authentic samples analysis

The method was later applied to the re-analysis of 68 blood specimens using DBS. A comparison between results in whole blood analysis, performed by NMS Labs, and DBS analysis is presented in **Table 15**.

12 drugs included in the scope of this method were found in the re-analyzed postmortem and DUID cases. Acetylfentanyl, alprazolam, cocaine, diazepam, dibutylone, fentanyl, FiBF, furanylfentanyl, methamphetamine, methoxyacetylfentanyl, mitragynine, N-ethyl-pentylone and U-47700 were detected. Examples of positive results are presented in **Figures 24** and **25**. A high rate of correct match between positivity in whole blood and in DBS was found for the majority of these compounds, with 10 drugs presenting a match of 81.3% or higher.

Table 15. Toxicological findings in postmortem and DUID specimens

	Number of positive whole blood samples	Number of positive DBS samples	None detected in DBS samples	% Match
Acetylfentanyl	3	1	2	33.3
Acrylfentanyl	1	0	1	0.0
Alprazolam	19	12	7	63.1
Cocaine	14	13	1	92.9
Diazepam	1	1	0	100
Dibutylone	1	1	0	100
Fentanyl	17	14	3	81.3
FiBF	9	8	1	88.9
Furanylfentanyl	16	16	0	100
Methamphetamine	13	11	2	84.6
Methoxyacetylfentanyl	6	6	0	100
Mitragynine	3	3	0	100
N-ethyl-pentylone	3	3	0	100
U-47700	13	12	1	92.3

False-negative results in DBS samples, in relation to whole blood results, were found, in a small number of samples, for 8 drugs: acetylfentanyl, acrylfentanyl, alprazolam, cocaine, fentanyl, FiBF, methamphetamine and U-47700. However, the re-analysis in DBS was not performed in a subsequent fashion, after whole blood testing, introducing some variations. As these samples were collected in 2016 and 2017 and stored until the re-analysis in 2019, a stability decrease may have occurred for some of these analytes, still in whole blood, which should be expected after a period of one or two years. In addition, some of these drugs were quantitated in 2016/2017 in whole blood at concentrations lower than the LOD of the method using DBS. Analysis of peripheral blood by UPLC-MS/MS from Case #42 showed fentanyl at 0.94 ng/mL and analysis of DBS sample by UPLC-QTOF resulted negative for fentanyl. Similar results (negative in DBS and levels lower than LOD in liquid blood) were observed in 1 sample for acetylfentanyl, 2 samples for alprazolam and 2 samples for fentanyl.

Figure 24. An example of positive result for fentanyl in a DBS sample (Case #4)

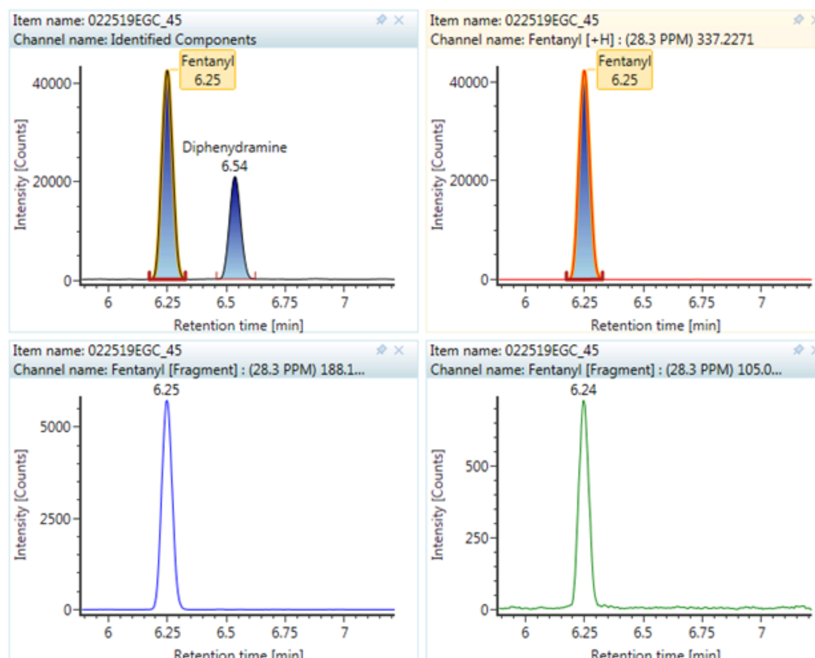
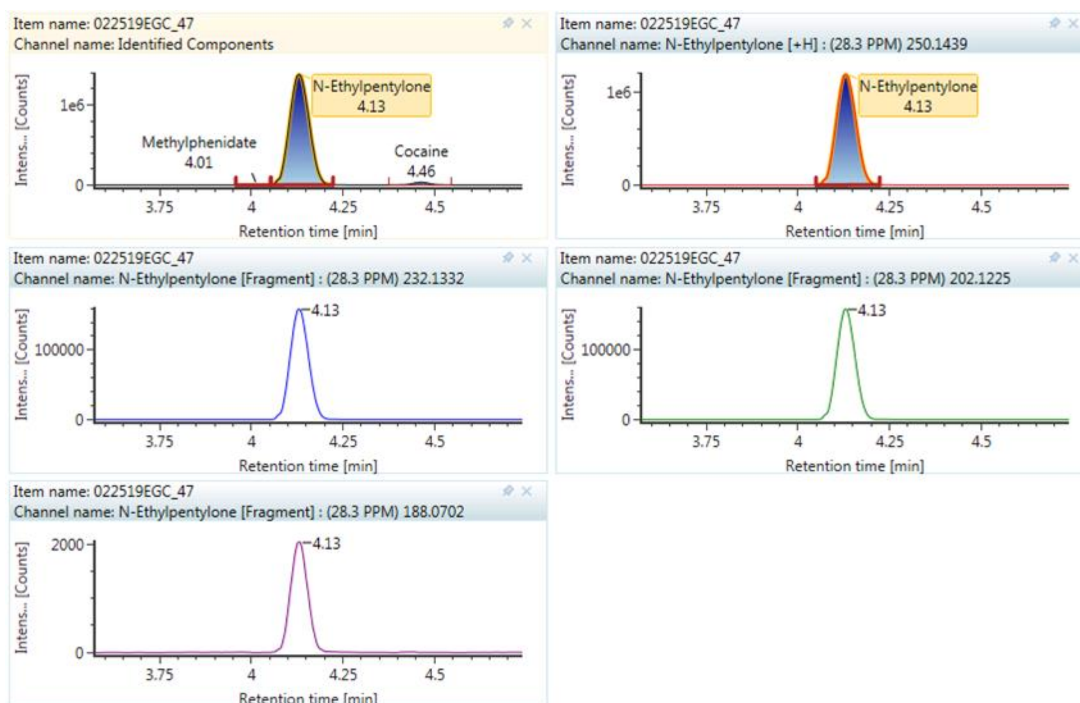


Figure 25. An example of positive result for N-ethyl-pentylone in a DBS sample (Case #6)



Results shown in **Table 16** summarize data regarding drugs identified in DBS that were not previously found in whole blood tested by NMS Labs.

Table 16. Summary of drugs detected in DBS but not detected in previous whole blood analysis performed by NMS Labs

Case Number	Drug detected in DBS but not in whole blood	Comments
6	Cocaine	Whole blood tested for cocaine. Cocaine was not found.
11	Cocaine	
27	Cocaine	
33	Diazepam	Whole blood tested for diazepam. Diazepam was not found.
36	Alprazolam	Whole blood was not tested for alprazolam. Urine was presumptively positive for benzodiazepines without confirmation in whole blood.

In these four cases (3 for cocaine and 1 for diazepam), the corresponding whole blood samples were screened by NMS Labs but resulted negative. In these cases, a possible cross contamination might occurred for DBS samples, especially for cocaine and diazepam, which are drugs commonly found in forensic laboratories. On the other hand, these findings can be a result of better recovery of these drugs in DBS. Alprazolam was detected in DBS sample from Case #27 but the corresponding whole blood sample was not previously tested for diazepam; urine was tested by NMS Labs using enzyme immunoassay (EIA), resulting in positive for benzodiazepines. However, confirmation was not performed in whole blood. In this sense, our findings in DBS sample from Case #27 cannot be assigned as a false-positive, as whole blood was not tested.

Another important aspect to discuss is that different sources of postmortem blood were tested using DBS, showing good results. Cardiac, peripheral, femoral, cavity, iliac, subclavian and pleural blood were tested using DBS and results were similar to those obtained in whole blood. The source of blood seems not to be a limitation but a homogenization of whole blood is strongly required, as coagulated blood can affect the spotting process, generating deviations, especially for quantitative measurements.

In relation to case demographics, blood specimens were collected from United States (from states of Louisiana, Colorado, Nevada, Illinois, New Jersey, Connecticut, New York, Tennessee, California, Hawai, Georgia, Pennsylvania, Vermont, Maine, Idaho, South Carolina, Indiana, Florida and Kansas), and Canada. Individuals were 14

female, 46 male and 8 had no gender provided, with age between 17 and 74 years, and 10 had no information about age.

In summary, based on these findings, DBS is a very interesting alternative matrix for toxicological testing of drugs of abuse and NPS in postmortem blood. Different drug classes have been identified, even after a long time storage in whole blood. However, for mitragynine, DBS is a good approach for testing but it is limited for long-term storage, as mitragynine was not stable in DBS. The reduced number of false negative results shows the potential of DBS, but a high sensitive technique should be adopted. The findings support also the use of DBS for sample mining, in order to investigate analyzed samples searching for the prevalence of new drug.

4.3. Development and validation of a screening method for synthetic cannabinoids in paper DBS using UPLC-Triple Quadrupole-MS/MS.

4.3.1. Method development

Based on the previous qualitative method, the same extraction procedure was used for synthetic cannabinoids extraction from DBS, with a few modifications, including not using buffers and using only methanol for extraction.

Initially, the previous UPLC-QTOF method developed for drugs of abuse and NPS was tested for synthetic cannabinoids identification in DBS but the detection was not successful. Following that, a second method was tested, using a Shimadzu (Japan) Nexera XR® UHPLC coupled to a Sciex (Framingham, MA) TripleTOF® 5600+ QTOF and a previously developed method for synthetic cannabinoids described elsewhere (KROTULSKI et al., 2019). One, two or three spots were tested but none of target synthetic cannabinoids were detected in DBS at 0.5, 1 and 2 ng/mL. A third approach was tested, using a Triple Quadrupole UPLC-MS/MS, and the sensitivity of the method was increased, enabling the detection of target compounds. An example of chromatogram of a DBS sample (4 ng/mL) is presented in **Figure 26.**

Figure 26. MRM chromatogram of synthetic cannabinoids in an extracted DBS sample at 4 ng/mL

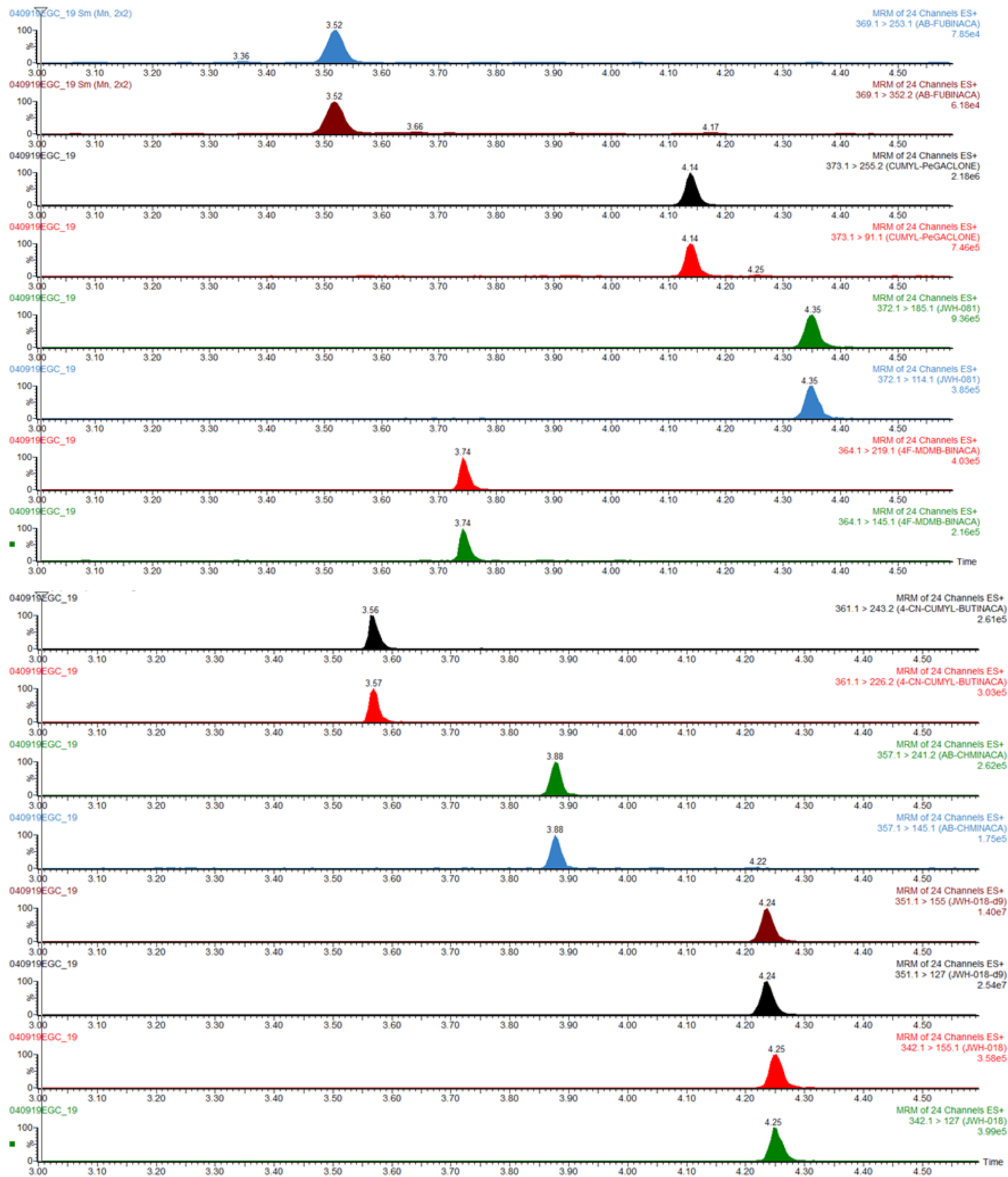
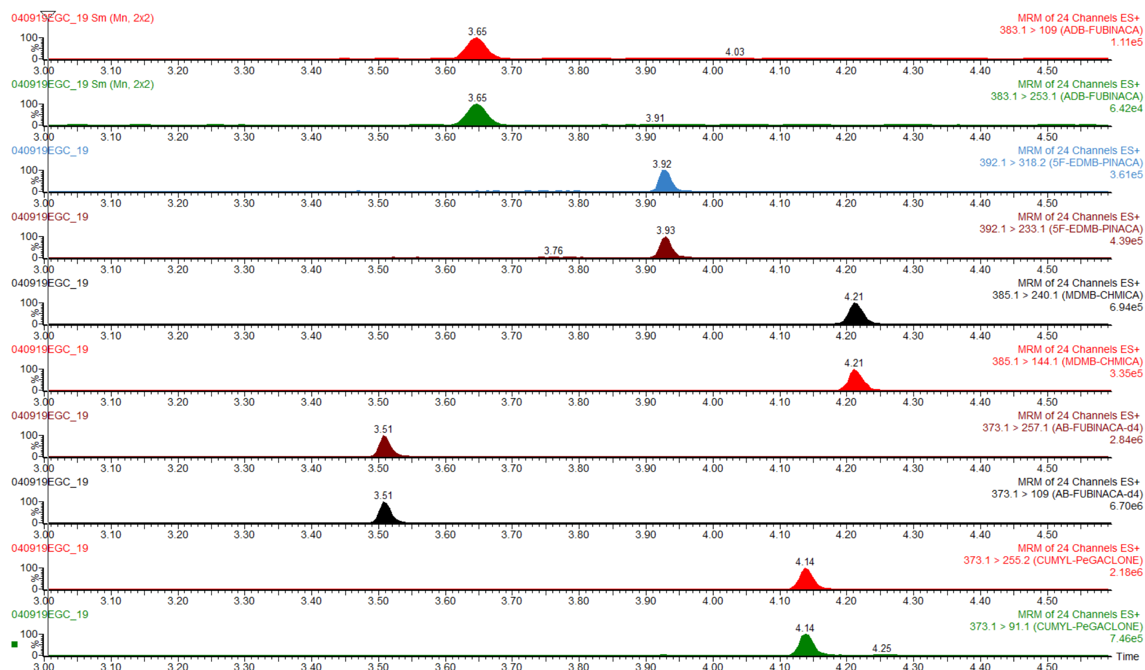


Figure 26. MRM chromatogram of synthetic cannabinoids in an extracted DBS sample at 4 ng/mL (conclusion)



4.3.2. *Method validation*

The qualitative method for screening synthetic cannabinoids in DBS was successfully validated and the results for LOD, recovery and matrix effects are shown in **Table 17**.

The LOD of target synthetic cannabinoids in DBS were 0.5, 1 or 2 ng/mL, which are consistent with the reported concentrations of some of these compounds in whole blood (**Table 22**). There are still no reports of blood levels of 4-CN-CUMYL-BUTINACA, 4F-MDMB-BINACA and 5F-EDMB. 4F-MDMB-BINACA was detected in death investigations and DUID investigations qualitatively only (KROTULSKI et al., 2019). In the literature, there is only one method for some natural and synthetic cannabinoids analysis in DBS, which reported an LOD of 0.05 ng/mL for JWH-018 and 0.03 ng/mL for JWH-081 (PROTTI et al., 2017); in one case JWH-081 was quantitated at 7.8 ng/mL in capillary DBS, 7.5 ng/mL in venous DBS and 13.5 ng/mL in plasma (PROTTI et al., 2017).

Table 17. Validation data: LOD, recovery and matrix effects

Compound	Retention Time (min)	LOD (ng/mL)	Recovery (%)	Matrix Effects (%)
4-CN-CUMYL-BUTINACA	3.57	0.5	78.4	-27.4
4F-MDMB-BINACA	3.75	1.0	91.2	-34.7
5F-EDMB-PINACA	3.92	1.0	86.4	-17.5
AB-CHMINACA	3.87	1.0	73.2	-20.8
AB-FUBINACA	3.51	2.0	92.9	-28.0
ADB-FUBINACA	3.64	2.0	82.6	-29.4
CUMYL-PEGACLONE	4.13	0.5	79.1	-2.07
MDMB-CHMICA	4.20	0.5	85.3	-22.0
JWH-018	4.31	0.5	72.2	-23.9
JWH-081	4.35	0.5	73.2	-16.1

In general, the LOD reached for each synthetic cannabinoids in DBS is acceptable, considering the analysis of authentic samples (**Table 18**). However, as most of synthetic cannabinoids are found in very low concentrations in blood, a very sensitive technique is recommended for DBS analysis.

Table 18. Comparison of LOD of synthetic cannabinoids using DBS-based method and the reported levels for the same drugs in blood

Compound	LOD (ng/mL)	Reported levels in *postmortem* or *in vivo* blood
4-CN-CUMYL-BUTINACA	0.5	0.2 – 66.4 ng/mL (YETER et al., 2017)
4F-MDMB-BINACA	1.0	n.a.
5F-EDMB-PINACA	1.0	n.a.
AB-CHMINACA	1.0	0.6 – 10 ng/mL (PETERSON; COUPER, 2015) < 0.03 – 14 ng/mL (TYNDALL et al., 2015) 4.1 ng/mL (extrapolated) (ANGERER et al., 2017) 0.5 ng/mL (ROMAŃCZUK et al., 2018) 7.6 ng/mL (MAEDA et al., 2018)

Table 18. Comparison of LOD of synthetic cannabinoids using DBS-based method and the reported levels for the same drugs in blood (conclusion)

Compound	LOD (ng/mL)	Reported levels in *postmortem* or *in vivo* blood
AB-FUBINACA	2.0	1.6 - 44.3 ng/mL (ALLIBE et al., 2017)
ADB-FUBINACA	2.0	7.3 ng/mL (SHANKS; CLARK; BEHONICK, 2016) 0.08 ng/mL (KÓVACS et al., 2019)
CUMYL-PEGACLONE	0.5	< 1 – 5.4 ng/mL (AKNOUCHE et al., 2019)
MDMB-CHMICA	0.5	0.89, 1.1 and 4.1 ng/mL (HESS et al., 2017)
JWH-018	0.5	0.1 – 199 ng/mL (SHANKS; DAHN; TERRELL, 2012) 0.1 – 1.1 ng/mL (YEAKEL; LOGAN, 2013) 0.08 – 0.46 ng/mL (TUV et al., 2014) 0.3 – 0.8 ng/mL (ÖZTÜRK; YETER; ALPERTUNGA, 2015)
JWH-081	0.5	0.19 ng/mL (TUV et al., 2014) 13.5 ng/mL (PROTTI et al., 2017)

The extraction of synthetic cannabinoids from DBS using only 1 mL of solvent was efficient with recoveries ranging from 72.2% to 92.9%. Similar recoveries were found for JWH-018 and JWH-081 by Protti et al. (2017) in DBS, 80% to 87%, using 1 mL of methanol for extraction. Matrix effects were reduced, ranging from -34.7% to -2.07%. As no buffer was used in the extraction, these findings support that aqueous buffer may increase recoveries but also contributes to matrix effects (ion enhancement or suppression).

The stable-isotope ISTD, JWH-018-d_9 and AB-FUBINACA-d_4, were not responsible for any interference on target cannabinoids responses. In a similar way, the analytes did not interfere with responses of the ISTD. No matrix components from different blank blood sources spiked on card or from the paper interfered with the analysis of synthetic cannabinoids (**Figures 27** and **28**). None of the drugs tested as interferents caused any interference. No carry over was detected (**Figure 29**).

Figure 27. MRM chromatograms of blank paper card (without blood)

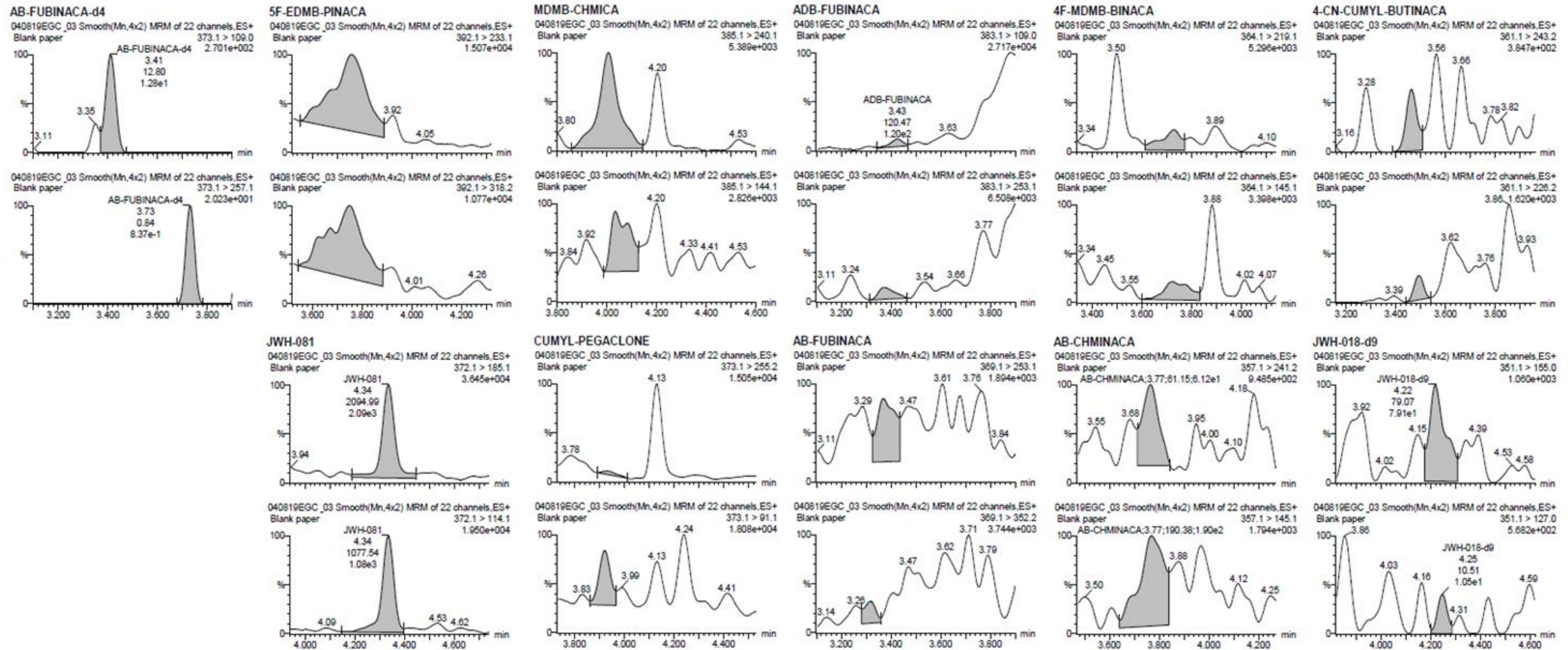


Figure 28. MRM chromatograms of blank DBS

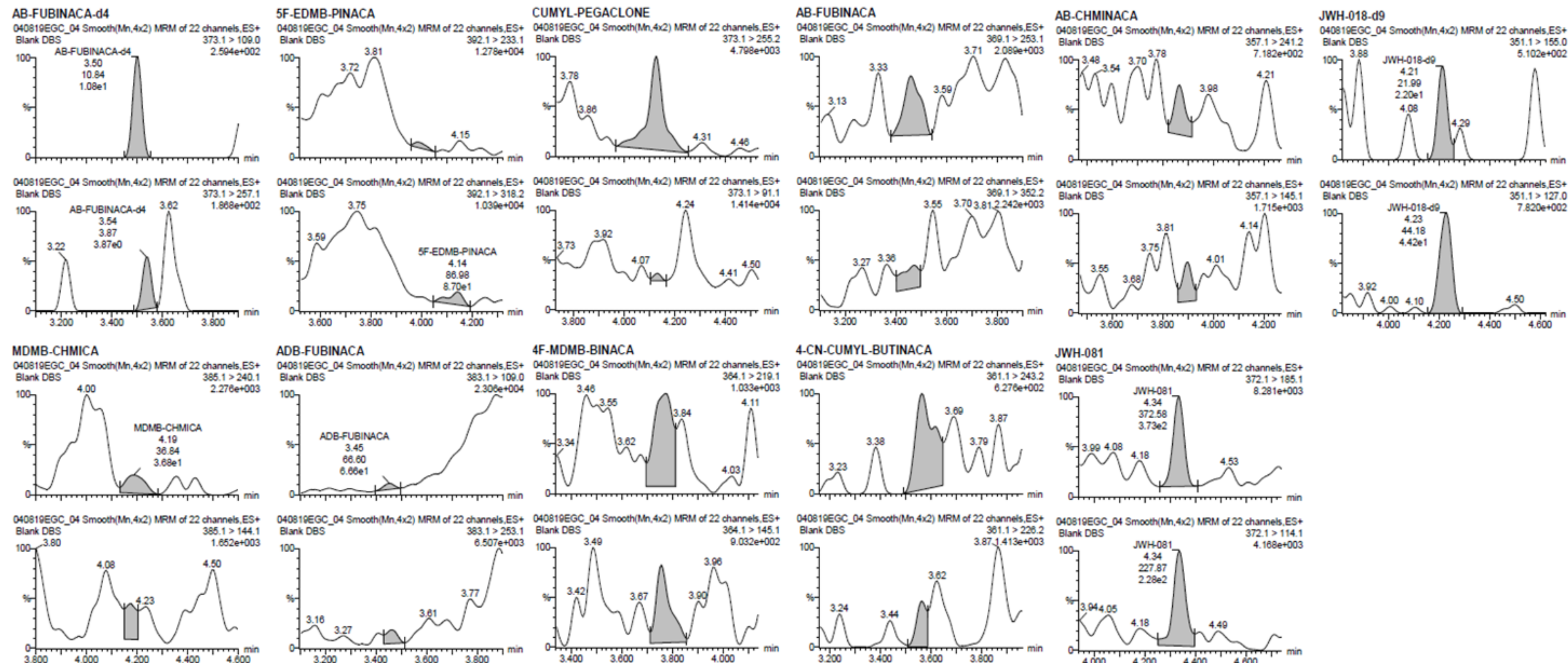
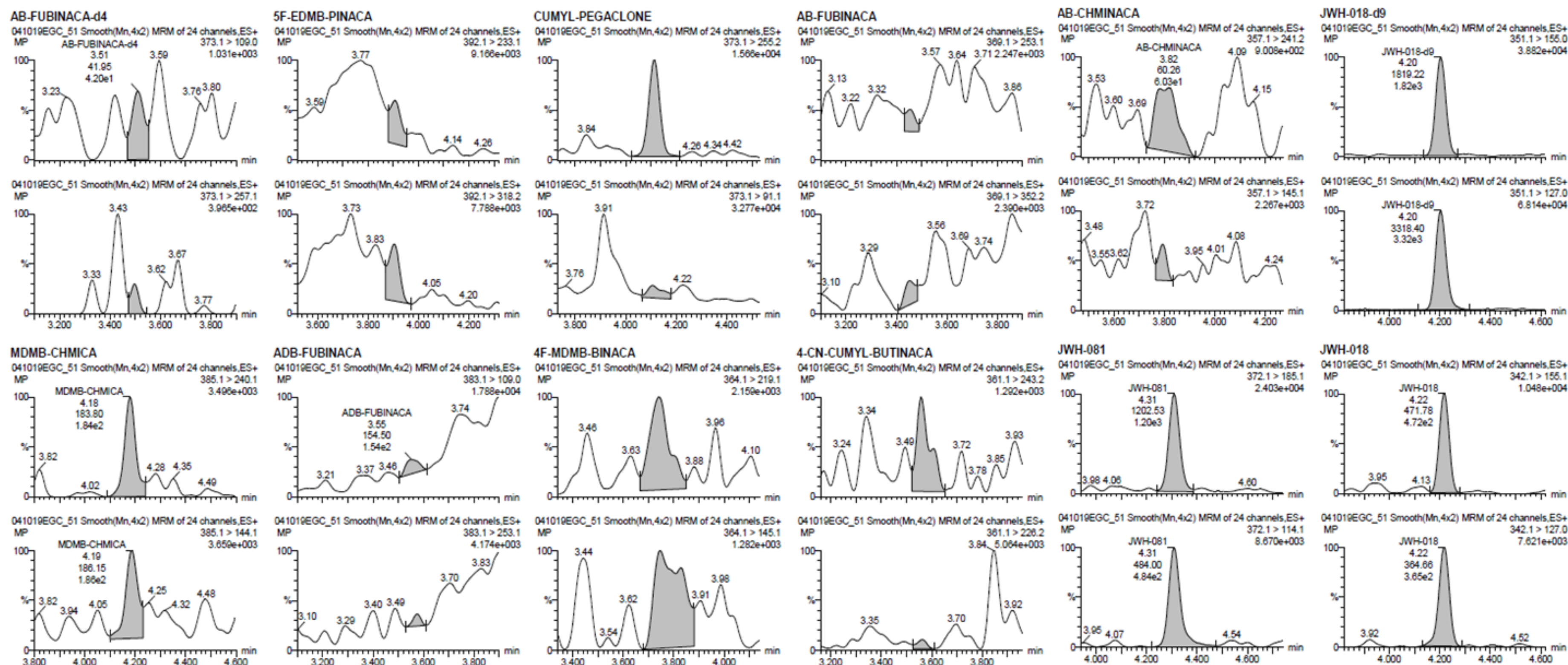


Figure 29. MRM chromatograms of mobile phase run after the analysis of a high concentrated DBS sample (100 ng/mL), showing no carry over. The peaks observed for JWH-018 and JWH-081 show very low areas.



4.3.3. Stability studies

All synthetic cannabinoids were still detectable in samples re-injected after 48h being kept in auto sampler at 10ºC. Although the method is qualitative, four compound presented a high variation in the response, in comparison to time zero. ADB-FUBINACA, MDMB-CHMICA and JWH-081 showed increased peak area imprecision for the low positive control after 48h. CUMYL-PEGACLONE presented an increased response after 48h. In the literature, synthetic cannabinoids extracted from blood were stable after processing for 24h or more at room temperature and up to 3 days at 4ºC (AMBROZIAK; ADAMOWICZC, 2018) (**Table 19**).

Table 19. Post-processing stability of synthetic cannabinoids after 48h in auto sampler

	4 ng/mL control	***% CV of peak area***	**10 ng/mL control**	***% CV of peak area***
4-CN-CUMYL-BUTINACA	98.3%	15.8	107%	8.02
4F-MDMB-BINACA	98.9%	17.8	104.6%	4.13
5F-EDMB-PINACA	93.2%	15.8	109%	4.06
AB-CHMINACA	74.7%	17.9	102%	6.05
AB-FUBINACA	118%	7.68	108%	1.67
ADB-FUBINACA	78.0%	22.8	78.9%	2.09
CUMYL-PEGACLONE	218%	15.7	236%	8.38
MDMB-CHMICA	102%	24.8	124%	8.08
JWH-018	102%	14.6	101%	5.54
JWH-081	162%	26.9	138%	19.5

All target synthetic cannabinoids were detectable in DBS specimens after 24h or 20 days, at room temperature or at -20ºC. In the literature, there is only one report of synthetic cannabinoids stability in DBS. This study reported that JWH–018, JWH-073, JWH–250, JWH–200, HU–211, CP 47,497, JWH-019, JWH-122, JWH-081, AM-2201 were stable in DBS at room temperature, after 1, 2, 3, 7, 15 and 30 days (PROTTI et al., 2017). In blood, 4-CN-CUMYL-BUTINACA was stable up to 3 days at room temperature, 4°C and −20°C (YETER, 2017). AB-FUBINACA was stable at 22°C, 4°C

and −20°C over 12 weeks in blood (FORT et al., 2017). In serum, at - 20ºC and 4ºC, AB-CHMINACA and ADB-FUBINACA were stable for 105 days and MDMB-CHMICA for 31 days (HESS et al., 2017).

4.4. Development and validation of a confirmatory and quantitative method for six synthetic cathinones in paper DBS using UPLC-Triple Quadrupole-MS/MS

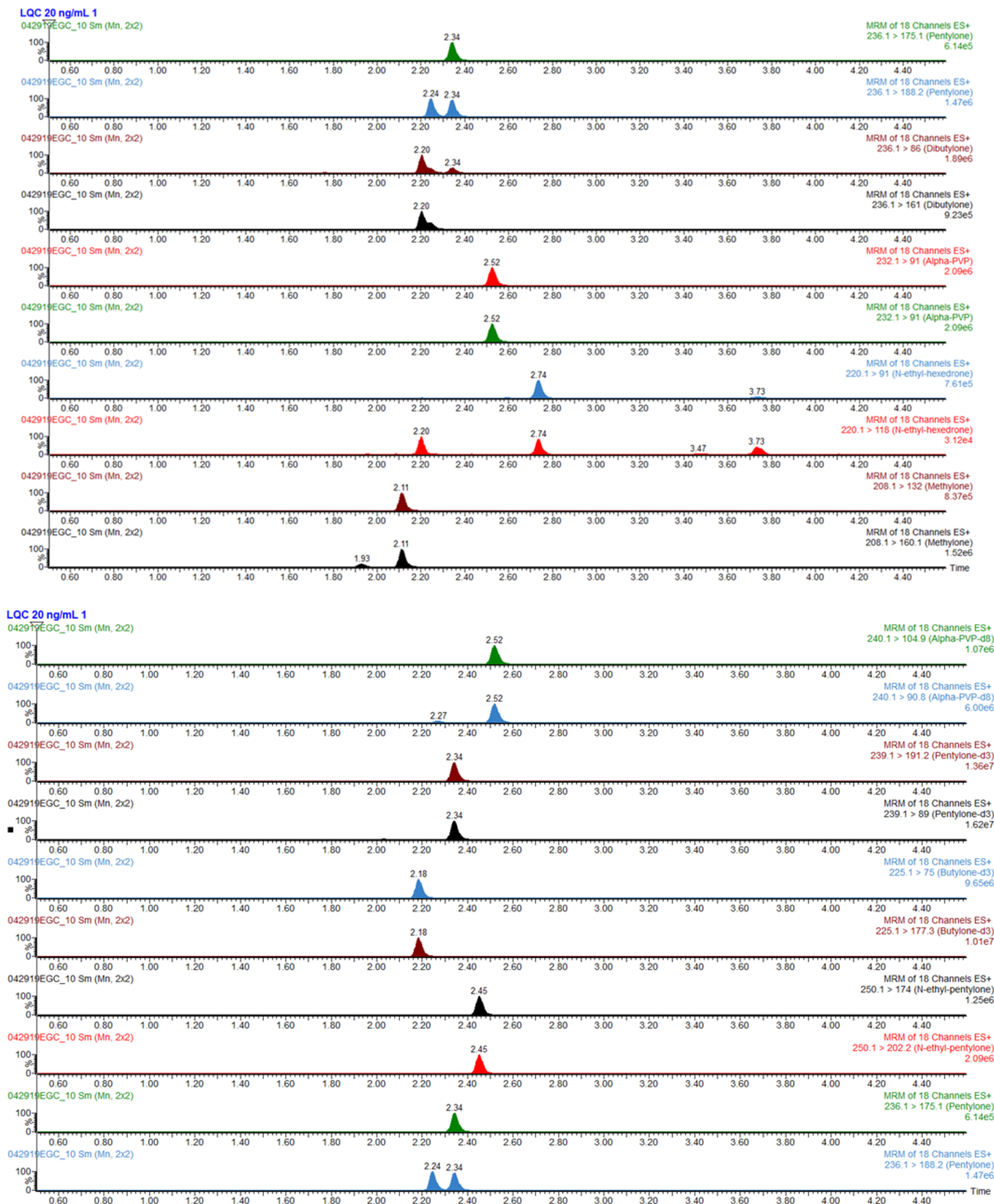
4.4.1. Method validation

Good chromatography was obtained for the cathinones and their ISTD **(Figure 30).** For all cathinones, a quadratic polynomial fitting was used for calibration model, resulting in average coefficient of correlations higher than 0.99 over five days. LLOQ for all target cathinones was 10 ng/mL, assessed over three days and using three different sources of whole blood for DBS preparation, resulting in imprecision and bias lower than 20% **(Table 20).** The LOD was 1 ng/mL for all cathinones, except N-ethyl-hexedrone, which showed unacceptable results for LOD testing.

Table 15. Validation data: calibration model and LLOQ

Compound	Coefficient of Correlation (R^2)	LLOQ	
		Average Imprecision (% CV)	Average Bias (%)
Alpha-PVP	0.9973	15.7	10.5
Methylone	0.9971	15.9	3.19
Dibutylone	0.9973	13.8	4.13
N-ethyl-hexedrone	0.9986	16.9	19.3
N-ethyl-pentylone	0.9984	13.8	7.5
Pentylone	0.9981	15.0	2.37

Figure 30. MRM chromatograms of DBS sample (LQC, 20 ng/mL)



The method presented acceptable intra and inter-assay imprecisions and bias over five days in triplicate, at three levels (LLOQ, LQC and HQC) **(Table 21)**. However, the results for methylone and N-ethyl-hexedrone were not acceptable, with these compounds exhibiting higher bias and imprecision.

Table 16. Validation data: average bias and imprecision (assessed over five runs)

Compound		Bias (%)	Intra-Assay Imprecision (% CV)	Inter-Assay Imprecision (% CV)
Alpha-PVP	LLOQ	6.47	9.22	16.1
	LQC	4.43	13.0	14.5
	HQC	6.37	8.17	12.3
Methylone	LLOQ	11.0	8.95	13.8
	LQC	11.2	9.67	10.9
	HQC	20.0	13.3	16.4
Dibutylone	LLOQ	5.67	7.14	14.2
	LQC	6.80	11.3	12.2
	HQC	11.9	10.2	12.8
N-ethyl-hexedrone	LLOQ	9.13	8.37	14.7
	LQC	7.80	9.73	10.4
	HQC	8.10	5.99	9.44
N-ethyl-pentylone	LLOQ	6.87	10.2	15.5
	LQC	8.80	12.1	13.0
	HQC	11.5	5.44	7.49
Pentylone	LLOQ	8.33	8.82	15.2
	LQC	10.2	11.0	12.0
	HQC	15.2	7.51	11.1

The method described showed high recoveries (higher than 85.1%) for methylone, dibutylone, N-ethyl-pentylone and pentylone. Alpha-PVP and N-ethyl-hexedrone showed reduced recoveries, between 69 – 77%. Matrix effects ranged between -23.4% and 97.7% (**Table 22**).

Table 17. Validation data: recovery and matrix effects

Compound		Recovery (%)	Matrix Effects (%)
Alpha-PVP	LQC	69.0	35.2
	HQC	71.1	97.7
Methylone	LQC	89.6	-23.4
	HQC	86.1	-6.05
Dibutylone	LQC	89.4	42.7
	HQC	89.8	30.8
N-ethyl-hexedrone	LQC	77.0	64.0
	HQC	74.8	157
N-ethyl-pentylone	LQC	90.4	25.6
	HQC	88.6	40.7
Pentylone	LQC	88.6	0.26
	HQC	85.1	16.5

Figures 31 – 34 show that no interferences from the paper or from the blood or carry over were observed for any of the target cathinones. None of the compounds tested for interferences produced any interferents peak for all target cathinones.

Figure 31. MRM chromatograms of blank paper card (without blood): no interferents peaks are observed for target cathinones or ISTD.

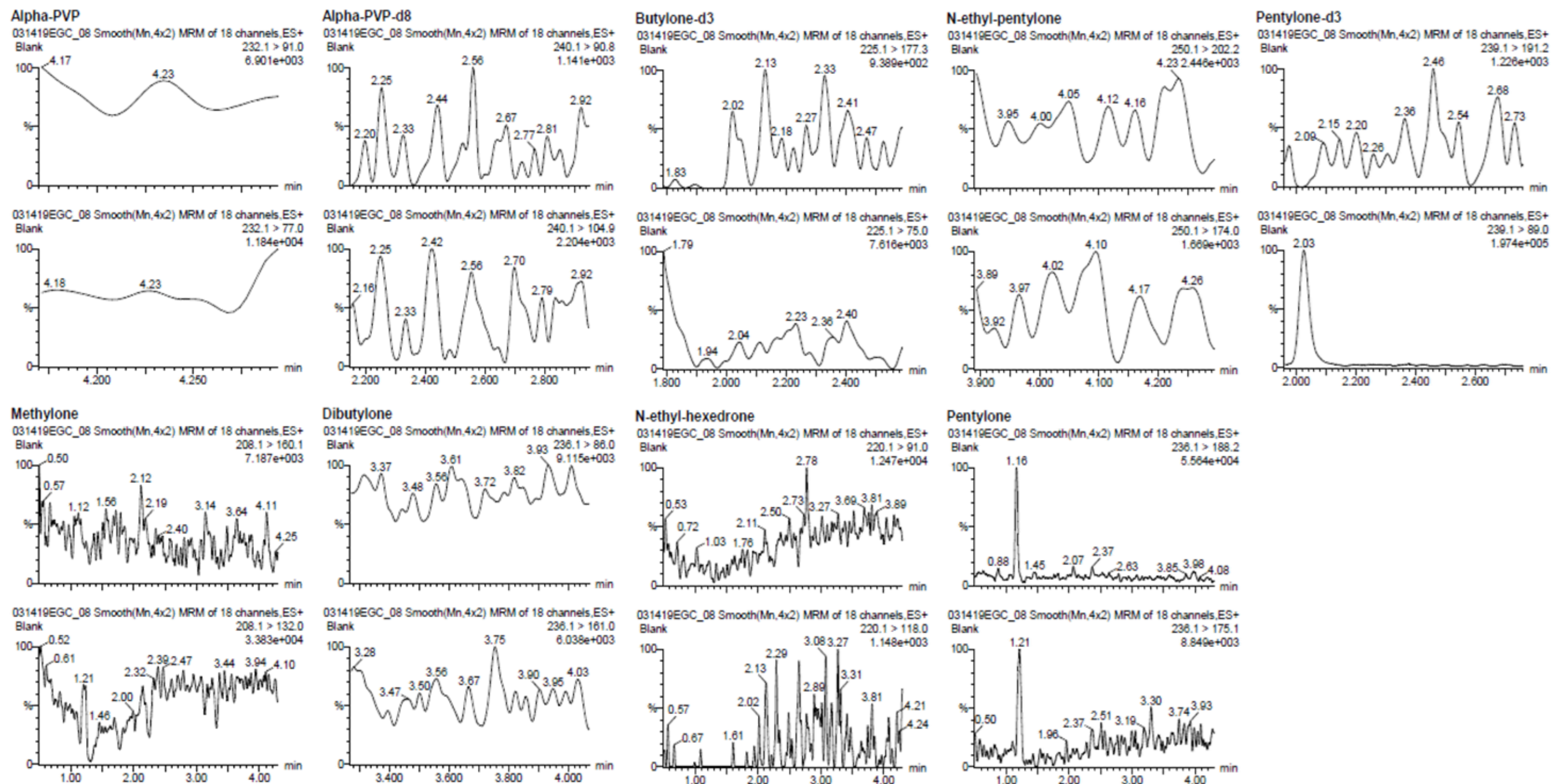


Figure 32. MRM chromatograms of blank DBS: no endogenous blood components are interfering with target cathinones or ISTD detection.

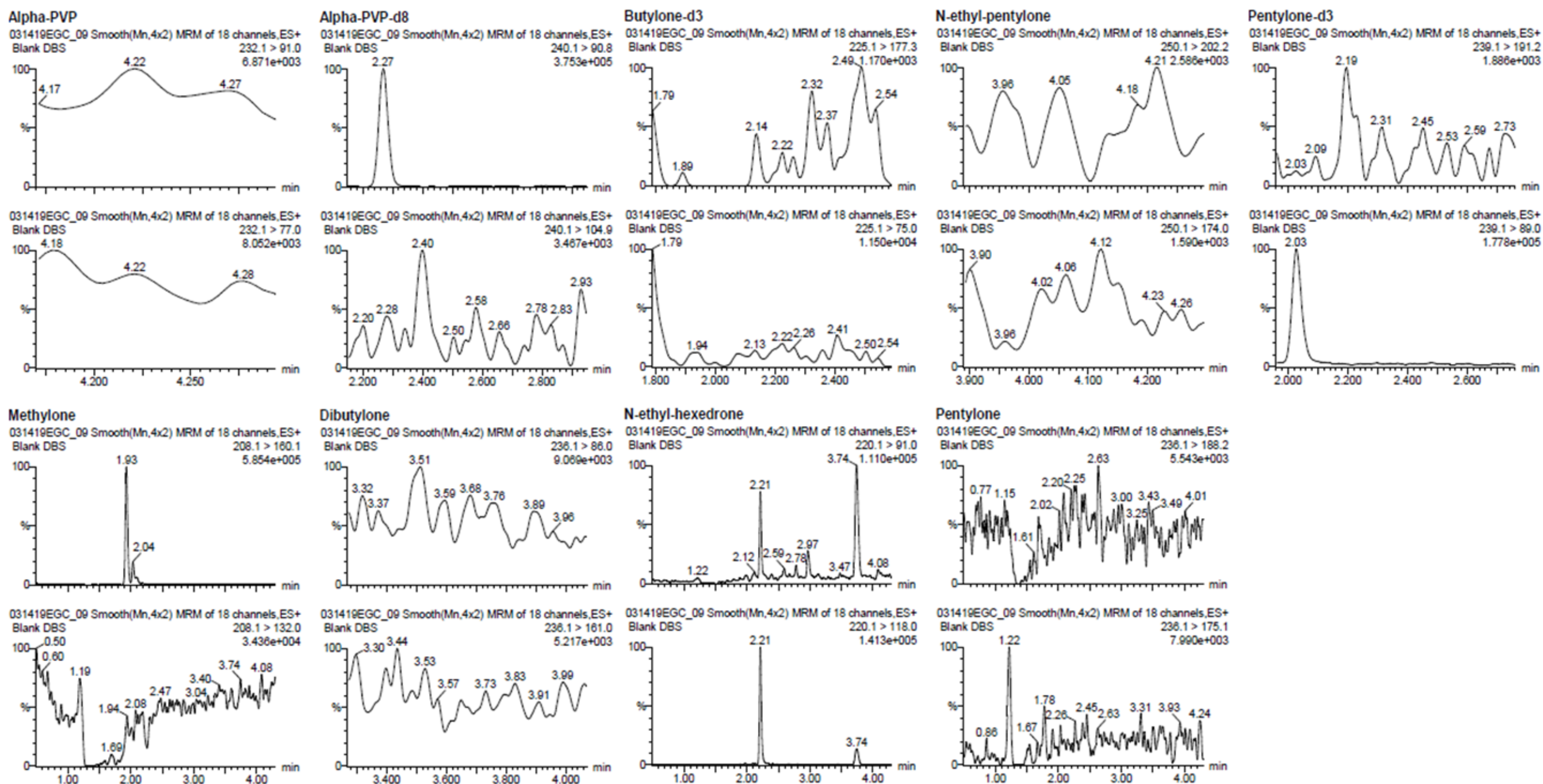


Figure 33. MRM chromatograms of mobile phase run after the analysis of a high concentrated DBS sample (500 ng/mL), showing no carry over.

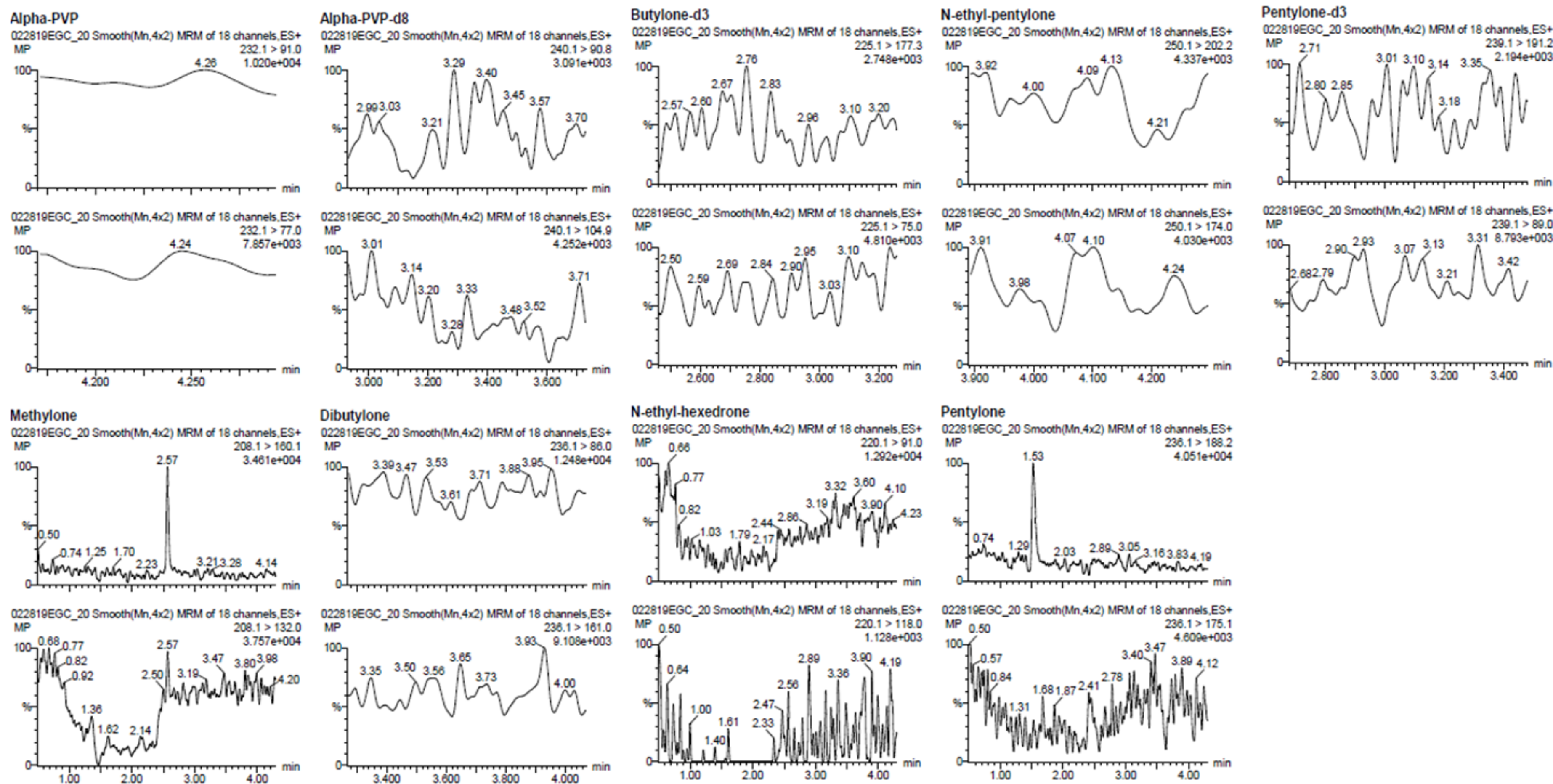
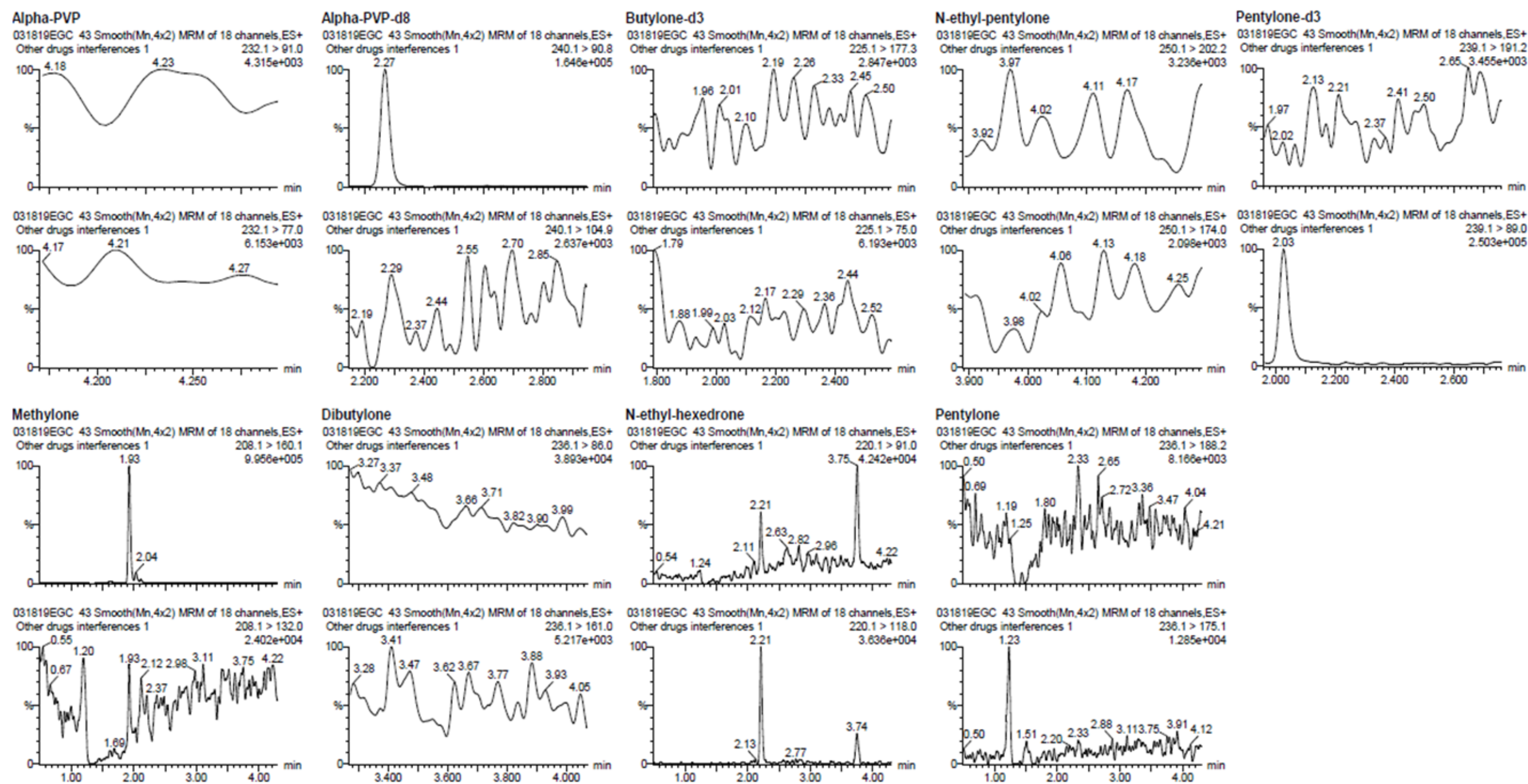


Figure 34. MRM chromatograms of DBS sample spiked with potential interferences: no interference detected for any of target compounds or ISTD.



All cathinones were stable 4 days after processing, at 10ºC (**Table 23**).

Table 23. Post-processing stability of synthetic cathinones in DBS

Compound	24h		4 days	
	LQC	HQC	LQC	HQC
Alpha-PVP	91.4%	96.2%	104%	109%
Methylone	89.5%	94.4%	103%	109%
Dibutylone	89.3%	96.9%	96.3%	109%
N-ethyl-hexedrone	86.3%	100%	108%	115%
N-ethyl-pentylone	87.3%	96.6%	97.8%	104%
Pentylone	87.8%	97.3%	97.0%	102%

Based on previous results with the qualitative method, a quantitative stability study was performed for cathinones for 24h **(Table 24).** For qualitative analysis, as described before, all drugs were still detected in DBS, with variations being observed for quantitation. All compound presented acceptable stability at room temperature and in freezer. Methylone, N-ethyl-hexedrone and pentylone exhibited a reduced stability (lower than 70%) at room temperature, with these three compounds presenting increased stability at -20ºC.

Table 24. Stability of synthetic cathinones in DBS

Compound	Room Temperature		-20ºC	
	CV of Peak Area (%)	Stability	CV of Peak Area (%)	Stability
Alpha-PVP	11.1	83.8%	2.91	99.5%
Methylone	20.8	34.5%	4.21	58.5%
Dibutylone	11.3	100%	2.72	88.9%
N-ethyl-hexedrone	5.83	34.7%	1.39	79.5%
N-ethyl-pentylone	10.4	73.0%	1.59	80.2%
Pentylone	15.0	52.1%	2.66	62.4%

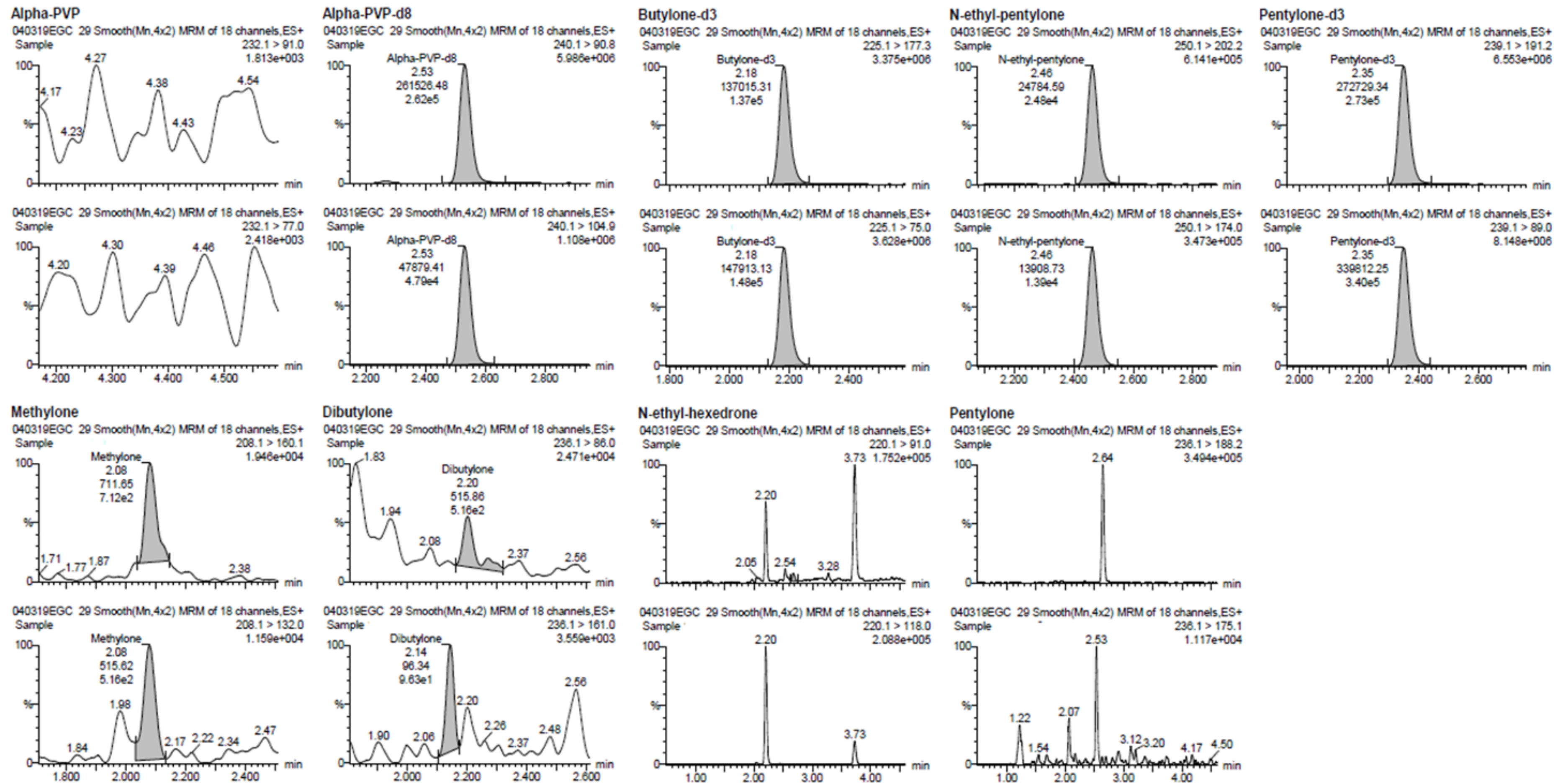
4.4.2. Authentic specimens analysis

An example of positive identification is presented in **Figure 35.** Additional case demographics are presented in **Table 25**. Samples were positive for dibutylone and N-ethyl-pentylone. Although the number of cases is reduced (five cases), the range of concentrations found in authentic samples using DBS is broad. However, for quantitation, additional considerations are needed. In this method, the whole spot was used for extraction, eliminating the hematocrit influence. If just a section of the spot is used, hematocrit effects should be evaluated. In addition, capillary and venous blood may present different concentrations for the same drug, and this need to be taken into account.

Table 25. Case samples data

Case	Type of blood	Location	Age	Gender	Findings in DBS
1	Femoral	VT	32	Female	Dibutylone, 264.2 ng/mL
2	Pheripheral	NY	Not given	Not given	N-ethyl-pentylone, 16.9 ng/mL
3	Pleural	NY	35	Male	Dibutylone, below LLOQ, and N-ethyl-pentylone, 443.2 ng/mL
4	Not specified	PA	Not given	Male	N-ethyl-pentylone, 10.8 ng/mL
5	Whole blood	FL	29	Male	N-ethyl-pentylone, 43.6 ng/mL

Figure 35. MRM chromatogram of a DBS specimen positive for N-ethyl-pentylone (16.9 ng/mL)



5. CONCLUSIONS

- Paper DBS are a good, reliable and interesting technique for drugs and NPS testing. Both qualitative and quantitative methods using DBS provided good results, with a few exceptions in the quantitative method (such as N-ethyl-hexedrone and methylone), enabling detection at low levels, increased stability and good correlation with whole blood testing results for different classes of drugs of abuse, including the NPS.

- The stability of drugs is not a limitation in DBS, but based in these findings, storage under low temperatures is suggested, as some drugs are not stable for long periods at room temperature.

- The main limitation of DBS is the need for a high sensitivity technique, such as QTOF or Triple Quadrupole Mass Spectrometers, whereas GC-MS is limited to the analysis of drugs at high concentrations.

- The method of DBS supported in salt is an interesting alternative approach for DBS, removing the paper and replacing it by a salt tablet. However, it is important to highlight that this concept require a high sensitive technique for the analysis and the process may be time-consuming.

- DBS offer many advantages that can help the rapid testing of novel synthetic drugs (such as designer benzodiazepines, opioids, synthetic cannabinoids, synthetic cathinones, phenetylamines and derivatives of PCP) as well as known drugs (as cocaine, MDMA, methamphetamine and LSD), or providing the possibility of shipping samples to other laboratories. This could be very helpful in forensic routine to analyze known and unknown drugs in a very fast, practical and low cost fashion.

CHAPTER 3. Analysis of 2,4-DNP in blood and urine using GC-MS

1. INTRODUCTION

2,4-DNP is a compound with many potential applications in industry. Over the last years, the abuse of 2,4-DNP due to its weight loss properties has become a serious concern, especially because of its severe adverse effects, including death. A number of deaths have been reported, making necessary the development and validation of a method for both qualitative and quantitative analysis of 2,4-DNP in biological specimens.

2. OBJECTIVES

Development and validation of analytical method using GC-MS for qualitative and quantitative analysis of 2,4-DNP in blood and urine and analysis of six postmortem specimens.

3. MATERIALS AND METHODS

3.1. Reagents and materials

Reference material of 2,4-DNP at a concentration of 5 mg/mL in methanol was obtained from Supelco (Bellefonte, PA, USA). Stable-isotope reference material 2,4-DNP-d_3 at 1 mg/mL in methanol was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TCMS) was purchased from Cerilliant (Round Rock, TX, USA). N-butyl-chloride was obtained from Honeywell (Morris Plains, NJ, USA). Hydrochloric acid was purchased from VWR Chemicals (Radnor, PA, USA). Human blank blood was purchased from Bio IVT (Hicksville, NY, USA).

3.2. Standard solutions, controls and calibrators

Standard stock solutions of 2,4-DNP were prepared at concentrations of 1000 mg/L and 100 mg/L in methanol, by diluting the reference material, and stored at −20°C in amber glass vials. Standard stock solution of 2,4-DNP-d_3 at concentration of 100 mg/L in methanol was prepared from the reference material by dilution, and stored at −20°C in amber glass vials. Calibrators at concentrations of 3, 5, 10, 30, 50 and 100 mg/L were prepared by adding aliquots of standard stock solutions into 500 µL of blank blood. Quality controls were prepared at concentrations of 3 mg/L (LLOQ), 8 mg/L (LQC) and 80 mg/L (HQC) were prepared in 500 µL of human blood or urine.

3.3. Sample preparation

500 µL of blood or urine were spiked with 50 µL of 100 µg/mL 2,4-DNP-d_3 solution and agitated using vortex. 1 mL of 0.1 M HCl and 3 mL of n-butyl-chloride were added to the samples and extraction was performed using orbital-agitation for 15 min at room temperature. Samples were then centrifuged for 5 min at 3500 rpm at 10°C, the supernatant was transferred to a clean glass tube and evaporated to dryness under a 12-psi compressed air flow at 35°C. The dried extract was reconstituted with 100 µL BSTFA-1% TCMS and derivatized for 30 min at 80°C. After derivatization, the mixture was transferred to a clean glass vial, capped using a crimper and 2 µL were injected into the GC-MS system.

3.4. GC-MS analysis

GC-MS analyzes were performed using an Agilent 7890A gas chromatograph coupled to Agilent 5975C mass spectrometer (Palo Alto, CA, USA). Hydrogen was used as carrier gas. The flow rate was set at 1.6 mL/min. A HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm) was used for chromatographic separation. GC operated in splitless mode and using a temperature program, starting at 100°C, with a holding time of 1 min, and then increased to 250°C, at 30°C/min, with a final hold time of 2.5 minutes. Total run time was 8.5 minutes. MS operated by electron impact ionization mode. Temperatures of injector, MS source and MS quadrupole were set to 265, 230 and 150°C, respectively. Data was acquired using Selected Ion Monitoring (SIM). The following ions were monitored: m/z 137, 195 and 241 for 2,4-DNP and m/z 198 and 244 for 2,4-DNP-d_3.

3.5. Method Validation

Analytical validation followed ASB guidelines (ASB, 2017). Calibration model, accuracy, precision, LLOQ, LOD, dilution integrity, selectivity, carryover and stability were assessed. Calibration model was evaluated over 5 days using a 6-point calibration curve and acceptable correlation coefficient was higher than 0.99. Accuracy and precision were assessed over 5 days for LLOQ, LQC and HQC. For precision, acceptable values were equal or lower than 15%. For accuracy, acceptable bias were equal or lower than 15% for the LLOQ and lower than 20% for LQC and HQC. LOD and LLOQ were tested over 3 days, using 3 different sources of blank blood in duplicate. Acceptance criteria for LOD was a signal-to-noise ratio of 3:1 and an acceptable target ion-ratio. For LLOQ, criteria were an acceptable target ion-ratio and precision equal or lower than 15% and accuracy equal or lower than 20%. Dilution integrity was assessed over 3 days by dilution of the highest calibrator (100 mg/L) 2 and 10 times, in triplicate, and analyzing precision and accuracy.

Recovery was performed for LQC and HQC, in triplicate. The assays were conducted as described in Section 3.2.5. Potential matrix interferences for 2,4-DNP were assessed using 10 different sources of blank blood. Interferences from the target compound or the ISTD were investigated by the analysis of 5 blank samples containing 2,4-DNP or 2,4-DNP-d_3 only. Potential interferences from over than 20 commonly found drugs in toxicology casework were investigated by the analysis of 3 blank blood samples spiked with a mix containing all these drugs at a high concentration. Carryover was

evaluated by injecting an extracted blank sample after the analysis of the highest calibrator (100 mg/L).

Long-term stability of 2,4-DNP in blood and urine samples were performed for LQC and HQC, after 24h, 3 days, 7 days, 15 days and 30 days, for samples kept at room temperature, refrigerated (4°C) and frozen (-20ºC). Post-processing stability was assessed with re-analysis of processed samples after 12h in autosampler at room temperature, comparing the quantification results at time zero and after 12h.

3.6. Authentic Specimens

Blood and urine specimens collected from cases suspected to be related to weight loss drugs use or specifically to 2,4-DNP use, based on investigation findings and medical reports, were obtained through a call made to the National Association of Medical Examiners (NAME) and to NMS Labs (Willow Grove, PA). Medical Examiner's Offices in the USA and NMS Labs (Willow Grove, PA, USA) gently provided specimens to CFSRE laboratory for testing. Blood and urine specimens were collected from six death investigations occurred between 2016 and 2018, in Minnesota, Georgia, North Carolina, Florida, California and Texas. Specimens were analyzed for 2,4-DNP, using the method described above, and were also screened for other illicit, therapeutic and emerging drugs (alkaline or neutral compounds) by UPLC-QTOF (MOHR; FRISCIA; LOGAN, 2016).

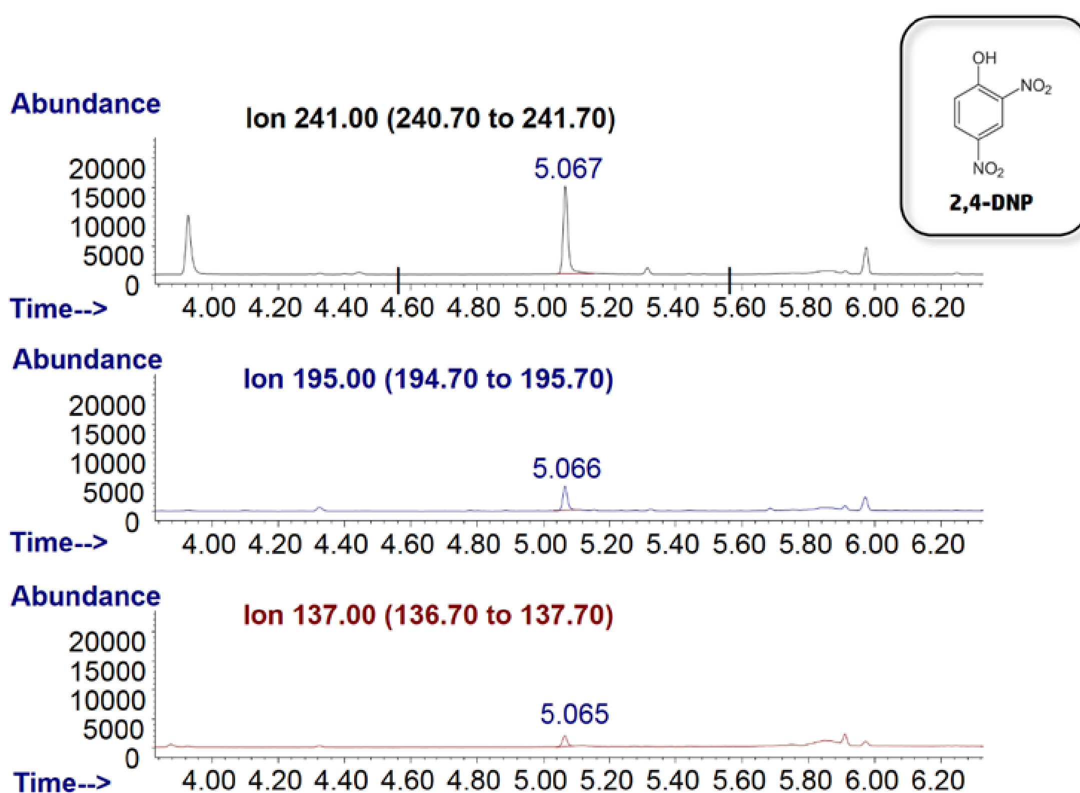
4. RESULTS AND DISCUSSION

4.1. Method Development

The analysis of nitrophenols, such as 2,4-DNP, can be properly performed by GC, with appropriate sensitivity. However, the chemical structure of nitrophenols, especially with the presence of hydroxyl groups, can cause the interaction of these compounds with active sites on the capillary column or the injector (KIM et al., 1993). The consequence is the retention of nitrophenols in the GC system and the reduction of sensitivity. In this context, for method development, two different strategies were tested: the analysis of 2,4-DNP with and without derivatization using helium and hydrogen as carrier gases. The detection of underivatized 2,4-DNP occurred only at elevated concentrations (higher than 100 mg/L) and using helium as carrier gas. The analytical performance using derivatization with BSTFA (with 1% TCMS) was improved, resulting in better sensitivity and chromatography. Two different volumes of BSTFA (50 and 100 µL) and the use of

ethyl acetate for reconstitution of 2,4-DNP prior derivatization were tested. The best results were found by the direct reconstitution with 100 µL of BSTFA, without any additional solvents. A chromatogram of derivatized 2,4-DNP obtained by GC-MS analysis using hydrogen as carrier gas is showed in **Figure 36**.

Figure 36. Selected ion chromatograms of 2,4-DNP (at 5.067 minutes) obtained from a blood sample



Another consideration is regarding the IS. Initially, 4-nitrophenol was used as ISTD for GC-MS analysis. However, after several quantitative tests, it was observed that 4-nitrophenol affected precision and bias. For this reason, a deuterated ISTD, 2,4-DNP-d_3 has been used in replacement to 4-nitrophenol.

4.2. Method Validation

The method presented acceptable analytical performance, according to validation guidelines. The calibration model presented the best fitting by quadratic polynomial method with 1/x weighting factor and an average correlation coefficient of 0.998 (3 – 100 mg/L). Method showed acceptable precision and accuracy in blood (**Table 26**).

Table 26. Average accuracy and precision assays

	LLOQ	LQC	HQC
Intra-assay imprecision (% CV)	3.81	3.56	10.7
Inter-assay imprecision (% CV)	5.85	3.68	10.6
Bias (%)	1.18	6.97	8.29

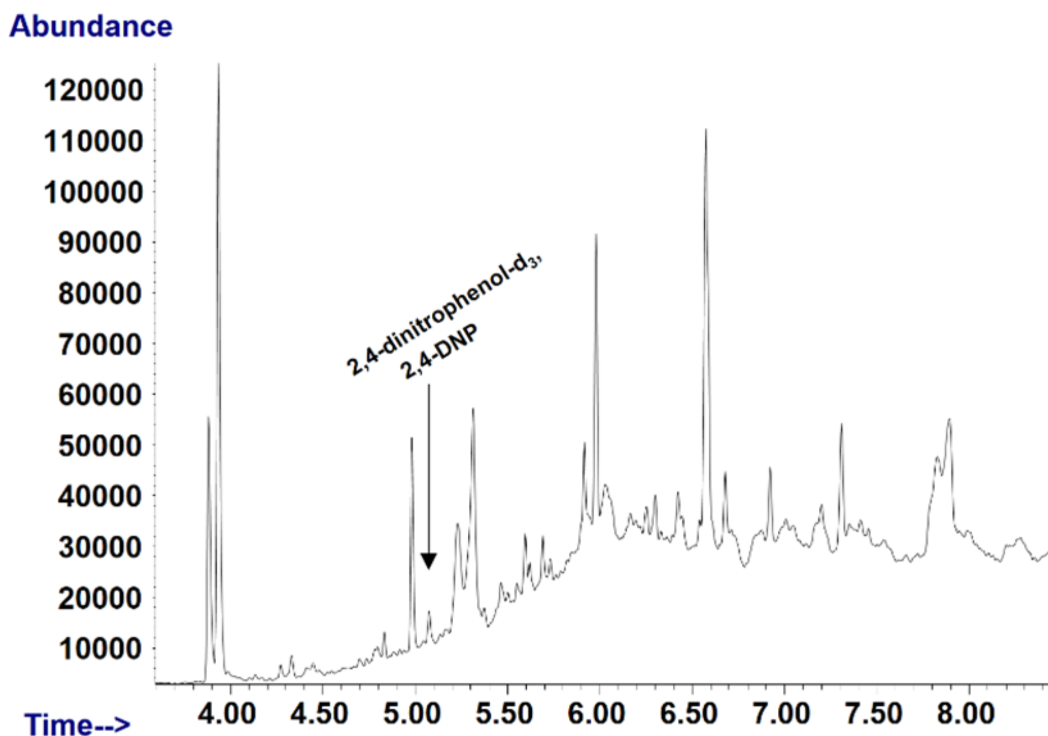
For urine, a matrix matching approach was adopted and LLOQ and both LQC and HQC were quantitated using a calibration curve prepared with blood calibrators. Method also presented acceptable precision/accuracy in urine analysis, with average biases of -3.88% at the LLOQ, -19.3% at the LQC and -11.0% at the HQC. The intra-assay imprecision in urine was 4.26% for the LLOQ, 3.24% for the LQC and 1.74% for the HQC. These findings support the use of a matrix matching approach for quantitation of 2,4-DNP in urine specimens by the use of calibration curve made with blood calibrators. The dilution controls presented acceptable bias and imprecision after dilution two and ten times (**Table 27**).

Table 27. Dilution integrity assays: results

	Blood			Urine		
Dilution	**Bias (%)**	**Inter-Assay Imprecision (% CV)**	**Intra-Assay Imprecision (% CV)**	**Bias (%)**	**Inter-Assay Imprecision (% CV)**	**Intra-Assay Imprecision (% CV)**
1:2	6.24	6.54	10.7	16.1	12.3	9.44
1:10	2.43	4.82	6.09	19.7	14.5	12.8

The sample preparation method using HCl and n-butyl-chloride exhibited a satisfactory efficiency of 92.1% (84.6% for LQC and 99.6% for HQC). Regarding potential interferences, no endogenous components from blood or from the stable-isotope internal standard, 2,4-DNP-d_3, were detected. In addition, no interferences from common drugs of abuse, therapeutic drugs or pesticides were detected **(Figure 37)**. No carryover was detected by injecting an extracted blank sample after running the highest calibrator (100 mg/L).

Figure 37. Chromatogram of blood samples spiked with drugs of abuse, medical drugs and pesticides, showing no interferents peaks for 2,4-DNP or the ISTD.



4.3. Stability Studies

2,4-DNP showed a 12h post-processing stability of 97.9% for LQC and 116% for HQC. Results from stability studies were plotted using Statistica® software and summarized in **Figure 38**. 2,4-DNP was found stable at 4ºC or -20ºC in both blood and urine, showing a slight reduction in the analytical response after 14 days of storage. However, at room temperature, 2,4-DNP shows instability, with a decrease in the response overtime in blood and urine.

4.4. Authentic Specimens Analysis

The analyzes of the six cases with 2,4-DNP suspect involvement revealed three cases positive for 2,4-DNP. The additional three cases from Florida, California and Texas had no 2,4-DNP present in the specimens available for analysis. Illustrative chromatograms authentic postmortem specimens positive or negative for 2,4-DNP are presented in **Figures 39** and **40**.

Figure 38. Results of 2,4-DNP stability studies in blood and urine specimens: (A) blood, at room temperature; (B) blood, at 4ºC; (C) blood, at – 20ºC; (D) urine, at room temperature; (E) urine, at 4ºC; (F) urine, at -20ºC.

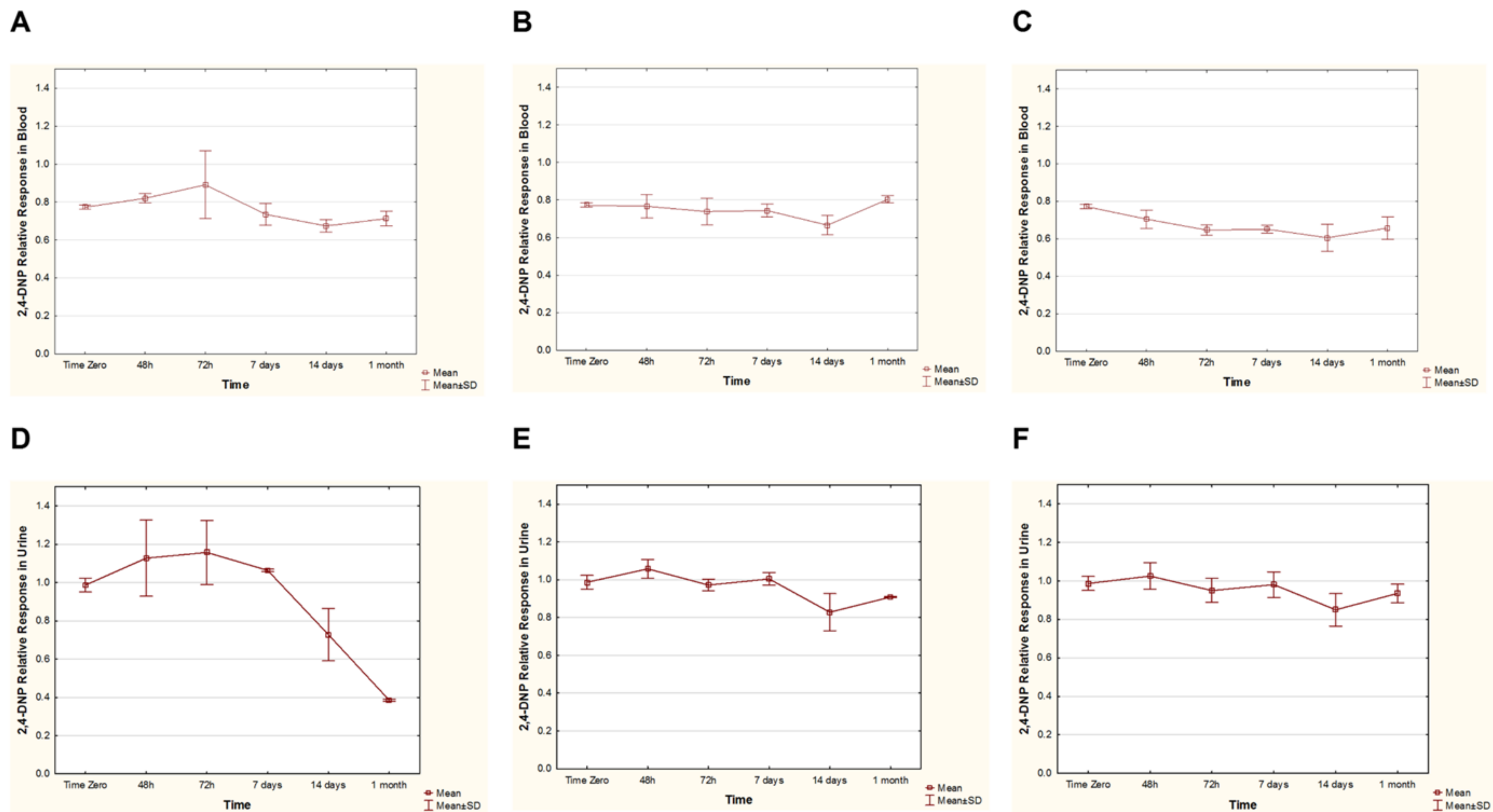


Figure 39. Selected ion chromatograms of authentic postmortem specimens (from Case 2) positives for 2,4-DNP: (A) iliac blood at 14.6 mg/L and (B) urine at 89.5 mg/L.

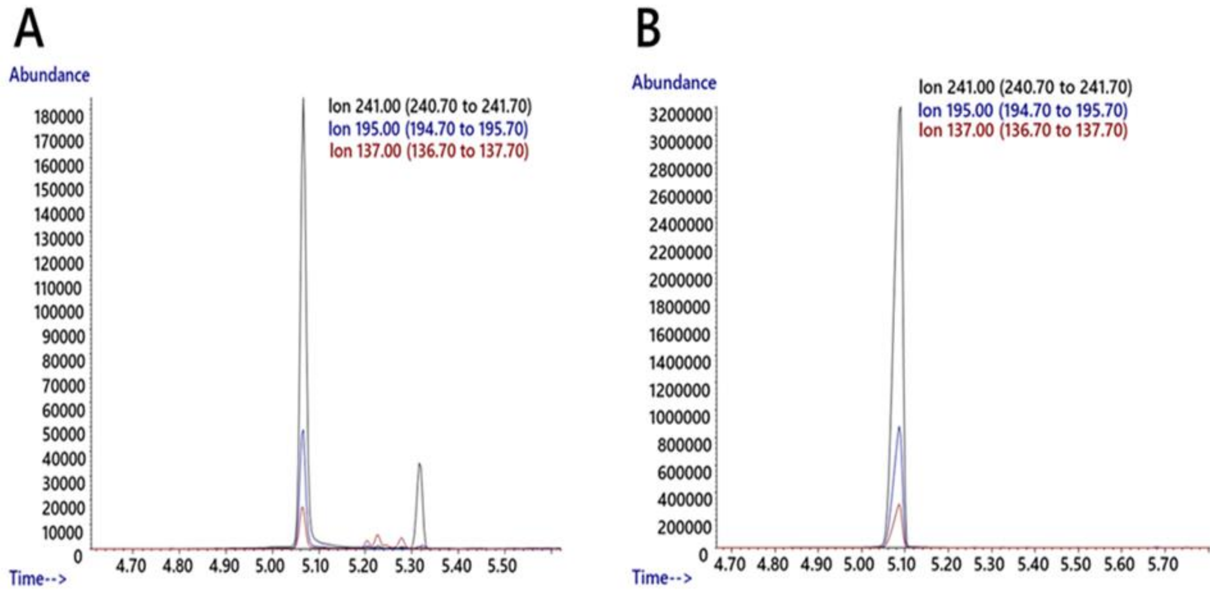
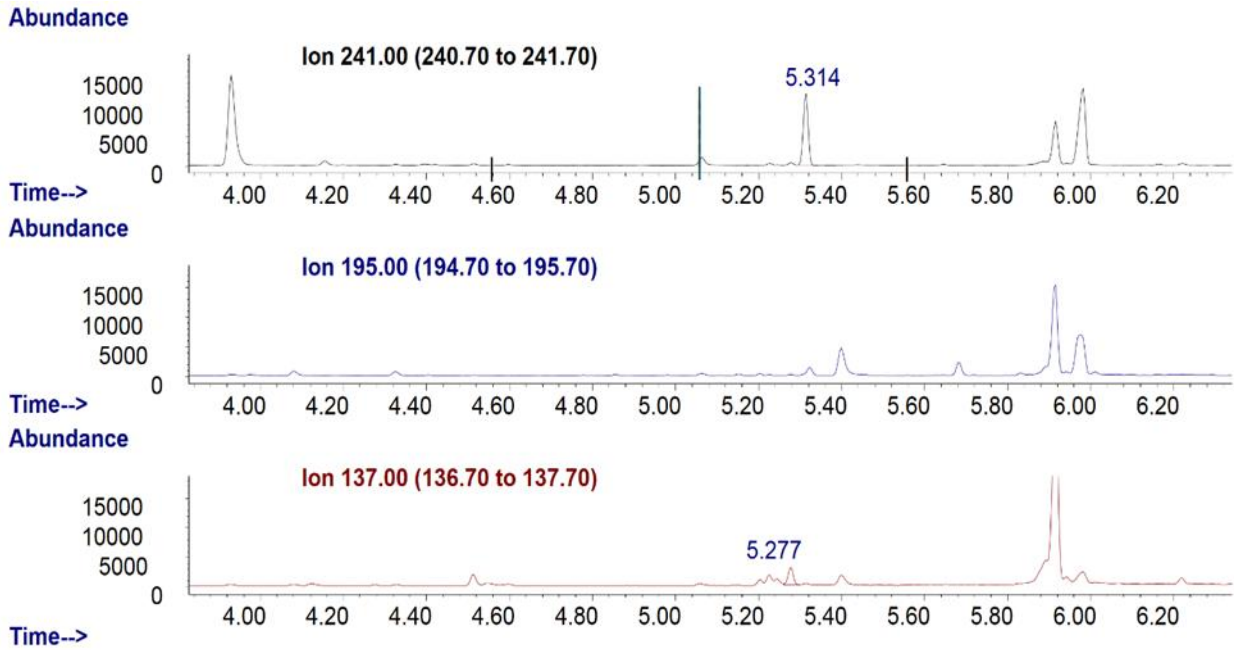


Figure 40. Selected ion chromatograms of authentic postmortem blood specimen (from Florida, USA) negative for 2,4-DNP.



4.4.1. Case 1 (Minnesota, USA)

A 21-year-old male presented to the hospital after the ingestion of about 3 mL of a "wintergreen oil". He showed shortness of breath, sweating, tachycardia and elevated body temperature, then suffered a cardiac arrest and could not be resuscitated. The individual had a past overdose after using the supposed novel opioid agonist MT-45 and 2-methyl-2-butanol (2M2B), purchased online. After his death, a relative stated that the decedent had been consuming a thermogenic supplement in the two weeks before the death and reported feeling hot. Investigations in the decedent's home found a bottle of 2,4-DNP with a label from "Aldrich" company. Analysis revealed 2,4-DNP in specimens collected at the admission and during the autopsy. Concentrations of 2,4-DNP in the admission blood and urine were 114 and 220 mg/L, respectively. The urine specimen collected at admission presented a very bright yellow coloration. 2,4-DNP concentrations in postmortem blood and urine were 68.2 mg/L and 79.2 mg/L. Methylphenidate was also found in postmortem blood and urine specimens.

4.4.2. Case 2 (Georgia, USA)

A male was found death with suspect of 2,4-DNP involvement. Iliac blood and urine specimens collected from autopsy were both positive for 2,4-DNP. The concentrations of 2,4-DNP in blood and urine were 14.6 mg/L and 89.5 mg/L, respectively. Additional analysis revealed temazepam, norclozapine, alprazolam, lidocaine, diazepam, venlafaxine, nordiazepam, boldenone, hydroxyzine and O-desmethylvenlafaxine in postmortem blood. In postmortem urine, hydroxyzine, monoethylglycinexylidide (MEGX), venlafaxine, norclozapine, boldenone, lidocaine, alprazolam, nordiazepam, O-desmethylvenlafaxine, diazepam, lamotrigine and temazepam were also found. An interesting finding in both postmortem blood and urine was the presence of boldenone, an anabolic-androgenic steroid (AAS) used in bodybuilding.

4.4.3. Case 3 (South Carolina, USA)

A female died and had 2,4-DNP in her purse. She stated allegedly been using 2,4-DNP for weight loss. Analysis of postmortem blood and urine revealed the presence of 2,4-DNP. Concentration of 2,4-DNP in postmortem urine was 61.6 mg/L. In blood, 2,4-DNP was detected below the LLOQ (at 3 mg/L). Additional findings in postmortem blood

were the presence of naloxone, lorazepam, venlafaxine and O-desmethylvenlafaxine. In postmortem urine, lorazepam, naloxone, venlafaxine, O-desmethylvenlafaxine and caffeine were also found.

5. CONCLUSIONS

The abuse of 2,4-DNP over the last years is another component of the complex problem of the emergence of novel drugs of abuse. There are many reports in the media showing death cases related to 2,4-DNP and for this reason medical examiners and toxicologists need to be aware of the possibility of this compound in routine analysis. The method developed and validated for analysis of 2,4-DNP is an useful tool for investigation of this compound in blood and urine, consisting in a simple, fast and sensitive method. The use of deuterated IS increased the analytical performance of the method and it is well recommended over other potential IS. Regarding the analysis of authentic specimens, although the limited number of cases analyzed in this work, the range of concentrations is wide, which can be attributed to the dose administered, time between ingestion and death and the purity of 2,4-DNP in the ingested material.

CHAPTER 4. *In vitro* metabolism of novel synthetic cathinones

1. INTRODUCTION

Synthetic cathinones are one of the most commonly reported classes of NPS. Many countries have synthetic cathinones found in forensic casework, in seized drug and toxicological samples. In 2018 alone, the Center for Forensic Science Research and Education (CFSRE), in Willow Grove, PA, United States, identified seven novel synthetic cathinones, in seized material. Some of these drugs included N-ethyl-hexylone, tertylone, 3,4-DCEC and 4-MDEC. Although not all these drugs have been found in toxicological samples to date, this does not exclude the possibility of people using these drugs, intentionally or not (as a contaminant of other drugs) in the future. Another synthetic cathinone commonly reported by Drug Enforcement and Administration (DEA) in seized samples is N-ethyl-hexedrone, and an intoxication with this drug and ADB-FUBINACA was recently reported (KOVÁCS ET AL., 2019). In this context, it is important to collect as much information as possible regarding these cathinones, including their analytical performance and metabolic profile in biological systems, which can help in the investigation of in vivo or post mortem cases in the future.

2. OBJECTIVES

The goal of this study was to study the metabolism of 3,4-DCEC, 4-MDEC, N-ethyl-hexylone, N-ethyl-hexedrone and tertylone using HLM and HRMS.

3. MATERIAL AND METHODS

3.1. Reagents, materials and solutions

HLM pooled from 50 different individual donors, mixed genders (20 mg/mL) were purchased from ThermoFisher Scientific (Waltham, MA, USA) and stored at −80°C. N-ethyl-hexylone, N-ethyl-hexedrone and tertylone were purchased from Cayman Chemical (Ann Arbor, MI, USA) and prepared at 1 mg/mL in methanol. 3,4-DCEC and 4-MDEC were obtained from seized material and prepared at 1 mg/mL in methanol. Diazepam (1 mg/mL) was purchased from Cerilliant (Round Rock, TX, USA). Nicotinamide Adenine Dinucleotide Phosphate (NADPH) sodium salt was purchased from Cayman. Monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4) and magnesium chloride ($MgCl_2$) were obtained from Sigma-Aldrich (St Louis, MO, USA). LC-MS grade acetonitrile and water were purchased from Honeywell (Morris Plains, NJ, USA). Sodium hydroxide was obtained from VWR (Radnor, PA, USA). Phosphate buffer (100 mM) was prepared by mixing 1.7 g of Na_2HPO_4, 12.15 g of NaH_2PO_4 and 2.033 g of $MgCl_2$ in 1 L of LC-MS grade water; pH was adjusted to 7.4 with NaOH. NADPH solution was prepared by dissolving 16.7 mg of NADPH sodium salt in 2 mL of LCMS grade water and stored in refrigerator.

3.2. *In vitro* assays

The assays were performed with two reaction mixtures, a negative control (without NADPH) and a drug standard control (without NADPH and HLM). A set of reaction positive control was performed along with all assays. The assays were performed in triplicate, over three different days. For incubations, 5 µL of drug standard in methanol was dried and reconstituted in equivalent volume of phosphate buffer/acetonitrile mixture (1:1, v/v). Phosphate buffer (pH 7.4) and 5 µL of drug solution were added to appropriate test tubes. 50 µL of 10 Mm NADPH solution were added to reaction mixtures. 25 µL of HLM were added to all test tubes except for drug standard control. Samples were placed in water bath at 37°C, under agitation, and incubated for 2h. After incubation, metabolic reactions were stopped by adding 500 µL of LC/MS grade acetonitrile. Samples were transferred to microcentrifuge tubes and centrifuged at 10,000 rpm for 5 min. After centrifugation, samples were transferred to glass test tubes, dried at 35°C in TurboVap® for 10 to 20 min to remove some of the solvent and moved to microcentrifuge tubes with

filter. Samples were centrifuged at 10,000 rpm for 5 min and transferred to vials with glass inserts for analysis via UPLC-QTOF.

3.3. Instrumental analysis

Analyzes were performed via Shimadzu (Kyoto, Japan) Nexera XR Ultra High Performance Liquid Chromatograph coupled to SCIEX (Framingham, MA, USA) TripleTOF® 5600+ QTOF Mass Spectrometer. Chromatographic separation was performed a Phenomenex® Kinetex C18 column (50 mm × 3.0 mm, 2.6 µm) using a reverse phase gradient, at flow rate of 0.4 mL/min. According to a previously published method (Krotulski et al., 2018b), mobile phases used were ammonium formate 10 mM, pH 3 (A) and methanol/acetonitrile 50:50 (B) and the gradient conditions are summarized in **Table 28**.

Table 28. Gradient conditions of chromatographic method

Time (min)	MPA (%)	MPB (%)
Initial	95	5
13	5	95
15.5	95	5

QTOF operated under positive electrospray ionization. Acquisition and fragmentation of precursor ions were performed by TOF scan (35 ± 15 eV). Data acquisition and processing were performed by Information Dependent Acquisition (IDA).

IDA is based on data-dependent acquisition mode, capturing a single precursor ion in the first quadrupole (Q1) window. In this method, ion intensity was monitored and Q1 isolated the 16 most intense ions in a cycle (> 100 cps). IDA provides increased specificity and certainty in the identification of product ions (KROTULSKI et al., 2018b).

3.4. Data Processing

SCIEX PeakView® Software Version 2.2 was used for spectral analysis and data examination. SCIEX MetabolitePilot™ Software Version 2.0 was used for the identification of metabolites, using data generated from previous examination on PeakView®.

For the identification of metabolites, generic and compound-specific parameters were used. Drug standard controls were used to add the compound to the software library, including name, chemical formula, polarity, exact mass and reference spectrum (KROTULSKI et al., 2018b). A set of 46 Phase I biotransformations combined with specific software criteria for positive identification were used for the investigation of metabolism patterns in reaction mixtures. Data were processed according to the criteria and potential metabolites were sorted by percent peak area and combined score of mass defect, isotope pattern, MS/MS spectra and mass accuracy (KROTULSKI et al., 2018c). Criteria were as follows: percent peak area higher than 0.1% and combined score higher than 75%. In addition, retention time and TOF spectra were also analyzed to assess if the potential metabolite was consistent with chemical structure and if the proposed metabolic reaction was coherent (KROTULSKI et al., 2018b). Chemical structure of metabolites was generated by the software or manually, according to the proposed reaction and the structure of the parent compound. Fragments for each metabolite were highlighted. Metabolites that have structural isomers due to reactions occurring in different sites of the molecule were not distinguishable using this method but they were noted (KROTULSKI et al., 2018b).

4. RESULTS AND DISCUSSION

4.1. 3,4-DCEC

3,4-DCEC produced a protonated peak with mass of 246.0448 Da in 5.78 min **(Figure 41)**. The most prominent fragments found for 3,4-DCEC are 230.0485 Da, 150.0227 Da, 202.0180 Da, 193.0648 Da and 115.0540 Da **(Figure 42)**.

Figure 41. Chromatogram of a reaction mixture incubated with 3,4-DCEC

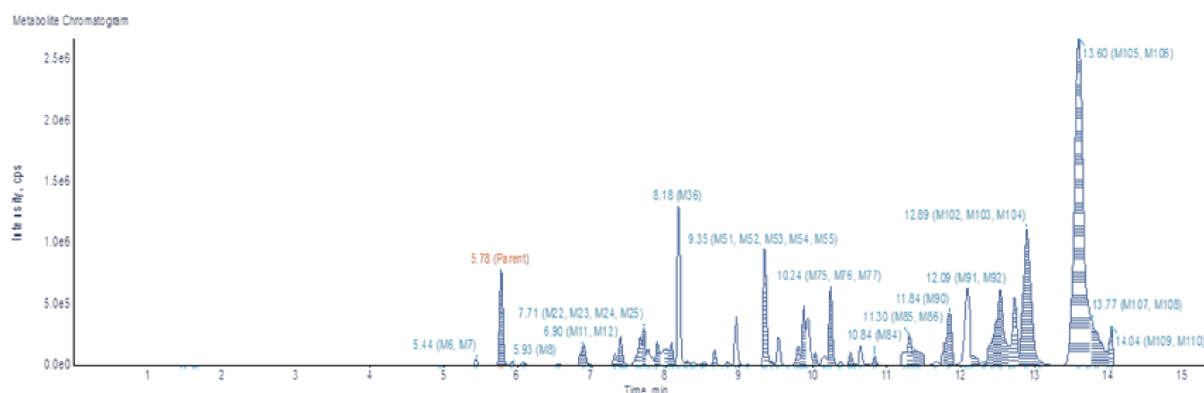
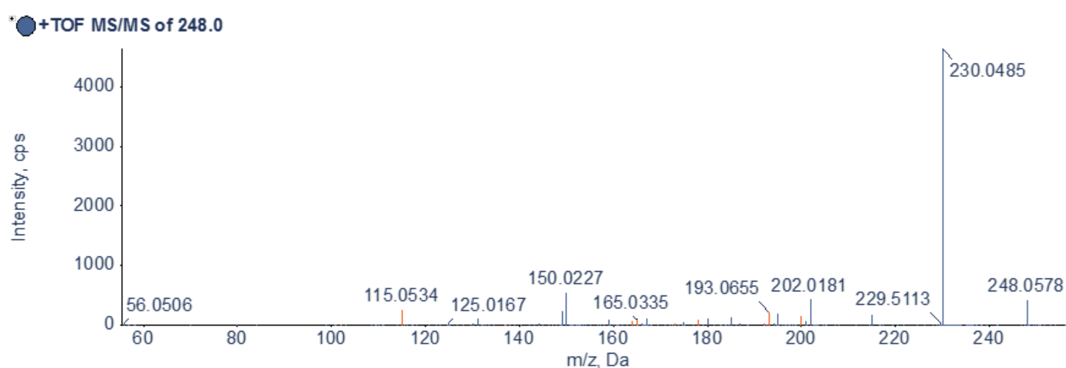


Figure 42. TOF-MS/MS spectra of 3,4-DCEC



In vitro assays showed three major metabolites for 3,4-DCEC. The results for all metabolites are summarized in **Table 29** and **Figure 43**. Metabolite 1 (**M1**), 2-amino-1-(3,4-dichlorophenyl)propan-1-one, was formed through N-deethylation, with loss of ethyl group (C_2H_4) by the parent compound. Metabolite 2 (**M2**), 1-(3,4-dichlorophenyl)-2-(ethylamino)propan-1-ol, was produced by reduction of carbonyl group of 3,4-DCEC to a hydroxyl group. The formation of metabolite 3 (**M3**), 1-(3,4-dichloro-2-hydroxy-phenyl)-2-(ethylamino)propan-1-one, occurred by oxidation in the aromatic ring, by introduction of hydroxyl group.

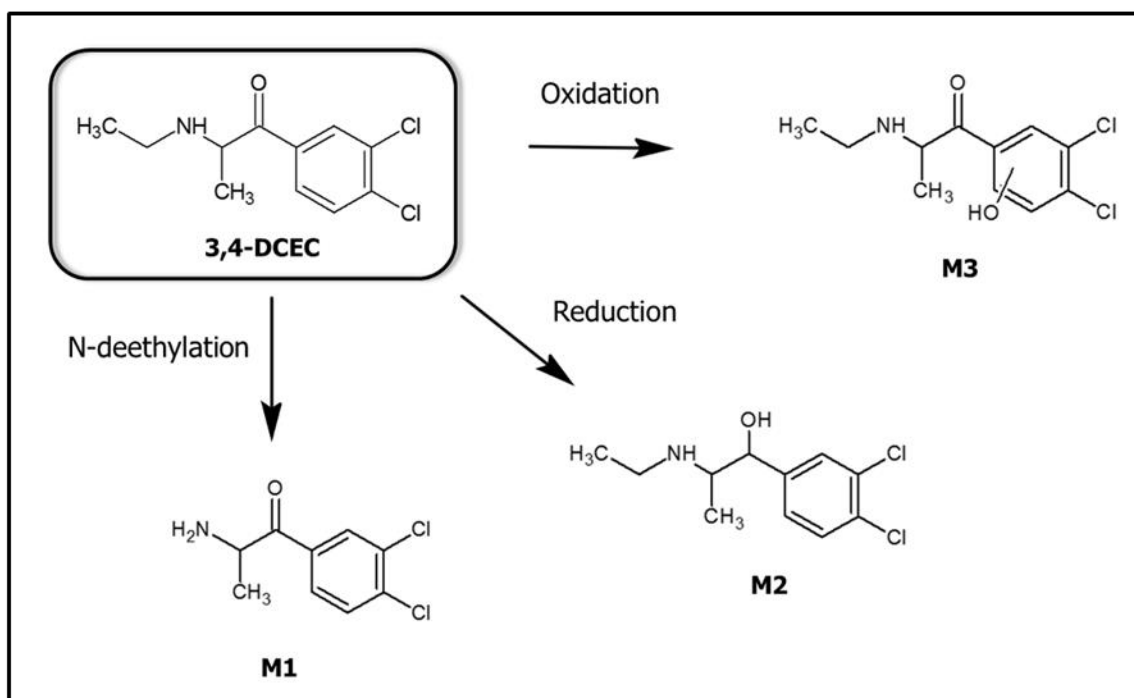
Table 29. Summary of in vitro metabolites of 3,4-DCEC

Metabolite	Name	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
	Parent	$C_{11}H_{13}Cl_2NO$	245.0374	245.0376	1.0	5.78	2.64×10^6	230.0485 150.0227 202.018 193.0648 115.054
M1	N-deethylation	$C_9H_9Cl_2NO$	217.0061	217.0065	1.9	5.44	1.18×10^5	165.034 202.000 174.989 164.025 102.046
M2	Hydrogenation (Reduction)	$C_{11}H_{15}Cl_2NO$	247.0531	247.0530	-0.3	5.93	6.83×10^4	230.0487 248.0596 150.023 202.0176 149.0143

Table 29. Summary of in vitro metabolites of 3,4-DCEC (conclusion)

Metabolite	**Name**	**Formula**	**$[M + H]^+$ (Da) Theoretical**	**$[M + H]^+$ (Da) Experimental**	**Mass error (ppm)**	**Average retention time (min)**	**Average peak area**	**Accurate fragment mass (Da)**
M3	Oxidation	$C_{11}H_{13}Cl_2NO_2$	261.0323	261.0326	1.1	8.08	1.91×10^4	172.9552 144.9599 262.0392 165.0334 209.0604

Figure 43. Proposed metabolism for 3,4-DCEC



Although no cases of 3,4-DCEC poisoning have been reported so far, the metabolism of 3,4-DCEC can be compared with other similar drugs. *In vitro*, 3,4-DCEC showed three major Phase I metabolites. Some of these metabolites were formed by the metabolic pathways as those metabolites of other similar cathinones, such as mephedrone, methcathinone and 3,4-dimethylmethicathinone (DMMC). **M1** is a major metabolite formed *in vitro* and the same reaction was also observed for DMMC (SHIMA et al., 2013) and mephedrone (POZO et al., 2015). The hydroxyl metabolite (**M2**) was formed through reduction of ketone group and the same reaction was also observed for methcathinone (PAUL; COLE, 2001), buphedrone, 4-methylbuphedrone, pentedrone, 4-methyl-*N*-ethylcatinone (4-MEC), N-ethylbuphedrone, ethcathinone and fluoromethcathinone (URALETS et al., 2014), 3,4-dimethylmethcathinone (DMMC) (SHIMA et al., 2013; URALETS et al., 2014), and mephedrone (URALETS et al., 2014; POZO et al., 2015). On the other hand, metabolites formed through *N*-deethylation followed by reduction of the ketone group were reported for DMMC and mephedrone in the literature. As this pattern of metabolism was not detected *in vitro* for 3,4-DCEC but reported for similar compounds, it is recommended to search for this metabolite *in vivo* or *post mortem*.

4.2. 4-MDEC

4-MDEC exhibited a peak in 5.01 min, corresponding to the protonated ion m/z 220.1699 **(Figure 44)**. The most intense fragments observed for 4-MDEC are: m/z 119.0848, 100.1119, 147.0802, 117.0697, 220.1701, 91.0543 and 72.081 **(Figure 45)**.

Figure 44. Chromatogram of a reaction mixture incubated with 4-MDEC

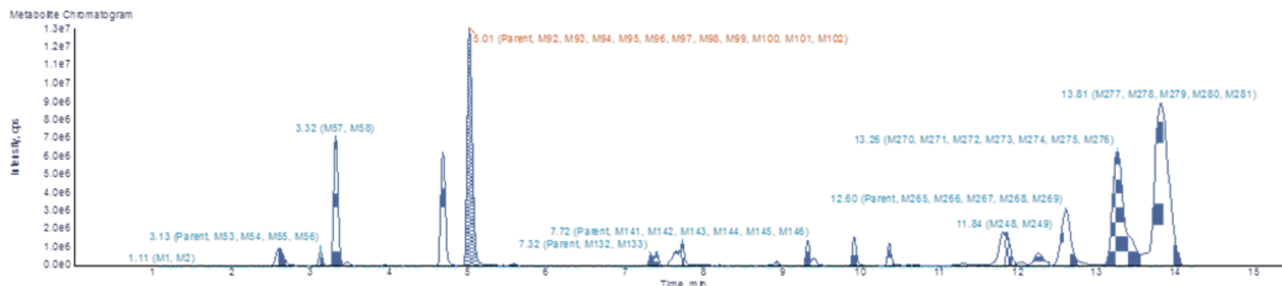
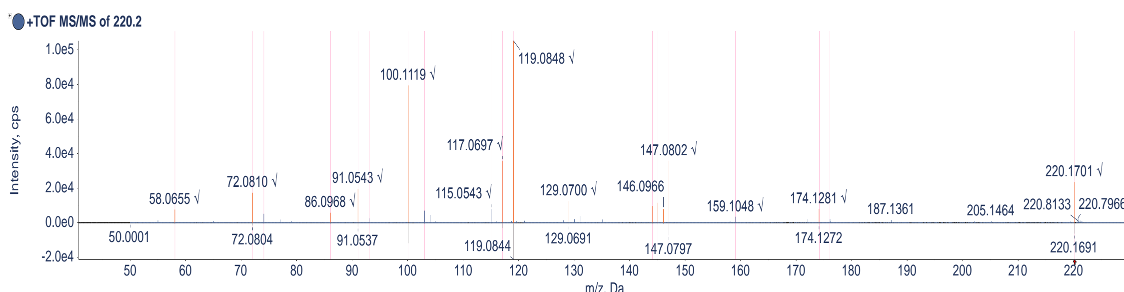


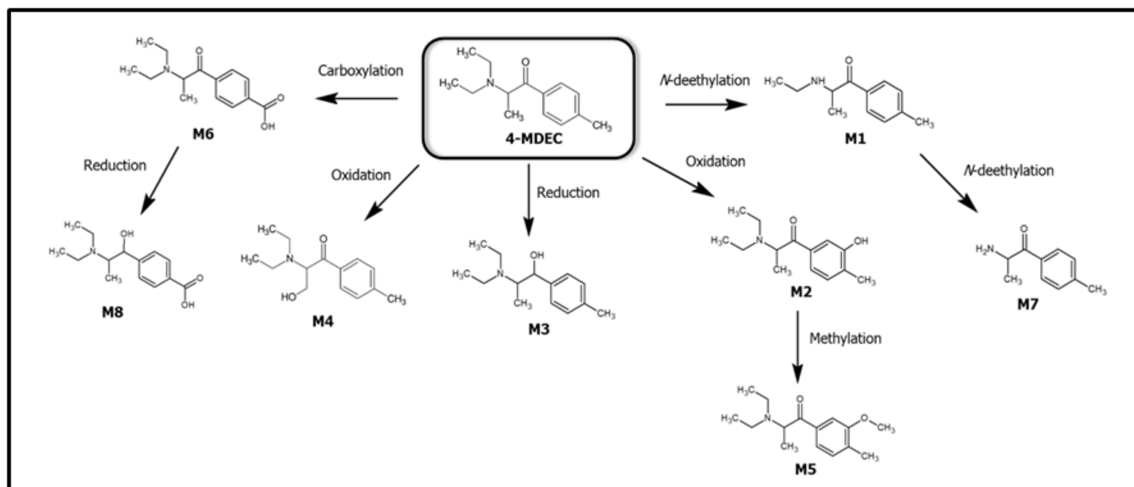
Figure 45. TOF-MS/MS spectra of 4-MDEC



Eight metabolites of 4-MDEC have been identified *in vitro*. The results for all metabolites are summarized in **Table 30** and **Figure 46**. **M1**, 2-(ethylamino)-1-(4-methylphenyl)propan-1-one, was biosynthesized through a N-deethylation reaction. The metabolite 2-(diethylamino)-1-(3-hydroxy-4-methylphenyl)propan-1-one, **M2**, was obtained by a oxidation reaction on aromatic ring. A third metabolite, **M3**, 2-(diethylamino)-1-(4-methylphenyl)propan-1-ol, resulted from a reduction reaction on carbonyl group, to a alcohol. Another proposed metabolite is **M4**, 2-(diethylamino)-3-hydroxy-1-(4-methylphenyl)propan-1-one, which was product of oxidation on the lateral carbonic chain. The metabolite 2-(diethylamino)-1-(3-methoxy-4-methylphenyl)propan-1-one (**M5**) was obtained by oxidation on aromatic ring followed by methylation on the hydroxyl group, generating a ether group bonded to the aromatic ring. **M6** (4-[2-(diethylamino)propanoyl]benzoic acid) was formed by demethylation followed by carboxylation on the aromatic ring of 4-MDEC. The minor metabolite **M7**, 2-amino-1-(4-methylphenyl)propan-1-one, was a product of eliminating the two ethyl groups on the nitrogen atom, resulting in a primary amine. Another minor metabolite (**M8**), 4-[2-

(diethylamino)-1-hydroxypropyl]benzoic acid, is a result of demethylation and carboxylation on aromatic ring combined with the reduction of carbonyl group to hydroxyl.

Figure 46. Proposed metabolism of 4-MDEC



The metabolites of 4-MDEC found *in vitro* share some similarities with other cathinones' metabolites. Four 4-MDEC metabolites (**M1, M3, M6** and **M7**) were formed by the same reactions as four mephedrone metabolites (POZO et al., 2015). Metabolites, **M1, M3, M6**, **M7** and **M8** share the same metabolic pathway as four metabolites of DMMC (SHIMA et al., 2013). The reduction of the ketone group that resulted in **M3** was also observed for cathinone and methcathinone (PAUL; COLE, 2001) and for buphedrone, 4-methylbuphedrone, pentedrone, 4-methyl-N-ethylcathinone, N-ethylbuphedrone, flephedrone and ethcathinone (URALETS et al., 2014).

Table 30. Summary of *in vitro* metabolites of 4-MDEC

Parent/Metabolite	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
Parent	$C_{14}H_{21}NO$	219.1623	219.1627	0.4	4.99	6.14×10^{7}	119.0848 100.1119 147.0802 117.0697 220.1701
M1	$C_{12}H_{17}NO$	191.1310	191.1313	1.1	4.66	1.48×10^{7}	144.0804 131.0734 119.0851 146.0956 159.1040
M2	$C_{14}H_{21}NO_2$	235.1572	235.1578	1.6	3.28	1.95×10^{7}	105.0697 100.112 133.0648 236.165 145.065

Table 30. Summary of in vitro metabolites of 4-MDEC (continuing)

Parent/Metabolite	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
M3	$C_{14}H_{23}NO$	221.1780	221.1778	-0.5	5.05	7.02×10^{6}	204.1747 189.1513 174.1278 131.0851 105.0698
M4	$C_{14}H_{21}NO_2$	235.1575	235.1578	2.0	5.41	2.00×10^{5}	88.0762 100.1125 236.1652 119.0849 60.045
M5	$C_{15}H_{23}NO_2$	249.1729	249.1733	1.3	3.93	1.59×10^{5}	250.1807 100.1119 147.0804 119.0853 149.0961

Table 30. Summary of in vitro metabolites of 4-MDEC (conclusion)

Parent/Metabolite	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
M6	$C_{14}H_{19}NO_3$	249.1365	249.1368	0.9	3.47	6.55×10^5	250.1442 105.0699 100.112 133.0641 149.0595
M7	$C_{10}H_{13}NO$	163.0997	163.0999	0.7	4.17	7.36×10^4	130.065 146.0966 119.0854 91.0543 117.0696
M8	$C_{14}H_{21}NO_3$	251.1521	251.1525	1.0	3.99	4.18×10^4	252.1597 88.0759 100.1122 135.0417 60.0441

4.3. N-ethyl-hexedrone

N-ethyl-hexedrone produced a protonated ion with mass 219.1619 Da (average retention time: 5.78 min), with the following fragments: 118.0639, 130.0639, 91.0532, 146.0952 and 145.0879 Da **(Figures 47** and **48)**.

Figure 47. Chromatogram of a reaction mixture incubated with N-ethyl-hexedrone

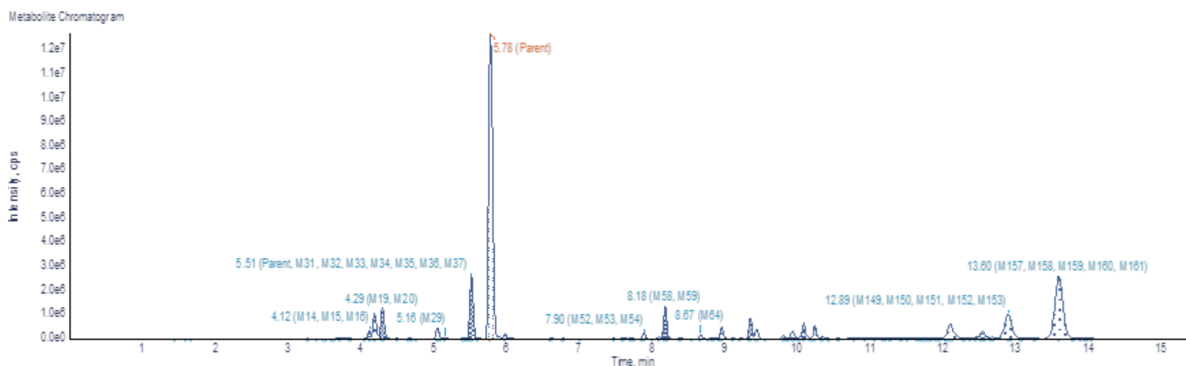
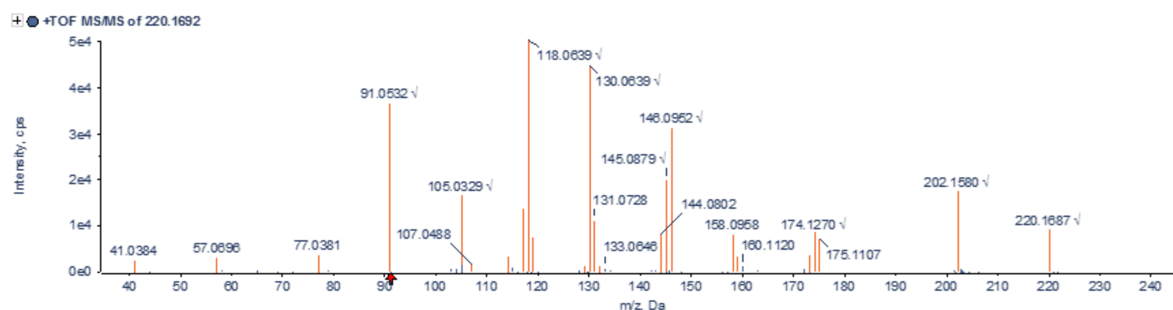


Figure 48. TOF-MS/MS spectra of N-ethyl-hexedrone



Five metabolites have been identified for N-ethyl-hexedrone (**Table 31** and **Figure 49**). Metabolite **M1** called 2-amino-1-phenylhexan-1-one was a product of N-deethylation, with loss of ethyl group. Metabolite **M2** is a product of the reaction of oxidation on aromatic ring, introducing a hydroxyl group at any of five carbon atoms available for bonding; an illustrative name for this metabolite, considering the substitution at third atom of carbon is 2-(ethylamino)-1-(4-hydroxyphenyl)hexan-1-one. Metabolite **M3**, (2Z)-2-(ethylamino)-1-phenylhex-2-en-1-one, was obtained by the desaturation at carbon number two, introducing a double bond in the molecule. The fourth metabolite, **M4**, 2-amino-1-phenylhexan-1-ol, is a product of the reaction of N-deethylation followed by reduction of ketone group to alcohol group. The last metabolite identified *in vitro* is **M5**, 2-(ethylamino)-1-[4-(hydroxymethyl)phenyl]hexan-1-one, which was product of

oxidation and methylation at the aromatic ring, introducing a hydroxymethyl group in the ring.

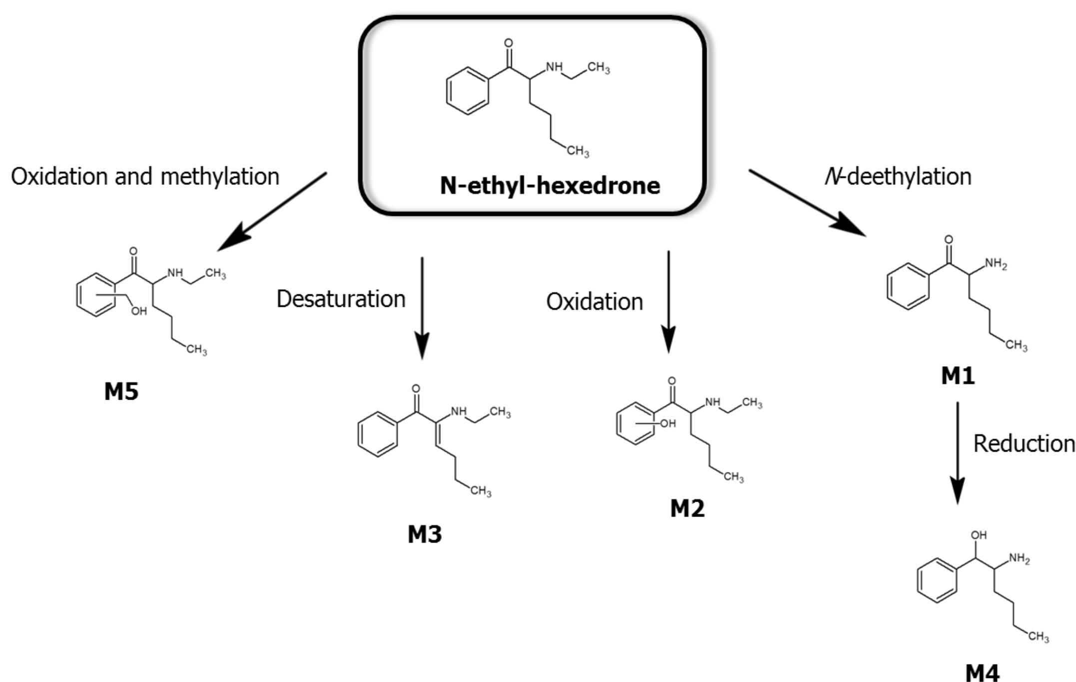
Table 31. Summary of *in vitro* metabolites of N-ethyl-hexedrone

Parent/Metabolite	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
Parent	$C_{14}H_{21}NO$	219.1623	219.1619	-1.2	5.78	4.21×10^{7}	118.0639 130.0639 91.0532 146.0952 145.0879
M1	$C_{12}H_{17}NO$	191.1310	191.1312	0.5	5.51	7.11×10^{6}	118.0644 91.0538 174.127 130.0646 117.0569
M2	$C_{14}H_{21}NO_2$	235.1572	235.1575	1.0	4.16	2.34×10^{6}	158.0961 218.1537 236.1642 162.0914 145.0995

Table 31. Summary of *in vitro* metabolites of N-ethyl-hexedrone (continuing)

Parent/Metabolite	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
M3	$C_{14}H_{19}NO$	217.1467	217.1469	0.6	5.41	$1.04{\times}10^5$	158.0961 218.154 130.0649 91.0541 144.0808
M4	$C_{12}H_{19}NO$	193.1466	193.1467	0.16	5.46	$4.53{\times}10^4$	176.1433 91.0542 117.0697 120.0801 106.0652
M5	$C_{15}H_{23}NO_2$	249.1728	249.1731	1.6	4.66	$3.93{\times}10^3$	202.1586 250.1818 214.1593 175.1115 187.1108

Figure 49. Proposed metabolism for N-ethyl-hexedrone



In vitro metabolism of N-ethyl-hexedrone has shown similarities with metabolic profile of other synthetic cathinones as 4-MDEC and 3,4-DCEC. The reduction of N-ethyl-hexedrone to an ephedrine derivative was observed in **M4**, as reported in cathinone (PAUL; COLE, 2001). The hydroxymethyl metabolite found for N-ethyl-hexedrone (**M5**) was also reported for mephedrone, as well as the *N*-deethylated metabolite (**M1**) and a hydroxyl metabolite (**M4**) (POZO et al., 2015). Metabolites obtained through the same reactions as **M1**, **M4** and **M5** were also reported for DMMC (SHIMA et al., 2013). The oxidation observed in the benzene ring of N-ethyl-hexedrone occurred in DMMC but introducing a carboxyl group instead a hydroxyl group (SHIMA et al., 2013).

4.4. Tertylone

Tertylone presented a protonated peak of 250.1441 Da at average retention time of 4.93 min **(Figure 50)**. The five most intense ions formed presented accurate masses of 118.0639, 176.0698, 147.0420, 57.06940 and 194.0805 Da **(Figure 51)**.

Figure 50. Chromatogram of a reaction mixture incubated with tertylone

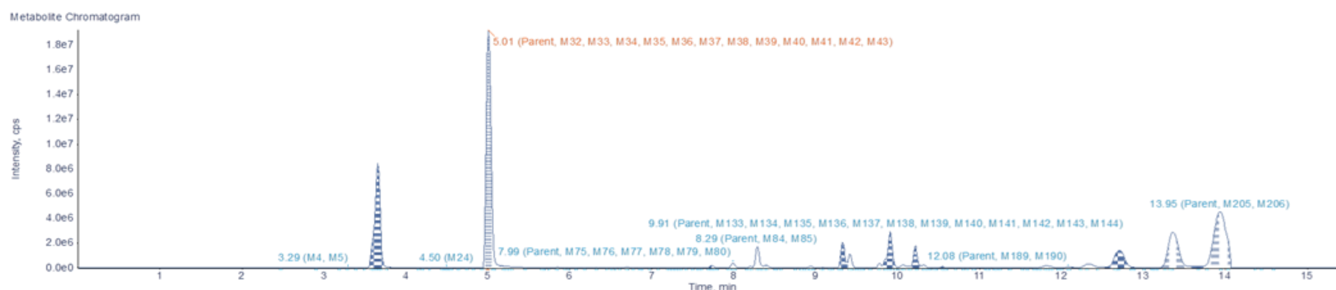
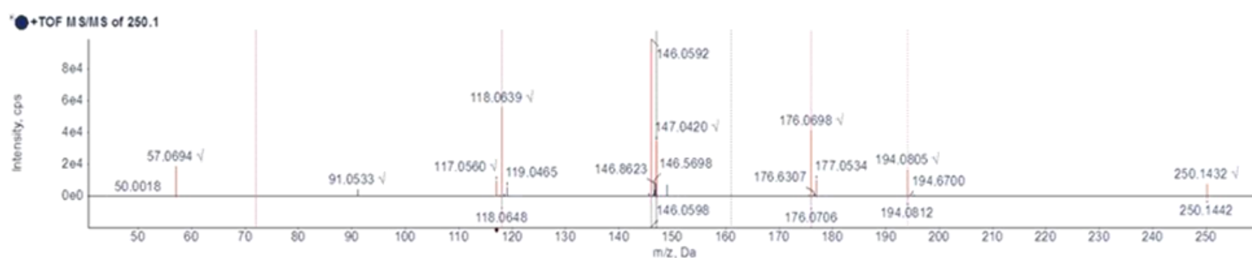


Figure 51. TOF-MS/MS spectra of tertylone



Tertylone presented five major Phase I metabolites *in vitro* (**Figure 52 and Table 32**). **M1** named 2-(*tert*-butylamino)-1-(3,4-dihydroxyphenyl)propan-1-one was produced by demethylenation, with break of methylenedioxy bond in the aromatic part of the molecule. A second metabolite, **M2** (2-amino-1-(3,4-dihydroxyphenyl)propan-1-one) was obtained by demethylenation and *N*-dealkylation, with loss of tert-butyl group bonded to nitrogen atom. **M3**, 2-amino-1-(1,3-benzodioxol-5-yl)propan-1-one, was obtained by loss of tert-butyl group bonded to nitrogen atom, converting the tertiary amine into a primary amine. A minor metabolite, **M4**, (1-(1,3-benzodioxol-5-yl)-2-(tert-butylamino)-3-hydroxypropan-1-one), is product of oxidation occurred at side chain of tertylone, specifically on methyl group. Another minor metabolite (**M5**) called 1-(1,3-benzodioxol-5-yl)-2-(tert-butylamino)prop-2-en-1-one resulted from a desaturation at side chain of tertylone.

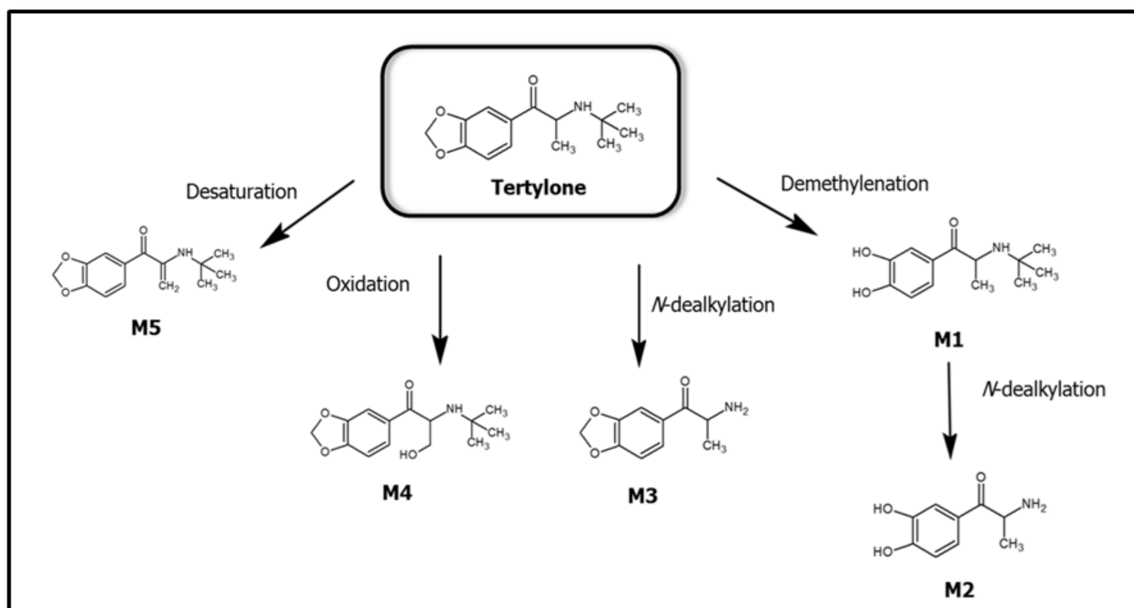
Table 32. Summary of *in vitro* metabolites of tertylone

Parent/Metabolite	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
Parent	$C_{14}H_{19}NO_3$	249.1364	249.1369	0.1	4.93	7.49×10^7	118.0639 176.0698 147.042 57.06940 194.0805
M1	$C_{13}H_{19}NO_3$	237.1364	237.1371	1.7	3.59	1.58×10^7	146.0593 118.0642 164.0700 57.06960 117.0566
M2	$C_9H_{11}NO_3$	181.0738	181.0736	-0.08	3.59	3.21×10^5	118.0641 146.0594 117.0562 164.0697 91.0533

Table 32. Summary of *in vitro* metabolites of tertylone (conclusion)

Parent/Metabolite	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
M3	$C_{10}H_{11}NO_3$	193.0739	193.0736	-1.6	5.5	1.16×10^5	146.0597 118.0645 117.0564 176.0703 91.0537
M4	$C_{14}H_{19}NO_4$	265.1314	265.1308	0.9	5.82	7.18×10^4	210.0764 192.0652 175.0621 162.0538 149.0225
M5	$C_{14}H_{17}NO_3$	247.1208	247.1206	-1.05	4.21	3.21×10^4	248.1281 149.059 119.0478 146.0583 176.0707

Figure 52. Proposed metabolism for tertylone



Tertylone presented an *in vitro* metabolism similar to N-ethyl-pentylone. Metabolites **M1** and **M3** of tertylone were formed through demethylenation and N-dealkylation, respectively, reactions that were also observed for N-ethyl-pentylone (KROTULSKI et al., 2018c). The hydroxylation of tertylone (**M4**) was also found for N-ethyl-pentylone (KROTULSKI et al., 2018c) and, in both cathinones, this is a minor metabolite. As these metabolites of N-ethyl-pentylone were also found in authentic blood and oral fluid specimens, it is likely that the metabolites found for tertylone can be found in authentic specimens as well.

4.5. N-ethyl-hexylone

N-ethyl-hexylone exhibited a protonated peak with mass 263.1524 Da at average retention time of 5.89 min, and the major fragments were m/z 135.0430, 216.1373, 189.0804, 114.1267 and 149.0224 Da **(Figures 53** and **54)**.

Figure 53. Chromatogram of a reaction mixture incubated with N-ethyl-hexylone

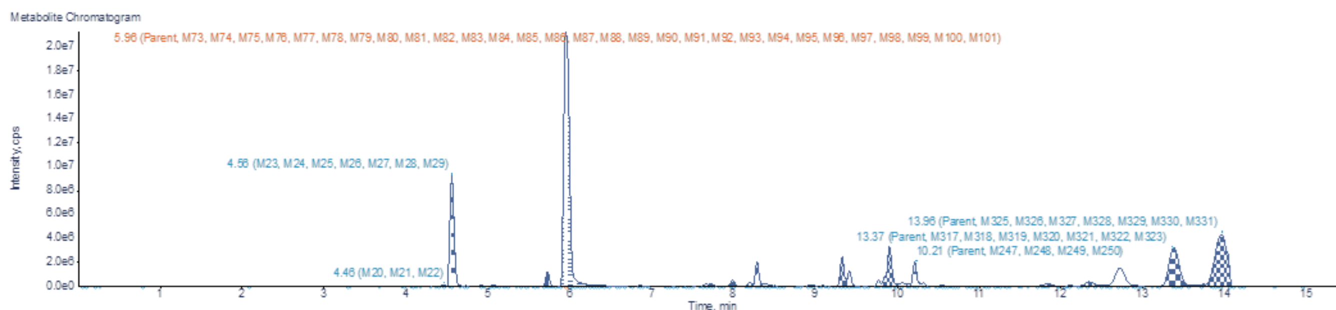
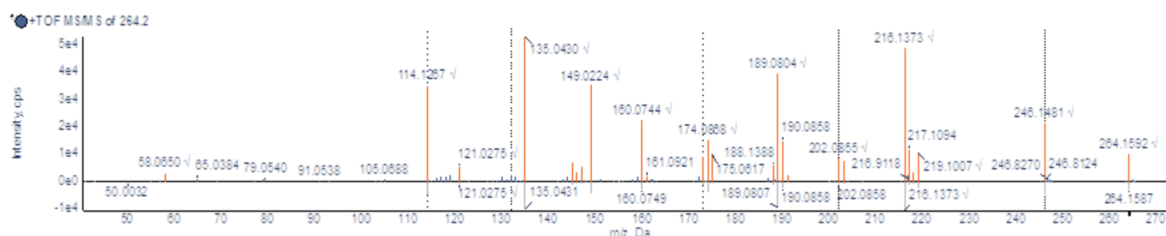


Figure 54. TOF-MS/MS spectra of N-ethyl-hexylone



Six major Phase I metabolites have been identified for N-ethyl-hexylone (**Figure 55**). Metabolite **M1**, 1-(3,4-dihydroxyphenyl)-2-(ethylamino)hexan-1-one, was formed through demethylenation, in the methylhenedioxy ring. Metabolite **M2**, 2-amino-1-(1,3-benzodioxol-5-yl)hexan-1-one, was a product of *N*-deethylation. Metabolite **M3**, 1-(1,3-benzodioxol-5-yl)-2-(ethylamino)-4-hydroxyhexan-1-one, was a product of oxidation on side chain, introducing a hydroxyl group to the molecule. The metabolite **M4** named 2-amino-1-(3,4-dihydroxyphenyl)hexan-1-one is a combination of previously described metabolic reactions, demethylenation and *N*-deethylation. Metabolite **M5** (2-amino-1-(1,3-benzodioxol-5-yl)-5-hydroxypentan-1-one) was product of N-deethylation followed by substitution of terminal methyl group on side chain by a hydroxyl group. Metabolite **M6** (1-(1,3-benzodioxol-5-yl)-2-(ethylamino)hexan-1-ol) was obtained by the reduction of ketone group in the parent structure to a hydroxyl group. The summary of all six metabolites is presented at **Table 33**.

Table 33. Summary of *in vitro* metabolites of N-ethyl-hexylone

Parent/Metabolite	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
Parent	$C_{15}H_{21}NO_3$	263.1521	263.1524	-0.2	5.89	8.66×10^7	135.043 216.1373 189.0804 114.1267 149.0224
M1	$C_{14}H_{21}NO_3$	251.1521	251.1528	2.0	4.26	1.08×10^7	123.0434 177.0778 216.1377 174.0908 162.0542
M2	$C_{13}H_{17}NO_3$	235.1208	235.1212	1.25	5.66	1.32×10^6	188.1073 146.06 160.1123 135.0441 218.118

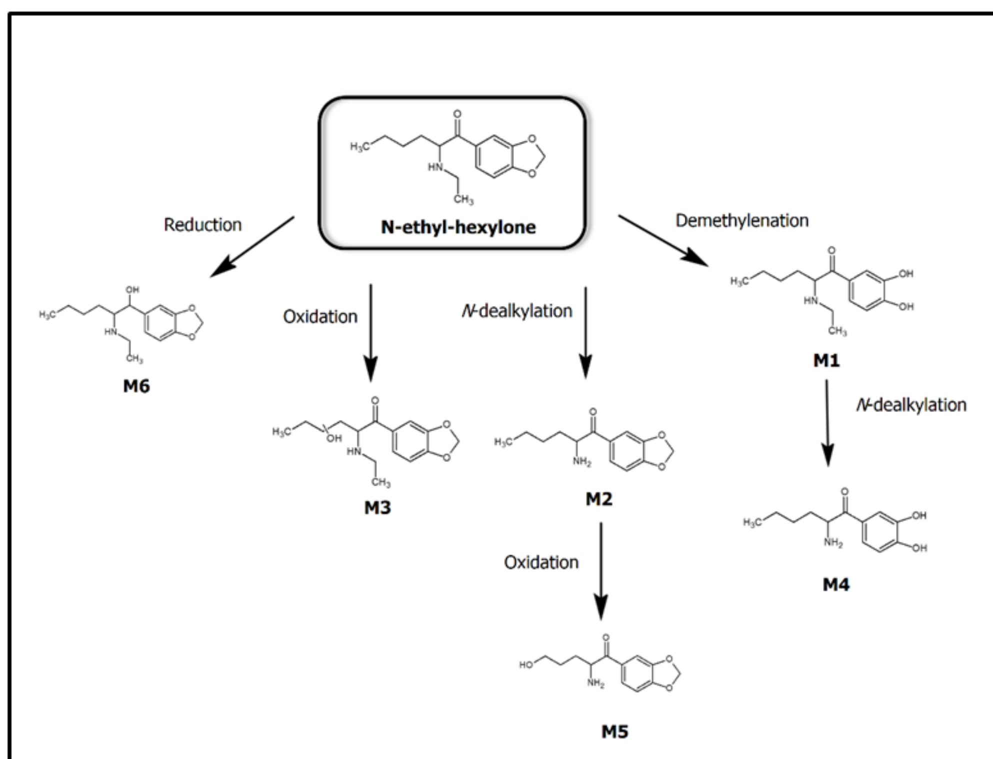
Table 33. Summary of *in vitro* metabolites of N-ethyl-hexylone (continuing)

Parent/Metabolite	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
M3	$C_{15}H_{21}NO_4$	279.1470	279.1471	0.7	5.83	1.94×10^5	280.1548 206.0815 176.0701 149.0236 202.0857
M4	$C_{12}H_{17}NO_3$	223.1208	223.1208	1.6	4.25	6.65×10^4	206.1178 188.1068 146.0584 123.044 160.1108
M5	$C_{13}H_{17}NO_4$	251.1158	251.1154	0.7	5.27	3.21×10^4	174.0542 234.112 186.0919 161.0455 189.0885

Table 33. Summary of *in vitro* metabolites of N-ethyl-hexylone (conclusion)

Metabolite	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
M6	$C_{15}H_{21}NO_4$	265.1678	265.1673	-2.1	4.76	1.94×10^5	266.1754 248.1632 123.0442 128.1446 207.0975

Figure 55. Proposed metabolism for N-ethyl-hexylone



As expected, N-ethyl-hexylone presented a metabolic profile very similar to N-ethyl-pentylone, as they have a structural difference of only one CH_2 group. N-ethyl-pentylone exhibited metabolites similar to N-ethyl-hexylone metabolites **M1**, **M2**, **M3** and **M6** (KROTULSKI et al., 2018c).

5. CONCLUSIONS

- The findings provided for the first time a set of metabolites of five novel synthetic cathinones found in the United States, 3,4-DCEC, 4-MDEC, N-ethyl-hexedrone, tertylone and N-ethyl-hexylone.

- A comparison with other synthetic cathinones metabolism reported in the literature revealed that as these drugs share some core structures, they also have similar metabolites formed by the same metabolic reactions.

- The findings of this study show the importance of reporting the metabolism of novel synthetic cathinones, which is a powerful tool for analysis of toxicological samples as well as to study the metabolism of other cathinones, enabling comparisons.

CHAPTER 5. GENERAL CONCLUSIONS

This work has explored the analytical and forensic aspects of novel drugs of abuse, used for recreational purposes (such as the NPS) or for body image enhancement (such as 2,4-DNP). As new drugs, methods for qualitative or quantitative analysis in biological fluids were needed. For NPS, a set of methods based on DBS technique was developed, proposing a new, easy and low cost approach, which increases the stability of drugs in the matrix as well as make the collection easier and possible to perform in a crime scene, using GC-MS or LC-MS/MS, according to the target drugs. In drug-related deaths investigations, the methods provided in this work can provide an alternative strategy for forensic laboratories, making easier shipping or storing specimens, without compromising the stability of target compounds. For example, a laboratory with no capability for these analyses can make DBS and ship these samples to another laboratory that has instrumentation for the needed analyses. Additionally, DBS could be implemented in roadside testing, to complement oral fluid testing. Future research may be performed to include or replace target compounds in these methods, since the prevalence of these NPS changes over time and between different regions, states and countries; further research can also be performed to extend these method to other fluids (e.g. dried plasma spots, dried urine spots) or to other analytical platforms (e.g. automated systems, LC-QTRAP systems). Regarding 2,4-DNP, since no methods were available in the literature, a method for analysis in blood and urine was developed, with acceptable validation and confirmation of three authentic cases in the United States. This is a modern method that has several advantages and can be easily implemented in forensic casework.

In vitro metabolism studies were also performed for some recently emerged NPS, which have been found in seized drug samples in the United States. Although these drugs have not been found in authentic specimens so far, this study provided a set of potential biomarkers of exposure to these NPS, which can be used for monitoring new cases or for data mining. The findings from this study can help to predict metabolic pathways for future analogs of the cathinones studied in this work, suggesting potential biomarkers for the in vivo metabolism of these analogs.

In this work, novel analytical approaches have been explored and they have potential to be used in routine STA. The STA consists in performing initially screening tests, followed by the confirmatory tests and, if necessary, quantitative analysis. Therefore, DBS can be implemented in STA as an alternate analytical approach for blood testing, for screening, confirmation or quantitation purposes. In a similar way, other specimens such as urine and oral fluid can be also analyzed using dried matrix spots approaches. In addition, *in vitro* metabolism assays can be performed along with STA standard tests to study potential metabolites of new

drugs and these potential metabolites can be investigated especially in urine specimens. If possible, a comparison between *in vitro* and *in vivo* or *post-mortem* specimens is very useful and recommended. For 2,4-DNP analysis, as performed in this work, it is recommended to perform additional testing for other drugs. During the STA in a 2,4-DNP investigation, it is beneficial to perform both acidic and basic extractions to search not only for 2,4-DNP (acidic compound) but also for basic drugs, such as stimulants and anabolic steroids. These additional findings can be very important for the management of intoxications or deaths investigations.

The emergence of NPS and other new drugs have changed the practice of Forensic Toxicology around the world. In the past, drug testing was performed in forensic laboratories usually focusing on a large number of analytes but the list of investigated compounds in toxicological analysis was usually fixed or slightly variable. The emergence of novel drugs has changed the scenario in the laboratories, with toxicologists and chemists now searching for known and completely unknown compounds in case samples. As consequence, analytical and interpretive issues have arisen from this new scenario. The interpretation of toxicological findings related to a new drug may be not straightforward, since reference values, clinical reports or metabolism patterns may be lacking for a new drug of abuse. A recommended strategy is to promote as possible the dissemination of experiences and results among forensic community. For example, a laboratory facing a series of cases involving a new cannabinoid can be helped by the recommendations of another laboratory that has seen cases involving the same compound before. Regarding analytical aspects, if a NPS has been reported in forensic casework, laboratories need to develop or to update their methods in order to include this new analyte in the scope, which requires reference materials. It is very important for forensic laboratories to be able to acquire these standards, since they are a critical component in method development, and good policies need to be adopted by public health agencies in the governments to make easier this process. Second, some NPS require high sensitivity techniques such as LC-QTOF and LC-MS/MS and not all laboratories have this instrumentation in house for performing these analyses in many countries, such as Brazil. In addition, novel bioanalytical strategies such as *in vitro* studies and automated systems can provide many analytical advantages in forensic laboratories but the costs for having these technologies are elevated. For this reason, supporting and funding Forensic Sciences initiatives and programs, including in collaborations with other countries, are powerful and necessary strategies to promote the excellence in forensic investigations.

REFERENCES

[1] ADAMS, A. J. et al. "Zombie" Outbreak Caused by the Synthetic Cannabinoid AMB-FUBINACA in New York. **The New England Journal of Medicine**, v. 376, n. 3, p. 235-242, 2017.

[2] AGENCIA NACIONAL DE VIGILÂNCIA SANITÁRIA (ANVISA). Relatório de Atividades 2017/2018 do Grupo de Trabalho para Classificação de Substâncias Controladas

[3] AKNOUCHE, F. et al. Testing for SGT-151 (CUMYL-PEGACLONE) and its Metabolites in Blood and Urine after Surreptitious Administration. **Journal of Analytical Toxicology**, in press, 2019.

[4] ALLIBE, N. et al. Case reports: Four concomitant non-fatal intoxications with AB-FUBINACA and MDMA. **Toxicologie Analytique et Clinique**, v. 29, n. 1, p. 101-110, 2017.

[5] AMBACH, L. et al. Quantification of cocaine and cocaine metabolites in dried blood spots from a controlled administration study using liquid chromatography-tandem mass spectrometry. **Drug Testing and Analysis**, v. 11, n. 5, p. 709-720, 2019.

[6] AMBACH, L. et al. Rapid and simple LC-MS/MS screening of 64 novel psychoactive substances using dried bloodspots. **Drug Testing and Analysis**, v. 6, n. 4, p. 367-375, 2014.

[7] AMBROZIAK, A.; ADAMOWICZC, P. Simple screening procedure for 72 synthetic cannabinoids in whole blood by liquid chromatography–tandem mass spectrometry. **Forensic Toxicology**, v. 36, n. 2, p. 280 – 290, 2018.

[8] AMERICAN ACADEMY OF FORENSIC SCIENCES STANDARDS BOARD (ASB). **Standard Practices for Method Validation in Forensic Toxicology**. ASB Standard 036, 1st edition, 2017.

[9] AMERICAN ACADEMY OF FORENSIC SCIENCES STANDARDS BOARD (ASB). **Guidelines for Opinions and Testimony in Forensic Toxicology**. ASB Standard 037, 1st edition, 2019.

[10] ANDRADE-EIROA, A. et al. Solid-phase extraction of organic compounds: A critical review (Part I). **TrAC Trends in Analytical Chemistry**, v. 80, p. 641-654, 2016.

[11] ANGERER, V. et al. Three fatalities associated with the synthetic cannabinoids 5F-ADB, 5F-PB-22, and AB-CHMINACA. **Forensic Science International**, v. 281, p. e9 – e15, 2017

[12] AVEDSCHMIDT, S. et al. Acetyl Fentanyl: Trends and Concentrations in Metro Detroit. **Journal of Forensic Sciences**, v. 64, n. 1, p. 149 – 153, 2019.

[13] BAKOTA, E. et al. Fatal Intoxication Involving 3-MeO-PCP: A Case Report and Validated Method. **Journal of Analytical Toxicology**, v. 40, n. 7, p. 504 – 510, 2016.

[14] BAKOTA, E. L. et al. Case Reports of Fatalities Involving Tianeptine in the United States. Journal of Analytical Toxicology, v. 42, n. 7, p. 503 – 509, 2018.

[15] BANG, I. C. Ein verfahren zur mikrobestimmung von blutbestandteilen. **Biochem Ztschr.**, v. 49, p. 19-39, 1913.

[16] BARRIOS, L. et al. Death following ingestion of methylone. **International Journal of Legal Medicine**, v. 130, n. 2, p. 381 – 385, 2016.

[17] BARROSO, M. et al. Hair: a complementary source of bioanalytical information in forensic toxicology. **Bioanalysis,** v. 3, n. 1, p. 67–79, 2011.

[18] BARTLETT J., BRUNNER M., GOUGH K. Deliberate poisoning with dinitrophenol (DNP): an unlicensed weight loss pill. **Emergency Medicine Journal**, v. 27, n. 2, p. 159-160, 2010.

[19] BORDIN, D. M. et al. Técnicas de preparo de amostras biológicas com interesse forense. **Scientia Chromatographica** v. 7, n. 2, p. 125-143, 2015.

[20] BOWDEN, J. A. et al. Enhancement of chemical derivatization of steroids by gas chromatography/mass spectrometry (GC/MS). **Journal of Chromatography B**, v. 877, p. 3237–3242, 2009.

[21] BOX, G. E. P.; BEHNKEN, D. W. Some new 3 level designs for the study of quantitative variables. **Technometrics**, v. 2, p. 455–475, 1960.

[22] BRANDON, E. F. A. et al. An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. **Toxicology and Applied Pharmacology**, v. 189, p. 233-46, 2003.

[23] BRERETON, R. G. Chemometrics: Data Analysis for the Laboratory and Chemical Plant. John Wiley & Sons: Chichester, 2003.

[24] BUDD, R. D.; LIU, Y. Phencyclidine concentrations in postmortem body fluids and tissues. **Journal of Toxicology Clinical Toxicology**, v. 19, n. 8, p. 843 – 850, 1982.

[25] BYARD, R. W.; BUTZBACH, D. M. Issues in the interpretation of postmortem toxicology. **Forensic Science, Medicine and Pathology**, v. 8, n.3, p. 205–207, 2012.

[26] CANDIOTI, L. V. et al. Experimental design and multiple response optimization. Using the desirability function in analytical methods development. **Talanta**, v. 124, p .123 – 138, 2014.

[27] CARLIER, J. et al. In Vitro Metabolite Profiling of ADB-FUBINACA, A New Synthetic Cannabinoid. **Current Neuropharmacology**, v. 15, n. 15, p 682 – 691, 2017.

[28] CASPAR, A. T. et al. Metabolism of the tryptamine-derived new psychoactive substances 5-MeO-2-Me-DALT, 5-MeO-2-Me-ALCHT, and 5-MeO-2-Me-DIPT and their detectability in urine studied by GC-MS, LC-MSn , and LC-HR-MS/MS. **Drug Testing and Analysis,** v. 10, n. 1, p. 184-195, 2018.

[29] CAYMAN CHEMICAL. Available at: https://www.caymanchem.com/. Accessed: 31/07/2019.

[30] CAYMAN CHEMICAL. Utopioids, 2018. Available at: https://www.caymanchem.com/news/utopioids. Accessed: 31/07/2019.

[31] CAYMAN STANDARDIZED NAMING OF SUBSTITUTED FENTANYLS GUIDE, 2019. Available at: https://www.caymanchem.com/literature/standardized-naming-of-substituted-fentanyls Accessed: 31/07/2019.

[32] CENTER FOR FORENSIC SCIENCE RESEARCH AND EDUCATION (CFSRE). Information Regarding "Santa Muerte" Stamped Drug Seizures Causing Hospitalizations in the Greater Philadelphia Area. Available at: https://www.forensicscienceeducation.org/wp-content/uploads/2018/07/Santa-Muerte-Philadelphia.pdf

[33] CHEPYALA, D. et al. Sensitive screening of abused drugs in dried blood samples using ultra-high-performance liquid chromatography-ion booster-quadrupole time-of-flight mass spectrometry. **Journal of Chromatography A**, v. 1491, p. 57 – 66, 2017.

[34] CHESSER, R. et al. Distribution of synthetic opioids in postmortem blood, vitreous humor and brain. **Forensic Science International**, v. 305, 2019

[35] COOPER, G. A. A.; KRONSTRAND, R.; KINTZ, P. Society of Hair Testing guidelines for drug testing in hair. **Forensic Science International**, v. 218, n. 1–3, p. 20-24, 2012.

[36] CUNHA, K. F.; EBERLIN, M. N.; COSTA, J. L. Development and validation of a sensitive LC–MS/MS method to analyze NBOMes in dried blood spots: evaluation of long-term stability. **Forensic Toxicology**, v. 36, n. 1, p. 113–121, 2018a.

[37] ________________________________. Long-term stability of synthetic cathinones in dried blood spots and whole blood samples: a comparative study. **Forensic Toxicology**, v. 36, p. 424–434, 2018b.

[38] CUTLER, C.; HUDSON, S. In vitro metabolism of the novel synthetic opioid agonist cyclopropylfentanyl and subsequent confirmation in authentic human samples using liquid chromatography-high resolution mass spectrometry. **Drug Testing and Analysis**, 2019, in press.

[39] DAMS, R. et al. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: Influence of ionization type, sample preparation, and biofluid. **Journal of the American Society for Mass Spectrometry**, v. 14, p. 1290–1294, 2003.

[40] DARKE, S. et al. Clinical and Autopsy Characteristics of Fatal Methamphetamine Toxicity in Australia. **Journal of Forensic Sciences**, v. 63, n. 5, p. 1466 – 1471, 2017

[41] DARKE, S.; TOROK, M.; DUFLOU, J. Circumstances and toxicology of sudden or unnatural deaths involving alprazolam. **Drug Alcohol and Dependence**, v. 138, p. 61 – 66, 2014.

[42] DE GIOVANNI, N.; FUCCI, N. The Current Status of Sweat Testing For Drugs of Abuse: A Review. **Current Medicinal Chemistry**, v. 20, p. 545 – 561, 2013.

[43] DE JONG, L. A. A.; OLYSLAGER, E. J. H.; DUIJST, W. L. J. M. The risk of emerging new psychoactive substances: The first fatal 3-MeO-PCP intoxication in The Netherlands. **Journal of Forensic and Legal Medicine**, v. 65, p. 101 – 104, 2019.

[44] DE MARTINIS, B. S. et al. Development and validation of a disk solid phase extraction and gas chromatography-mass spectrometry method for MDMA, MDA, HMMA, HMA, MDEA, methamphetamine and amphetamine in sweat. **Journal of Chromatography B**, v. 852, n. 1 – 2, p. 450 – 458, 2007.

[45] DE ZEEUW, R. A. Drug screening in biological fluids: The need for a systematic approach. **Journal of Chromatography B**, v. 689, p. 71 – 79, 1997.

[46] DÉGLON, J. et al. Rapid LC–MS/MS quantification of the major benzodiazepines and their metabolites on dried blood spots using a simple and cost-effective sample pretreatment. **Bioanalysis**, v. 4, n. 11, p. 1337–1350, 2012.

[47] DESROSIERS, N. A.; HUESTINS, M. A. Oral Fluid Drug Testing: Analytical Approaches, Issues and Interpretation of Results. **Journal of Analytical Toxicology**, v. 43, n. 6, p. 415 – 433, 2019.

[48] DEROUX, S. J.; SGARLATO, A.; MARKER, E. Phencyclidine: a 5-year retrospective review from the New York City Medical Examiner's Office. **Journal of Forensic Sciences**, v. 56, n. 3, p. 656-659, 2011.

[49] DERRINGER G, SUICH R. Simultaneous optimization of several response variables. **Journal of Quality Technology**, v. 12, p. 214–9, 1980.

[50] DIAO, X. et al. Human Hepatocyte Metabolism of Novel Synthetic Cannabinoids MN-18 and Its 5-Fluoro Analog 5F-MN-18. **Clinical Chemistry**, v. 63, n. 11, p. 1753 – 1763, 2017.

[51] DIAO, X.; HUESTIS, M. New Synthetic Cannabinoids Metabolism and Strategies to Best Identify Optimal Marker Metabolites. **Frontiers in Chemistry**, v. 7, n. 109, 2019.

[52] DINIS-OLIVEIRA, R. J. et al. Suicide by hanging under the influence of ketamine and ethanol. **Forensic Science International**, v. 202, n. 1-3, p. e23-e27, 2010.

[53] DINIS-OLIVEIRA, R. J. et al. Collection of biological samples in forensic toxicology. **Toxicology Mechanisms and Methods**, v. 20, n. 7, p. 363 – 414, 2010.

[54] DOMINGO, O. et al. Mitragynine concentrations in two fatalities. **Forensic Science International**, v. 271, e1 – e7, 2017.

[55] DRUG ENFORCEMENT ADMINISTRATION (DEA)**. 2018 National Drug Threat Assessment**. United States: 2018a.

[56] ____. **2018 Annual Emerging Threat Report**. United States: 2018b.

[57] DRUID, H. D.; HEARN, W. L. Strategies for Post-Mortem Toxicology Investigation. In: KARCH, S. B. (ed). **Drug Abuse Handbook**. 2nd edition. Boca Raton: CRC Press, 2007. Chapter 12.

[58] DRUMMER, O. H. Postmortem toxicology of drugs of abuse. **Forensic Science International**, v. 142, n. 2 -3, p. 101-113, 2004.

[59] _________________. Introduction and Review of Collection Techniques and Applications of Drug Testing of Oral Fluid. **Therapeutic Drug Monitoring**, v. 30, n. 2, p. 203 – 206, 2008.

[60] _________________. Good Practices in Forensic Toxicology. **Current Pharmaceutical Design**, v. 23, n. 36, 2017.

[61] DRUMMER, O. H. et al. Interpretation of postmortem forensic toxicology results for injury prevention research. **Injury Prevention**, v. 19, p. 284-289, 2013.

[62] DUNN, W. B.; ELLIS, D. I. Metabolomics: Current analytical platforms and methodologies. **Trends in Analytical Chemistry**, v. 24, n. 4, 2005.

[63] ELLEFSEN, K. N. et al. 4-Methoxy-α-PVP: in silico prediction, metabolic stability, and metabolite identification by human hepatocyte incubation and high-resolution mass spectrometry. **Forensic Toxicology**, v. 34, p. 61 – 75, 2016.

[64] ELLIOTT, S. P. MDMA and MDA concentrations in antemortem and postmortem specimens in fatalities following hospital admission. **Journal of Analytical Toxicology**, v. 29, n. 5, 296-300, 2005.

[65] ESHLEMAN, A. J. et al. Neurochemical pharmacology of psychoactive substituted N-benzylphenethylamines: High potency agonists at 5-HT2A receptors. **Biochemical Pharmacology**, v. 158, p. 27-34, 2018.

[66] EUROPEAN MONITORING CENTRE FOR DRUGS AND DRUG ADDICTION (EMCDDA). **The misuse of benzodiazepines among high-risk opioid users in**

Europe. Available at: http://www.emcdda.europa.eu/publications/pods/benzodiazepines_en (Accessed: 19/09/2019)

[67] ____. **European Drug Report 2019: Trends and Developments**. Luxembourg: Publications Office of the European Union, 2019.

[68] FARRELL, L. J.; KERRIGAN, S.; LOGAN, B. K. Recommendations for toxicological investigation of drug impaired driving. **Journal of Forensic Sciences**, v. 52, n. 5, p. 1214 – 1218, 2007.

[69] FARINA, M.; YONAMINE, M.; SILVA, O. A. One-step liquid–liquid extraction of cocaine from urine samples for gas chromatographic analysis. **Forensic Science International**, v. 127, p. 204–207, 2002.

[70] FASINU, P.; BOUIC, P. J.; ROSENKRANZ, B. Liver-Based In Vitro Technologies for Drug Biotransformation Studies - A Review. **Current Drug Metabolism**, v. 13, p. 215-224, 2012.

[71] FAVRETTO, D. et al. LC-ESI-MS/MS on an ion trap for the determination of LSD, iso-LSD, nor-LSD and 2-oxo-3-hydroxy-LSD in blood, urine and vitreous humor. **International Journal of Legal Medicine**, v. 121, n. 4, p. 259 – 265, 2007.

[72] FERRARI JUNIOR, E.; CALDAS, E. D. Simultaneous determination of drugs and pesticides in postmortem blood using dispersive solid-phase extraction and large volume injection-programmed temperature vaporization-gas chromatography–mass spectrometry. **Forensic Science International**, v. 290, p.318-326, 2018.

[73] FERREIRA, S. L. C. et al. Box-Behnken design: An alternative for the optimization of analytical methods. **Analytica Chimica Acta**, v. 597, p. 179–186, 2007.

[74] FINKELSTEIN, M. J. et al. Analysis of Acetyl Fentanyl in Postmortem Specimens by Gas Chromatography-Mass Spectrometry (GC-MS): Method Validation and Case Report. **Journal of Analytical Toxicology**, v. 43, n. 5, p. 392 – 398, 2019.

[75] FLANAGAN, R. J. et al. Micro-extraction techniques in analytical toxicology: short review. **Biomedical Chromatography**, v. 20, p. 530 – 538, 2006.

[76] FOGARTY, M. F.; PAPSUN, D. M.; LOGAN, B. K. Analysis of Fentanyl and 18 Novel Fentanyl Analogs and Metabolites by LC-MS-MS, and report of Fatalities Associated with Methoxyacetylfentanyl and Cyclopropylfentanyl. **Journal of Analytical Toxicology**, v. 42, n. 9, p. 592 – 604, 2018.

[77] FORT, C. et al. Stability of Synthetic Cannabinoids in Biological Specimens: Analysis Through Liquid Chromatography Tandem Mass Spectrometry. **Journal of Analytical Toxicology**, v. 41, n. 5, p. 360–366, 2017.

[78] GILL, J. R. et al. Ecstasy (MDMA) deaths in New York City: a case series and review of the literature. **Journal of Forensic Sciences,** 47, n. 1, p. 121–126, 2002.

[79] GRAFINGER, K. E. et al. In vitro phase I metabolism of three phenethylamines 25D-NBOMe, 25E-NBOMe and 25N-NBOMe using microsomal and microbial models. **Drug Testing and Analysis**, v. 10, n 10, p. 1607-1626, 2018.

[80] GRAFINGER, K. E. et al. Study of the in vitro and in vivo metabolism of the tryptamine 5-MeO-MiPT using human liver microsomes and real case samples. **Drug Testing and Analysis**, v. 10, n. 3, p. 562 – 574, 2018.

[81] GRAPP, M. et al. Systematic forensic toxicological analysis by liquid-chromatography-quadrupole-time-of-flight mass spectrometry in serum and comparison to gas chromatography-mass spectrometry. **Forensic Science International**, v. 287, p. 63 – 73, 2018.

[82] GRUNDLINGH, J. et al. 2,4-dinitrophenol (DNP): a weight loss agent with significant acute toxicity and risk of death. **Journal of Medical Toxicology**, v. 7, n. 3, p. 205 – 212, 2011.

[83] GUTHRIE, R.; SUSI, A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants, **Pediatrics**, v. 32 p. 338–343, 1963.

[84] HASSAN, Z. et al. Novel Psychoactive Substances-Recent Progress on Neuropharmacological Mechanisms of Action for Selected Drugs. **Frontiers in Psychiatry**, v. 8, n. 152, 2017.

[85] HE, Y; CONCHEIRO-GUISAN, M. Microextraction sample preparation techniques in forensic analytical toxicology. **Biomedical Chromatography**, v. 33, n. 1, 2019.

[86] HEPLER, B. R.; ISENSCHMID, D. S. Specimen Selection, Collection, Preservation and Security. In: KARCH, S. B. (ed). **Drug Abuse Handbook**. 2nd edition. Boca Raton: CRC Press, 2007. Chapter 12.

[87] HERNANDES, V. V.; BARBAS, C.; DUDZIK, D. A review of blood sample handling and pre-processing for metabolomics studies. **Electrophoresis**, p. 1 – 10, 2017.

[88] HESS, C. et al. Freeze-thaw stability and long-term stability of 84 synthetic cannabinoids in serum. **Drug Testing and Analysis**, v. 9, p. 1506–1511, 2017.

[89] HESS, C. et al. Simultaneous detection of 93 synthetic cannabinoids by liquid chromatography-tandem mass spectrometry and retrospective application to real forensic samples. **Drug Testing and Analysis**, v. 9, n. 5, p. 721 – 733, 2017.

[90] HØISETH, G.; TUV, S. S.; KARINEN, R. Blood concentrations of new designer benzodiazepines in forensic cases. **Forensic Science International**, v. 268, p. 35 – 38, 2016.

[91] HOLBOROW, A.; PURNELL, R. M.; WONG, J. F. Beware the yellow slimming pill: fatal 2,4-dinitrophenol overdose. **BMJ Case Reports**, April, 2016.

[92] HOUNOUM, B. M.; BLASCO, H.; EMOND, P.; MAVEL, S. Liquid chromatography–high-resolution mass spectrometry-based cell metabolomics: Experimental design, recommendations, and applications. **Trends in Analytical Chemistry**, v. 75, p. 118 – 128, 2016.

[93] HSIAO, A. L. et al. Pediatric Fatality Following Ingestion of Dinitrophenol: Postmortem Identification of a ‘‘Dietary Supplement’’. **Clinical Toxicology**, v. 43, p. 281–285, 2005.

[94] HSU, R. et al. Gas chromatography electrospray ionization mass spectrometry analysis of trimethylsilyl derivatives. **Journal of Mass Spectrometry**, v. 51, p. 693–698, 2016.

[95] HUSSAIN, J. N.; MANTRI, N.; COHEN, M. M. Working Up a Good Sweat – The Challenges of Standardising Sweat Collection for Metabolomics Analysis. **The Clinical Biochemist Reviews**, v. 38, n. 1, p. 13 – 34, 2017.

[96] INGELS, A. M. E.; LAMBERT, W. E.; STOVE, C. P. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. **Analytical and Bioanalytical Chemistry**, v. 398, n. 5, p. 2173 – 2182, 2010.

[97] JONES, A. W.; HOLMGREN, A. Concentrations of alprazolam in blood from impaired drivers and forensic autopsies were not much different but showed a high prevalence of co-ingested illicit drugs. **Journal of Psychopharmacology**, v. 27, n. 3, p. 276 – 281, 2013.

[98] JOYE, T. et al. Liquid chromatography-high resolution mass spectrometry for broad-spectrum drug screening of dried blood spot as microsampling procedure. **Analytical Chimica Acta**, v. 1063, p. 110-116, 2019.

[99] KAHL, J. H. et al. Quantitative Analysis of Fentanyl and Six Fentanyl Analogs in Postmortem Specimens by UHPLC-MS-MS. **Journal of Analytical Toxicology**, v. 42, n. 8, p. 570-580, 2018.

[100] KAHL, J. M. M.; CUNHA, K.; COSTA, J. L. Development and validation of an analytical method for the determination of fentanyl and its analogues in dried blood spots (DBS). In: XXI BRAZILIAN CONGRESS OF TOXICOLOGY AND XV TIAFT

LATIN-AMERICAN REGIONAL MEETING, 2019, Águas de Lindóia, SP. **Abstract Book**, Águas de Lindóia: 2019, p. 219.

[101] KARCH, S. B. (Editor). **Drug Abuse Handbook.** 2ª edição. CRC Press: Boca Raton, 2006. 1288 p.

[102] KEEVIL, B. G. The analysis of dried blood spot samples using liquid chromatography tandem mass spectrometry. **Clinical Biochemistry**, v. 44, n. 1, p. 110 – 118, 2011.

[103] KIM, T.; JONGKI, H.; JAESEONG, R.; KANGJIN, K. Analysis of Nitrophenols with Gas Chromatography/Mass Spectrometry by Flash Heater Derivatization. **Bulletin of the Korean Chemical Society**, v. 14, n. 5, p. 529-530, 1993.

[104] KINTZ, P. Hair Analysis in Forensic Toxicology: An Updated Review with a Special Focus on Pitfalls. **Current Pharmaceutical Design**, v. 23, n. 36, p. 5480 – 5486, 2017.

[105] KINTZ, P. et al. Murdered while under the influence of 3-MeO-PCP. **International Journal of Legal Medicine**, v. 133, n. 2, p. 475-478, 2019.

[106] KOPEC, K. T.; FREIERMUTH, C.; MAYNARD, S.; BEUHLER, M. Dinitrophenol (DNP) Fatality Associated with a Falsely Elevated Salicylate Level: a Case Report with Verification of Laboratory Cross Reactivity. **Journal of Medical Toxicology**, v. 14, n. 4, p. 323 – 326.

[107] KOVÁCS, K. et al. Fatal intoxication of a regular drug user following N-ethyl-hexedrone and ADB-FUBINACA consumption. **Journal of Forensic and Legal Medicine**, v. 65, p. 92 – 100, 2019.

[108] KRAEMER, M. et al. Death cases involving certain new psychoactive substances: A review of the literature. **Forensic Science International**, v. 298, p. 186-267, 2019.

[109] KROTULSKI, A. J. et al. Dibutylone (bk-DMBDB): Intoxications, Quantitative Confirmations and Metabolism in Authentic Biological Specimens. **Journal of Analytical Toxicology**, v. 42, n. 7, p. 437-445, 2018a.

[110] ___________________. et al. Metabolism of novel opioid agonists U-47700 and U-49900 using human liver microsomes with confirmation in authentic urine specimens from drug users. **Drug Testing and Analysis**, v. 10, n. 1, p. 127-136, 2018b.

[111] ___________________. et al. N-Ethyl Pentylone (Ephylone) Intoxications: Quantitative Confirmation and Metabolite Identification in Authentic Human Biological Specimens, **Journal of Analytical Toxicology**, v. 42, n. 7, p. 467-475, 2018c.

[112] ___________________. et al. 4F-MDMB-BINACA: A New Synthetic Cannabinoid Widely Implicated in Forensic Casework. **Journal of Forensic Sciences**, v. 64, n. 5, 2019.

[113] KUEPPERS, V. B.; COOKE, C. T. 25I-NBOMe related death in Australia: A case report. **Forensic Science International**, v. 249, p. e15-e18, 2015.

[114] KUPKE, I. R.; KATHER, B.; ZEUGNER, S. On the composition of capillary and venous blood serum. **Clinical Chemistry Acta**, v. 112, n. 2, p. 177 – 185, 1981.

[115] LARSEN, L. G.; HALBERG, L.; BAKKE, S. A. Fatal course following poisoning with an illegal weight-reducing agent. **Ugeskr Laeger,** v. 177, n. 37, V04150329, 2015.

[116] LAVILLA, I. et al. Greener derivatization in analytical chemistry. **Trends in Analytical Chemistry**, v. 61, p. 1–10, 2014.

[117] LEE, H. C. H. et al. 2,4-Dinitrophenol: A threat to Chinese body-conscious groups. **Journal of the Chinese Medical Association**, v. 77, p. 443-445, 2014.

[118] LILLSUNDE, P. Analytical Techniques for Drug Detection in Oral Fluid. **Therapeutic Drug Monitoring**, v. 30, n. 2, p. 181-187, 2008.

[119] LOGAN, B. K. Past perspectives and future directions in forensic toxicology. In: MARTELL, D. A. (ed); **The Future of Forensic Science.** Hoboken, NJ, USA: John Wiley & Sons, 2019. Chapter 11.

[120] LOGAN, B. K. **Characteristics of Working Models for Rapid Notification**. Presentation at Novel Psychoactive Substances (NPS) Symposium, November, 13th – 14th, 2018.

[121] LOGAN, B. K. et al. Reports of Adverse Events Associated with Use of Novel Psychoactive Substances, 2013-2016: A Review. **Journal of Analytical Toxicology**, v. 41, n. 7, p. 573 – 610, 2017.

[122] LOGAN, B. K.; WEISS, E. L.; HARRUFF, R. C. Case report: distribution of methamphetamine in a massive fatal ingestion. **Journal of Forensic Sciences**, v. 41, n. 2, p. 322 – 323, 1996.

[123] MAEDA, H. et al. AB-CHMINACA-induced sudden death from non-cardiogenic pulmonary edema. **Clinical Toxicology (Philadelphia, PA)**, v 56, n. 2, p. 143 – 145, 2018.

[124] MARGALHO, C. et al. Determination of "new psychoactive substances" in postmortem matrices using microwave derivatization and gas chromatography-mass spectrometry. **Journal of Chromatography B**, v. 1020, p. 14 – 23, 2016.

[125] MARTUCCI, H. F. H.; Distribution of furanyl fentanyl and 4-ANPP in an accidental acute death: A case report. **Forensic Science International**, v. 283, p. e13 – e17, 2018.

[126] MATSON, M.; SCHENK, N. Fatality of 33-Year-Old Man Involving Kratom Toxicity. **Journal of Forensic Sciences**, 2019, *in press*.

[127] MATSUTA, S. ET al. Development of a simple one-pot extraction method for various drugs and metabolites of forensic interest in blood by modifying the QuEChERS method. **Forensic Science International**, v. 232, p. 40–45, 2013.

[128] MAURER, H. H. Systematic toxicological analysis procedures for acidic drugs and/or metabolites relevant to clinical and forensic toxicology and/or doping control. **Journal of Chromatogtaphy B**, v. 733, n. 1-2, p. 3-25, 1999.

[129] ______________. Current role of liquid chromatography–mass spectrometry in clinical and forensic toxicology. **Analytical and Bioanalytical Chemistry**, v. 388, n. 7, p. 1315–1325, 2007.

[130] ______________. Pitfalls in drug testing by hyphenated low- and high-resolution mass spectrometry. **Drug Testing and Analysis**, v. 12, p. 172–179, 2020.

[131] MCKENZIE, C. et al. Chemical synthesis, characterisation and in vitro and in vivo metabolism of the synthetic opioid MT-45 and its newly identified fluorinated analogue 2F-MT-45 with metabolite confirmation in urine samples from known drug users. **Forensic Toxicology**, v. 36, n. 2, p. 359 – 374, 2018.

[132] MENA BRAVO, A.; LUQUE DE CASTRO, M. D. Sweat: A sample with limited present applications and promising future in metabolomics. **Journal of Pharmaceutical and Biomedical Analysis**, v. 90, p. 139– 147, 2014.

[133] MERCIECA, G. et al. Rapid and simple procedure for the determination of cathinones, amphetamine-like stimulants and other new psychoactive substances in blood and urine by GC-MS. **Journal of Pharmaceutical Biomedical Analysis**, v. 149, p. 494 – 501, 2018.

[134] MERCOLINI, L.; MANDRIOLI, R.; GERRA, G. et al. Analysis of cocaine and two metabolites in Dried Blood Spots by liquid chromatography with fluorescence detection: A novel test for cocaine and alcohol intake. **Journal of Chromatography A**, v. 1217, p. 7242–7248, 2010.

[135] MERCOLINI, L.; PROTTI, M. Biosampling strategies for emerging drugs of abuse: towards thefuture of toxicological and forensic analysis. Journal of Pharmaceutical and Biomedical Analysis, v. 130, p. 202–219, 2016.

[136] MICHELY, J. A. et al. Biotransformation and detectability of the new psychoactive substances N,N-diallyltryptamine (DALT) derivatives 5-fluoro-DALT, 7-methyl-DALT, and 5,6-methylenedioxy-DALT in urine using GC-MS, LC-MSn, and LC-HR-MS/MS. **Analytical and Bioanalytical Chemistry**, v. 409, n. 6, p. 1681-1695, 2017.

[137] MIRANDA, E. J. et al. Two deaths attributed to the use of 2,4-dinitrophenol. **Journal of Analytical Toxicology**, v. 30, n. 3, p. 219 – 222, 2006.

[138] MITCHELL-MATA, C. et al. Two Fatal Intoxications Involving 3-Methoxyphencyclidine. **Journal of Analytical Toxicology**, v. 41, n. 6, p. 503-507.

[139] MOGLER, L. et al. Phase I metabolism of the recently emerged synthetic cannabinoid CUMYL-PEGACLONE and detection in human urine samples. **Drug Testing and Analysis**, v. 10, n. 5, p. 886-891, 2018.

[140] MOHR, A. L. A.; FRISCIA, M.; LOGAN, B. K. Identification and prevalence determination of novel recreational drugs and discovery of their metabolites in blood, urine and oral fluid. **National Criminal Justice Reference Service**, Document Number 250338, p. 1–24, 2016.

[141] MOLNÁR, B.; MOLNÁR-PERL, I. The role of alkylsilyl derivatization techniques in the analysis of illicit drugs by gas chromatography. **Microchemical Journal**, v. 118, p. 101–109, 2015.

[142] MONTGOMERY, M.; LEBEAU, M.; MORRIS-KUKOSKI, C. New hair testing conclusions. **Journal of Analytical Toxicology**, v. 41, n. 2, p. 161 – 162, 2017.

[143] MOODY, M. T. et al. Analysis of fentanyl analogs and novel synthetic opioids in blood, serum/plasma, and urine in forensic casework. D**rug Testing and Analysis**, v. 10, p. 1358–1367, 2018.

[144] MORETTI, M.; FRENI, F; TOMACIELLO, I. et al. Determination of benzodiazepines in blood and in dried blood spots collected from postmortem samples and evaluation of the stability over a three month period. **Drug Testing and Analysis**, p. 1–9, 2019.

[145] MUELLER, R. K. General approach of Systematic Toxicological Analysis - Current status and future. In: MANGIN, P.; LUDES, B. (eds) **Acta Medicinæ Legalis Vol. XLIV 1994**. Berlin: Springer, 1994.

[146] NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY (NIST). **NIST/SEMATECH e-Handbook of Statistical Methods**. Chapter 5: Process Improvement. Available at: http://www.itl.nist.gov/div898/handbook/ (Accessed 11 July, 2019).

[147] NISBET, L. A. et al. Gas Chromatography-Mass Spectrometry Method for the Quantitative Identification of 23 New Psychoactive Substances in Blood and Urine. **Journal of Analytical Toxicology**, v. 43, n. 5, p. 346–352, 2019.

[148] ODOARDI, S.; ANZILLOTTI, L.; STRANO-ROSSI, S. Simplifying sample pretreatment: application of dried blood spot (DBS) method to blood samples, including postmortem, for UHPLC-MS/MS analysis of drugs of abuse. **Forensic Science International**, v. 243, p. 61 – 67, 2014.

[149] OLIVEIRA, R.; HENION, J.; WICKREMSINHE, E. Fully-automated approach for online dried blood spot extraction and bioanalysis by two-dimensional-liquid chromatography coupled with high-resolution quadrupole time-of-flight mass spectrometry. Analytical Chemistry, v. 86, p. 1246−1253, 2014.

[150] ÖZTÜRKA, Y. E.; YETER, O.; ALPERTUNGA, B. Validation of JWH-018 and its metabolites in blood and urine by UPLC–MS/MS: Monitoring in forensic cases. **Forensic Science International**, v. 248, p. 88 – 93, 2015.

[151] PAPASEIT, E. et al. Mephedrone Concentrations in Cases of Clinical Intoxication. **Current Pharmaceutical Design**, v. 23, n. 36, 2017.

[152] PAPSUN, D. et al. Case Series of Novel Illicit Opioid-Related Deaths. **Academic Forensic Pathology**, v. 7, n. 3, p. 477 – 486, 2017.

[153] PAPSUN, D. et al. The Trouble With Kratom: Analytical and Interpretative Issues Involving Mitragynine. **Journal of Analytical Toxicology**, v. 43, n. 8, 2019.

[154] PAUL, B. D.; COLE, K. A. Cathinone (Khat) and Methcathinone (CAT) in Urine Specimens: A Gas Chromatographic-Mass Spectrometric Detection Procedure. **Journal of Analytical Toxicology**, v. 25, n. 7, p. 525-530.

[155] PEDERSEN, A. J. et al. In vitro metabolism studies on mephedrone and analysis of forensic cases. **Drug Testing and Analysis**, v. 5, n. 6, p. 430 – 438, 2013.

[156] PERKINS, R. G. A Study of the Munitions Intoxications in France. **Public Health Reports (1896-1970)**, v. 34, n. 43, p. 2335-2374, 1919.

[157] PETERS, F. T. et al. Method Development in Forensic Toxicology. **Current Pharmaceutical Design**, v. 23, p. 5455-5467, 2017.

[158] PETERSON, B. L.; COUPER, F. J. Concentrations of AB-CHMINACA and AB-PINACA and Driving Behavior in Suspected Impaired Driving Cases. **Journal of Analytical Toxicology**, v. 39, n. 8, p. 642 – 647, 2015.

[159] PILGRIM, J. L.; WOODFORD, N.; DRUMMER, O H. Cocaine in sudden and unexpected death: a review of 49 post-mortem cases. **Forensic Science International**, v. 227, n. 1 – 3, p. 52 – 59, 2013.

[160] POKLIS, J. L. et al. Postmortem detection of 25I-NBOMe [2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine] in fluids and tissues determined by high performance liquid chromatography with tandem mass spectrometry from a traumatic death. **Forensic Science International**, v. 234, p. e14 – e20, 2014.

[161] POLETTINI, A. et al. Fully-automated systematic toxicological analysis of drugs, poisons, and metabolites in whole blood, urine, and plasma by gas chromatography-full

scan mass spectrometry. **Journal of Chromatography B**, v. 713, n. 1, p. 265 – 279, 1998.

[162] POWELL, J. W.; PRESNELL, S. E. Loperamide as a Potential Drug of Abuse and Misuse: Fatal Overdoses at the Medical University of South Carolina. **Journal of Forensic Sciences**, 2019 Jun 20. doi: 10.1111/1556-4029.14115.

[163] POZO Ó. J. et al. Mass spectrometric evaluation of mephedrone in vivo human metabolism: identification of phase I and phase II metabolites, including a novel succinyl conjugate. **Drug Metabolism and Disposition**, v. 43, p. 248-257, 2015.

[164] PRATA, V. M.; EMÍDIO, E. S.; DOREA, H. S. New catalytic ultrasound method for derivatization of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid in urine, with analysis by GC-MS/MS. **Analytical Bioanalytical Chemistry**, v. 403, p. 625–632, 2012.

[165] PRESLEY, B. C.; LOGAN, B. K.; JANSEN-VARNUM, S. A. In vitro Phase I metabolism of indazole carboxamide synthetic cannabinoid MDMB-CHMINACA via human liver microsome incubation and high-resolution mass spectrometry. **Drug Testing and Analysis**, 2019, *in press*.

[166] RAB, E.; FLANAGAN, R. J.; HUDSON, S. Detection of fentanyl and fentanyl analogues in biological samples using liquid chromatography-high resolution mass spectrometry. **Forensic Science International**, v. 300, p. 13-18, 2019.

[167] REISFILED, G. et al. Unexpected Urine Drug Testing Results in a Hospice Patient on High-Dose Morphine Therapy. **Clinical Chemistry**, v. 55, n. 10, 2009.

[168] RICHARDS-WAUGH, L. L. et al. Deaths Involving the Recreational Use of α-PVP (α-pyrrolidinopentiophenone). In: 65TH AMERICAN ACADEMY OF FORENSIC SCIENCES MEETING, 2013, Washington, D.C. **Procedings.** Washington: AAFS, 2013, p. 521-522. Available at: http://www.aafs.org/sites/default/files/pdf/ProceedingsWashingtonDC2013.pdf

[169] RICHARDSON, T. Pitfalls in Forensic Toxicology. **Annals of Clinical Biochemistry**, v. 37, p. 20 – 44, 2000.

[170] RICKLI, A. et al. Receptor interaction profiles of novel psychoactive tryptamines compared with classic hallucinogens. **European Neuropsychopharmacology**, v. 26, n. 8, p. 1327 – 1337, 2016.

[171] ROMAŃCZUK, A. et al. Aspects related to analysis and medico-legal assessment in the context of the presence of synthetic cannabinoids in biological material based on the example of AB-CHMINACA. **Archiwum Medycyny Sądowej i Kryminologii**, v. 68, n. 4, p. 266-280, 2018.

[172] RYONA, I.; HENION, J. A book-type dried plasma spot card for automated flow-through elution coupled with online SPE-LC-MS/MS bioanalysis of opioids and stimulants in blood. **Analytical Chemistry**, v. 88, p. 11229−11237, 2016.

[173] SADONES, N. et al. Do capillary dried blood spot concentrations of gamma-hydroxybutyric acid mirror those in venous blood? A comparative study. **Drug Testing and Analysis**, v. 7, p. 336–340, 2015.

[174] SADONES, N. et al. Microwave-assisted on-spot derivatization for gas chromatography –mass spectrometry based determination of polar low molecular weight compounds in dried blood spots. **Journal of Chromatography A**, v. 1465, p. 175–183, 2016.

[175] SASTRE, C. et al. Post Mortem Redistribution of Drugs: Current State of Knowledge. **Current Pharmaceutical Design**, v. 23, n. 36, 2017.

[176] SCIENTIFIC WORKING GROUP FOR THE ANALYSIS OF SEIZED DRUGS (SWGDRUG). **SWGDRUG Recommendations - Version 8.0**, 2019. Available at: http://swgdrug.org/Documents/SWGDRUG%20Recommendations%20Version%208_FINAL_ForPosting_092919.pdf

[177] SCHIFANO, F. et al. Abuse of Prescription Drugs in the Context of Novel Psychoactive Substances (NPS): A Systematic Review. **Brain Sciences**, v. 8, 4, 2018.

[178] SCHUMMER, C. et al. Comparison of MTBSTFA and BSTFA in derivatization reactions of polar compounds prior to GC/MS analysis. **Talanta**, v. 77, p. 1473–1482, 2009.

[179] SCHWILKE, E. W. et al. Opioid Disposition in Human Sweat after Controlled Oral Codeine Administration. **Clinical Chemistry**, v. 52, n. 8, p. 1539–1545, 2006.

[180] SEGURA, J.; VENTURA, R.; JURADO, C. Derivatization procedures for gas chromatographic–mass spectrometric determination of xenobiotics in biological samples, with special attention to drugs of abuse and doping agents. **Journal of Chromatography B**, v. 713, p. 61–90, 1998.

[181] SEO, H. et al. Metabolic profile determination of 25N-NBOMe in human liver microsomes by liquid chromatography-quadrupole time-of-flight mass spectrometry. **International Journal of Legal Medicine**, v. 133, n. 3, p. 833 – 841, 2019.

[182] SHANKS, K. G.; CLARK, W.; BEHONICK, G. Death Associated With the Use of the Synthetic Cannabinoid ADB-FUBINACA. **Journal of Analytical Toxicology**, v. 40, n. 3, p. 236 – 239, 2016.

[183] SHANKS, K. G.; DAHN, T.; TERRELL, A. R. Detection of JWH-018 and JWH-073 by UPLC–MS-MS in Postmortem Whole Blood Casework. **Journal of Analytical Toxicology**, v. 36, n. 3, p. 145–152, 2012.

[184] SHIMA, N. et al. Urinary excretion and metabolism of the newly encountered designer drug 3,4-dimethylmethcathinone in humans. **Forensic Toxicology**, v. 31, p. 101-112, 2013.

[185] SIGMA-ALDRICH. Available at: https://www.sigmaaldrich.com/. Accessed: 31/07/2019.

[186] SIMÕES, S. S.; AJENJO, A. C.; DIAS, M. J. Dried blood spots combined to an UPLC-MS/MS method for the simultaneous determination of drugs of abuse in forensic toxicology. **Journal of Pharmaceutical and Biomedical Analysis**, v. 147, p. 634-644, 2018.

[187] SKOPP, G. Preanalytic aspects in postmortem toxicology. **Forensic Science International**, v. 142, n. 2 – 3, p. 75 – 100, 2004.

[188] SMITH, M. P.; BLUTH, M. H. Forensic Toxicology: An Introduction. **Clinics in Laboratory Medicine**, v. 36, p. 753–759, 2016.

[189] SNOW, N. H.; BULLOCK, G. P. Novel techniques for enhancing sensitivity in static headspace extraction-gas chromatography. **Journal of Chromatography A**, v. 1217, n. 16, p. 2726 – 2735, 2010.

[190] SOFALVI, S. et al. An LC-MS-MS Method for the Analysis of Carfentanil, 3-Methylfentanyl, 2-Furanyl Fentanyl, Acetyl Fentanyl, Fentanyl and Norfentanyl in Postmortem and Impaired-Driving Cases. **Journal of Analytical Toxicology**, v. 41, n. 6, 2017.

[191] STIMPFL, T.; VYCUDILIK, W. Automatic screening in postmortem toxicology. **Forensic Science International**, v. 142, n. 2-3, p. 115-125, 2004.

[192] STOVE, C. P. et al. Dried blood spots in toxicology: from the cradle to the grave? **Critical Reviews in Toxicology**, v. 42, n. 3, p. 230-243, 2012.

[193] STRANO-ROSSI, S. et al. Metabolism of JWH-015, JWH-098, JWH-251, and JWH-307 in silico and in vitro: a pilot study for the detection of unknown synthetic cannabinoids metabolites. **Analytical and Bioanalytical Chemistry**, v. 406, p. 3621, 2014.

[194] SWORTWOOD, M. J. et al. First metabolic profile of PV8, a novel synthetic cathinone, in human hepatocytes and urine by high-resolution mass spectrometry. **Analytical and Bioanalytical Chemistry**, v. 408, n. 18, p. 4845 – 4856, 2016.

[195] ______________. et al. In vitro, in vivo and in silico metabolic profiling of α-pyrrolidinopentiothiophenone, a novel thiophene stimulant. **Bioanalysis**, v. 8, n. 1, p. 65-82, 2016.

[196] TEMPORAL. K. H. et al. Metabolic Profile Determination of NBOMe Compounds Using Human Liver Microsomes and Comparison with Findings in Authentic Human Blood and Urine. **Journal of Analytical Toxicology**, p. 1 – 12, 2017.

[197] TERADA, H. Uncouplers of Oxidative Phosphorylation. **Environmental Health Perspectives**, v. 87, p. 213-218, 1990.

[198] TEWARI, A. et al. Weight loss and 2,4-dinitrophenol poisoning. **British Journal of Anesthesia**, v. 102, n. 4, p. 566-567, 2009.

[199] THE INTERNATIONAL ASSOCIATION OF FORENSIC TOXICOLOGISTS (TIAFT). Laboratory guidelines for toxicological analysis. **TIAFT Bulletin**, V 31, n. 4, p. 23 – 26, 2001.

[200] TIMMERMAN, P. et al. EBF recommendation on the validation of bioanalytical methods for dried blood spots. **Bioanalysis**, v. 3, n. 14, p. 1567 – 1575, 2011.

[201] TUV, S. S. et al. Prevalence of synthetic cannabinoids in blood samples from Norwegian drivers suspected of impaired driving during a seven weeks period. **Accident Analysis & Prevention**, v. 62, p. 26 – 31, 2014.

[202] TYNDALL, J. A. et al. An outbreak of acute delirium from exposure to the synthetic cannabinoid AB-CHMINACA. **Clinical Toxicology**, v. 53, n. 10, p. 950 – 956, 2015.

[203] TYRKKÖ, E. et al. In silico and in vitro metabolism studies support identification of designer drugs in human urine by liquid chromatography/quadrupole-time-of-flight mass spectrometry. **Analytical and Bioanalytical Chemistry**, v. 405, n. 21, p. 6697 – 6709, 2013.

[204] UNITED NATIONS OFFICE ON DRUGS AND CRIME (UNODC). **Current NPS Threats, Volume I**. March, 2019. Available at: https://www.unodc.org/pdf/opioids-crisis/Current_NPS_Threats_-_Volume_I.pdf. Accessed: 19/09/2019.

[205] ____. **World Drug Report 2016**. Available at: https://www.unodc.org/wdr2016/. Accessed: 21/10/2019.

[206] ____. **Recommended Methods for the Identification and Analysis of Synthetic Cathinones in Seized Materials**. 2015. Available at: https://www.unodc.org/documents/scientific/STNAR49_Synthetic_Cathinones_E.pdf

[207] URALETS V. et al. Testing for designer stimulants: metabolic profiles of 16 synthetic cathinones excreted free in human urine. **Journal of Analytical Toxicology**, v. 38, n. 5, p. 233-241, 2014.

[208] VAN SCHOOR, J.; KHANDERIA, E.; THORNILEY, A. Dantrolene is not the answer to 2,4-dinitrophenol poisoning: more heated debate. **BMJ Case Reports**, v. 11, e225323, 2018.

[209] VENKATAKRISHNAN, K.; VON MOLTKE, L. L.; GREENBLATT, D. J. Human Drug Metabolism and the Cytochromes P450: Application and Relevance of In Vitro Models. **Journal of Clinical Pharmacology**, v. 41, p. 1149-1179, 2001.

[210] VERPLAETSE, R; HENION, J. Quantitative determination of opioids in whole blood using fully automated dried blood spot desorption coupled to on-line SPE-LC-MS/MS. **Drug Testing and Analysis,** v. 8, p. 30–38, 2016.

[211] VERSCHRAAGEN, M. et al. Post-mortem cases involving amphetamine-based drugs in the Netherlands: Comparison with driving under the influence cases. **Forensic Science International**, v. 170, n. 2–3, p. 163-170, 2007.

[212] VUCKOVIC, D. Current trends and challenges in sample preparation for global metabolomics using liquid chromatography-mass spectrometry. **Analytical and Bioanalytical Chemistry**, v. 403, p. 1523 – 1548, 2012.

[213] WAGNER, M. et al. The use of mass spectrometry to analyze dried blood spots. **Mass Spectrometry Reviews**, v. 35, n. 3, p. 361–438, 2016.

[214] WANG, C.; WALKER, A. E. Fatal Mitragynine-Associated Toxicity in Canada: A Case Report and Review of the Literature. **Academic Forensic Pathology**, v. 8, n. 2, p. 340–346, 2018.

[215] WARTHIN, A. S. A fatal case of toxic jaundice caused by dinitrophenol. **Bulletin International Association of Medical Museums**, v.7, p. 123–126, 1918.

[216] WELLS, R. Recent advances in non-silylation derivatization techniques for gas chromatography. **Journal of Chromatography A**, v. 843, p. 1–18, 1999.

[217] WIERGOWSKI, M. et al. Identification of novel psychoactive substances 25B-NBOMe and 4-CMC in biological material using HPLC-Q-TOF-MS and their quantification in blood using UPLC–MS/MS in case of severe intoxications. **Journal of Chromatography B**, v. 1041 – 1042, p. 1 – 10, 2017.

[218] WILHELM, A. J. et al. Therapeutic Drug Monitoring by Dried Blood Spot: Progress to Date and Future Directions. **Clinical Pharmacokinetics**, v. 53, p. 961–973, 2014.

[219] WOHLFARTH, A. et al. Metabolic characterization of AH-7921, a synthetic opioid designer drug: in vitro metabolic stability assessment and metabolite identification, evaluation of in silico prediction, and in vivo confirmation. **Drug Testing and Analysis**, v. 8, n. 8, p. 779-791, 2016.

[220] WOLF, B. C. et al. Alprazolam-Related Deaths in Palm Beach County. **The American Journal of Forensic Medicine and Pathology**, v. 26, n. 1, p. 24-27, 2005.

[221] WORLD ANTI-DOPING AGENCY (WADA). **Prohibited List 2019.** Available at: https://www.wada-ama.org/en/content/what-is-prohibited. Accessed in 06/06/2019.

[222] WRIGHTON, S. A.; RING, B. J.; VANDENBRANDEN, M. The use of in vitro metabolism techniques in the planning and interpretation of drug safety studies. **Toxicologic Pathology**, v. 23, n. 2, 1995.

[223] WYMAN, J. F. Principles and procedures in forensic toxicology. **Clinics in Laboratory Medicine**, v. 32, n. 3, p. 493 – 507, 2012.

[224] YAREMA, M. C.; BECKER, C. E. Key concepts in postmortem drug redistribution. **Clinical Toxicology (Phila.)**, v. 43, n. 4, p. 235 - 241, 2005.

[225] YEAKEL, J. K.; LOGAN, B. K. Blood Synthetic Cannabinoid Concentrations in Cases of Suspected Impaired Driving. **Journal of Analytical Toxicology**, v. 37, n. 8, p. 547–551, 2013.

[226] YEN, M.; EWALD, M. B. Toxicity of Weight Loss Agents. **Journal of Medical Toxicology**, v. 8, n. 2, p. 145–152, 2012.

[227] YETER, O. Identification of the Synthetic Cannabinoid 1-(4-cyanobutyl)-N-(2-phenylpropan-2-yl)-1H-indazole-3-carboxamide (CUMYL-4CN-BINACA) in Plant Material and Quantification in Post-Mortem Blood Samples. **Journal of Analytical Toxicology**, v. 41, n. 9, p. 720 – 728, 2017.

[228] ZACK, F. et al. Death within 44 days of 2,4-dinitrophenol intake. **International Journal of Legal Medicine**, v. 130, p. 1237–1241, 2016.

[229] ZAKARIA, R. Advantages and Challenges of Dried Blood Spot Analysis by Mass Spectrometry Across the Total Testing Process, **EJIFCC**, v. 27, n. 4, p. 288 – 317, 2016.

[230] ZAWILSKA, J. B. An Expanding World of Novel Psychoactive Substances: Opioids. **Frontiers in Psychiatry,** v. 8, n. 110, 2017.

[231] ZAWILSKA, J. B.; WOJCIESZAK, J. An expanding world of new psychoactive substances-designer benzodiazepines. **Neurotoxicology**, v. 73, p. 8-16, 2019.

[232] ZHANG, D.; LUO, G.; DING, X.; LU, C. Preclinical experimental models of drug metabolism and disposition in drug discovery and development. **Acta Pharmaceutica Sinica B**, v. 2, n. 6, p. 549 – 561, 2012.

ANNEX

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PAPER

TOXICOLOGY

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Analysis of 2,4-Dinitrophenol in Postmortem Blood and Urine by Gas Chromatography–Mass Spectrometry: Method Development and Validation and Report of Three Fatalities in the United States[†,‡]

ABSTRACT: 2,4-dinitrophenol (2,4-DNP) is a compound used in the early 1900s as a weight-loss drug but later prohibited due to its severe adverse effects, including death. It has however been attracting interest, due to its weight-loss properties, and appears to be re-emerging in forensic casework. As 2,4-DNP is available for use in industry and as a pesticide and easily accessible online, the dissemination of this drug can be fast. The compound exerts its effects through inhibition of ATP synthesis, and corresponding thermogenic energy loss which can be fatal. A method for qualitative and quantitative analysis of 2,4-DNP in blood and urine specimens using GC-MS with hydrogen as carrier gas is described. The method was validated and displayed acceptable performance parameters with linearity (R^2 higher than 0.998), inter-assay imprecision (lower than 10.6%), intra-assay imprecision (lower than 10.7%), and extraction efficiency (92.1%). Stability of 2,4-DNP in blood and urine was studied, and the drug was stable up to 30 days refrigeration or frozen. Six cases in United States suspected to be related to 2,4-DNP were analyzed. Three cases were found to be positive for 2,4-DNP. Concentrations of 2,4-DNP were in the range of 61.6–220 mg/L in urine and <3–114 mg/L in blood. Based on our findings, we suggest that medical examiners and forensic toxicologists be aware of the reappearance of 2,4-DNP, including this compound as a target in death investigations related to weight-loss drugs.

KEYWORDS: forensic science, 2,4-dinitrophenol, gas chromatography, mass spectrometry, postmortem toxicology, weight-loss drugs

2,4-dinitrophenol (2,4-DNP) is a phenolic compound which has been used in industry for manufacturing dyes, and as an herbicide and pesticide since the 1900s (1,2). In appearance, it is a bright yellow powder, with a characteristic odor and low solubility in water (3). In the 1930s, 2,4-DNP was also used as weight-loss drug, and some studies reported that consuming a daily dose of between 300 and 400 mg of 2,4-DNP over 2 weeks would increase the basal metabolic rate of a person between 36–95% (4). This use, however, was prohibited in 1938 because of severe adverse effects, including nausea, vomiting, hyperthermia, rhabdomyolysis, agitation, shortness of breath, cataracts, reduction of urinary volume, renal failure, coma, and death (4–6).

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2,4-DNP acts as a cellular oxidative phosphorylation uncoupler and is a chemical ionophore (3,7–9). Energy produced during the Krebs Cycle is not stored as adenosine triphosphate (ATP) but released in form of excess heat, due to the unbalance in the proton gradient induced by 2,4-DNP (6,8). The basal metabolism rate (including metabolism of lipids) is increased, as well as oxygen consumption, and heart rate (6).

Some studies have also characterized the metabolic profile of 2,4-DNP in vivo and in vitro. In mouse, 2,4-DNP undergoes biotransformation to 2-amino-4-nitrophenol (2A-4NP), 2-nitro-4-aminophenol (2N-4AP), and 2,4-diaminophenol (2,4-DAP) by reduction (6,10). In rat liver homogenates, 4-amino-2-nitrophenol (4A2NP) and 2-amino-4-nitrophenol (2A4NP) metabolites were identified (11). In humans, 2A-4NP was the only metabolite identified in blood. The metabolites 2A-4NP, 2N-4AP, DNP glucuronide, DNP sulfate, and 2A-4NP glucuronide were detected in urine. In blood, the metabolites of 2,4-DNP have a short half-life after ingestion, and the blood/urine ratio is high, suggesting that the best biomarker for 2,4-DNP exposure in blood is the parent drug rather than the metabolites (6).

In the past decade, many reports of 2,4-DNP re-emerging as a weight-loss drug associated with lethal intoxication cases have been reported in several countries as United States (US)(5), Italy (6), United Kingdom (UK)(3), and China (12). The number of clinical cases caused by 2,4-DNP intoxication, with high mortality,

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