

## **SOLID PHASE EXTRACTION in CLINICAL & FORENSIC TOXICOLOGY**

*by Sarah Willie*

An important step in the development of a bio-analytical method is the extraction of the compounds of interest from the biological matrix, as this will have implications on the overall sensitivity and selectivity of the method. Conventionally, liquid-liquid and solid-phase extraction methods are chosen. Liquid-liquid extraction is time-consuming, difficult to automate, requires high-purity solvents and can result in the formation of emulsions with incomplete phase separations with the latter leading to impure extracts due to the presence of traces of water. In addition, safe disposal of toxic solvents may be problematic and expensive [1]. Solid phase extraction (SPE) separates and concentrates analytes from a liquid matrix by partitioning the analytes between a solid and a liquid phase. SPE aims to remove interfering compounds with good recovery of analytes and reproducible results. In addition, it should facilitate the rapid and efficient simultaneous processing of multiple samples [2]. SPE also has disadvantages which include low reproducibility (due to differences among column batches), difficulties in standardizing the use of a vacuum and the variable intensity of drying steps. Furthermore, SPE material can be expensive and the optimisation of an SPE procedure is not always simple.

When developing an SPE method, suitable sorbent material and washing and eluting solvents have to be selected according to the characteristics of the analytes and of the matrix. In addition, the extract should be compatible with the final analytical procedure (HPLC or GC). An SPE procedure consists of four important steps: column conditioning, sample loading, column washing and elution. In the conditioning step a solvent (typically methanol), followed by an aqueous buffer, is passed through the sorbent bed to wet the packing material and to solvate the functional groups of the sorbent. The solvation of the sorbent is necessary for reproducible interaction with the analytes of interest, and it may also remove any contaminants already present on the column. After conditioning the column, the matrix containing the analytes is loaded and the analytes are retained onto the sorbent bed. The loading conditions depend on the choice of sorbent as discussed below. After retention of the analytes, a wash step utilising aqueous buffers and/or solvents is passed through the sorbent to elute interferences present in the interstitial spaces of the column, whilst the analytes are still retained. In the eluting step, a solvent is selected that disrupts the analyte-sorbent interaction, resulting in elution of the analytes. A good eluent should be strong enough to elute the compounds of interest in a limited volume, however, the stronger the eluent used, the more interfering (matrix) compounds co-elute. Therefore, an optimized wash step is very important [3, 4].

**SPE sorbents and modes of interaction:** Different kinds of interactions can occur between the analytes of interest and the active sites on the sorbent [1]. These include hydrophobic interactions such as Van Der Waals forces and hydrophilic interactions such as dipole-dipole, induced dipole-dipole, hydrogen bonding and  $\pi$ - $\pi$  interaction. Other mechanisms include electrostatic attractions between charged groups on the compound and a charged group on the sorbent surface, as well as molecular recognition mechanisms [3]. The SPE sorbents that involve only one of these interactions are reversed phase, normal phase, ion-exchange, immuno-affinity and molecularly imprinted polymers (MIPs), while mixed modes combine several interaction mechanisms. In addition, restricted-access matrix sorbents (RAM) combine hydrophobic, ionic or affinity interactions, with the exclusion of large matrix components

such as proteins by selection of pore size or chemical repulsion, using an appropriate hydrophilic coating on the sorbent surface.

Not only are the chemical characteristics of the functional groups on the sorbent materials important, but also the characteristics of the back-bones on which these functional groups are attached. The back-bones are either silica- or polymer based. Silica-based columns tend to contain a limited number of unbound or 'free' silanols, which provide polar, acidic patches on the column surface. This results in secondary hydrophilic or ionic interactions when using reversed phase and ion-exchange columns. These secondary reactions could be of interest, but are not reproducible as the number of free silanol groups can change from batch to batch. For normal phase sorbents, secondary hydrophobic interactions can occur, due to the small alkyl chains that support the functional groups. The silica-based sorbents have a large variation of functional groups available, are relatively inexpensive and are stable within a pH range of approximately 2–7.5. The polymer-sorbents (e.g., styrene-divinylbenzene) are more hydrophobic, more retentive and stable within a pH range of 0–14, with no secondary interactions observed. Newer polymers such as Oasis HLB combine hydrophilic and hydrophobic interactions. They do not always require a conditioning step and are able to extract analytes over a large polarity range. However, due to their hydrophilic character, they can retain water, necessitating a longer drying step, especially if gas chromatography (and derivatisation) is used as the final analytical method [2, 5].

*Reversed phase sorbents:* Reversed phase sorbents are mainly utilized for the extraction of apolar compounds from polar matrices such as plasma, using hydrophobic interaction mechanisms. The compounds are eluted with a less polar organic solvent that disrupts the Van Der Waals forces. Apolar sorbents such as C18 are widely used in clinical and forensic toxicology as they are broad-spectrum sorbents, leading to extraction of a whole range of compounds such as fluoroquinolones, cannabinoids, and nicotine from various biological samples (e.g. plasma, brain tissue) [6-8].

*Adsorption stationary phases and normal phase sorbents:* Adsorption stationary phases consist of unmodified sorbent materials such as pure silica, magnesium silicate, diatomaceous earth or alumina. Silica and alumina sorbents adsorb water, so they should be kept dry before use and no aqueous matrix should be applied. Water will deactivate hydrogen binding sites, resulting in reduced retention of the analytes and variable recoveries. Normal phase sorbents are created when polar functional groups such as cyano-, primary amine- or diol-functions are bonded to the silica surface. These sorbents extract more polar compounds from an apolar matrix through hydrophilic interactions. Elution with a polar organic solvent is necessary to disrupt the hydrophilic interactions. In clinical applications these sorbents are mostly utilized to purify apolar extracts (e.g., hexane) of solid matrices [1, 3].

*Ion-exchange sorbents:* Ion-exchange sorbents focus on ionic interactions between the analytes of interest and the functional groups on the sorbent. Based on this mechanism, negatively (anion exchange) and positively (cation exchange) charged compounds can be extracted from a biological matrix. When using this extraction mode, the pH at the loading, washing and elution steps is important. At the loading step on an anion (cation) exchange sorbent, the pH should be two pH units higher (lower) than the pKa of the compound and two pH units lower (higher) than the sorbent pKa. At this pH, more than 99% of the functional groups are charged. At the elution step, the pH is chosen so that the functional groups on the compound and/or sorbent are not charged, resulting in disruption of the ionic interactions. The anion exchange sorbent consists of quaternary amines, with a pKa greater than 14 or aliphatic aminopropyl groups bonded to a silica surface, with a pKa of 9.8. When using a cation exchange mode, the choice between a weak (carboxylic acid with pKa 4.8) or a strong cation exchanger (sulphonic acid with pKa <1) can be made. The ionic strength of the buffer and the flow-rate should also be considered. Ion-exchange sorbents are used to isolate groups of either basic or acidic drugs from biological matrices. The use of SCX for extraction of antidepressants from plasma, recently published by our research group, is such an example [9]. Mixed mode sorbents combining a cation or anion exchange mode and a hydrophobic

sorbent are used for screening of drugs in forensic toxicological analysis. Because the substances present are not known in advance, the extraction procedure must isolate a range of compounds and remove interfering substances from the matrix. By using different eluting conditions, extracts containing neutral, basic and acidic drugs are produced separately [10].

*Molecular recognition mechanisms:* Immuno-affinity sorbents and molecularly imprinted polymers (MIPs) use molecular recognition mechanisms to selectively extract analytes of interest at trace levels from matrices with a high level of interfering compounds. Because immuno-affinity sorbents and MIPs are ‘tailor-made’ with high selectivity for a target molecule or structural analogues, these sorbents are not useful for toxicological screening, but they result in highly sensitive and specific methods. Caro and Marcé et al. have reviewed the application of MIPs for the extraction of compounds such as propranolol, verapamil, ibuprofen and tramadol in various matrices such as plasma, urine and serum [11]. Immuno-affinity sorbents consist of biological antibodies covalently linked to silica, controlled-size glass particles, agarose or other soft gels. However, these biological antibodies can be denatured after contact with an organic solvent or, if stored incorrectly, fungi. Therefore, the sorbent should be preserved in a buffer solution containing sodium azide and stored in a cooled environment (4°C). In addition, these sorbents are expensive and only a limited variety of immuno-sorbents are commercially available. As a result of these draw-backs, MIPs were produced using template molecules to create a cavity in a polymer, which will ‘recognise’ the target molecule. A specific cavity is created with the same dimensions and interaction mechanisms of the analyte of interest. MIPs are stable in organic solvents, at higher temperatures and over a wider pH range compared to immuno-affinity sorbents. They are prepared rapidly and easily [12]. For both kinds of sorbents, it may be difficult to remove the target molecules completely and residual material may cause cross-over. When using MIPs, the SPE conditions are comparable to reversed phase sorbent conditions. However, for immuno-affinity sorbents milder conditions (pH and organic content) are necessary [1, 5].

*Restricted-access matrix sorbents:* Restricted-access matrix sorbents (RAM) are used in clinical toxicology to exclude large molecules such as proteins and to extract small analytes using hydrophobic, ionic or affinity interactions. Rbeida et al. used RAM sorbents containing hydrophilic diol and diethylaminoethyl groups that exhibit weak anion exchange capacities. As a result, acidic compounds such as naproxen, ibuprofen and diclofenac were extracted from plasma [13]. A cation-exchange restricted access material (diol groups combined with sulphonic acid groups) was developed to determine basic drugs such as atropine and sotalol [14]. Direct injection of plasma or serum samples onto a chromatographic system is possible with RAM sorbents, which facilitates automation [15]. RAM sorbents do not retain proteins, while conventional solid phase sorbents may be clotted by proteins remaining on top of the sorbent bed. If compounds are highly protein bound, analyte recovery can be lower, as the active sites of the compounds that would normally interact with the sorbent are not available for this interaction. The majority of proteins are large molecules, prohibiting the penetration of the sorbent pores. As a result, the drug is carried through the sorbent bed by the protein instead of being retained on the column. Sonication, centrifugation and sample dilution in combination with a slow sorbent pass-through of the sample all seem to demolish the protein binding of drugs. Furthermore, addition of salt or changing the pH can also modify the protein binding. Protein precipitation using organic solvents is another strategy. However, under these conditions there is no longer an exact measurement of the initial physiologically bound versus unbound fraction [1, 5].

**Automation:** One of the major advantages of SPE is the possibility of automation. Automation results in higher sample throughput, improved precision and accuracy, and minimal operator intervention. These lead to safer sample handling and time-saving procedures. However, carry-over and less control over the system can result in systematic errors, while sample stability can be problematic if the samples are analysed sequentially [16]. Several new SPE formats and sorbents have been developed in order to be more compatible with automation or to create a higher sample throughput. Solid-phase-extraction sorbents that

do not need a conditioning step and new SPE formats such as the 96-well plate are created for rapid sample preparation, resulting in high sample throughput. Another trend is the miniaturisation of the sorbent beds, resulting in a small elution volume, hence highly concentrated extracts. Examples of these micro-scale formats are the SPE pipette tip and the solid-phase micro-extraction microfiber [1, 2].

In addition, SPE can be performed off-line or on-line. For off-line SPE, the sample preparation is separate from the chromatographic analysis, while on-line SPE is directly connected to the chromatographic system. Important advantages of the on-line techniques include the decreased risk of sample contamination, the suitability for full automation and the analysis of the complete sample (the whole extract is analysed), leading to improved sensitivity. When automating an SPE method one has to keep in mind that a manual SPE method can not be transferred directly. There are differences in pressures, flow-rates and solvent compositions (stability!) that need to be optimized again.

*On-line systems for liquid chromatography:* The most common on-line SPE system for liquid chromatography (LC) involves the application of a small precolumn located in a six-port high-pressure switching valve. This method is referred to as 'column-switching'. When the sample is injected, it is pre-concentrated on a precolumn that is pressure resistant and has small dimensions to avoid band broadening in space. The interferences are then washed from the precolumn and flushed to waste. The valve switches to elute the analytes onto the analytical column. To avoid band-broadening in space, a back flush desorption mode can be used. However, this may create blockages on the analytical column when biological samples are analysed. The column-switching method has limitations, such as the necessary use of small pre-columns containing a limited amount of sorbent, resulting in a small breakthrough volume. In addition, the pre-column sorbent and the analytical column should be complementary, resulting in a restricted choice of sorbents (mostly C18 sorbents).

Another possibility is to use the six-port valve as an interface for switching arrangements (Fig. 1). In this system the precolumn is not placed in the valve and the pre-concentration can be achieved in low- or high-pressure modes. The advantage of this method is that larger pre-columns can be used [12, 17]. On-line SPE-LC is very well suited for analysis of biological fluids such as urine and plasma, especially in combination with RAM sorbents [17].

*On-line systems for gas chromatography:* While on-line coupling of SPE with LC is a robust system, on-line coupling of SPE with GC is more complicated, due to the incompatibility between the aqueous solvents used for SPE and the stationary phases used in the GC system. In addition, the need for derivatisation and small injection volumes make on-line SPE-GC more complex. However, several systems have been developed for on-line SPE-GC methods, using six-port valves in combination with a drying gas, as well as a solvent vapour exit. In large-volume transfer the solvent evaporation technique is critical for obtaining sharp peaks. On-column and loop-type interfaces are usually employed with a retention gap and fully concurrent solvent evaporation techniques, respectively (Fig. 2). Several methods are already described using online SPE-GC (e.g., trazodone and benzodiazepines in plasma) with improved detection limits, better repeatability, shorter analysis time and lower consumption of organic solvents [18]. However, due to practical considerations, on-line SPE combined with GC is not always robust. Therefore, a new technology has been developed combining solid phase extraction with GC. Solid-phase microextraction (SPME) involves the use of a microfiber immersed into the sample or the headspace of the sample for sorption of the analytes, followed by direct desorption in the inlet of the gas chromatographer. This technique is an equilibrium technique: not all of the analytes present in the matrix are extracted by the coated fiber. Other variations of the SPME technique are the 'stir-bar' and in-tube SPME. The 'stir-bar' utilizes a stirring bar that absorbs analytes from aqueous matrices, while the in-tube SPME is an adsorbent just before the GC-column that is desorbed by temperature increase [1, 18]. SPME is gaining a lot of interest in the toxicological field and various methods to extract drugs and poisons from biological samples, such as whole blood, plasma, urine, hair and breath are published [19, 20].

**Reflections on the use of SPE in clinical and forensic toxicology:** The trend in clinical toxicology is to simplify manipulations and to reduce the time necessary for sample preparation. Miniaturization, high-throughput systems and automation of SPE are of interest in this field. However, these new and automated methods are not frequently used in routine applications due to the need for further optimisation, the higher instrumental cost and increased complexity of the system. Off-line automatic workstations are often applied because they reduce workload and offer flexibility. This flexibility can be relevant in avoiding problems during derivatisation or due to matrix precipitation during analysis. As most analytes need derivatisation prior to GC analysis, this can be the limiting factor in the on-line development of an SPE-procedure in clinical toxicology. However, several on-line derivatisation procedures have been reported (e.g., in the GC precolumn inlet [18]).

Another problem encountered with on-line procedures is the high protein content of plasma and blood. These proteins can precipitate after contact with buffers or solvents and can block the SPE tubes as well as the analytical column. Possible solutions are a pH change of the sample, a denaturation or precipitation step, and the use of RAM sorbents with the latter leading to on-line procedures.

While off-line automation is frequently applied in clinical toxicology, on-line procedures must gain robustness and be less complex to become a more valuable tool in everyday routine analysis.

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#### **References:**

- [1] V Walker, et al.; *Ann. Clin. Biochem.*; 39 (2002), 464.
- [2] CW Huck et al.; *J. Chromatogr. A*; 885 (2000), 51.
- [3] KC Van Horne; *Sorbent extraction technology*; Analytichem International; 1985, 142p
- [4] EM Thurman and MS Mills; *Solid Phase Extraction: Principles and Practice*; J.D. Winefordner; 1998, 344p
- [5] K Pyrzyńska; *Chem. Anal*; 48 (2003), 781
- [6] D Kowalczyk, et al.; *J. Liq. Chromatogr. R T*; 26 (2003), 1731
- [7] J Teske, et al. ; *J. Chromatogr. B.*; 772 (2002), 299
- [8] R Dawson, et al.; *Toxicol Mech Method*; 12 (2002), 45
- [9] S Wille, et al.; *J Chromatogr. A*; 1098 (2005) 13
- [10] JP Franke, et al. ; *J Chromatogr. B*; 713 (1998), 51
- [11] E Caro, et al.; *TRAC*; 25 (2006), 143
- [12] M. Hennion; *J. Chromatogr. A*; 856 (1999), 3
- [13] O Rbeida, et al.; *J. Chromatogr. A*; 1030 (2004), 95
- [14] P Chiap, et al.; *J Chromatogr. A*; 975 (2002); 145
- [15] S Souverain; *J. Chromatogr. B*; 801 (2004), 141
- [16] DT Rossi, et al. ; *J. Chromatogr. A*; 885 (2000), 97
- [17] K Pyrzyńska, et al. ; *Crit. Rev. Anal. Chem.*; 32 (2002), 227.
- [18] T Hyötyläinen, et al.; *J. Chromatogr. B*; 817 (2005), 13
- [19] L Juntting, et al. ; *Forensic Sci Int.* 97 (1998), 93
- [20] S Ulrich; *J. Chromatogr. A*; 902 (2000), 167

#### **About Sarah Willie**

Sarah Wille (Sarah.Wille@UGent.be) joined the Laboratory of Toxicology at the Ghent University (Belgium) after she obtained her Pharmacist degree at the same institution in 2002. In the Lab of Prof. W. Lambert, she is working on her Ph.D. project, concerning the monitoring of new generation antidepressants in biological fluids with GC-MS, while she helps with the analysis of various (post-mortem) samples using GC-techniques.

Guidance of students during analytical exercises and thesis work is another task she likes to do. She already published 3 peer-reviewed publications and being a member of TIAFT, she has attended several international meetings where she presented her work on solid-phase extraction of antidepressants. Currently, she became a member of IATDMCT and hopes to join the meeting in Nice, as there will certainly be a lot of interesting subjects to discuss and people to meet. In her spare time, she plays the violin, likes to go to the movies and to spend time with her friends and family.

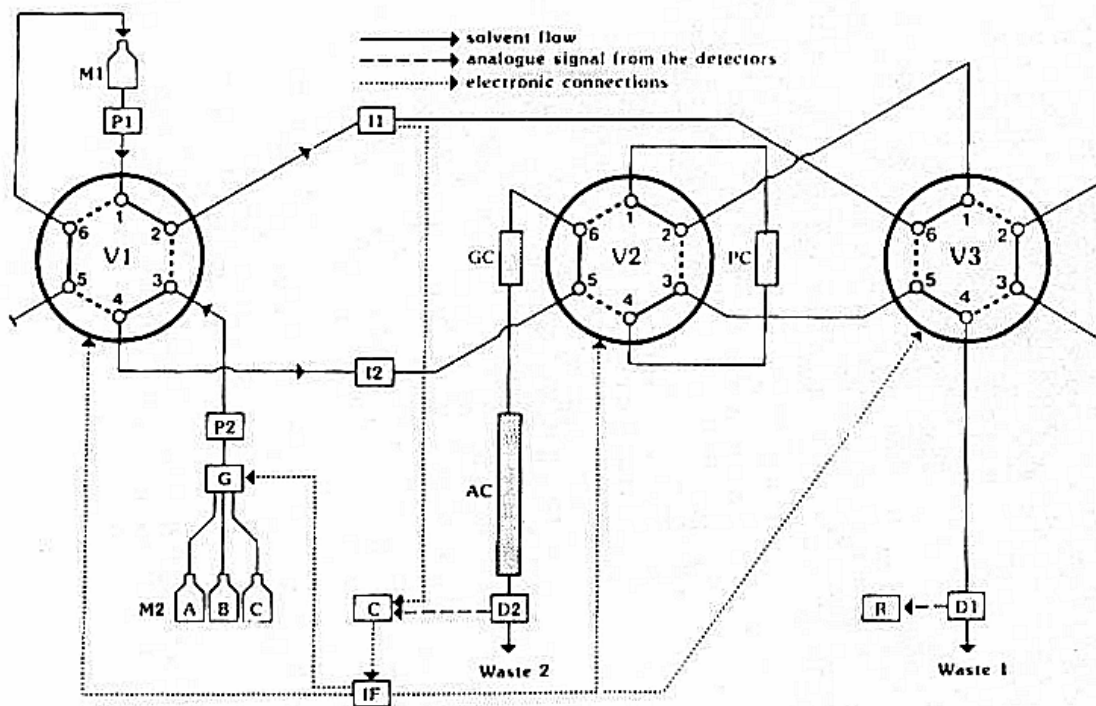


Fig 1: Schematic representation of the column switching system. PC: pre column; GC: guard column; AC: Analytical column. *Reprinted with permission from Elsevier from R Wyss & F Bucheli, J Chromatog, 1988;456:33.*

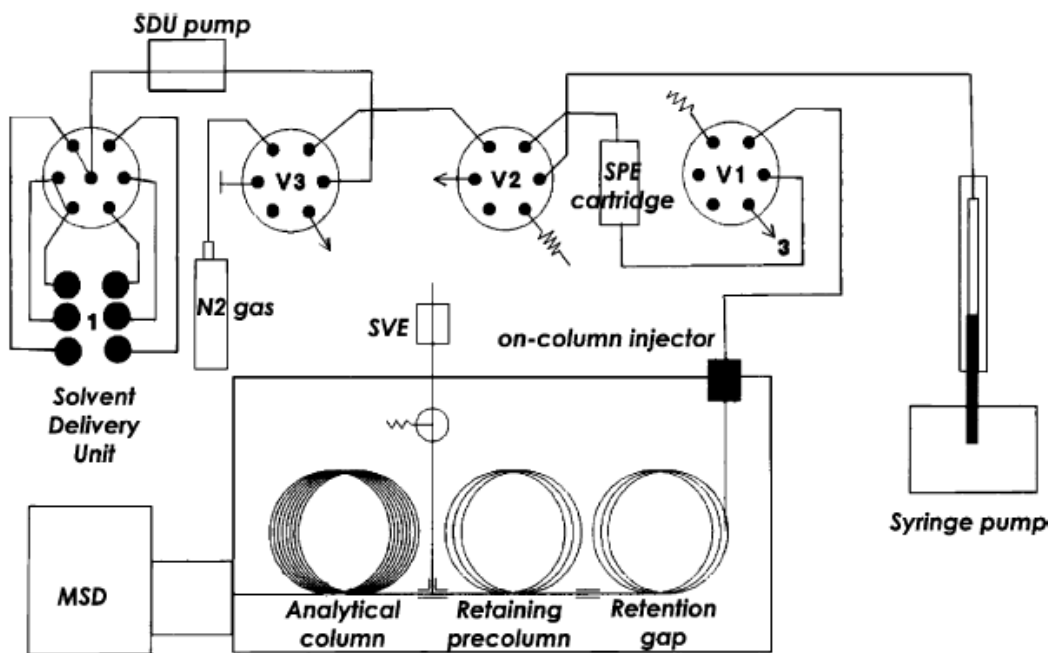


Fig 2: Construction of an on-line SPE-GC system consisting of three switching valves (V1-V3), two pumps (SDU pump and syringe pump) and a GC system equipped with an SVE, an MS detector, a retention gap, a retaining precolumn and an analytical GC column. *Reprinted with permission from Elsevier from AJH Louter et al. J Chromatog A 1996;725:67.*