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# Recommendations on Sample Preparation of Biological Specimens for Systematic Toxicological Analysis

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# *ISSUE*

*Upon selecting the appropriate specimen (1), the isolation of toxicologically relevant compounds from the biological matrix is essential for their successful detection and identification in systematic toxicological analysis (STA).*

*The following guidelines on sample preparation for the systematic toxicological analysis of biological specimens provide recommendations for sample pre-treatment and sample extraction. Due to the large number of toxicologically relevant compounds in STA, there is no one particular standard procedure for a comprehensive sample preparation. Different procedures that have been published in the literature are often complementary because they focus on a different spectrum of targeted analytes. These procedures can be run in parallel or in sequence, and the analytical toxicologist has to decide how they should be applied in each individual case to be investigated. Therefore, sample preparation - like sample collection - is case-dependent in STA.*

# BACKGROUND

STA is the application of an adequate analytical strategy for the detection and identification of as many as possible potentially toxic compounds and their metabolites in biological samples. This usually involves the detection and identification of a a “general unknow” as opposed to the confirmation or exclusion of an expected substance from a finite list.

In cases involving drug-facilitated crimes, attempted and accidental poisonings, and in cases of mysterious deaths, the most frequently used liquid specimens for STA are urine and blood. In cases involving a living subject serum or plasma and in postmortem cases, vitreous humor and cerebrospinal fluid may be used as well. In the field of postmortem toxicology, additional specimens such as gastric content, bile, different tissues, and hair samples are frequently analyzed.

Urine is the best specimen for comprehensive drug and poison screening (2). The accumulation of drugs, poisons, and their metabolites in urine will often result in higher concentrations than in blood, aiding in their detection; moreover drugs and poisons can be extracted from urine for days or longer. A disadvantage of urine specimens has to be recognized in instances where death occurs very rapidly after exposure to a drug or poison. In these cases, the urine specimen may test negative for the causative agent, so caution must be used in evaluating results in such cases and alternative specimens must also be analyzed.

In blood, serum, plasma, vitreous humor, cerebrospinal fluid and tissue samples, the expected concentrations are often much lower than in urine but, due to the shorter time-window for their detection, there is an additional possibility for the interpretation of a possible intoxication or an impairing effect at the time of sampling or the moment of death.

In order to detect phase II metabolites of drugs and poisons as well, a further pre-treatment step with a hydrolytic cleavage of the conjugate bond (de-conjugation) with strong acids, bases, or enzymes must be performed prior to extraction. Care has to be taken to avoid the degradation of analytes and the formation of by-products and artifacts (due to the possibly too harsh conditions of sample pretreatment).

Because the physico-chemical properties of toxicologically relevant compounds differ greatly, non-selective extraction procedures are needed which cover a wide variety of possible target compounds. Different methods have been applied to isolate drugs from biological specimens; the most frequently used procedures are liquid-liquid extraction (LLE), and solid-phase extraction (SPE).

In order to monitor the whole STA procedure, internal standards that mimic the physico-chemical properties of the possible analytes have to be added at the earliest possible stage (prior to sample pre-treatment).

The recommendations that follow are specific to cases in which STA will be performed on biological specimens.

# RECOMMENDATIONS

## 1.0 - INTERNAL STANDARDS

For any STA procedure, the internal standards should represent acidic, neutral, and basic compounds of different polarity and volatility, during the steps of sample pre-treatment, extraction, fractionation, purification, concentration, derivatization (if applied), separation, and detection. Therefore, multiple internal standards should be added at the earliest possible stage in a way that homogeneity of the sample is guaranteed and protein precipitation by the organic solvent added with the internal standard is prevented. The added concentration should be representative for the case under investigation (e.g. between therapeutic and toxic concentration). Stable isotope-labeled internal standards are recommended when mass spectrometry is applied as a detectiontechnique; active drugs should only be used as internal standards when no alternative is available and after proofing their absence in the sample.

## 2.0 - HEADSPACE ANALYSIS AS PART OF STA (3-5)

Volatile substances are often implicated in forensic cases as a result of substance abuse, accidental intake, for physiological reasons, therapeutic use, industrial exposure, or criminal utilization.

Additional screening for volatile substances is an indispensable part of STA because these substances are generally not detected after usual sample preparation by LLE or SPE.

Volatile compounds are mostly eliminated, unchanged, via exhalation. Blood can therefore be used to detect these substances. Often a simple dilution with a buffer is the only sample preparation needed.

When static headspace GC is used as the identification and detection method, special attention should be paid to the choice of conditions for sample pre-treatment (sample pH, ionic strength, incubation temperature) and GC-temperature and GC-column (polarity, film thickness) in order to optimize extraction yields and separation capacity.

When classical headspace equipment is not available, static solid-phase micro extraction (SPME) is an inexpensive, easy to use alternative. Different SPME fiber types allow the adsorption of volatile and semi-volatile compounds onto the fiber, from which they are thermodesorbed in the GC injector. The choice of fiber type, the conditions for sample pre-treatment (sample pH, ionic strength, stirring, incubation time and temperature) as well as headspace and desorption conditions (time, temperature, and fiber carry-over) need to be considered during development of the procedure.

An enhancement of extraction yields is possible using dynamic extraction methods, such as solid-phase dynamic extraction (SPDE) and in-tube extraction (ITEX). However, they require special equipment and automation.

# 3.0 - SAMPLE EXTRACTION

## 3.1 - Sample Hydrolisis

Beta-glucuronidation of hydroxyl-, carboxyl-, amino- and thiol-groups as well as sulfation of hydroxyl- and aminogroups are the major phase II metabolism pathways. The resulting glucuronated and sulfated conjugates are

relatively stable. For a proper detection of the parent compound and phase I metabolites, hydrolysis is needed, and this procedure is therefore frequently applied to urine samples.

Hydrolysis can be performed in a strong acidic or basic milieu. Incubation at 100Â°C accelerates the hydrolysis and yields almost complete cleavage. Standard procedures use 37% hydrochloric acid for 15 min at 100Â°C. When time is a limiting factor, this type of hydrolysis is often preferred. However, the disadvantage is the destruction of compounds sensitive to strong acidic or alkaline conditions, such as benzodiazepines, cocaine, acetylated opiates, etc. Therefore the application of strong acids or bases for hydrolysis should be restricted to emergency cases, where fast results are essential.

In all other cases enzymatic hydrolysis should be favored: Although more time-intensive, cleaner extracts can be achieved and, due to mild hydrolysis-conditions, stability of the analytes as well as a minimum of artifacts can be expected.

Different types of enzymes are commercially available, but the most frequently used are Beta-glucuronidase from E. coli or Helix pomatia, sometimes combined with arylsulfatase. In order to achieve reliable results it is crucial to pay attention to the pH and temperature optima of the different preparations of purified glucuronidase and sulfatase.

Beta-glucuronidase from E. coli provides the largest pH optimum of all glucuronidases, which is situated between 5.5 and 7.5, whereas Beta-glucuronidase from Helix pomatia works best between pH 4.5 and 5.5. The temperature optimum for Beta-glucuronidase from E. coli and Helix pomatia lies at 50°C and 60°C, respectively. Beta-glucuronidase-arylsulfatase from Helix pomatia provides the advantage of the cleavage of glucuronide and sulfate conjugates at the same time, but the glucuronidase activity is not as high as in the E. coli preparation. Moreover, E. coli solution leads to cleaner extracts in comparison to Helix pomatia Beta-glucuronidase/ arylsulfatase.

A typical procedure for the enzymatic hydrolysis of glucuronides is to mix 1 mL of urine with internal standards (glucuronides) and 1 to 2 mL of buffer and then to add Beta-glucuronidase (approx. between 1.000 and 20.000 units per mL urine) and - when necessary - sulfatase and incubate at 37° overnight (approx. 16 h), or at least 90 min at 50°C. After incubation, the pH of the solution is adjusted appropriately for liquid-liquid or solid-phase extraction.

The cleavage of ether groups such as morphine-6-glucuronide generally takes more time than hydrolysis of phenolic glucuronides like morphine-3-glucuronide.

In general glucuronides should also be added as internal standards when a hydrolysis step is applied.

## 3.2 Sample Pre-Treatment

Immunoassays and some analytical instruments (such as LC-MS) can handle direct application of urine samples (sometimes after dilution) or precipitated blood. These are rapid and simple procedures, but immunoassays are restricted to a very limited number of target analytes, and the co-injected large amount of matrix compounds can interfere with the ionization process in LC-MS (e.g. ion suppression). Moreover, when precipitation is applied - or occurs unnoticed during sample preparation - analytes can be lost by adsorption and occlusion. In STA such approaches are risky, because neither the target-compounds nor the composition of the matrix are known at the beginning of the analysis. Therefore sample preparation is a key issue in STA, and can only be simplified when the toxic compounds can easily be separated from the less volatile matrix components (e.g. gases and volatile compounds) or special extraction devices can be applied (such as solid-phase micro extraction; SPME) as described above. However, the isolation of the important group of less volatile drugs (such as most drugs-of-

abuse, pharmaceuticals, herbicides and pesticides) generally requires more complex procedures for sample preparation.

Samples that are comparable to a purely aqueous solution (such as urine) require only a minimum of sample pretreatment (such as dilution and centrifugation) for further LLE or SPE. Biological specimens that contain proteins (such as blood, vitreous humor, and cerebrospinal fluid) require special attention and often a more intensive pre-treatment (such as additional protein precipitation - bearing the risk of analyte loss) before extraction. In all cases where homogeneity of specimens is questionable (this is true for nearly all postmortem specimens) and in the case of solid specimens (such as tissue samples), homogenization with a mechanical blender (such as Ultra-Turrax Tube Drive, IKA, Staufen, Germany or gentleMACSâ„¢ Dissociator, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) or with scissors (followed by enzymatic/proteolytic digestion with trypsin or other peptidases and subsequent filtration for elimination of crude residues) is necessary prior to any further pre-treatment and extraction.

A high protein content of the sample will result in emulsions with LLE and, together with other insoluble particulate materials, may impede the flow of the specimens through the SPE cartridges and adsorb the analytes. Different techniques to remove proteins by precipitation have been applied. An example would be the drop-by-drop addition of an organic solvent such as acetonitrile, methanol, or acetone (about three times the volume of sample) while vortexing. As mentioned before, protein precipitation bears the risk of adsorption and occlusion of target compounds, resulting in low recovery rates and, in the worst case, in failure to detect the "general unknown" .

Simply diluting or homogenizing the sample with an appropriate buffer (such as 0.05 M phosphate buffer pH 7.4; by a ratio 1:5 to 10; v/v) can therefore be the preferable solution. This common approach facilitates the specimens flow through a SPE cartridge by reducing the samples' viscosity. In addition, the sample may be treated in an ultrasonic bath, and after centrifugation and/or filtration the resulting supernatant is then used for the extraction. This approach is also applicable to putrefied postmortem tissue samples if dilution is increased (more than 1:10).

## 3.3 Liquid-Liquid Extraction (LLE).

LLE is recommended as a fast, inexpensive and efficient procedure and works especially well with biological fluids with very low protein content. Moreover, it is based on well-defined thermodynamic relationships and has a wide dynamic range (6).

The extraction should be performed at various pH-values (e.g. pH 2 to 3 and pH 8 to 9) and the pH of the sample has to be strictly controlled, therefore adjustment with buffer solutions is recommended:

- Acidic buffer (such as sodium dihydrogen phosphate)
- Weakly basic buffer (such as sodium hydrogen carbonate) (for amphoteric drugs, such as morphine, the basic phase should not exceed the pH of 9)
- Saturation with neutral salts is recommended (e.g. NaCl)
- A phase ratio (organic/aqueous) of one to two should be the aim in order to avoid co-extraction of a large amount of interferences

The following mixtures of solvents have been recommended for use in STA procedures (7,8):

- Diethyl ether/ethyl acetate (1:1; v/v)
- Dichloromethane/isopropanol/ethyl acetate (1:1:3; v/v/v)

It should be kept in mind that these two solvents have been applied mainly for the screening of urine; when used with other matrices (e.g. tissue samples) dirty extracts can be expected. Therefore additional solvents or mixtures and special extraction procedures might be necessary, including derivatization techniques (such as acetylation, methylation, and silylation) (9-12).

For an example of the consecutive extraction of neutral, acidic and basic compounds, see the following comprehensive extraction scheme for STA:

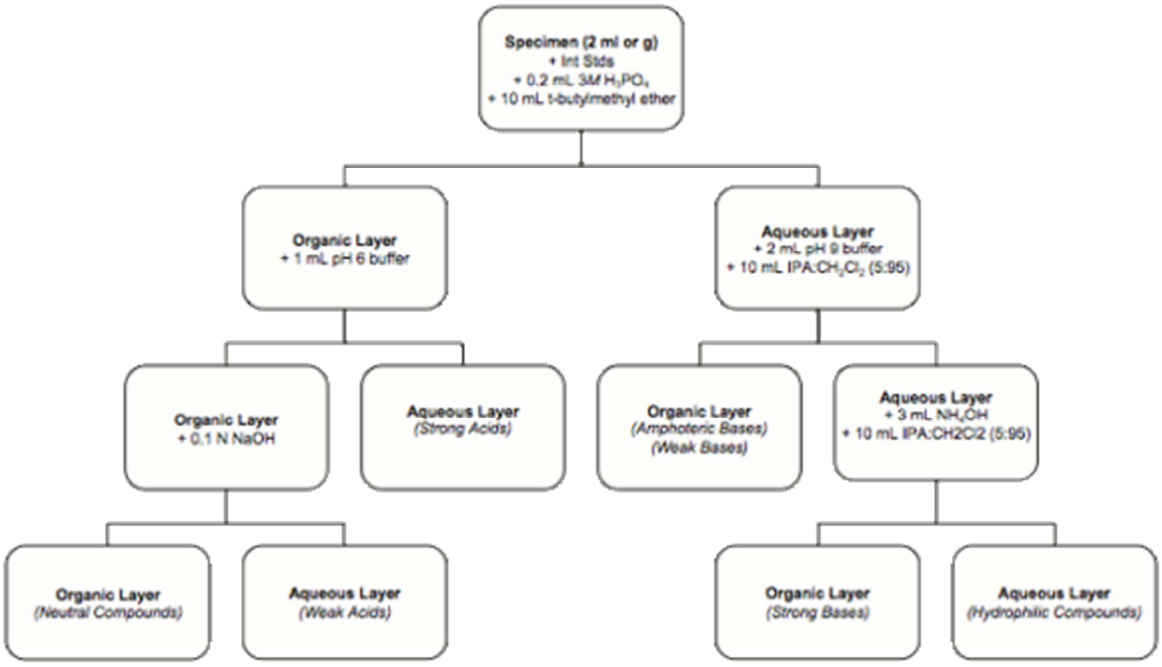


**Figure 1:** LLE Scheme for STA (click on picture to enlarge)

Emulsions: During LLE, stabile emulsions can occur. In this case the problem might be overcome by either an increase of the phase ratio (organic/aqueous), or saturation with neutral salts, or freezing of the aqueous phase, or the application of supported LLE (adsorption of the aqueous phase on diatomaceous earth before extraction with water immiscible organic solvent).

## 3.4 Solid-Phase Extraction (SPE)

SPE may be used as an alternative to LLE, especially when specimens containing high amounts of protein have to be extracted, or automation of the whole extraction process is needed. SPE has been successfully applied in postmortem toxicology to extract body fluids and tissue samples, when appropriate sample dilution was used (20). Sample pre-treatment (as described above) is essential in SPE to allow continuous flow of the specimens through the SPE cartridges and to avoid clocking.

SPE yields high extraction efficiency, which accommodates smaller sample sizes, thereby reducing solvent consumption. The analytes are isolated from the aqueous sample onto a solid sorbent; for STA preferably a sorbent that shows a minimum of selectivity. These sorbents can be based on either surface-modified silica with a non-polar retention mechanism (such as RP2 up to RP18), or they are polymerbased, also with a hydrophobic character. Some polymeric sorbents additionally show hydrophilic characteristics via polar-modification.

Based on these sorbents, so-called â€œmixed-mode sorbentsâ€œ with additional electrostatic interactions (such as cation exchange) have been developed. They are frequently applied for STA, because they allow obtaining a sequential isolation of acidic/neutral compounds and basic compounds. The two extracts are usually analyzed separately in order to facilitate the proper separation and detection of basic compounds, as the basic extract is cleaner than the acidic/neutral one. Additionally, washing- and elution-steps have to be optimized to avoid discrimination of certain analytes.

Examples for the application of SPE in STA can be found in the literature (13-23).

## 3.5 Derivatization

To enlarge the spectrum of detectable substances when GC is applied as a detection-technique, derivatization can be performed prior to or during the extraction.

An important prerequisite for this approach is the availability of reference data (e.g. retention times, mass spectra) for the corresponding derivatives of toxicologically relevant compounds. Trimethylsilylation is one of the most common derivatization techniques for GC analysis. However, acetylation (e.g. with acetic anhydride) for basic and neutral compounds and methylation (e.g. with diazomethane) for acidic drugs prior to or during the extraction have been proposed (9,11,12,24-26).

Silylating agents are able to react with a wide spectrum of functional groups (hydroxyl-, carboxyl-, amidic-, and amino-groups) and for this reason silylation is particularly suitable for STA. However, silyl-derivatives are very sensitive to moisture and therefore the reaction should occur under strictly anhydrous conditions. Efficient and reproducible silylation may be obtained with a mixture of MSTFA-toluene (1:4 v/v) containing 5% TMCS prepared just before use and filtered over anhydrous sodium sulphate (30 min at 75Â°C or 1 min in a microwave oven, 2450 MHz, 750 W). A further advantage of silylating agents is that they do not require evaporation of the excess reactant prior to GC separation (24).

## 3.6 General Remarks

Every extraction procedure consists of several steps and each one of them can aid or impair the overall performance. For example, the removal of solvents needs special attention, because some semi-volatile compounds (such as amphetamines) can easily be lost. It is therefore crucial to evaluate the concentration-step and all other steps of the chosen extraction procedure carefully. Substances with different physico-chemical properties should be used for extensive testing under laboratory conditions to ensure the procedures' "fitness for purpose" and to explore its limits.

# SUMMARY

These guidelines should assist forensic toxicologists in the process of developing an adequate strategy for sample preparation, because this is a crucial part of the STA procedure. The isolation and enrichment of toxicologically relevant compounds is the prerequisite for their subsequent detection and identification. There is not one single procedure that covers all toxicologically relevant substances, therefore complementary techniques for extraction have to be combined.

Since no selective clean-up is possible in STA, a large number of interferences from the sample matrix will be co-extracted. Therefore powerful analytical procedures are necessary to ensure sufficient separation and accurate detection and identification of the unknown compounds.

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