Sample Preparation

Solid Phase Extraction – Overview

Wherever you see this symbol, it is important to access the on-line course as there is interactive material that cannot be fully shown in this reference manual.
Aims and Objectives

Aims

- To introduce the terminology associated with Solid Phase Extraction (SPE)
- To explain the chemistry and physical properties associated with SPE sorbents and substrates
- To outline principles associated with choosing sorbent mass in SPE to optimise analyte recovery
- To explain the different steps in a Solid Phase Extraction protocol
- To fully explore each protocol step and investigate sorbent selection, eluent solvent strength optimisation and the use of pH and ionic additives to control selectivity in SPE
- To highlight specific issues with Pharmaceutical Sample Preparation

Objectives

At the end of this Section you should be able to:

- Select Solid Phase Extraction Sorbents for particular analyte extractions
- Choose appropriate sample preparation strategies for various analyte and sample matrix types
- Properly condition and equilibrate sorbents to maximise analyte retention and minimise interference retention
- Choose the correct sample loading, washing and elution conditions to effect the best selectivity from a Solid Phase Extraction Protocol
Content

Solid Phase Extraction –Overview  3
SPE Terminology  5
SPE Sorbent Physical Properties  8
SPE Sorbent Substrates  10
SPE Sorbent Surface Chemical Nature  12
Choosing Sorbent Mass  16
Protocol Steps in SPE  16
SPE Sample Pretreatment  17
SPE Column Conditioning  18
SPE Column Equilibration  20
Sample Loading  22
Column Washing  22
Analyte Elution  23
Pharmaceutical SPE  24
Solid phase extraction, or SPE, is perhaps the most powerful sample preparation technique in common use today. Among SPE’s strengths are selectivity, flexibility, and high automation potential.

SPE involves the use of a chromatographic sorbent in a column format. A sample is passed through the column bed, analytes retain on the sorbent while the sample matrix liquid passes through, then the sorbent bed is washed to remove undesired interferences, and the purified analytes subsequently eluted from the column.
SPE may also be used to retain interferences, allowing analytes to pass unretained through the sorbent bed.

SPE products are available in a variety of formats to accommodate different sample sizes and applications. The original format used most commonly in pharmaceutical sample preparation is a small syringe barrel, containing 100 milligrams or less of sorbent material. This has more recently been somewhat displaced by a 96-well plate format, well suited for automation and supported by many commercial automation platforms.
**SPE Terminology**

“SPE column” refers to the extraction device used to execute the SPE protocol. Also referred to as the SPE “cartridge”, or for the 96-well format, the “extraction plate”.

“Sorbent” refers to the chromatographic material or packing used to implement the SPE procedure. The sorbent is typically entrained in the SPE column.

“Matrix” refers to the liquid present in the SPE sorbent bed at any time. The matrix may be either a protocol solvent or the sample itself.

“Retention” is the phenomenon whereby the analytes are attracted to and held by the active chemistry within the sorbent bed.

“Elution” is the disruption of the attractive interaction between the analytes and the sorbent bed, resulting in the analytes coming free from the sorbent and out of the SPE column.
The elution process

“Breakthrough” describes the analyte passing through the SPE column unretained during sample application, especially when the desired result is retention.

“Capacity” refers to the mass of retained compounds that may be held by a given mass of sorbent.
Low versus high capacity systems.

“Selectivity” refers to the ability of a given sorbent/extraction protocol to isolate the analytes preferentially from the rest of the sample components.
“Efficiency” refers to the ability of a sorbent to capture a given mass of analytes in a narrow chromatographic band on the sorbent bed.

**SPE Sorbent Physical Properties**

Virtually all commercial SPE materials are irregular-shaped rigid particles, with nominal sizes ranging from 8 to 70 microns, although the 40-60 micron range is by far the most common. One reason for the use of 40-60 micron particles is they allow for reasonable flow rates through the sorbent bed with nominal impetus applied to the sample (usually vacuum or pressure). Smaller particles require higher pressure for processing, with a few notable exceptions, in particular the disk and powder-based disk products, which have very short bed paths, thereby offering less total resistance to flow than the typical sorbent bed. In these formats smaller particles may be used.
Most SPE materials are fully porous in nature. The use of small porosity sorbents (for example, nominal porosity of 60 Angstroms) has the concurrent benefit of very high surface area (as high as 500 m$^2$ per gram) for active adsorption, thus offering the highest capacity possible per mass of sorbent. Typical absolute capacity values are on the order of a few percent retained compounds per mass of sorbent (for example, 5 milligrams retained compound per 100 milligrams of sorbent), although in certain special cases the capacity may be as high as 25%.
SPE Sorbent Substrates

SPE sorbents may be broadly divided into two different classes of material — silica-based sorbents and polymer sorbents.

Silica-based sorbents start with activated silica as a substrate, to which a functional group is bonded. The respective specific functional group bonded to the silica imparts the primary retentive properties to the sorbent, although the base silica also plays an active role in many extractions. Many commercial silica-based sorbents exist, which are named using acronyms that describe the primary character of the functional group on the silica. Common sorbent types are C18, C8, C6, C4, C2, phenyl, cyclohexyl, cyanopropyl, aminopropyl, diethyl amine, diol, propylsulfonic acid, phenylsulfonic acid, propyl carboxylic acid, propyltrimethyl amine, and unbonded silica.

Polymer—based sorbents also are available in a wide range of chemistries, covering a broad spectrum of polarities. The most non-polar sorbents are often based on styrene-divinyl benzene copolymers, sometimes further modified to create ion-exchange sorbents through amination or sulfonation. Other polymers incorporate polar functional groups into the polymer, which make the sorbents water-wettable, and offers additional possibilities for retention mechanisms.

There are a number of additional SPE sorbent chemistries available, including activated carbon, alumina, and florisil. However, these materials tend to have less well-controlled surface properties, and therefore have not been as successful commercially as the silicas and polymers.
SulphonatedstyreneDVB structure

QuataminestyreneDVB structure
SPE Sorbent Surface Chemical Nature

In reality the surface of the silica used for SPE sorbents is heterogeneous with several forms of silanol species present. Each of the different groups show different selectivity, with geminal species being the most reactive.

As well as the monofunctional bonding chemistry discussed on the previous page, some manufacturers use trifunctional silanes (an organosilane with three chlorine atoms). These phases show greater homogeneity after the surface species has been applied and give the surface a greater degree of protection to extremes of pH.

Trifunctional bonding chemistry gives rise to a polymeric surface, having a higher carbon loading and fewer silanol groups than the monofunctional phases.
Mixed mode interaction between the analyte, the C2 sorbent ligand AND the unreacted surface silanol group.

No matter what type of bonding chemistry is used, due to steric factors involved in binding the surface species, the number of unreacted (or residual) silanol species that remain on the sorbent surface is high (>50% for a typical C18 bonded phase). These silanol species are able to interact with analyte molecules in certain circumstances. In some cases this can be a disadvantage and unexpected retention effects and poor efficiency may be observed. In other instances, when combined with shorter chain length hydrophobic species (C2, C4 etc.) these silanol species may be used to effectively retain analyte species using both hydrophobic and electrostatic interactions. This approach is known as ‘mixed mode’ and will be discussed later.

Trifunctional ligands – surface binding chemistry
At a solution pH of around 4 the silanol species on the silica surface will be around 50% ionized and so the solution pH can be adjusted to ensure full ionization (~pH2) or full ion suppression (~pH6).

Where secondary silanol interactions are not required many manufacturers employ end capping techniques. This process involves binding sterically small trimethylchlorosilane species to the silica surface to target the remaining surface silanol groups and ‘cap’ them with a hydrophobic group. This leads to a more homogeneous silica surface and increases the hydrophobicity of the phase — leading to more efficient retention of hydrophobic analytes.
It should be noted that silica sorbents show instability to hydrolysis at extremes of pH. Below pH 2 the bonded surface species are susceptible to hydrolysis and efficiency of the sorbent is drastically reduced on extended exposure to solutions below pH2. Above pH 8 the silica itself is susceptible to hydrolysis and again the sorbent will show rapid deterioration upon extended exposure to solution pH>8. Certain sorbents are more susceptible to hydrolysis than others and those with which particular care must be taken are shown.
Choosing Sorbent Mass

In reality the choice of sample size and sorbent mass is dependant upon two important factors — the first is the expected concentration of the sample and the second is the choice of analytical technique for the separative stage of the analysis.

The analytical technique will have an impact on both the sample size, the volume of the cartridge and the sorbent mass, as well as the choice of the elution solvent. Where MS detection techniques are available, the sensitivity of the analysis is often improved.

To estimate the sample size required for HPLC-UV / MS analysis one must determine the sensitivity of the analytical method. The information shown assumes an analytical range of 0.25 — 10 ppm (injected solution concentration) for HPLC — UV and a limit of detection of 20ppb for HPLC-MS.

Table 1. Sample volume/sorbent mass

<table>
<thead>
<tr>
<th>HPLC-UV analysis. Sample concentration</th>
<th>Sample volume (mL)</th>
<th>Elution volume (mL)</th>
<th>Cartridge size/sorbent mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 — 2 ppm</td>
<td>50 - 100</td>
<td>3 - 6</td>
<td>6mL / (0.5-1)g</td>
</tr>
<tr>
<td>2 — 10 ppm</td>
<td>2 -10</td>
<td>0.5 - 2</td>
<td>(1-3)mL / (100-200)mg</td>
</tr>
<tr>
<td>HPLC-MS analysis (required to achieve eluent conc 20ppb)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20ppb</td>
<td>3</td>
<td>3</td>
<td>500mg / (3-6)mL</td>
</tr>
<tr>
<td>20ppb</td>
<td>0.5</td>
<td>0.5</td>
<td>100mg / 1mL</td>
</tr>
<tr>
<td>20ppb</td>
<td>0.2 — 0.5</td>
<td>0.2 — 0.5</td>
<td>(25-50)mg / (0.5-1)mL</td>
</tr>
<tr>
<td>20ppb</td>
<td>0.075 - 200</td>
<td>0.075 - 200</td>
<td>10-25mg/Micro-titre plate</td>
</tr>
</tbody>
</table>

For HPLC-MS the detection limit is calculated assuming an absolute detection limit of 1ng minimum detectable mass on column and a maximum injection size of 50ml leading to a sample concentration of 20ng/ml (i.e. 20ppb).

If your instrumental range differs from that given here, multiply the required sample volume by the appropriate factor.

If greater sensitivity is required, the final elution volume can be concentrated. The concentration factor gained through this post elution concentration can also be used to calculate a reduction in the sample volume required.

When using anion or cation exchange sorbents it should be noted that these resin types are capable of retaining 0.3 — 0.5 milliequivalents per gram of analyte (i.e. 0.3 — 0.5 mM per gram of sorbent for singly charged ions). The calculation shown gives and approximate indication of sorbent mass (minimum bed size) for ion exchange sorbents.

\[
\text{Minimum bed mass(mg)} = \frac{\text{Sample volume(mL)}}{\text{Molecular weight analyte}} \times \frac{\text{Approx. analyte Conc(mg/L)}}{\text{Analyte charge(absolute value)}} \times 3
\]

Protocol Steps in SPE

Most extraction protocols in SPE involve six separate steps. The first step is pre-treatment of the original sample to optimize the sample chemistry for the SPE procedure to be applied. The other five steps involve passing a solvent or the optimized sample through the extraction column or cartridge in order to create a particular chemical environment.
Within this environment, analytes and contaminants may partition either into the solvent passing through the column, or partition onto the chromatographic sorbent in the column. If the analytes and contaminants partition differentially, a separation of the analytes from the contaminants is brought about.

The five steps involving the extraction column are typically referred to as:

1. Column conditioning
2. Column equilibration
3. Sample loading
4. Column washing
5. Analyte elution

**SPE Sample Pretreatment**

Before a sample is applied to an SPE column for extraction, it is often necessary to pretreat the sample to make it compatible with the SPE procedure and optimize the sample chemistry to promote analyte retention.

Sample pretreatment typically involves three primary elements — particulate removal, sample chemistry adjustment to facilitate retention, and freeing bound analytes. Particulate removal is normally accomplished through a simple filtration. In some cases, simply passing the sample through the SPE column will remove particulates via the frits in the column. Particulates may also be removed by centrifugation prior to sample application to the column.

**Particulate removal**

Adjusting the sample chemistry to promote retention may involve a pH adjustment, or dilution with a solvent or buffer that will facilitate retention via the SPE mechanism being
employed. Since retention is a function of not only the analyte characteristics and the sample solvent, but also the specific chromatographic mechanism selected for the SPE procedure, the exact sample modifications can vary considerably from one protocol to the next. For an analyte to retain on a sorbent surface, it must be free in solution, versus being bound to other sample components. Two main examples of this binding are binding to sample particulate components, and binding to proteins, especially common in pharmaceutical applications.

**Sample chemistry and bound analytes**

**SPE Column Conditioning**

The first column step in a typical extraction protocol is referred to as column conditioning. The function of this step is to “activate” or “wet” the chromatographic sorbent to allow proper phase interface with the sample to be applied.

Many extraction sorbents are extremely hydrophobic, and will not wet to an aqueous sample. In this situation, the most common conditioning involves application of a water-miscible organic solvent to the column. The most common solvents employed for this purpose are methanol, acetonitrile, or tetrahydrofuran. These solvents function in a surfactant capacity, allowing interaction of the hydrophobic sorbent with the highly polar aqueous sample.

The sorbents most likely to require a conditioning step are either very hydrophobic bonded silicas, such as C18, or very hydrophobic polymer resin sorbents, such as styrene divinyl benzene.
Non–conditioned (activated) sorbent

Good analyte retention with conditioned sorbent

Good polar analyte achieved with hydrophilic sorbents without conditioning
If an extraction sorbent is water-wettable, a column conditioning step may not be necessary. When using any particular commercial extraction sorbent for aqueous samples, it is important to confirm with the manufacturer whether the sorbent in use is water-wettable or not. If the manufacturer cannot supply this information, the user should compare a proposed extraction protocol with and without a conditioning step. If there is no difference in the results, most likely the sorbent is water-wettable, and a conditioning step may not be required in the final protocol.

**SPE Column Equilibration**

The second column step in a typical extraction protocol is referred to as column equilibration. This step is typically performed just prior to application of the sample. The function of column equilibration is to create a sorbent chemistry environment as similar to the sample as possible. This is required so that the sample chemistry itself does not change the extraction environment over the course of the sample application. If the sorbent is not equilibrated properly, irreproducible results and poor analyte recoveries are often seen.

One important parameter to be addressed in column equilibration is pH. If pH is important to the effectiveness of the extraction chemistry, the sample pH should be adjusted properly in both the sample pretreatment step and the column conditioning step. If a sample is pretreated to a pH, for example, of 3.5, and the column is equilibrated at a different pH, for example 7.5, during the course of the sample application to the column, the pH of the column environment will gradually shift from 7.5 to 3.5. Therefore, the initial portion of the applied sample will experience a different extraction environment than the latter portion of the sample. This can result in irreproducible results and poor analyte recoveries.
It is also important to ensure that the column equilibration solvent is fully miscible with the sample to be applied or precipitation of sample components may occur, possibly blocking the flow through the column.
Sample Loading

The third step in a typical SPE protocol is application to the column of the sample to be extracted. The single most important element of this step is the linear velocity of the sample as it passes through the column.

The linear velocity of the sample application is a function of two individual elements — the flow rate at which the sample is applied, and the diameter of the column to which the sample is being applied. For a given flow rate, a wider column will offer a slower linear velocity than will a narrow column.

The importance of an adequately slow linear velocity relates to the residence time of the analytes in the extraction sorbent. This residence time must be sufficient for the necessary chromatographic interaction to occur. Since this interaction is spatially dependent (in other words, active functional groups on the analyte must orient properly relative to the functional groups on the extraction sorbent), sample application with too small a residence time may result in analyte breakthrough.

The speed of sample application also is a function of the particular extraction mechanism being employed. This will be addressed in greater detail in the section on specific extraction mechanisms.

Column Washing

The fourth step in a typical SPE protocol is washing the sorbent to selectively remove undesired contaminant species from the sorbent, while leaving the target analytes retained on the sorbent. This step is usually performed using a solvent that has higher elution strength than the solvent in which the sample was applied to the column, relative to the particular extraction mechanism being employed.

In many cases, the wash solvent is a “weaker” version of the elution solvent — that is, the elution solvent diluted with a weaker eluent. This ensures miscibility of all the solvents in the extraction protocol, resulting in the fastest equilibration of each protocol step with the next, and the greatest reproducibility.
One common error in SPE protocols is to use wash solvents that are too weak. Philosophically, the concept of a wash solvent is to remove as many of the undesired contaminants as possible. Many SPE users employ very weak elution solvents due to a concern for eluting analytes during wash steps. Unfortunately, this often results in poor quality extracts, since many of the contaminants are left on the column during the wash, and subsequently eluted along with the desired analyte in the elution step. In general, this issue leads to the following important conclusion — wash steps should be performed with the strongest solvent possible that does not elute the analyte. This ensures removal of the greatest amount of contaminants, resulting in the cleanest possible extract.

**Analyte Elution**

The final step in a typical SPE protocol is recovery of the desired analytes from the sorbent column by elution with a suitable solvent. The types of solvents used for elution must be capable of disrupting all of the retentive interactions between functional groups on the analytes and functional groups on the extraction sorbent. An elution solvent which disrupts only a portion of the interactions between the analyte and the sorbent will bring about only partial elution, resulting in irreproducible results and often poor analyte recoveries.

Many modern SPE procedures employ sorbents that are specifically designed to exhibit multiple retention mechanisms. Such sorbents are often referred to as “mixed mode” sorbents, and are extremely popular in pharmaceutical applications, for extraction of basic and acidic drugs. Analyte elution from these surfaces often requires especially “strong” elution solvents, to ensure complete recovery of the analytes from the sorbents.
Having said all of the above, one all too common error in SPE protocols is to use elution solvents that are too strong. The elution step is an additional opportunity for sample cleanup by leaving undesired contaminants behind on the sorbent during elution. Use of a very strong elution solvent will tend to elute these contaminants along with the desired analytes.

This situation leads to another important conclusion — elution steps should be performed with the weakest solvent possible that provides complete elution of the analytes. This leaves behind on the sorbent the greatest possible amount of contaminants, resulting in the cleanest possible extract.

**Pharmaceutical SPE**

SPE is a technique that is particularly well-suited for pharmaceutical sample preparation prior to bioanalysis. The most common matrices encountered in pharmaceutical bioanalysis are aqueous biological samples, such as animal plasma, serum and other body fluids (preclinical trials), human plasma, serum and urine, and aqueous buffers used for cell and tissue culture. Sample sizes range from 25 microliters for small animal plasma samples, up to several milliliters for human urine samples.
Typical sample volumes

Pharmaceutical analytes of interest cover a wide range of chemical properties, and include very non-polar drugs, basic and acidic drugs, and highly polar compounds such as small peptides and glucuronide metabolites. The very wide range of analyte chemistries requires commensurate flexibility in sample preparation methods by SPE, including the use of hydrophobic, polar, ion-exchange, and chelation mechanisms to effect sample cleanup.

Analytes

The wide range of analyte chemistries also dictates a need for rapid and facile method development to accommodate the large number of new drug leads passing through the discovery process. Also popular are "generic" methods, which can be applied to many classes of analyte, with only small variations.
In order to simplify the complex selection of choices in pharmaceutical SPE, it is convenient to break the different protocols into mechanistic classes, and examine the extraction process for each individual class in detail.

Polar sorbent types
Non polar sorbent types

- C18
- C8
- C2
- Phenyl

Anion/cation sorbent types

- SAX
- DEA
- CBA
- PRS