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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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 microsomas 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 microsomas 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 -20°C, 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 -20°C. O método confirmatório para análise de catinonas sintéticas em DBS também apresentou bons resultados, com R² 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 -20°C, except mitragynine. 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 -20°C. 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-methyldiethylcathinone (4-MDEC), 3,4-dichloroethylcathinone (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.

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 overdose, 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 known 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 be 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 DUI 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 detect 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 degrade 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 occur 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 complex 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; CONCEIRO-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 CB₁ and CB₂ 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 CB₁ 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, butyrylfentanyl 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 $\alpha 1$), anxiolytic effects (subunit $\alpha 2$) and myorelaxant effects (subunits $\alpha 2$, $\alpha 3$ and $\alpha 5$) (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-synthesized 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 40°C), 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	<p>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.</p> <p>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.</p>	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 immunoassays, 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 -20°C and -80°C (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 4°C and -20°C 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 -80°C (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		<i>In vitro</i> 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	<i>In vitro</i> 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 <i>Cunninghamella elegans</i> (C. <i>elegans</i>)	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 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.

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