Stability of synthetic cathinones in clinical and forensic toxicological analysis—Where are we now?

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Abstract
Understanding the stability of analyzed drugs in biological samples is a crucial part for an appropriate interpretation of the analytical findings. Synthetic cathinones, as psychoactive stimulants, belong to a major class of new psychoactive substances. As they are subject to several degradation pathways, they are known to clinical and forensic toxicologists as unstable analytes in biological samples. When interpreting analytical data of synthetic cathinones in biological samples, analysts must be aware that the concentration of analytes may not accurately reflect the levels at the time they were acquired owing to many factors. This review provides (i) an overview of the current scientific knowledge on the stability of synthetic cathinones and/or metabolites in various human biological samples with a focus on factors that may deteriorate their stability—such as storage temperature, length of storage, matrix, pH, type of preservatives, concentration of analytes, and the chemistry of the analytes—and (ii) possible solutions on how to avoid such degradation. The PubMed database as well as Google Scholar was thoroughly searched to find published studies on the stability of synthetic cathinones since 2007 by searching specific keywords. A total of 23 articles met the inclusion criteria and were included in this review. Synthetic cathinones that carry methylenedioxy or N-pyrrolidine ring showed higher degradation resistance over other substituted groups. Acidification of samples pH plays a crucial role at increasing the stability of cathinones even with analytes that were frequently considered as poorly stable. This review also provides several recommendations for best practice in planning the experimental design, preservation, and storage conditions in order to minimize synthetic cathinones' degradation in human biological samples.

KEYWORDS
new psychoactive substances, pH, preservatives, stability, synthetic cathinones
INTRODUCTION

In clinical and forensic toxicology investigations, instability of analytes under the influence of various conditions may occur in biological samples. The loss of analytes may take place owing to chemical degradation, enzymatic metabolism, or the existence of interfering substances due to matrix degradation, involving (but not limited to) improper sample storage conditions, handling, and/or transportation.

Stability studies may help to determine the time frame that must be taken into account between sample collection and analysis, or to determine the ideal storage conditions for a sample to be preserved for a purpose of drug testing in order to minimize or reduce sample loss, thus not significantly compromising the analytical results. In toxicology laboratories, the stability of classical drugs of abuse (i.e., amphetamine, benzodiazepines, cannabinoids, cocaine, codine, and morphine) in biological samples has been widely described.

One of the reasons of increased interest in investigating the stability of classical drugs is certainly that their existence in these samples may lead to sanctions for the users, or if the workplace drug testing (WDT) guidelines require certain laboratories to store all confirmed positive samples for a minimum of 1 year, for a potential retesting. Thus, drug stability in biological samples is an important consideration for assuring accurate reports of drug concentration and/or its metabolites.

Over the past decade or so, new psychoactive substances (NPSs) gained a high popularity on the illicit drug markets, possibly because of the reducing purity and availability of classical stimulant drugs such as amphetamine, 3,4-methylenedioxyamphetamine (MDMA), or cocaine. Besides the latter reasons, additional factors may motivate the use including the expansion of online and virtual markets, cost, and legal status. Furthermore, NPSs may not be included in conventional laboratory drug testing screening; indeed, there is often lack of a reliable immunoassays screening for NPSs owing to their structural similarity of classical drugs. Moreover, as NPSs continuously appear on the drug market every year as structural isomers or analogs, drug testing laboratories often cannot keep track for all of them—having to continuously update their existing analytical methods and constantly obtain new reference materials, which is often time-consuming and expensive. The latter are sometimes commercially unavailable. The existence and availability of drugs that may not be initially controlled inevitably offer an attractive alternative to recreational drug users. One of the reasons is the desire to minimize the risk of being prosecuted for illicit drug use.

By far, the NPS class of synthetic cathinones (SCs), derived from naturally occurring cathinone found in the leaves of Khat, became an attractive alternative stimulant group of drugs to accompany the classical banned stimulant compounds. These recreational drugs are also known as “legal highs” as they were initially legal in Europe and the UK and sold under deceptive names, for example, “bath salts,” “research chemicals,” or “plant food.” The first attempts for the synthesis of SCs started in the end of 1920s with methcathinone and mephedrone in 1928 and 1929, respectively. Thereafter, a few other derivatives have been designed and investigated for possible therapeutic use, for instance, bupropion, which plays an important role in the treatment of depressive disorders. Recreational SCs use only began in the mid-2000s when methylone emerged on the illicit drug market in the Netherlands and Japan. Since then, new SC derivatives began to appear in 2007 via online drug forums. Starting from 2009, the National Poisons Information Service (NPIS) had a notable rise in the number of inquiries related to SCs.

Currently, the number of SCs reported for the first time to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) over the past decade includes 138 substances, clearly showing a continuous circumvention of existing legislations of stimulant drugs of abuse.

Generally, NPS drug stability knowledge including SCs is relatively limited. Often, stability studies are mainly evaluated as a part of laboratories method validation; hence, it can be very hard to retrieve stability data by a systematic literature search. This ultimately makes it difficult for clinical and forensic toxicologists to recover the desired information. To the best of authors’ knowledge, a review on the stability of SCs in human biological samples has never been reported and should be a useful document to guide future research and appropriate sample handling for clinical and forensic cases involving potential SC use.

This article highlights key findings from previously published studies focusing on the stability of SCs in biological samples by searching the scientific literature since 2007. The choice of this starting year is based on the evidence of first seizures of SCs (i.e., mephedrone) in Europe, which is considered the point when SCs were starting to emerge on the illicit drug markets. Another reason is that other researchers have summarized the literature for stability in human biological samples before 2007 including SCs. This review was restricted to studies concerning human samples only to accurately simulate the real conditions. Ultimately, this review aims to provide insights of current knowledge about the stability of SCs in biological samples and offers a critical assessment on the limited studies conducted in the mentioned time period to understand limitations and to provide suggestions and improvements in future work.

MATERIALS AND METHODS

The most relevant findings of SCs in the available literature concerning their in vitro stability studies in human biological samples were reviewed. Different factors were used as inclusion or exclusion criteria of search selection to extrapolate the required scientific information. Published articles between January 1, 2007, and March 20, 2020, were conducted via searching of PubMed. Google Scholar was also used to identify any further relevant study. In addition, articles with at least one of the following criteria were excluded: (1) publications not in English; (2) studies that contained unclear data or interpretation; and (3) studies not performed in
| Compound name | Chemical structure | Reference |
|---------------|--------------------|-----------|
| 2-MMC         | ![2-MMC Chemical Structure](image1) | 51        |
| 3-CEC         | ![3-CEC Chemical Structure](image2) | 51        |
| 3-CMC         | ![3-CMC Chemical Structure](image3) | 51        |
| 3-FMC         | ![3-FMC Chemical Structure](image4) | 1,12,34,48|
| 3-MMC         | ![3-MMC Chemical Structure](image5) | 51        |
| 3,4-DMMC      | ![3,4-DMMC Chemical Structure](image6) | 1,12,33,48,51|
| 4-CEC         | ![4-CEC Chemical Structure](image7) | 51        |
| 4-CMC         | ![4-CMC Chemical Structure](image8) | 41,51     |
| 4-CPD         | ![4-CPD Chemical Structure](image9) | 51        |
| Compound name | Chemical structure | Reference |
|--------------|--------------------|-----------|
| 4-EMC        | ![Chemical structure](image) | 1,12,39,51 |
| 4-FMC        | ![Chemical structure](image) | 1,12,31,33,39,41,43,48,49 |
| 4-MEC        | ![Chemical structure](image) | 1,12,33,36,39,44,48,51 |
| 4-MPD        | ![Chemical structure](image) | 51 |
| Benzedrone   | ![Chemical structure](image) | 33,46 |
| Buphedrone   | ![Chemical structure](image) | 1,12,33,34,39,45 |
| Cathinone    | ![Chemical structure](image) | 31,33,39,44,45,48,51 |
| Diethylcathinone | ![Chemical structure](image) | 33,48 |
| Ethcathinone | ![Chemical structure](image) | 1,12,31,33,45,51 |

(Continues)
| Compound name                  | Chemical structure | Reference                  |
|-------------------------------|--------------------|----------------------------|
| Mephedrone                    | ![Mephedrone](image) | 1.12,31-35,37,39,41,43-46,48,49,51 |
| Methcathinone                 | ![Methcathinone](image) | 1.12,31,33,34,39,45,48     |
| Methedrone                    | ![Methedrone](image) | 1.12,31,33,34,39-41,43,48,49,51 |
| N-Ethylbuphedrone             | ![N-Ethylbuphedrone](image) | 41                         |
| N-Ethylhexedrone              | ![N-Ethylhexedrone](image) | 51                         |
| N-Propylpentedrone            | ![N-Propylpentedrone](image) | 51                         |
| N,N-Dimethylcathinone         | ![N,N-Dimethylcathinone](image) | 48                         |
| Pentedrone                    | ![Pentedrone](image) | 1.12,33,39,51              |
| N-Alkylated-methylenedioxy    | ![N-Alkylated-methylenedioxy](image) | 1.12,31,33,34,44,46,48,51  |
| Compound name        | Chemical structure | Reference     |
|----------------------|--------------------|---------------|
| Dibutylone           | ![Chemical structure](image) | 41            |
| Dimethylone          | ![Chemical structure](image) | 41            |
| Ephylone             | ![Chemical structure](image) | 51            |
| Ethylone             | ![Chemical structure](image) | 1,12,33,41,44,48 |
| Eutylone             | ![Chemical structure](image) | 1,12,41,51    |
| Methylone            | ![Chemical structure](image) | 1,12,31,33,34,41,43–45,48,49,51 |
| Pentylone            | ![Chemical structure](image) | 1,12,33,34,46,48,51 |
| N-Pyrrolidine-methylenedioxy | ![Chemical structure](image) | 3,4-MDPHP 51 |
| 3,4-MDPHP            | ![Chemical structure](image) |             |
| MDPBP                | ![Chemical structure](image) | 1,12,33,41,51 |
| Compound name | Chemical structure | Reference |
|---------------|--------------------|-----------|
| MDPPP         | ![MDPPP structure](image) | 33,39     |
| MDPV          | ![MDPV structure](image) | 1,12,32–34,39,41,43–45,48,49,51 |

### N-Pyrrolidine with/without ring substituents

| Compound name | Chemical structure | Reference |
|---------------|--------------------|-----------|
| 4-Cl-α-PVP    | ![4-Cl-α-PVP structure](image) | 51        |
| 4-F-α-PHP     | ![4-F-α-PHP structure](image) | 51        |
| 4-MPBP        | ![4-MPBP structure](image) | 1,12,33,41 |
| MPHPP         | ![MPHP structure](image) | 51        |
| Naphyrone     | ![Naphyrone structure](image) | 1,12,33,34,39,45,48,51 |
| PV9           | ![PV9 structure](image) | 51        |
| Pyrovalerone  | ![Pyrovalerone structure](image) | 1,12,33,39 |
| Compound name | Chemical structure | Reference |
|---------------|--------------------|-----------|
| α-PBP         | ![α-PBP structure](image) | 41        |
| α-PHP         | ![α-PHP structure](image) | 51        |
| α-PiHP        | ![α-PiHP structure](image) | 51        |
| α-PPP         | ![α-PPP structure](image) | 33        |
| α-PVP         | ![α-PVP structure](image) | 1,12,33,39,45,51 |

**Metabolites**

- 4-Carboxy-mephedrone
  - ![4-Carboxy-mephedrone structure](image)
  - 35,40
- 4-Methylephedrine (dihydro-mephedrone)
  - ![4-Methylephedrine structure](image)
  - 33,35,39,40
- Buphedrone ephedrine (dihydro-buphedrone)
  - ![Buphedrone ephedrine structure](image)
  - 33

(Continues)
human samples. The search was conducted by applying the following keywords: “synthetic cathinone” or “cathinone derivative” or “stability of novel psychoactive substance” or “stability of new psychoactive substance” or “stability of synthetic cathinones” or “legal highs” or “bath salts” or “designer drugs.” Further, combining keywords, for example, “AND,” “NOT,” and “OR,” was also used to help with databases using Boolean operator.

As a result, 23 found studies of NPSs and/or SCs assessed the stability of certain SCs in human biological samples and fulfilled the above criteria, of which 12 studies were exclusively focused on the investigation of SC stability, whereas the other studies performed the stability as a part of method validation. So far, 58 SCs and metabolites have been described in the literature with respect to their stability data, which are summarized in Table 1.

### RESULTS AND DISCUSSION

#### 3.1 Stability investigations for SCs in conventional matrices: Whole blood, plasma, serum, and urine

In a method development and validation study of selected SCs and ephedrines in whole blood samples, Sørensen investigated the stability of cathinone, methcathinone, ethcathinone, mephedrone, 4-fluoromethcathinone (4-FMC), methedrone, methylone, and butylone, stored for 6 days at 5°C and 20°C. One set of samples was preserved with sodium fluoride/potassium oxalate (NaF/KOx) (pH 7.4) and another set with NaF/citrate buffer (pH 5.9). After 5 days of storage at 20°C, the concentrations of unsubstituted and ring-substituted secondary amines were lost by >70% in samples preserved with NaF/KOx, compared with less than 40% loss in samples.
with NaF/citrate buffer. The other SCs, that is, methylenedioxy SC types, were also degraded, however at a lower level (5% loss). In contrast, at 5°C, all analytes that were preserved with NaF/KOx were relatively stable up to 3 days; but thereafter, cathinone, methcathinone, ethcathinone, methedrone, and 4-FMC were less stable. The researchers pointed out that the degradation rate of SCs increased with increasing blood pH level but also varied with chemical structures. Notably, all ephedrine analytes were stable over the whole study under the same storage conditions, additives, and pH. This finding highlights the increased stability of the hydroxy-group-containing ephedrines compared with β-keto group-containing analytes, namely, SCs.

In published methods for the detection and quantification of selected SCs and their related ephedrines in human whole blood, Amman et al.48 evaluated the stability of cathinone, N,N-dimethylcathinone, 3,4-dimethylmethcathinone (3,4-DMMC), ethylone, methylene, 4-methylethcathinone (4-MEC), butylone, 4-FMC, mephedrone, methedrone, methcathinone, diethylcathinone, methylole, methylenedioxyxyprovalerone (MDPV), naphrynone, 3-fluoromethcathinone (3-FMC), cathinone, and pentylene. This work included freeze/thaw (F/T) stability (−20°C/room temperature [RT]) assessment over eight cycles and long-term stability over 6 weeks at −20°C, all at low and high concentrations of 30 and 900 ng/ml, respectively. The authors found all analytes to be stable under the studied conditions.

The stability of mephedrone and MDPV (including other NPSs) was further investigated by Johnson and Botch-Jones32 in various biological samples, as these analytes were encountered for the first time in these authors’ laboratory. Both analytes were spiked in human whole blood, urine, and serum samples and then stored for 14 days at −20°C (freezer), 4°C (refrigerator), and 22°C (RT). MDPV appeared to be fairly stable in all samples under the above conditions with the exception of whole blood where minor degradation (around 10%) occurred on Day 4 when left at 22°C. However, according to the authors, this small degradation could be attributed to analytical variation error rather than actual instability. Mephedrone appeared to be the least stable when compared with MDPV. Although mephedrone remained stable in all samples when stored at −20°C for 14 days, significant loss of concentrations was observed when left at 22°C in whole blood, plasma, and urine on Days 2, 4, and 7, respectively. Mephedrone was stable in all samples over the 14 days when stored at 4°C except for whole blood, where about 30% of its concentration on Day 7 was lost.

Several other authors studied the stability of SCs and their metabolites in urine. Conceiroke et al.33 reported the method development and validation including a stability study of cathinone, methcathinone, 4-FMC, methylene, ethcathinone, α-pyrolidinopropiophenone (α-PPP), buphedrone, ethylene, methedrone, diethylcathinone, 3,4-methylenedioxy-α-pyrolidinopropiophenone (MDPPP), butylone, mephedrone, 4-FMC, 3,4-methylenedioxy-α-pyrolidinobutiophenone (MDPBP), pentedrone, pentylene, α-pyrolidinopentiophenone (α-PVP), 4-methyl-α-pyrolidinobutiophenone (4-MPBP), MDPV, pyrovalerone, benzedrone, and naphrynone, nor-mephedrone, dihydro-4-MEC, buphedrione, dihydrophendrine, and 4-methylphendrine. The stability study included short-term stability for spiked urine that stored in the dark over 24 h at RT, 72 h at 4°C, and F/T stability over three cycles. The authors reported diethylcathinone, MDPBP, MDPBP, α-PVP, 4-MPBP, and MDPV as well as all metabolites (except for nor-mephedrone) to be stable at RT for 24 h. In addition, all analytes were stable at 4°C for 72 h and after three F/T cycles. The only exceptions were benzodrone at both 4°C for 72 h and after three F/T cycles (degradation of >22% and >26%, respectively) and naphrynone that showed instability after three F/T cycles (degradation of >22%).

In the validation of a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method in urine published by Al-Saffar et al.,34 the first attempt to investigate the stability of methcathinone, buphedrione, mephedrone, 3-FMC, 4-FMC, methedrone, methylene, butylene, pentylene, MDPV, and naphrynone was conducted by storing the calibration standards along the spiked samples at −20°C. The authors noted that calibration standards were unstable, and therefore, standards were freshly prepared at the time of analysis. In this study, spiked urine samples were stored for 3 months at −20°C (freezer), 6°C (refrigerator), and 22°C (RT). Methedrone, methylene, butylene, and naphrynone were relatively stable at 22°C for 24 h, but on Week 1, they showed a significant degradation (40%) to nearly complete degradation with trace remaining of 0.1%. Only MDPV remained fairly stable for the entire study except for the third month at 22°C, reinforcing the hypothesis that the introduction of either methylenedioxy groups or most likely constraining the basic nitrogen in a five-membered ring increases resistance of SCs to degradation pathways. In contrast, buphedrione, 3-FMC, and 4-FMC were unstable under all the given conditions.

In a pharmacokinetics study, Olesti et al.35 evaluated the stability of mephedrone and its metabolites including nor-mephedrone, N-succinyl-nor-mephedrone, dihydro-mephedrone, and 4-carboxymephedrone in human urine and plasma. The stability study included F/T stability over two cycles at 4°C and at −20°C for 1, 3, and 6 weeks. As a result, all compounds were stable under the above-assessed conditions.

Soh and Elliott36 evaluated the stability of 13 NPSs, including one SC (4-MEC) in human whole blood and plasma at RT (20–23°C). In whole blood, 4-MEC was unstable on Day 1 (about 60% loss) and became undetectable after 14 days. In plasma sample, 4-MEC shows a considerable decrease of 54% in its concentration after 14 days. However, a trace amount of 4-MEC remained detectable up to 37 days. Further analysis was conducted to determine the breakdown products. The authors concluded that dihydro-4-MEC resulting from keto reduction was the only breakdown product in both samples.

As previous studies reported that mephedrone is subject to degradation in blood samples, Busardó et al.37 attempted to reduce analyte losses by using different preservatives in both antemortem (AM) and postmortem (PM) blood samples. In this work, mephedrone was spiked in AM and PM blood samples unpreserved, preserved with 3% ethylenediaminetetraacetic acid (EDTA), or preserved with 1.67%/0.2% NaF/KOx. The stability was assessed over 185 days at
A comprehensive and systematic study on the stability of SCs displaying secondary amines (methcathinone, ethcathinone, pentedrone, and buphedrone), secondary amines with ring substituents (3-FMC, 4-FMC, methyline, 4-ethylmethylcathinone [4-EMC], ethylene, methedrone, butylene, mephedrone, etylene, 4-MEC, pentyline, and 3,4-DMMC), and tertiary amines (α-PVP, MDPBP, 4-MPBP, MDPV, pyrovalerone, and naphyrone) in whole blood and urine was reported by Glicksberg and Kerrigan.\(^1\) Unpreserved whole blood and preserved urine (1% NaF) were assessed under various conditions. The analytes spiked at 100 and 1,000 ng/ml and monitored over 6 months at −20°C, 4°C, 20°C, and 32°C. Moreover, the influence of pH was also evaluated by adjusting the pH of urine to either 4 or 8. In addition, analysis of variance (ANOVA) was also used to determine statistical significance (p = 0.05). Consequently, apart from the analyte concentrations, this study revealed that stability was found to be highly affected by other factors such as storage temperature, pH, and the chemistry of the analytes. Tertiary amines were the most stable under the given conditions than secondary amines, including ring-substituted secondary amine. Among others, 3-FMC was found to be the most unstable with significant degradation observed after only few hours of storage, whereas MDPV and MDPBP were the most stable. Furthermore, it was noted that degradation rate decreased with lower storage temperature for all analytes, even for the most unstable ones. In addition, these analytes were found to be fairly stable in acidic urine (pH 4) when compared with those in alkaline urine (pH 8). Markedly, by comparing the most unstable analyte with the most stable with acidic and basic pH at −20°C (Figure 1), respectively, it can be observed that both analytes were relatively stable over 165 days and that their degradation rate are closely similar. Presumably, the stability of SCs at lower temperatures is highly dependent on the pH value rather than the type of analyte.

A long-term stability study on 22 SCs and related metabolites in unprocessed urine samples was carried out by Alsenedi and Morrison.\(^3,12\) The analytes covered in this study are cathinone, methcathinone, buphedrone, pentedrone, 4-FMC, mephedrone, 4-MEC, 4-EMC, methedrone, α-PVP, pyrovalerone, MDPPP, MDPV, naphyrone, 4-methylphendrine (mephedrone metabolite), and dihydro-4-MEC. As a result, all of these analytes proved to be stable when stored at −20°C for 201 days. However, all analytes were significantly degraded at 4°C after 21 days and at RT after 2 days. In this report, however, neither 4°C nor RT was tested for tertiary amine groups, but according to the authors, these SC types were estimated to be stable under the above-described conditions.

Czerwinska et al.\(^4\) investigated the stability of mephedrone and its Phase I metabolites, including dihydro-mephedrone, dihydro-nor-mephedrone, hydroxytolyl-mephedrone, 4-carboxy-mephedrone, and nor-mephedrone in whole blood samples. The samples were collected from drug-free donors in Vacutainer tubes containing 0.25% NaF/0.1% KOx as additives and stored at −20°C and 4°C for a 10-day study period. Their findings showed that

**FIGURE 1** Stability of MDPV, 3-FMC, MDPBP, and methcathinone in urine samples over 165 days of storage at −20°C and at pH of 4 or 8. Reproduced from Glicksberg and Kerrigan\(^5\)
dihydro-mephedrone and dihydro-nor-mephedrone were stable over the whole study under all the storage conditions. By contrast, both mephedrone and hydroxytolyl-mephedrone were found to be relatively unstable under the given conditions, whereas 4-carboxy-mephedrone and nor-mephedrone showed the poorest stability. Overall, dihydro-mephedrone followed by dihydro-nor-mephedrone was consistently the most stable of the investigated analytes, and 4-carboxy-mephedrone represents the most unstable metabolite, with <60% remaining at both temperatures. Comparing 4-carboxy-methylene represents the most unstable metabolite, with was consistently the most stable of the investigated analytes, and Overall, dihydro-mephedrone followed by dihydro-nor-mephedrone was expected by the greater biochemical complexity of blood compared with urine matrix, but also again highlighting the critical role of the pH in maintaining sample integrity. As mentioned, clearly the acidity of pH plays a crucial role at increasing the stability of SCs even with those analytes that are frequently regarded as poorly stable (Figure 2). A further experiment was conducted to compare the effect of repeated thawing and freezing samples on the stability of analytes. From their findings, similar results were obtained for repeated thawing (24 F/T cycles) with samples not thawed.

3.2 Stability investigations for SCs in alternative samples

Whereas the existing literature mainly describes the stability of SCs in blood, urine, plasma, and serum samples, Strano-Rossi et al. investigated the stability of 4-MEC, methylenedione, mephedrone, and MDPV in unpreserved oral fluid (OF) after eight F/T cycles (for 2 months at −20°C).

De Castro et al. studied the stability of methedrone, methylene, mephedrone, MDPV, and 4-FMC in authentic human OF. Spiked OF was either unpreserved (neat OF) or preserved with Quantisal™ buffer and after three F/T cycles. The only exceptions were methedrone and mephedrone at low quality control (QC) (0.6 ng/ml) when kept for 72 h on an autosampler (6°C), with a degradation of 30.8% and 20.7%, respectively.

Mecorelli et al. reported a study via LC–MS/MS and volumetric absorptive microsampling (VAMS™) for SCs in dried urine, plasma, and OF spots, which also included stability data. The investigation of cathinone, methylene, ethylene, butylene, mephedrone, 4-MEC, and MDPV revealed them to be stable in all samples for 7 days at RT.

Miller et al. investigated the stability of cathinone, methcathinone, buphedrone, mephedrone, 4-MEC, MDPV, methylone, naphyrone, α-PVP, and ethcathinone in human OF. Analytes were spiked at low and high QC (2.5 and 150 ng/ml, respectively) with

![Figure 2](image-url) Stability examples of 4-FMC (secondary amine) and MDPV (tertiary amine) in urine (pH 5) and blood (pH 7), respectively, over 165 days at −26°C. Reproduced from Adamowicz and Malczyk"
neat OF (unpreserved), OF preserved with Quantisal™ buffer, or with Oral-Eze™ and stored in the dark at −20°C, 4°C, and RT for 1 month and after three F/T cycles. At RT, only MDPV remained stable for 1 month in neat OF and Oral-Eze buffer samples, whereas few analytes, namely, methylone, α-PVP, and MDPV, remained stable in Quantisal. Furthermore, the poorest stability was observed in neat OF followed by Oral-Eze under the same temperature. At 4°C, only MDPV and α-PVP were found to be stable after 1 month in neat OF, followed by buphedrone, 4-MEC, mephedrone, methylene, MDPV, and α-PVP in Oral-Eze samples. On the other hand, all analytes in Quantisal remained quite stable after 1 month except for methcathinone, buphedrone, and naphyrone at low concentrations. At −20°C, no instability was observed for analytes in the three sample types except for cathinone and naphyrone in Oral-Eze at a low concentration. Only naphyrone was found to be unstable at low QC after three F/T cycles in the three types of samples. However, buphedrone and cathinone also showed instability in Quantisal and Oral-Eze at low QC under this condition, respectively. Most of the analyte levels in all sample types were significantly decreased after storage at RT for over 1 month, of which several analytes were completely lost.

Da Cunha et al.46 compared the stability of butylone, benzodrone, mephedrone, and pentylone in whole blood and dried blood spots (DBSs) during storage at −20°C, 4°C, and RT for 90 days. Both samples were unpreserved and fortified at 30 and 750 ng/ml. At RT, a marked instability was found in all analytes when stored for 90 days in both samples. However, analytes remained detectable in DBS over the 90 days but undetectable in whole blood. At −20°C for 90 days, all analytes had no or only minor effects on the concentrations in both samples. Likewise, 4°C for 90 days led to no significant losses for butylone and pentylone in both samples but to a significant to complete degradation for mephedrone and benzodrone under the same storage temperature. The authors concluded that degradation rate of analytes in whole blood is generally higher than that observed in DBS, indicating the usefulness of DBS as an alternative matrix to whole blood. As with the previous studies, butylone and pentylone were generally the most stable, whereas mephedrone followed by benzodrone showed to be the least stable.

Recently, Czerwinska et al.47 published a first method for the detection of mephedrone and its metabolites in fingerprints by LC–MS/MS and paper spray–mass spectrometry (MS). The stability of mephedrone and dihydro-mephedrone was carried out as a part of method validation. They compared the stability to freshly prepared solution at −20°C and 4°C for 1 and 4 weeks. All analytes were unstable under the given conditions. On Week 4 at −20°C, both mephedrone and dihydro-mephedrone lost 38–41% and 63–67% of their baseline values, respectively. A comprehensive list of publications on stability studies of SCs illustrating various conditions in human samples is summarized in Table 2.

### 3.3 Stability of extracted samples on autosampler (from hour to days)

Investigations on processed (i.e. extracted) sample stability help to assess the integrity of an analyte, which has been extracted from a biological sample, for the period of its storage until the time of analysis or after initial injection. This is because instability of analytes may occur not only in biological samples but also in processed/extracted samples ready for analysis. It is therefore important to assess the impact of storage conditions against expected and/or unexpected situations that may arise when samples are left on an autosampler, for example, large batch size or technical faults of the instrument. Table 3 summarizes the key results of processed sample stability of SCs.

### 3.4 Analytical techniques and types of samples for evaluating the stability of SCs

In the case of SCs, the vast majority of these methods has been conducted by means of LC–MS1,12,31–35,37,38,40–46,48–50 with the exception of the method described by Busardò et al.,37 Alsenedi and Morrison,39 and Woźniak et al.,51 in which SCs and related metabolites were analyzed by gas chromatography–MS (GC–MS).

Whole blood and urine are the samples of choice for most stability testing owing to their common use in toxicology laboratories largely as a result of interpretative and detection window requirements. For example, whole blood can reflect the concentration of a drug/poison at the time of death, whereas urine samples represent longer detection windows and are characterized by the presence of major metabolites that can assist in determining drug history. Nevertheless, alternative samples like OF have gained importance in forensic settings owing to the short detection window and noninvasive sample collection for some situations (e.g. WDT and drug driving). The percentage of each matrix type from the reviewed literature to evaluate the stability of SCs is illustrated in Figure 3.

### 3.5 Study design and reporting of results

The evaluation of processed sample stability is provided as a part of validation parameters in a number of official guidelines such as the US Food and Drug Administration (FDA),52 the European Medicines Agency (EMA),53 and the Scientific Working Group for Forensic Toxicology (SWGTOX) (recently replaced by ANSI/ASB Standard 036).54,55 The latter is the most commonly used of these guidelines for the evaluation of SCs stability likely owing to its guidance on how to perform and evaluate validation parameters of analytical methods including detailed experimental design for stability. Furthermore, recommendations published by Peters et al.56 on how these guidelines are best applied to forensic and analytical toxicology may be helpful to evaluate the stability data. As an example, the authors recommend evaluating the stability under conditions of samples preparation to decide whether appropriate preservatives should be added to reduce/
| No. analyte | Conc. | Matrix and conditions | Temp. | Stability acceptable criteria | Length of time | Type of container | Main findings | Reference |
|------------|-------|-----------------------|-------|-------------------------------|----------------|------------------|---------------|-----------|
| 28 SCs     | 3 and 300 ng/ml | Urine (pH 7.6) | RT and three F/T | Stable within ±20% | 72, 24, and 48 h (on autosampler) | Polypropylene cryovials | - Unstable at RT after 24 h of storage except for tertiary amines | 33 |
| 4-MEC, methylene, mephedrone, and MDPV | 50 ng/ml | Unpreserved OF | 20°C (on autosampler) and eight F/T (at −20°C) | At RT: ratios between reference and stability samples within ±10% indicate stability; F/T cycles: negative slope significantly different from zero indicate instability | 4 weeks at RT, 2 months for eight F/T | N/S | - Stable | 42 |
| 22 SCs | 100 and 1,000 ng/ml | Unpreserved whole blood (pH ~ 7.4); urine with 1% NaF (pH 4 and 8) | −20°C, 4°C, 20°C, and 32°C | Stable within ±20% | 6 months | 100-ml nonsterile polypropylene containers for urine and 10-ml evacuated glass tubes for blood (no additives) | - More stable in acidic urine pH (4) | 1,12 |
| Five SCs | 30 (after three F/T for preserved OF) ng/ml | OF (neat or with Quantisal™ buffer) | 24 h at 4°C (for neat OF) and three F/T cycles (for both sample types) | Stable within ±15% | 72 h at 6°C on autosampler, 24 h at 4°C, and three F/T cycles | Salivette® and/or Quantisal™ OF collection devices | - Stable under all conditions except for mephedrone, methedrone, at low QC on autosampler (6°C) | 43 |
| Seven SCs | 250 ng/ml | Dried urine, plasma, and OF spots | RT | Stable within ±15% | 7 days | VAMS™ collection devices in a sealed polyethylene bags | - Stable under all conditions | 44 |

(Continues)
| No. analyte | Conc. | Matrix and conditions | Temp. | Stability acceptable criteria | Length of time | Type of container | Main findings | Reference |
|-------------|-------|-----------------------|-------|------------------------------|---------------|------------------|---------------|-----------|
| Mephedrone and metabolites | LLOQ (0.5/1/10 ng/ml), low QC (1/10/100 ng/ml), med QC (10/100/1,000 ng/ml), and ULOQ (100/1,000/10,000 ng/ml) | Urine and plasma | Two F/T cycles at 4°C and −20°C | Stable within ±20% | Two F/T cycles | Injection vials | - Stable | 35 |
| 17 SCs | 100 ng/ml | Blood (pH 7) and urine (pH 9) | RT, 5°C, −26°C, and 24 F/T cycles | Stable within ±15% | 6 months | 2 (for F/T) and 20-ml glass vials | - More stable in urine | 41 |
| 10 SCs | 2.5 and 150 ng/ml | Neat OF (unpreserved), OF preserved with Quantisal™ buffer, or with Oral-Eze™ | −20°C, 4°C RT, and three F/T cycles | Stable within ±20% | 1 month | 2-ml polypropylene cryotubes | - More stable in Quantisal at RT | 45 |
| Four SCs | 30 and 750 ng/ml | Whole blood and DBS | −20°C, 4°C and RT | Stable within ±15% | 90 days | N/S | - All unstable at RT | 46 |
| Mephedrone and five metabolites | 0.25 and 8 ng/ml | Whole blood (0.25% NaF/0.1% KOx) | −20°C and 4°C | Stable within ±10% | 10 days | NaF/KOx plastic Vacutainer tubes | - Both dihydro-mephedrone and dihydro-nor-mephedrone stable at all conditions | 40 |
| No. analyte | Conc. | Matrix and conditions | Temp. | Stability acceptable criteria | Length of time | Type of container | Main findings | Reference |
|------------|-------|-----------------------|-------|-------------------------------|---------------|------------------|---------------|-----------|
| Mephedrone | 1,000 ng/ml | AM and PM blood in unpreserved, 3% EDTA, or with 1.67%/0.2% NaF/KOx | −20°C, 4°C, and 20°C | The differences in mean concentrations between groups that were statistically significant at the 5% levels used to indicate instability | 185 days | 2-ml Eppendorf tubes | - Unstable at 4 and 20°C (less unstable in NaF/KOx) - Stable at −20°C (more stable in NaF/KOx) | 37 |
| 11 SCs | 1,000 ng/ml | Urine | −20°C, 6°C, and 22°C | N/S | 3 months | N/S | - More stable at −20°C - Tertiary amines (MD) consistently most stable - Buphedrone, 3-FMC, and 4-FMC unstable under all conditions | 34 |
| Mephedrone and MDPV | 1,000 ng/ml | Whole blood, urine, and serum | −20°C, 4°C and RT | N/S | 14 days | Glass container | - More stable at 20°C, followed by 4°C in all samples - Tertiary amines more stable than secondary amines | 32 |
| 18 SCs | 30 and 900 ng/ml | Whole blood | 4°C, −20°C, and eight F/T cycles | Stable within ±20% | 4°C for 24 h; eight F/T cycles and −20°C for 6 weeks | 2-ml Eppendorf tube | - Stable under all conditions | 48 |
| Mephedrone and dihydro-mephedrone | 50 ng/ml | Fingerprint (right thumb and index) | 4°C and −20°C | Stable within ±15% | 1 and 4 weeks | N/S | - Unstable under all conditions | 47 |
| No. analyte | Conc.         | Matrix and conditions                        | Temp.  | Stability acceptable criteria                                                                 | Length of time | Type of container                        | Main findings                                                                                           | Reference |
|------------|--------------|----------------------------------------------|--------|------------------------------------------------------------------------------------------------|----------------|----------------------------------------|--------------------------------------------------------------------------------------------------------|-----------|
| 10 SCs     | 2 and 500 ng/ml | OF preserved with Quantisal™ buffer          | 15°C   | Negative slope significantly different from zero indicate instability                           | 6 h            | Quantisal™ collection devices          | - Stable                                                                                               | 50        |
| 4-MEC      | 2,000 ng/ml  | Unpreserved whole blood and plasma           | RT     | N/S                                                                                          | 21 days for whole blood and 37 days for plasma | N/S                      | - Unstable under both conditions                                                                 | 36        |
|            |              |                                              |        |                                                                                                |                |                                        | - Higher degradation rate in blood compared with plasma                                               |           |
|            |              |                                              |        |                                                                                                |                |                                        | - Dihydro-4-MEC was the main degradation breakdown                                                   |           |
| Eight SCs  | 100 ng/ml    | Whole blood NaF/KOx (pH 7.4) or NaF/citrate buffer (pH 5.9) | 5°C and 20°C | N/S                                                                                         | 6 days         | NaF/KOx and/or NaF/citrate in Venosafe tubes | - More stable with NaF/citrate (pH 5.9) than NaF/KOx (pH 7.4)                                         | 31        |
|            |              |                                              |        |                                                                                                |                |                                        | - More stable at 5°C                                                                                  |           |
|            | 500 and 1,000 ng/ml | Urine                                    | −20°C  | Stable within ±20%                                                                            | 201 days       | N/S                      | - All stable at −20°C                                                                                  | 39        |
| 22 SCs     | including three metabolites                     |                                              |        |                                                                                                |                |                                        | - More stable in tertiary amines, followed by secondary amines (MD-substituted)                     |           |

Abbreviation: N/S, not specified.
| Name of analyte                                                                 | Conc.          | Matrix and conditions | Temp. of autosampler | Stability acceptable criteria                                                                 | Length of time (on autosampler) | Main findings                          | Reference |
|--------------------------------------------------------------------------------|----------------|-----------------------|----------------------|-----------------------------------------------------------------------------------------------|---------------------------------|----------------------------------------|-----------|
| Ethcathinone, \( N,N \)-dimethylcathinone, 3,4-DMMC, ethylone, methylone, 4-MEC, butylone, 4-FMC, mephedrone, methedrone, methcathinone, diethylcathinone, methylone, MDPV, naphyrone, 3-FMC, cathinone, pentylole | 30 and 900 ng/ml | Whole blood            | 4°C                   | Negative slope significantly different from zero indicate instability                       | 24 h with 2-h time interval between injections | Stable                                               | 48        |
|                                                                                   | 3, N/S and 300 ng/ml | Urine (pH 7.6)        | 4°C                   | Stable within ±20%                                                                          | 48 h                            | Stable (from −9.5 to 7.3%)                | 33        |
|                                                                                   | 1,000 ng/ml     | Urine                 | 12°C                 | N/S                                                                                         | 3 days                          | Stable (<4%)                            | 34        |
| Cathinone, ethcathinone, 2-methylmethcathinone (2-MMC), 3-methylmethcathinone (3-MMC), mephedrone, pentedrone, 4-CMC, 3-chloromethcathinone (3-CMC), 4-MEC, 4-EMC, 3,4-DMMC, 4-methylpentedrone (4-MPD), N-propylpentedrone, 3-chloroethcathinone (3-CEC), 4-chloroethcathinone (4-CEC), N-ethylhexedrone, methedrone, 4-chloropentedrone (4-CPD), \( \alpha \)-PVP, methylone, \( \alpha \)-pyrroldinoisohexanophenone | 2.5, 25, and 200 ng/ml | Whole blood            | RT                   | Stable within ±20% for medium and high QC; ±15% for low QC                                 | 12 and 24 h                     | Stable (9.8% and 14% for 12 and 24 h, respectively) | 51        |

(Continues)
| Name of analyte                          | Conc.     | Matrix and conditions | Temp. of autosampler | Stability acceptable criteria | Length of time (on autosampler) | Main findings | Reference |
|----------------------------------------|-----------|-----------------------|----------------------|------------------------------|---------------------------------|---------------|-----------|
| (α-PiHP), 4-fluoro-α-pyrrolidino hexanophenone (4-F-HP), butylone, α-pyrrolidino hexanophenone (α-PHP), etylone, pentylone, 4-chloro-α-pyrrolidinopentiophenone (4-c-α-PVP), ephylone, 4-methyl-α-pyrrolidino hexanophenone (MPHP), α-pyrrolidino octanophenone (PV9), MDPBP, MDPV, 3,4-methylenedioxy-α-pyrrolidinohexanophenone (3,4-MDPHP), naphryone | 50 ng/ml  | Unpreserved OF        | 20°C                 | Ratios between reference and stability samples within ±10% indicate stability | 4 weeks            | Stable       | 42        |
| MDPV, mephedrone, 4-FMC, methedrone, etylone, butylone, methcathinone, methylene, α-PVP, pyrovalerone | 2 and 500 ng/ml | OF preserved with Quantisal™ buffer | 15°C              | Negative slope significantly different from zero indicate instability | 6 h (with 1 h interval between injections) | Stable       | 50        |
| Methedrone, methylene, mephedrone, MDPV, 4-FMC | 0.6 and 150 ng/ml | Neat OF              | 6°C                  | Stable within ±15%            | After 72 h                     | Stable except for methedrone and mephedrone at 0.6 ng/ml (loss of 30.8% and 20.7%, respectively) | 43        |
| Cathinone, methcathinone, buphedrone, mephedrone, 4-MEC, MDPV, methylene, naphrynone, α-PVP, ethcathinone | 2.5 and 150 ng/ml | OF preserved with Quantisal™ buffer | 10°C                 | Stable within ±20%            | 24 h                           | Stable       | 45        |
| 4-FMC, methylene, mephedrone, methedrone, MDPV | 6, 30 (for 4-FMC) and 1500 pg/mg | Hair                 | 6°C                  | Stable within ±15%            | 72 h                           | Stable (<14.5% loss) | 49        |

Abbreviation: N/S, not specified.
prevent degradation if occurred. According to SWGTOX guideline, processed sample stability is often determined by comparing results of QC samples prepared from reference materials and then analyzing in triplicate before (reference samples) and after (stability samples) being exposed to different conditions to assess their stability against situations commonly encountered in the laboratory. In addition, it has been recommended to complete the stability evaluation at least at two concentration levels (low and high). At each concentration, average signal (i.e. absolute peak areas of analyte or ratios of peak areas of analyte to internal standard) of stability samples at each time interval is compared with average signal of reference samples. When comparing stability samples with reference samples, differences in the average signal should be higher or lower than the acceptance criterion of method accuracy (e.g. ±10% or ±20%) to consider the analyte as unstable at a given storage temperature. Where appropriate, it is recommended to perform the F/T stability over at least three cycles in triplicate.

In contrast, a slightly different approach to study processed sample stability (also for long-term testing) as recommended by FDA is that QCs at each testing point should be compared with QCs of freshly prepared calibrators and QCs and that analytes should be considered stable until the accuracy reach >±15%. In other words, the results of accuracy and precision of analyte of interest at each concentration were compared with those freshly prepared and analyzed to define the length of time that samples can be stable within acceptance criteria.

3.6 | Critical discussion

The reviewed studies clearly demonstrated that concentrations of many analytes are decreased under elevated storage temperatures. Moreover, the reported findings are comparable for most of chemical structures and draw similar conclusions; however, some studies reported different level of degradation for certain analytes and matrices as an influence of other factors such as additives and pH. In order to better explain such discrepancies among different stability studies, the chemical, enzymatic and bacterial activities need to be considered as well, as some considerations may need to be taken into account when establishing an experimental design. It worth noting that a number of reports were excluded in this review owing to limiting the stability to “stable” or “unstable” with no further information on storage conditions or concentration level of degradation.

3.6.1 | Storage temperature

Reviewed stability studies on SCs revealed minor to severe degradation based on storage temperature. The instability at RT was observed for the majority of analytes. The reviewed results indicated that degradation took place between 1 day and 1 week for most of the analytes. Conversely, refrigeration slowed degradation noticeably, extending stability from 3 days to 3 months. Moreover, freezing conditions offered a larger degree of stability than other conditions ranging from 2 weeks to 7 months. Despite significant differences in the rate of decomposition of SCs at each storage condition that can be attributed to other factors, in general, their stability was definitely higher at freezing conditions when compared with other storage conditions.

3.6.2 | Chemical structure

Besides the role of various storage conditions on the stability, reports by most authors strongly indicate high degradation of SC-type analytes featuring secondary amines (either with or without ring substituents) such as mephedrone, methedrone, 4-CMC, 4-MEC, and 4-FMC. It is worth noting that the positional isomers of the latter were slightly more stable than 3-FMC in this group. Tsujikawa et al. assessed the stability of methcathinone analogs having fluorine-substituted aromatic ring and explained the stability variance by applying the Hammett equation. As a result, higher decomposition rate constants of 3-FMC (meta-substituted) compared with 4-FMC (para-substituted) were observed. Other researchers also pay attention to the effect of different halogens on the stability of SCs. They found lower degradation rates of fluorine- than chlorine-containing SCs. Besides chlorine and fluorine analogs in the previous studies, other halogenated derivatives such as bromine and iodine might be anticipated to show a different degree of instability in biological samples. However, stability of these halogenated SCs has not been investigated in biological samples.

Secondary amine-containing SCs also possessing methylenedioxy group such as butylone, ethylone, methylone, and pentylyone had also experienced degradation but at lower level. N-Pyrrolidine without methylenedioxy significantly slowed degradation at different storage conditions and even slower for
N-pyrollidine-methylenedioxy, indicating that the methylenedioxy and/or the N-pyrollidine ring may play a role at preventing putative chemical and/or enzymatic degradation/biotransformation pathways of the parent drug in the matrix. Another suggestion was drawn by Glicksberg and Kerrigan that the inability of tertiary amines to undergo oxidative deamination may be attributed to its relative stability.

Most of the analytes (50 out of 58) in the reviewed articles are parent and not metabolites. All these parent SCs share a ketone functional group in their molecule. This ketone is readily reduced to hydroxyl group, that is, reduced SC metabolites. In previous experiments, only reduced metabolites (i.e. dihydro-mephedrone and dihydro-nor-mephedrone) were found to be stable in whole blood when stored at 4 and −20°C for 10 days, which is in good agreement with stability findings described by Concheiro et al. They found that dihydro-4-MEC, dihydro-buphedrone, and dihydro-mephedrone were fairly stable at RT for 24 h and at 4°C for 72 h in urine samples. In urine, however, Alsenedi and Morrison reported a degradation of dihydro-4-MEC by 22% after 24 h of storage at 4°C. Related data for stability of SC metabolites are currently limited, and hence, further stability studies on SC metabolites are needed to draw better image.

### 3.6.3 Concentration levels

The concentration of SCs of reported studies was limited to low and high QC's varying from 0.25 up to 2,000 ng/ml, frequently relying on concentrations that are incorporated in the method validation ranges. Despite the varied range of concentrations, Glicksberg and Kerrigan were the only one who compared the statistical differences between two different concentrations of SCs in whole blood and urine samples. This study found no statistical significant degradation of 100 and 1,000 ng/ml in blood stored at 4°C over 6 months. Likewise, for urine (pH = 4) when stored at RT over 6 months, demonstrating other factors may have an impact on the stability of SCs unrelated to the concentration. Whether one or more concentration is used, it is necessary to establish the concentrations on the basis of concentrations found in real AM as well as PM cases to reflect the reality of actual degradation.

### 3.6.4 Formation of breakdown products

It is obvious that most of the stability investigations of SCs in the literature has typically been limited to the parent compounds. A related issue is knowledge of instability products that may need to be monitored as part of the analytical method. This is especially the case, as is sometimes for SCs only metabolites are detected in the biological sample and not the parent analyte.

However, very few studies further investigated the degradation products that formed as a result of parent analyte instability of SCs. Of those, only Soh and Elliott confirmed the formation of dihydro-4-MEC from 4-MEC in biological samples (viz. whole blood and plasma), whereas other authors used nonbiological samples to monitor SC breakdown products. However, the concentration ratio of degradation of 4-MEC and formation of dihydro-4-MEC was not proportional, which may indicate the presence of other undetected products.

Thus, it would be ideal to carry out more studies of potential newly discovered breakdown products (resulting from in vitro instability), which alongside metabolites may possess longer detection window than the parent analyte. As mentioned earlier, the stability of dihydro-mephedrone and dihydro-nor-mephedrone metabolites over the parent analyte (i.e. mephedrone) is probably the most notable example.

### 3.6.5 Additives

After collection of whole blood samples, clotting (coagulation) can occur within 30 min in the collection tube. Consequently, blood samples can be collected in tubes containing an anticoagulant that inhibits/decreases clotting formation process by chelating calcium, which plays a major role in this process. EDTA, heparin, citrate, and KOx are commonly used anticoagulants in various clinical and forensic applications to stabilize blood in a liquid form. Some anticoagulants are often combined with a preservative such as NaF to inhibit glycolysis of blood glucose by exerting its effects on enolase. Furthermore, NaF may inhibit the activity of many other enzymes such as urease, cholinesterase, and also bacterial proliferation, thus reducing losses of nitroaromatic compounds. Addition of NaF to various biological samples (e.g. urine, bile, and vitreous humor) is a common practice in forensic work, as it helps protecting various drugs from degradation such as clonazepam, cocaine, and nitrazepam. Preferably, the concentration of 2% weight by volume (w/v) should be added.

As previously mentioned, in a study by Busardò et al., mephedrone was unstable in tubes containing no additives, whereas enhancement of stability was noted in tubes containing 3% EDTA. The greatest inhibition of mephedrone degradation was achieved in tubes containing 1.67%/0.2% (NaF/KOx). Likewise, Miller et al. indicated less stability of SCs in unpreserved OF in comparison with devices containing Quantisal™ buffer. In these two studies, additives demonstrated enhancement of SC stability to the samples compared with unprocessed ones. However, additives should be carefully chosen for such analytes. For instance, in a work by Sørensen, addition of NaF/citrate (pH 5.9) to AM whole blood showed better stability than NaF/KOx (pH 7.4). However, the concentration of preservatives in each tube was not specified in this study. Thus, it is not clear whether the use of NaF/citrate has a better stability, or the concentration of NaF/KOx was small (whereas in the previous study the increase of NaF concentration [1.67% w/v] showed better stability), or another factor, that is, the acidity of pH (5.9) in NaF/citrate, that may explain the
3.6.6 | pH

From previous studies, it is well understood that sample pH is a key factor that plays a crucial role in the stability of SCs. Many of the previous studies reported that acidification of samples pH revealed greater enhancement to the stability of SCs. For instance, in unpreserved urine samples at pH 5 stored at RT, naphyrine was reported to degrade with a half-life of 20 days, whereas in preserved (1% NaF) urine sample at pH 8 stored under the same temperature, it showed a half-life of 11 days. Consequently, increasing the sample pH significantly accelerated the rate of degradation in SCs and vice versa. However, the reviewed studies usually state the pH value at the beginning of the experiment; they implicitly assume that the pH was stable throughout the experiment. One stability investigation found that sample pH could change/increase during the experiment based on temperature conditions and/or matrix type. For example, blood and urine pH were initially measured and remained the same after 6 months of storage at −26°C. However, the pH in urine sample was increased from 5 to 7 after storage at 5°C and became more alkaline (pH 8) after storage at RT, possibly owing to bacterial activity. Such influences in pH may explain some variations in the stability of SCs in urine samples compared with other types of matrix at least for higher temperature conditions. Thus, the addition of antimicrobial agent (e.g., sodium azide) may tackle this problem and substantially reduce the sample alkalization. This step, however, has not yet been investigated for its potential effect on the stability of SCs in urine samples.

3.6.7 | Selection of collection container

Generally, the selection of sample container is based on the intended purpose in a way to assure that it does not compromise the analytical investigations. For example, the choice of container size should allow the sample to be as close to full as possible to reduce uncertainty about oxidative losses due to air trapped in the top of the container or “salting out” effects, which may occur if preservatives are added to the sample.

Disposable plastic containers and/or tubes are routinely used for the collection of clinical and forensic samples, especially for urine. If plastic containers are used, it is recommended to use polypropylene and not polystyrene because of the high susceptibility of the latter to crack when frozen. On the other hand, glass tubes are more suitable for long-term storage at low temperatures. The principal issue of glass is the possibility of breakage, but this concern can be reduced by using an appropriate storage rack.

Although the stability of SCs was not compared in these containers, the reported stability of blood concentrations of a variety of other illicit drugs collected in plastic and glass Vacutainer evacuated tubes was not deemed to be significant.

3.6.8 | Experimental design

In the literature, the experimental design for reporting the stability of SCs is highly variable. For example, whereas the majority of authors used two levels of concentration, few authors used three or four. Although analyte stability is recommended to be assessed at two concentration levels as described before, a number of authors used only one concentration. In most publications, it seems that the number of F/T cycles was in accordance with the abovementioned recommendations, that is, three F/T cycles. The majority of authors performed the F/T stability over three cycles. However, few others performed two cycles or eight cycles. The vast majority of authors reported their stability data using a predefined acceptance criteria; for example, average of stability samples must be within ±10%, ±15%, ±14.4 to ±14.9%, or ±20% of that of reference samples. Alternatively, Busardò et al. used a statistical approach first by assessing the mean concentration of samples over certain time intervals under various conditions. Subsequently, polynomial regression analysis was performed to check for statistically significant differences between groups when p value was lower than 0.05 (p < 0.05). Amaratunga et al. used regression analysis approach to assess the stability of processed samples by plotting the absolute peak areas of analytes against certain time intervals. Negative slope significantly different from zero (p < 0.05) were regarded unstable. In this test, it is necessary to plot the absolute peak areas instead of peak area ratios. This is because any degradation that may occur to peak area of internal standard would necessarily correct peak area ratio of analyte, causing inaccurate interpretation of degradation findings.

Glicksberg and Kerrigan used an ANOVA test to determine whether instability (concentration below 20%) of SCs was significantly dependent on analytes, temperature, pH, and/or concentration. The authors looked first at variances within group prior to the comparison between groups to confirm that differences within group were not significant. Soh and Elliott used another approach by calculating the percentage changes between target analytes and negative internal control that is known to be stable (amitriptyline) over respective times. In this approach, both target analyte and stable analyte were added to the samples at an equal concentration. The peak height ratios of target analyte to the peak height of amitriptyline were then plotted against certain time intervals. Subsequently, the percentage differences between ratios were monitored to identify any instability.

An important limitation of certain stability studies is that neither the acceptance limits nor statistical approaches for evaluating the stability are specified. As described above, only few authors have followed the standards for their stability testing, and therefore, stability reports present significant variations in the literature. This has turned out to be particularly true for the various differences in
acceptance criteria used in the literature, as it may lead to underestimation/overestimation of true changes. For example, certain analytes might be reported as unstable in one study, whereas the same analytes are found stable by another study owing to the wider acceptance criteria. Obviously, the need for fixed guidelines concerning the experimental designs and evaluation of stability results is important.

Although the procedure of SWGTOX describe processed sample stability, it was used to determine the stability for both short- and long-term under various conditions. Despite comparing the results with reference samples, it is recommended to start the short- and long-term stability with freshly prepared calibrators and QCs at each analysis day/time to confirm that any loss of analyte or variations in accuracy and precision is attributed to analyte instability and not to experimental conditions.

Regardless of the chosen approach, stability study is required after a full validation of analytical method for the previous reasons. Apart from that, the choice of analyte concentration should be based on concentration found in authentic cases or the pharmacokinetics of the analyte. Reed, proposed to use a biological sample from the species of interest, for example, human to reduce interspecies variability in analyte stability.

4 | CONCLUSION

The presented studies show that the majority of SCs are unstable under the common storage conditions in clinical and forensic laboratories. SC subgroups that carry N-alkylated (either unsubstituted or ring substituted) seem to have a poor stability in most studies even at low temperatures. The degree of stability varied between structural isomers as well as halogenated analogs. Preanalytical factors have great impact on the stability results. For example, acidification of the samples and addition of an appropriate preservative proved their ability to maximize stability. In cases where SCs are suspected to be encountered, samples must be stored at −20°C or lower with an appropriate preservative, for example, NaF (combined with anticoagulant in the case of blood) and acidic pH if not analyzed immediately to avoid degradation. However, more studies are required in terms of the effect of additives on the stability of SCs in biological samples. Extracted samples appear to be less susceptible to degradation regardless of the employed conditions. Moreover, differences between stability of repeated F/T cycles and freezing samples appeared to be insignificant.

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