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# Emerging procedures and solvents in biological sample pre-treatment

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ABSTRACT

The treatment of biological samples, especially from complex matrices, has consistently challenged analytical operators. The classic problems to be faced for any analysis, regardless of the origin of the sample, such as for example contamination and loss of analyte, in biological samples, are particularly emphasized. In particular, the main cause of the error is due to the degradation of the analyte which in several cases due to biological interaction. Many factors can influence the stability of drugs, chief among them the physicochemical properties of the drug, characteristics of the matrix, the tendency to conjugation/deconjugation, sample collection procedure, container characteristics (e.g., oxidation, adsorption), and the use of preservatives or other additives. The problem is severe in the toxicological and forensic fields, especially for analyzes considered "non-repeatable." In this review, we will explore all the major problems in the pre-extraction phase for the chemical-analytical aspect in the pharmacotoxicological and forensic fields.

## 1. Introduction

In recent years many efforts have been made to optimize analytical procedures for extraction from biological samples. The overview is comprehensive as many techniques have been applied by various authors, such as liquid-liquid extraction (LLE) [1], solid-phase extraction (SPE) [2], solid-phase microextraction (SPME) [3], Single-drop microextraction (SDME), Continuous-flow microextraction (CFME), Hollow fiber-based liquid phase microextraction (HF-LPME), dispersive liquid-liquid micro-extraction (DLLME) [4], microextraction by packed sorbet (MEPS) and fabric-phase sorptive extraction (FPSE) [2]. The extraction step is among the most critical for the correct assessment of analyte concentration. However, for the same purpose, it is of equal importance to avoid loss of the target drug in the steps immediately preceding extraction.

A reliable qualitative and quantitative toxicological/forensic analysis is essential in all relevant cases. Overestimation or underestimation of the analyte being sought can lead to unreliable results, false interpretations, and unwarranted conclusions. Aware of their responsibilities, most practitioners in the toxicology/forensic field take extensive measures to ensure the quality of analytical results by continuously optimizing the methods used in their laboratories [5]. Unfortunately, the reality is that even the most accurate, sensitive, thorough, and precise analytical method can yield a reliable result only in the sample analyzed at the time of analysis [6]. Depending on the various interactions that individual substances may have, drug concentrations could have changed considerably from sample acquisition to analysis. Knowledge of all possible interactions between target molecules and biological matrices is essential to provide the most plausible data possible. Based on our experience and the data reported in the literature [7,8], we thought it appropriate to focus on the 3 main pre-analysis factors that most compromise the result obtained: (1) the physicochemical properties of the drug, (2) the characteristics of the matrix, and (3) the use of preservatives or other additives (Fig. 1).

Poor stability of drugs is due in most cases to inherent chemical instability due to the presence of functional groups susceptible to trans-

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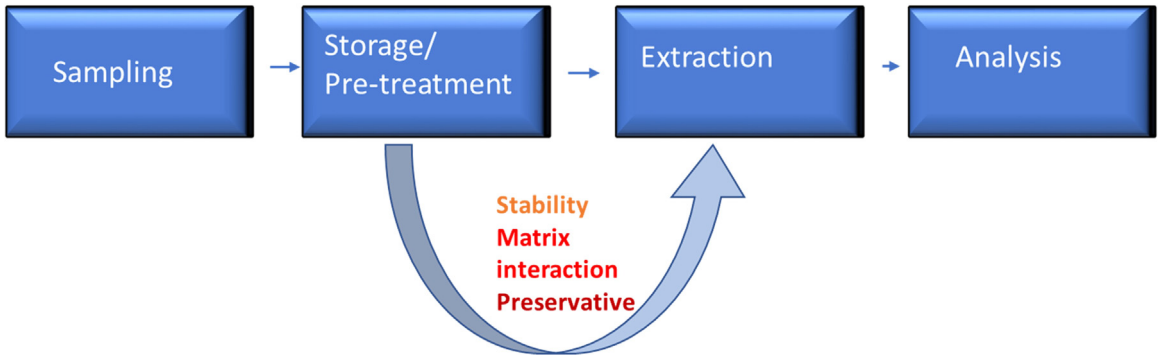


**Fig. 1.** Main steps of the analytical procedure and focus of the issues treated.

formation, such as esters (e.g., 6-acetyl morphine), or those that are easily oxidized or reducible (containing sulfur), but also to photolabile substances (e.g., lysergic acid diethylamide). In the field of forensic toxicology, many substances have been characterized for their stability profile [6,9–11]. However, it should be kept in mind that only in the last decade many new psychoactive substances (NPS) have been identified [12,13]. In fact, in addition to the classical structural analogs of known NPS "families," new, never-before-identified "cores" have been identified whose stability in the biological environment and interactions are still unknown [14,15]. We report here an overview of the studies carried out in the last five years relating to the stability of substances of pharmacological/forensic interest from the point of view of the "chemical structure" [16].

An imperative for achieving a proper analytical process is to block potential matrix interferences. The ideal process involves removing interferences so that the analyte of interest can be isolated, resulting in increased selectivity and reproducibility. The main interactions given by the matrix are due to residual enzyme activity in the biological tissue (autolysis) and putrefaction of the same [7]. These processes affect both sample composition and the integrity of tissues and fluids and can have consequences for drug analysis. The enzyme activities naturally present in the body may continue after tissue sampling, while the microorganisms present are responsible for putrefactive processes. Biological activities vary according to the various tissue; for example, they are very high in the pancreas and very slow in the heart and liver [17]. In postmortem blood sampling, a drastic decrease in blood pH immediately after death (down to 5.5) must be considered. Determination of low concentrations of analytes in biological matrices may be more straightforward if the continuous influx of matrix effects is blocked, hence the need for cleaner extracts. An overview of the main parameters to be controlled and the related actions taken to block them will be presented in this review.

During sampling and analysis, chemical processes may occur (photodecomposition, decomposition, microbial action, oxidation or reduction processes, etc.). Therefore, the samples must be stored throughout the period under suitable conditions, depending on the nature of the samples: darkness, low temperature, the addition of preservatives, antioxidants, or adjustment of the pH value [18]. The main alterations to be avoided concern contamination, alteration, and/or loss of analytes. The sample may alter its contents due to physical and chemical changes, such as adsorption on the container walls, volatilization, or precipitation of analytes. These problems to date are addressed and minimized by adding appropriate preservatives/stabilizers, chosen appropriately depending on the analyte. The relevant section will report the main substances used for this purpose.

## 2. Chemical stability of drugs

Many reviews on the stability of biologically active substances (drugs, drugs of abuse, etc.) classify these substances based on their pharmacological activity. Recently, some authors [19,20] have been making efforts to correlate chemical reactions/structures with molecular stability in the biological environment to implement predictivity on the causes of degradation/instability. Finding such, very useful studies are among the purposes of our review to highlight the correlation of the stability of molecules with their chemical structure. Recent examples of the stability of pharmacologically active molecules classified according to the characteristics of the "active" functional groups in the molecule will be given below.

### *2.1. Oxygenate compounds*

#### *2.1.1. Ester*

de Souza et al. recently reported data they obtained indicating that in a homologous linear series, methyl esters have the lowest hydrolytic lability in rat plasma. Comparison with higher homologs has been reported not only for model molecules but also for currently used drugs such as flumazenil and benzocaine, which contain a carboxyethyl ester, and their lower homologs containing a carboxymethyl ester. A greater tendency for hydrolysis in rat plasma was observed for the other homologs, isosteres, and retrofitters, than for hydrolysis catalyzed in basic environments. The authors correlated the hydrolytic lability of ester linkage to the carboxylesterase-rich biological medium (rat plasma and rat liver microsomes), suggesting that hydrolytic activity is increased just by the high concentration of these enzymes [21]. Similarly, Shervington and Ingham prove that ester analogs of anticancer drugs derivatized to increase their pharmacological activity lose that increase as they are easily hydrolyzed by the esterases present [22]. In the forensic field, the most analyzed molecule with ester functionality is certainly heroin. Jurado et al. demonstrate that this molecule is volatile in blood and not very stable in urine, despite preservatives and stabilizers. The hydrolysis of the ester bonds present is the cause of such instability and the authors suggest analyzing the metabolites derived from this molecule in parallel [23].

#### *2.1.2. Poly oxygenate compounds*

Compounds containing hydroxylated functional groups, such as alcohol, phenols, and carboxyl, are widely used in pharmaceutical and toxicological/forensic fields [24,25]. Most of these compounds contain multiple functional groups, and it takes work to classify them. It is appropriate to enter the classification category of the functional group involved in the degradative mechanism. For example, Doxorubicin, an extensively studied antineoplastic antibiotic [26–30], has several functional groups. In addition to its pharmacological activity, the molecule and its metabolites exhibit high toxicity, so continuous monitoring of concentrations is required. Siebel et al. recently reported [31] stability data in a biological matrix, demonstrating high substance degradation (Table 1) and complex degradative metabolism.

#### *2.1.3. Ethers*

Ethereal bonds are present in several drug structures and drugs of abuse. Although they are considered nonreactive compounds, they ex-

**Table 1**
Residual concentration of Doxorubicin (as%) at different storage temperatures.

| Temperature | 4 °C | - 20 °C | −80 °C |
|---|---|---|---|
| Period | 4 weeks | 1 year | 1 year |
| DOX (25 μg /L) | 13.3% | 67.4% | 91.7% |
| DOX (500 μg /L) | 3.7% | 63.2% | 90.1% |

hibit moderate instability, mainly when they belong to molecular structures that facilitate their degradation. For example, 2 antihistamines, diphenhydramine (DIPH) belonging to the first generation of antihistamines and bepotastine (BEPO) belonging instead to the second generation, were recently studied by Gumieniczek et al. [32]. The authors deduce from the experimental data collected that the instability is due precisely to the presence of the ether bonds. The decomposition of DIPH is studied under stress conditions, and it may be related to the cleavage of the ether bond, especially under acidic conditions (Fig. 2A). Similarly, supported by the results obtained, the decomposition of BEPO is proposed by breaking the ether bond with the formation of the relevant products (Fig. 2B).

### 2.2. Amines

It has been estimated that about 40% of small molecules are used as drugs containing amine (aliphatic or aromatic) functionalities. Among the significant contamination and degradation products related to these types of molecules is the development of nitrosamines [33]. Exposure to a nitrosating agent may be evident at the manufacturing stage, in the final formulation, or in the biological environment. In addition, enzyme activity related to molecules with such functionality can affect their stability [34].

#### 2.2.1. Aliphatic amines

The aliphatic amines class includes many substances with biological activity that can potentially be used for pharmacological activity but also as drugs of abuse. Examples include cathinones as primary amines, ephedrine as a secondary amine, natural (morphine, codeine), and synthetic (methadone, fentanyl) opioids as tertiary amines. The stability of these compounds has been extensively studied over the years [35] and is currently still of great interest [36–41]. Aldubayyan et al. [42] recently reported evaluation of the stability of some of these molecules in biological samples in a pervasive and comprehensive review. The study reveals that many compounds belonging to the primary amine class are unstable under standard storage conditions, also demonstrating a correlation between structure (substituents, alkylation, halogenation, etc.) and stability. The authors also point out that preanalytical factors have the most significant impact on stability. For example, adding acids (acidification of samples) and preservatives increases stability enormously.

#### 2.2.2. Aromatic amines

Aromatic amines represent a wide range of molecules used in pharmacology. They can be classified as exo- and hetero-cyclic, and compared with aliphatic analogs, they possess a higher intrinsic chemical stability [43,44]. Among the wide range of molecules studied, we have chosen here to report as an example the stability in biological samples of a drug that has been widespread for a long time but are still widely used today and for which continuous monitoring of concentration after administration is essential the folates. Because of their importance and use, folates have been extensively studied [45–47]. Recent literature reports differences regarding the stability of various forms of folates in biological samples. Paladugula et al. [48] conclude from the analysis of their data, obtained from serum samples fortified with ascorbic acid as a stabilizer, that this method provides acceptable stability for the various

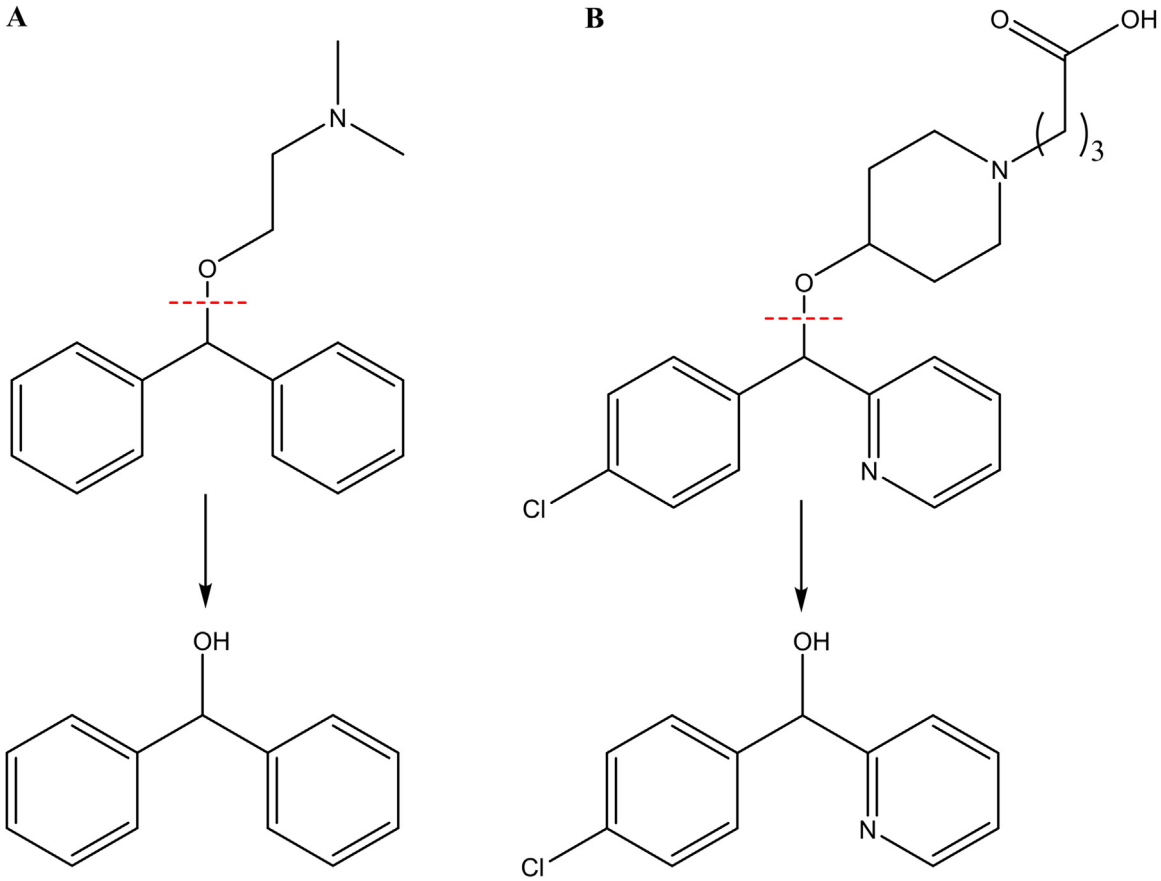


**Fig. 2.** Presumed degradation pathway of A) diphenhydramine (DIPH) and B) bepotastine (BEPO) (adapted from ref. 32).

forms of folates for time ≤4 months and during multi-year frozen storage of serum pools. In contrast, Schittmayer et al. [49] report the need to derivatize to the exocyclic amine group to ensure the molecule's stability for subsequent analysis.

### 2.3. Compounds containing sulfur

Sulfur can be present in molecular structures in various forms, depending on its oxidation state. Chemical and pharmacological properties but also stability will therefore depend on the "type" of sulfur-containing functionality. Various functionalities are found with a sulfur atom at diverse oxidation states, as illustrated by thioether, sulfoxide, sulfone, sulfonamide, sulfamate, and sulfamide functions. Major functional groups include sulfamides (e.g., sulfamethoxazole), S-onium (e.g., S-Adenosyl methionine), and Thiol (e.g., Cystine and Cysteine). Sulfonamides are among the oldest antibiotics, and consequently, their properties have been extensively studied [50–52]. They are considered relatively stable molecules, so much so that their accumulation is problematic for environmental reasons. [53]. Recently Ovung and Bhattacharyya [54] reported studies in which he demonstrates the interaction between sulfamide derivatives and several enzymes, thus correlating the well-known toxicity of this class of drugs with this activity. S-Adenosylmethionine represents one of the critical analytes in bioanalytics [55]. Its concentration levels are essential for diagnostic and pharmacological monitoring [56]. Unfortunately, the molecule is not very stable: at the physiological concentration (79 nM) it was stable for only 30 min of incubation, and the main degradation reaction (transformation of S-adenosylmethionine to S-adenosylhomocysteine) heavily alters the clinical significance associated with the analysis [57]. Currently almost all analytical methods involve derivatization reactions to obtain validated methods [56], but administration as a drug also suffers from the same instability problem and new formulations are currently being studied to minimize the effect [58].

### 2.4. Compounds containing phosphorous

Depending on their chemical structure, phosphorus-containing drugs can be classified into phosphorites, phosphonates, phosphinates, phosphoric amides, bisphosphonates, and others [59]. The derivatization of pharmacologically active molecules with phosphate groups is widely used, with the purpose being to increase their cellular uptake [60,61]. In contrast, the insertion of a phosphonate group increases their stability by generating a pro-drug [62]. The examples in the literature are countless; here, we decided to report data from an antiviral that has been on the market for a few years and used in the Sars-Cov2 pandemic. In the previous biennium (2020–2021), the possibility of having effective antivirals available has been a global need; the efforts that researchers have made from various fields have made it possible to safely use Remdesivir, both from a toxicological [61,63], standpoint and for analytical monitoring [64–66]. Demonstration of the drug's poor plasma-level stability [61] also, involving the phosphoramidite group [67] and simultaneous evaluation of its high efficiency as an antiviral has stimulated the study of new formulations for administration [68].

## 3. Matrix interactions

The stability of drugs in biological matrices under conditions of storage, handling, and analysis is essential in the analytical field [6,69,70]. Most data on the stability of drugs in biological matrices are often conducted as part of the development and validation of bioanalytical methods. Stability experiments in their respective publications are often the only data source on a particular drug's stability. However, since they are often etched within the paper and not reported in the abstract, it is very difficult to retrieve them from the literature. Storage of biological evidence at subzero temperatures is one of the methods currently used to prevent the degradation of analytes until analysis. However, previous studies have shown that freezing biological evidence does not stop degradation [18]. The main freezing preservation techniques will be discussed in Section 4. In this section, we report the 3 main processes that may influence the analyte concentration before pretreatment o direct analyses. In most cases, the main interactions between analyte and matrix are classified about the biological tissue from which the analyte is to be extracted; considering that the main interactions occur due to enzyme metabolism [71] and/or from reactions with other biological macro-molecules, we will describe the activity of the main classes of enzymes on drugs and drug-drug cross-reactivity. In addition, especially in the forensic field, it is necessary to consider normal *in vivo* biological processes and putrefactive processes that may arise after the blockage of vital functions.

### 3.1. Enzymatic degradation

Although decreased and under less-than-ideal conditions, enzyme activity continues even after the sample is taken. In the bioanalytical and forensic fields [72], this is a well-known issue, and many efforts are made to minimize these processes [69,70]. The primary enzymatic reactions that can most affect the concentrations of analytes in the sample are 3: hydrolysis, oxidation, and conjugation (Fig. 3).

However, it is not easy to hypothesize or determine the prevalent enzyme activity on a polyfunctionalized molecule. For indicative purposes, only the distribution of metabolites according to enzyme categories [71] is shown in the following graphic (Fig. 4).

From the data reported here, it is plausible to infer that most of the drugs administered will be metabolized according to these patterns. Although considerably slowed, enzyme activity continues after sample collection, which could certainly affect the accuracy of the result.

### 3.2. Cross interference

Analyte-matrix interactions not only include degradative aspects, but unfortunately can also affect the assays directly. The enzyme-linked immunosorbent assay (ELISA) technique and more recently the development of biosensors (i.e., sensors with biological probes such as enzymes) are typical examples of techniques that suffer the problem. The phenomenon of cross-interference, which is very significant in the biological field but is rare in the chemical field. Generally, this term refers to the interaction between proteins (often an enzyme) and the target analyte [73]. In the analytical field, the problem becomes relevant in the development of biosensors that rely on substrate-receptor interaction [74–76]. Recently Prabodh et al. have elegantly approached the problem of cross-interference in sensors through time-resolved competitive binding assays. In this way, despite the presence of cross-reactivity, the system can be used for the distinction and quantification of analytes [74].

### 3.3. Bacterial and microbiological decomposition

In routine analyses carried out in the diagnostic field, problems related to the degradation of analytes through putrefactive processes can be considered negligible. Opposed, however, is the weight of this aspect in the analysis of postmortem samples in the forensic field [77]. In addition to the autolysis phase, which is characterized by residual enzymatic activity on metabolites, the putrefactive phase of decomposition significantly affects the 'homeostasis of pharmacologically active substances and their metabolites [78]. During putrefactive processes, bacteria degrade tissues, and degradation products consisting of lipids, proteins, and carbohydrates can complicate the matrix and make it challenging to identify and quantify analytes. In addition, bacterial metabolism can cause changes in the concentration of the analyte being sought if the analyte is recognized as a substrate (e.g., ethanol) [79,80]. Except in a few rare cases, unfortunately, these processes have not been sufficiently studied, nor have drug degradation products. Because of this, analytical methods that can detect both the target molecule and its metabolites

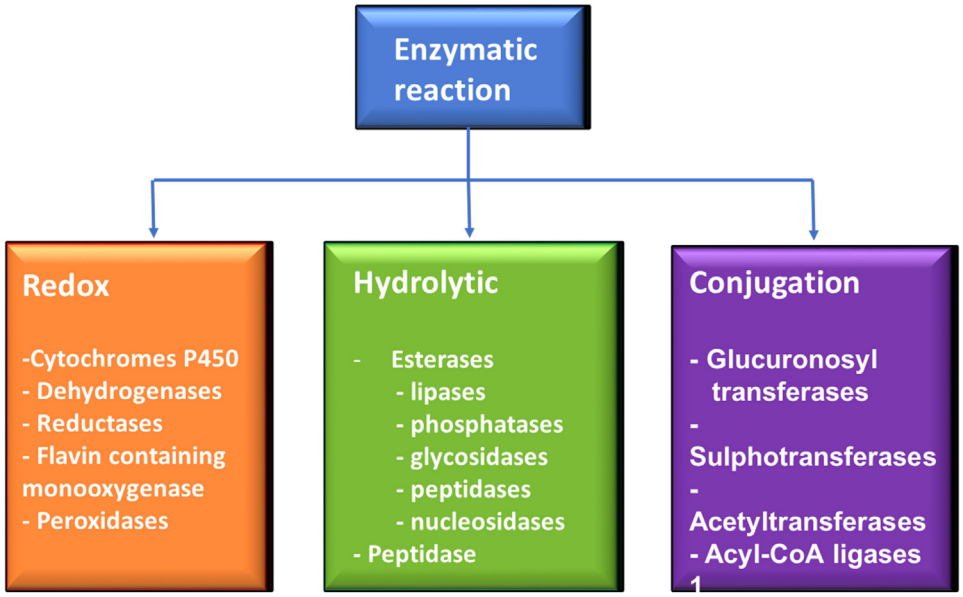


**Fig. 3.** Primary types of enzymatic reactions are involved in drug metabolism.

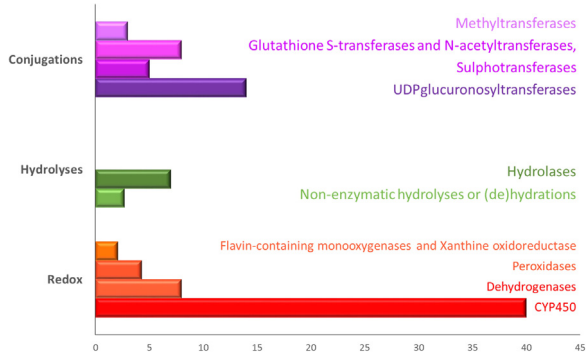


**Fig. 4.** Distribution of drugs according to enzyme families. Percentages given refer to 6967 occurrences of enzymes = 100%.

have also been underdeveloped; therefore, it is considerably difficult to obtain information to correlate the detected postmortem concentration with the actual concentration. Here we report an in-depth look at one of the main cases where degradative/fermentation processes heavily influence the concentration of an analyte: the postmortem determination of ethanol concentration. Determination of the concentration of ethanol in the human body at the time of death is one of the parameters that is always requested of the forensic toxicologist. To distinguish antemortem ethanol consumption from postmortem ethanol formation, analysis of the metabolites ethyl glucuronide (EtG) and ethyl sulfate (EtS), which are produced in living individuals enzymatically, is used today [81]. It has been estimated that only a small fraction (i.e., <0.1%) of the total dose of ingested ethanol is metabolized *in vivo* in the form of the EtG and EtS, so sensitive analytical methods are needed. Of paramount importance has been to show that EtG and ETS are not formed in samples fortified with ethanol [82,83]. Further developments, given the importance of this issue and its wide use in various toxicology laboratories, methods on alternative matrices to blood have recently been investigated [84], and excellent degrees of correlation have been obtained using humor *in vitro* [85,86] as an alternative biological matrix.

## 4. Storage and preservatives

The metabolic changes that occur after sampling are influenced by several variables and can dramatically affect the actual concentrations for several analytes. Although these processes are difficult to control, alterations in analyte concentrations due to improper *in vitro* preservation and/or storage are less tolerated [77]. Therefore, sample storage and physical conditions (e.g., temperature) during storage should be addressed. Of the following topics, general specimen preservation and storage procedures are highlighted.

The preservation of collected biological samples is critical and is sensitive to subsequent processing methods. Freshly obtained biological tissues that constitute the samples undergo both hypoxic ischemia and intrinsic enzymatic degradation, both capable of altering the concentration of analytes.

Both diagnostically and for research purposes, immediately after collection, samples must be processed immediately to prevent degradation of the analytes being sought. The consequences can invalidate all subsequent work, e.g., in the search for a potentially toxic molecule, it is essential to avoid its degradation into other metabolites/derivatives that could participate in the biological response. Sample treatment can be by physical process (typically temperature variation such as freezing) or chemical fixation/stabilization. For biological specimens, the main preservation techniques are 2: freezing (see Table 2) and formalin fixation. Freezing is the most widely used preservation technique both for classical and more recent analytical techniques, such as cryofixation [87]. Unfortunately, none of these techniques is free from tissue damage issues so problems in the accurate evaluation of the analytes of interest cannot be excluded [88–92]. To date, the best possible application of these techniques involves the use coupled with substances that minimize this damage, especially the use of cryoprotectives in freezing [93,94]. The most commonly used cryoprotectants are: DMSO, ethylene glycol, glycerol, propylene glycol, 2-methyl-2, 4-pentanediol (MPD), trehalose, formamide, glycerol 3-phosphate, proline, sorbitol, diethylene glycol, sucrose, triethylene glycol, and polymers [95,96]. The literature search for studies directed toward assessing possible interference between cryopreservatives and potential classes of analytes, or the interaction of the preservatives themselves with biological tissues has yielded few results [97,98], indicating that the issue has not yet been fully addressed in the scientific field.

No treatment technique is perfect for all applications; each technique has advantages and shortcomings. Therefore, procedures must be optimized for the specific application taking into account the nature of the analyte [99,100]. The following table (Table 2) shows the main techniques/substances used for these purposes.

The use of chemical preservatives (see Table 2, Part B) is often applied when the analytes to be searched belong to a restricted class of compounds and the goal is to block any reaction on them. Many ana-

**Table 2**
Main techniques/substances used for the physical process or chemical fixation/stabilization.

| Physical process (part A) | | Refs. |
|---|---|---|
| **Cool** | | |
| **Temperature ( ˚C)** | **Used for** | |
| −0,5 to −27 | Short-term DNA | [100,101] |
| −27 to −40 | middle- term DNA | [101] |
| −40 to −80 | DNA/RNA | [101] |
| −80 to −130 | for urine, blood, blood fractions (plasma, serum | [101] |
| −130 to −150 | tissues | [101] |
| **Warm** | | |
| 90–95 | Pathogen and enzyme disactivation | [102] |
| **Chemical process (part B)** | | |
| NaF, PMSF,BNPP, eserine, paraoxon, acetylcholine, dichlorvos, Ellman's reagent, DFP | Esterase/protease inhibitor | [103] |
| ascorbic acid, mercaptoethanol/propanol, Na metabisulfite,l-cysteine; EDTA as anticoagulant | Antioxidants | [103] |
| dithiothreitol, pyrosulfite | Reducing agents | [103] |
| Aprotinine, Dihydrouridine, THU | Enzyme inhibitors (peptidase and nuclease) | [103] |
| Formic, Hydrochloridric, O-phosphoric and succinic acid | acidifiers | [103] |

lytes that are substrates of enzymes, through inhibition of these (proteases/esterases, nucleases, etc.) are preserved. For example, Methylecgonidine [104], the biomarker for cocaine use, is transformed into Ecgonidine through the action of an esterase; the use of both sodium fluoride and phenylmethylsulfonylfluoride (PMSF) blocks cholinesterase and carboxylesterase activity, preventing this biotransformation.

For analytes that can be oxidized in biological tissues, the addition of antioxidants has been shown to be effective in blocking the reaction. For example, mitoxantrone (MTZ), an anthracene derivative with cytostatic action, has a half-life of about 70 h in plasma at room temperature, the degradation pathway involves oxidation of the phenylenediamine moiety to the corresponding quinoneimine [105]; addition of ascorbic acid blocks the oxidative process. Acylglucuronides are reactive metabolites of drugs containing a carboxylic acid moiety, such as diclofenac and ibuprofen. They are pH labile compounds susceptible to both hydrolysis and intramolecular acyl migration and are not stable under basic conditions.The degradation of acylglucuronides is negligible at acidic pH, and the use of acidifiers is fundamental to the quantification of these metabolic intermediates.

## 5. Conclusions

General knowledge of potential instability factors for different classes of compounds may help the bioanalyst to optimize methods/processes in order to stabilize the sample and prevent phenomena such as degradation, interconversion, and nonspecific binding. The sample must be stabilized immediately after the sample collection. However, unfortunately an operator not trained in "chemical" sample handling, especially in forensic cases, performs the sample collection sometimes.

This field, in recent years, has been mainly focused on the importance of "analyte loss" in the pre-extraction phase has been reevaluated for obtaining an accurate result. The stabilization phase of target analytes before their extraction from the sample can interest the bioanalytical (either as monitoring or as research) and forensic sectors. Given the heterogeneity of possible samples, it is very complex to develop a general protocol. This literature review was based on published work in various fields to enhance the most recent results, particularly between 2018 and 2022.

The 3 critical points that can heavily affect the analysis result by varying the concentration of analytes were evaluated chemically. Stability, biological interaction, and preservation methods are evaluated in several examples, collecting the most recent re-ported experiences. Drugs that contain ester functionality, sulfur atoms, or easily oxidized or reducible functions tend to decompose quickly. It is strongly recommended that they be stored at −20 ˚C or lower to avoid degradation.

In addition to the chemical degradation of analytes, interaction with the biological matrix, especially with enzymes, can also cause severe errors in analytical procedures.

Finally, stabilizers and preservatives must be carefully evaluated according to the chemical characteristics of the target analyte.

Although the attention paid to avoid "analyte loss" has increased significantly in the past 10–15 years, many improvements can still be made, such as by implementing studies with recovery tests related to the extractive phase and the pre-extractive phase .

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Data availability

Data will be made available on request.

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