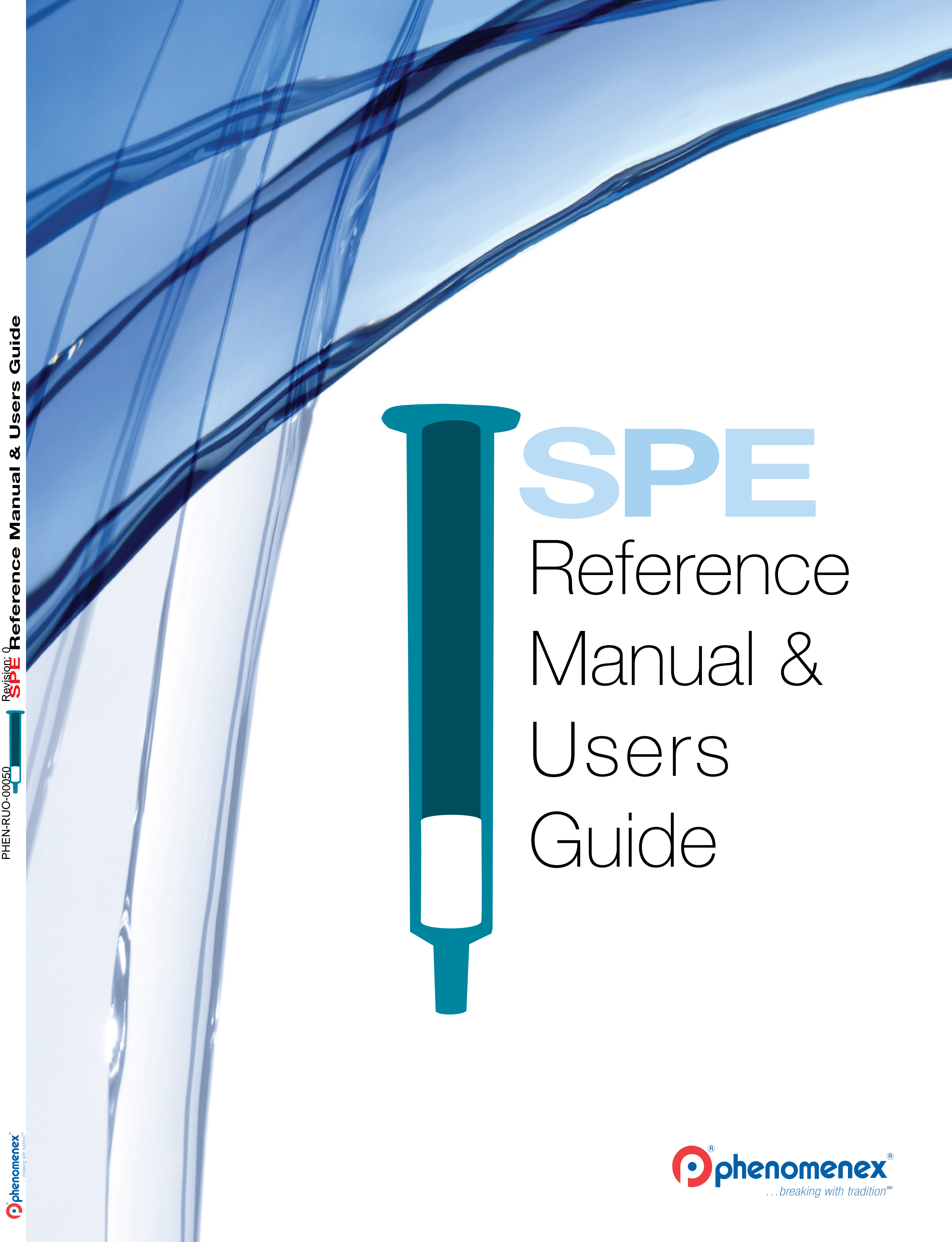

SPE
Reference Manual & Users Guide
phenomenex
...breaking with tradition
PHEN-RUO-00050
Revision: 0
SPE Reference Manual & Users Guide

1. OBJECTIVE AND PURPOSE OF THIS GUIDE

The objective of this manual is to provide analytical chemists with a practical bench-top guide to SPE. The goal is to teach the basic principles of SPE, and facilitate the process of choosing the proper sorbent, optimizing methods, and troubleshooting problems.

The initial sections of this guide contain general information about SPE sorbents, extraction mechanisms, and important background information on factors that should be considered before choosing a product or developing a method.

Included on this CD is Technical Note TN-0013, A Simple Approach to Fast and Practical Solid Phase Extraction (SPE) Method Development, that allows the chemist to choose the proper SPE column and sample pre-treatment based on the most basic information about the target analyte(s) and the sample/matrix.

The final sections of this guide include standardized, generic procedures that can be used once the sorbent has been chosen. Also included throughout this guide are practical and helpful hints for optimizing and customizing these basic protocols for each particular target analyte(s) and sample/matrix.

Finally, Appendix II contains a comprehensive "SPE Troubleshooting Guide" that can be used to resolve virtually any potential difficulty that may arise with any particular sample, analyte(s), column, or method.

When used in conjunction with other guidebooks from Phenomenex for Liquid and Gas chromatography (see Appendix III), this manual allows the chromatographer to develop, optimize and solve any separation problem, regardless of its complexity, and streamline the entire analytical procedure from sample preparation through the final analysis.

Phenomenex offers this SPE Users Guide as a practical aide for SPE method development, optimization, and troubleshooting. For a theoretical discussion of SPE, and chromatographic theory in general, we suggest that the reader consult the literature references (1-4) in Appendix III.

2. SOLID PHASE EXTRACTION OVERVIEW

Solid Phase Extraction (SPE) is a method for rapid sample preparation in which a solid stationary phase is typically packed into a syringe barrel (**Fig.1**) and used to selectively extract, concentrate, and purify target analytes prior to analysis by HPLC or GC.

During the last two decades, SPE has steadily gained acceptance within the analytical community and is now rapidly replacing traditional liquid:liquid extraction (LLE) as the sample preparation technique of choice for discriminating chromatographers around the world.

The efficacy and economy of SPE is now well documented in a staggering number of peer-reviewed journal articles and reviews. Literally, thousands of SPE applications are now available for the extraction of key analytes in the pharmaceutical, clinical/toxicological, environmental, and biomedical fields, and new methods continue to be developed on a daily basis.

3. THE GOAL OF SAMPLE PREPARATION

The ultimate goal of any sample preparation technique is to facilitate analysis and maximize sample throughput by concentrating the target analyte and removing impurities.

SPE accomplishes these goals because it:

1. *Eliminates otherwise co-eluted impurities and/or instrument-fouling particulates.*
2. *Concentrates analytes and improves sensitivity (and reduces LOD and LOQ).*
3. *Facilitates the rapid and efficient, simultaneous processing of multiple samples (either manually or via automation).*
4. *Enables solvent switching or buffer exchange prior to analysis.*
5. *Provides high extraction efficiencies, with quantitative recoveries of analytes and low levels of contaminants.*
6. *Provides consistent, reproducible results (provided that the extraction conditions are properly optimized and the sorbent surface chemistry is reproducible).*

By combining the tremendous separation power of classical liquid chromatography with a low cost, convenient, disposable cartridge, SPE effectively achieves these goals in a more direct, economical, and reproducible manner than traditional liquid:liquid extraction (LLE). Quite frankly, SPE simply works.

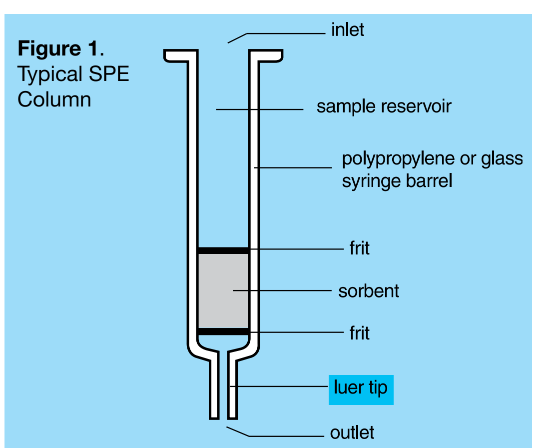


Figure 1. Typical SPE Column

See glossary *(p.45)* for definition

4. SPE VS. LLE

Since the mid-1970's when commercial SPE devices were first introduced, analysts have been switching from LLE to SPE for the simple reason that it provides better results with fewer complications. In fact, SPE is currently approved as an alternative to LLE for a large percentage of the official methods for the US EPA (United States Environmental Protection Agency).

SPE provides a number of critical benefits over LLE, including the following:

1. *Improved selectivity and specificity.*
2. *Higher recoveries.*
3. *Enhanced removal of interferences and particulates.*
4. *Greater reproducibility.*
5. *Reduced labor, ease of use, and the ability to rapidly process multiple samples and facilitate automation.*
6. *Greater flexibility in terms of solvent miscibility.*
7. *Elimination of emulsions.*
8. *Enormous decreases in solvent consumption and a concomitant reduction in hazardous waste.*
9. *More "environmentally-friendly" solvents, and reduced exposure of lab personnel to toxic and/or flammable solvents.*
10. *Concentration of key analytes, enhanced analytical sensitivity, reduced LOD and LOQ, improved analytical column longevity and resolution, and minimized instrument down time.*
11. *Ability to optimize separations using well-established principles of chromatographic theory and generic HPLC method optimization techniques.*

Clearly, SPE offers a variety of key technical, performance and economic benefits that classical LLE cannot match. SPE consistently provides cleaner extracts and recoveries that are significantly higher than those achieved with LLE, particularly with difficult-to-extract amphoteric and highly polar, water-soluble analytes.

5. SPE SORBENTS

The key element to any SPE product is the sorbent. The physiochemical properties of the sorbent determine extraction efficiency and the overall quality of the separation.

A. Sorbent Properties

SPE sorbents are available in a wide range of surface chemistries, pore sizes (60, 120, 300 Å), particle sizes (10, 40, 100 µm), and base supports (silica, alumina, polymers). **Table 1** summarizes the physiochemical properties of the most common SPE sorbents.

The optimal sorbent for any given extraction problem is dependent upon the properties of the target analyte, the sample/matrix composition, and other factors that are discussed below. Technical Note TN-0013 (included on this CD) and the next several sections of this guide are designed to help facilitate the process of choosing the proper sorbent based on each of these important factors.

B. Sorbent Specificity

Ideally, SPE sorbents function with affinity-like specificity, retaining only the target analyte(s) without binding any contaminants. The eluant from this "ideal" SPE sorbent contains only the analyte(s) of interest and is free of any extraneous "peaks". In most situations, however, typical SPE sorbents are not that selective. Unless the mobile phase and other extraction conditions have been properly optimized, or the analyte concentrations are fairly high, a wide range of contaminants can also be retained and affect the analysis.

As a result, it is extremely important that the sorbent chemistry is chosen carefully, and that each of the extraction conditions (sample loading, wash, and elution steps) are properly optimized. In fact, sorbent specificity is so strongly dependent upon the extraction conditions that the sample/matrix loading conditions can even determine which chromatographic mechanism predominates on a given sorbent (see Sections 7-D and 8-B). Consequently, the extraction conditions help determine sorbent specificity just as much as the surface chemistry itself. Details on the relative specificities and properties of each of the most common sorbents used for SPE are provided in Sections 7, 9 and 10.

C. Silica-based Sorbents

Silica-based SPE sorbents are the most popular because they are rigid, inexpensive, easy to derivatize and manufacture reproducibly, stable, and immune to shrinking and swelling in common aqueous and organic solvents.

The most popular SPE sorbents are chemically-modified silica particles with functional groups covalently attached to the surface (**Fig.2**). Modification of the silica surface dramatically alters chromatographic selectivity. For example, bare silica is extremely polar, retaining analytes via normal phase and cation-exchange mechanisms. By attaching a saturated hydrocarbon such as C18, the surface becomes hydrophobic (non-polar). Covalent attachment of a variety of other functional groups has been used to produce SPE sorbents with an enormous range of chromatographic selectivities.

Upon completion of the initial bonding reaction, silica-based sorbents may still contain a small quantity of unreacted or "free" silanols (**Fig.3**). These exposed silanols provide polar, acidic patches on the chromatographic surface that are capable of binding amines via hydrogen bonding and cation-exchange mechanisms. Since a number of important analytes are ionized

See glossary *(p.45)* for definition

under typical extraction conditions, interaction with these residual silanols may cause undesirable retention or low recoveries. In order to minimize these secondary interactions, the bonded phase is typically subjected to an "endcapping" reaction in which the residual silanols are methylated, typically with trimethylsilyl (TMS) groups.

However, it should be noted that in SPE, free silanols can often be used to facilitate the retention of polar analytes on *reversed phase* sorbents like C18, C8 and Phenyl. Non-endcapped phases provide the analyst with a powerful separation tool due to the mixed polarity of the sorbent, which contains both hydrophobic alkyl chains and polar, acidic silanol moieties. Hydrocarbon groups on the analyte interact with the hydrophobic alkyl chains, and the polar or charged functional groups on the analyte interact with the silanols, enhancing retention and affecting selectivity, often for the better.

D. Polymeric Sorbents

Polymer-based SPE sorbents or "resins" are typically composed of highly cross-linked polystyrene-divinylbenzene (PSDVB or SDB). In contrast to bare silica, native SDB is non-polar, and capable of strong hydrophobic and π-π interactions.

Like silica, the polarity of the SDB surface can be modified by the addition of various functional groups.

The newer generations of SDB resins are highly cross-linked, with negligible shrinking and swelling, the particle sizes are carefully controlled, and the polymers are cleaned extensively in order to reduce endogenous extractable contaminants.

Table 1 provides a list of the typical physical properties of common silica-based and polymer-based SPE sorbents.

Figure 2.
Structures of Common Silica-based Sorbents

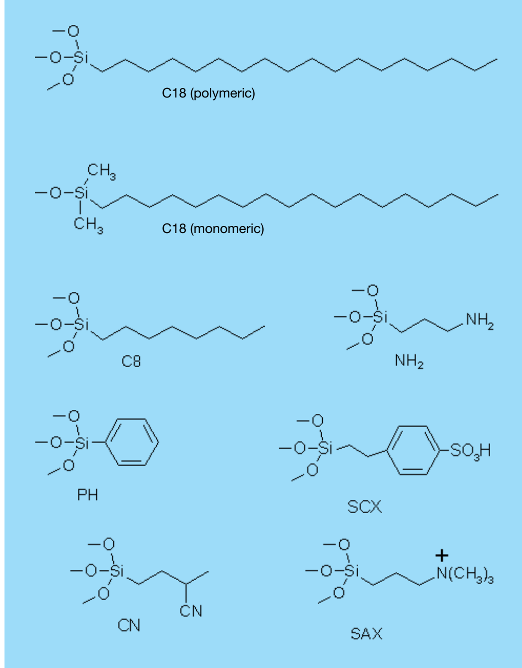


Figure 3.
C18 Bonded Silica Containing Free Silanols

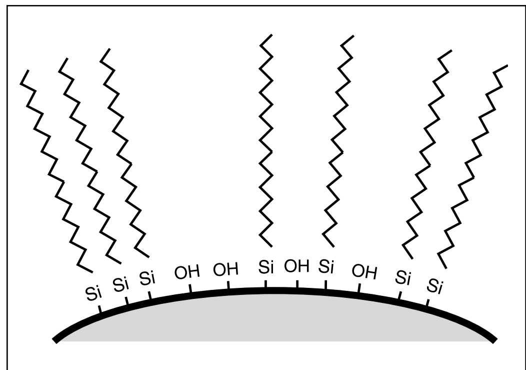


Table 1. Physiochemical Properties of Typical SPE Sorbents

Support	Phase	Surface Modification	% Carbon Range	% Nitrogen Range	% Sulfur Range	Surface Area Range (m²/g)	Particle Size Range (microns)	Pore Size Range (Angstrom)	Retention Mechanism
Silica	C18	Octadecyl (polymeric)	15 - 20	0	0	450 - 550	50 - 60	65 - 75	RP
Silica	C18	Octadecyl (monomeric)	12- 16	0	0	280 - 320	50 - 60	120 - 140	RP
Silica	C8	Octyl	8 - 12	0	0	450 - 550	50 - 60	60 - 75	RP
Silica	PH	Phenyl	8 - 11	0	0	450 - 550	50 - 60	60 - 75	RP
Silica	CN	Cyanobutyl	5 - 10	1 - 2	0	450 - 550	50 - 60	60 - 75	RP + NP
Silica	NH_2	Aminopropyl	3 - 6	1 - 2	0	450 - 550	50 - 60	60 - 75	NP + IEX
Silica	SCX	Phenylsulfonic acid	7 - 12	0	1 - 3	450 - 550	50 - 60	60 - 75	IEX
Silica	SAX	Me_3(propyl)ammonium Cl^-	4 - 8	0.5 - 1.2	0	450 - 550	50 - 60	60 - 75	IEX
Silica	Silica	Acidic, Neutral	0	0	0	250 - 600	50 - 60	60 - 75	NP
Alumina	Alumina	Acidic, Neutral, Basic	0	0	0	100 - 150	50 - 300	100 - 120	NP
Florisil	Florisil	None	0	0	0	300 - 600	50 - 200	60 - 80	NP
Polymer	SDB	None	90 - 93	0	0	500 - 1000	75 - 150	50 - 300	RP

RP = Reversed Phase, NP = Normal Phase, IEX = Ion-Exchange

See glossary *(p.45)* for definition

E. Surface Area

The surface areas for typical SPE sorbents range from 250 to 600 m^2/g. In fact, one gram of sorbent has about the same surface area as a basketball court.

F. Pore Size

The vast majority (98 %) of the surface area of SPE bonded phases is contained inside the pores. Most SPE sorbents have a mean pore size of only 60 or 70 Å. This effectively restricts the access of macromolecules greater than 15 kiloDaltons (kDa), limiting their ability to interact with the sorbent.

G. Particle Size

Most SPE products are based on 50 µm irregular silicas. These large particles provide low pressure drops and rapid flow rates without clogging. The efficiency of such large particles is relatively low, only about 5,000 plates per meter, or 5 % of those obtained with 5 µm HPLC packings. In fact, the typical SPE column only contains about 5 to 10 theoretical plates, which is less than one percent of that in a typical HPLC column. Despite these differences, however, SPE columns perform quite effectively, and this is due to the selectivity imparted by their surface chemistry, and as discussed in the following sections, the chromatographic operating conditions and a variety of optimization techniques that are typically employed.

6. SPE AND LIQUID CHROMATOGRAPHY

As with any analytical technique, the true versatility of SPE can only be realized and achieved by first understanding, and then properly controlling the physiochemical interactions that may exist between the sorbent and the sample/matrix components.

SPE relies upon the same basic chromatographic retention mechanisms and physiochemical interactions that are utilized in classical liquid chromatography and modern HPLC. The delicate interplay of these interactions between the analyte, sorbent and mobile phase is the driving force which is responsible for the separation power of all liquid chromatographic methods, including SPE. As in HPLC, the separations achieved with SPE can readily be optimized by the proper modification of the mobile phase conditions during the sample loading, wash, and elution steps, and by the judicious choice of the stationary phase.

In practice, however, SPE and HPLC often differ strongly in terms of the harshness of the mobile phase conditions utilized, and consequently, the degree and the strength of the interactions between the solutes and the stationary phase. In contrast to analytical HPLC, SPE more closely resembles the digital type of chromatography which is typically employed in preparative LC, where "on-off" type of step gradient conditions are frequently employed to selectively elute the contaminants from the target compound(s). With SPE, the goal is typically to adjust the mobile phase conditions to the point at which the analyte is either fully retained or fully eluted in only a few column volumes. In contrast, analytical HPLC takes advantage of relatively subtle, partial interactions that allow each of the analytes to differentially migrate along the length of the column in order to enhance resolution.

Despite these differences, however, the basic principles and interactions that govern both HPLC and SPE separations are, in fact, the same (*see Section 7*). Consequently, experienced chromatographers can readily utilize their understanding of chromatographic theory and HPLC optimization techniques in order to optimize SPE separations.

7. EXTRACTION MECHANISMS

SPE sorbents are most commonly categorized by the nature of their primary interaction or retention mechanism with the analyte of interest. The three most common extraction mechanisms used in SPE are *reversed phase, normal phase* and *ion-exchange* (**Fig.4**). **Table 1** provides a summary of the most common SPE sorbents and the major retention mechanisms employed on each. Details are provided below and in Section 10.

A. Reversed Phase

"Reversed phase" extractions are commonly used to extract hydrophobic or even polar organic analytes from an aqueous sample/matrix. Hydrocarbon chains on both the analyte and the sorbent are attracted to one another by low energy van der Waals dispersion forces. Common *reversed phase* sorbents contain saturated hydrocarbon chains such as C18 and C8, or aromatic rings such as Phenyl (PH) or SDB. Because reversed phase extractions are relatively non-specific, a wide range of organic compounds is typically retained. As a result, it is important to optimize the extraction conditions, particularly the composition of the wash solvent (*see Section 8-C*). Analytes are typically eluted with organic solvents such as methanol or acetonitrile, in combination with water, acids, bases, or other solvents and organic modifiers.

B. Normal Phase

"Normal phase" retention mechanisms are commonly employed to extract polar analytes from non-polar organic solvents. The retention mechanism is based on hydrogen bonding, dipole-dipole and π-π interactions between polar analytes and polar

See glossary *(p.45)* for definition

stationary phases such as silica, alumina and Florisil®. Highly specific normal phase extractions can be obtained by carefully optimizing the polarity of the conditioning solvent and the solvent(s) used to dilute and load the sample/matrix. Analytes can be eluted with the use of relatively low concentrations of polar organic solvents such as methanol or isopropanol, in combination with non-polar organic solvents.

C. Ion-Exchange

"Ion-exchange" mechanisms are used to extract charged analytes from low ionic strength aqueous or organic samples. Charged sorbents are used to retain analytes of the opposite charge. For example, positively charged analytes containing amines are retained on negatively charged *"cation-exchangers"* such as sulfonic or carboxylic acids. In contrast, negatively charged analytes containing sulfonic acid or carboxylic acid groups are retained on positively charged "*anion-exchangers*" containing any one of a variety of different amino groups. *Ion-exchange* mechanisms rely on specific, high-energy coulombic interactions between the sorbent and the analyte. Only species of the proper charge are retained by the column, so most matrix contaminants are simply rinsed away to waste during the loading and the wash steps. For this reason, cation-exchange SPE is commonly used for the extraction of basic compounds (drugs and other amines) from complex biological samples. Analytes are typically eluted with high ionic strength salts and buffers and/or strong acids or bases.

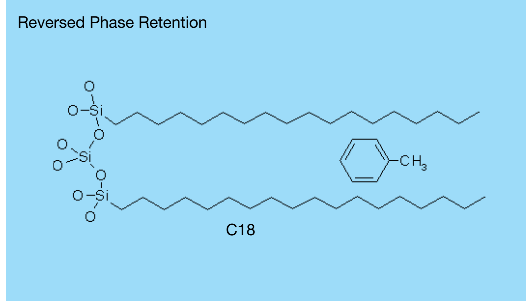
D. Sample/Matrix Composition Affects the Extraction Mechanism

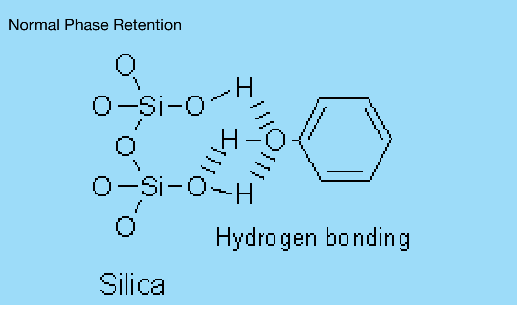
The specificity of any sorbent towards a given analyte is highly dependent upon the composition of the sample/matrix and the solvents used for dilution. Changes in the polarity, aqueous:organic composition, pH and ionic strength during the sample loading step can actually determine the chromatographic retention mechanism and selectivity in terms of which solutes and analytes are, in fact, retained and recovered.

Weak ion-exchangers provide the most dramatic example of the effects of the loading (and the conditioning) solvent conditions on chromatographic specificity. In the presence of a non-polar organic solvent-rich sample/matrix, these sorbents function in the *normal phase*, retaining polar analytes via hydrogen bonding and dipole-dipole interactions (and adsorption chromatography). Alternatively, with an aqueous sample/matrix at neutral pH values (pH 6-8), charged analytes may be retained by an *ion-exchange* mechanism. Finally, at pH extremes (<4 or >10) or in the presence of ion pair reagents or high salt concentrations, charged as well as neutral analytes may be retained on the alkyl spacer arm via a *reversed phase* mechanism.

Consequently, the same sorbent can be used to extract a wide range of different target analytes, and, depending upon the composition of the conditioning solvent and the sample/matrix, dramatic differences in selectivity, recovery and purity can be obtained.

Figure 4.
SPE Retention Mechanisms





Anion-Exchange Retention

Carboxylic Acid (Deprotonated)

Cation-Exchange Retention

SCX

Amine (protonated)

See glossary (p.45) for definition

E. Retentive vs. Non-Retentive SPE

The majority of SPE extractions are "retentive" since a sorbent retains the target analytes, while contaminants simply pass through the column to waste (**Fig.5A**). On the other hand, in a "non-retentive" extraction, the sorbent has no affinity for the analyte, but a high affinity for the sample contaminants. As a result, the analyte passes directly through the column without being retained, while the contaminants are bound (**Fig.5B**). Non-retentive extractions are particularly useful when the analyte is highly soluble in the sample/matrix and/or the dilution solvent and can not be easily partitioned out of solution onto a solid or a liquid phase. They are also simpler, since there is no need for an "elution" step; the analyte(s) is collected during the loading and wash steps (**Fig.5B**).

8. STEPS IN SPE PROTOCOLS

Sample preparation with SPE typically consists of 4 basic steps: conditioning, sample loading, wash, and elution (**Table 2**). As noted above, non-retentive extractions may only require 2 or 3 steps, since the analyte is effectively eluted during the sample loading and the wash steps. In contrast, retentive extractions typically consist of all 4 steps (**Fig. 5A**). In each step, the sample or mobile phases (which are either called wash or elution solvents) are passed through the sorbent and collected as desired.

Because SPE takes advantage of the same physiochemical interactions utilized in classical liquid chromatography and HPLC, the recovery and purity of the target analyte(s) can be optimized by adjusting the composition of the solvents used during each of these steps (sample loading, wash and elution). In this manner, chromatographers can utilize their experience in HPLC in order to utilize the full power of SPE and optimize the extraction efficiency.

A. Conditioning Step

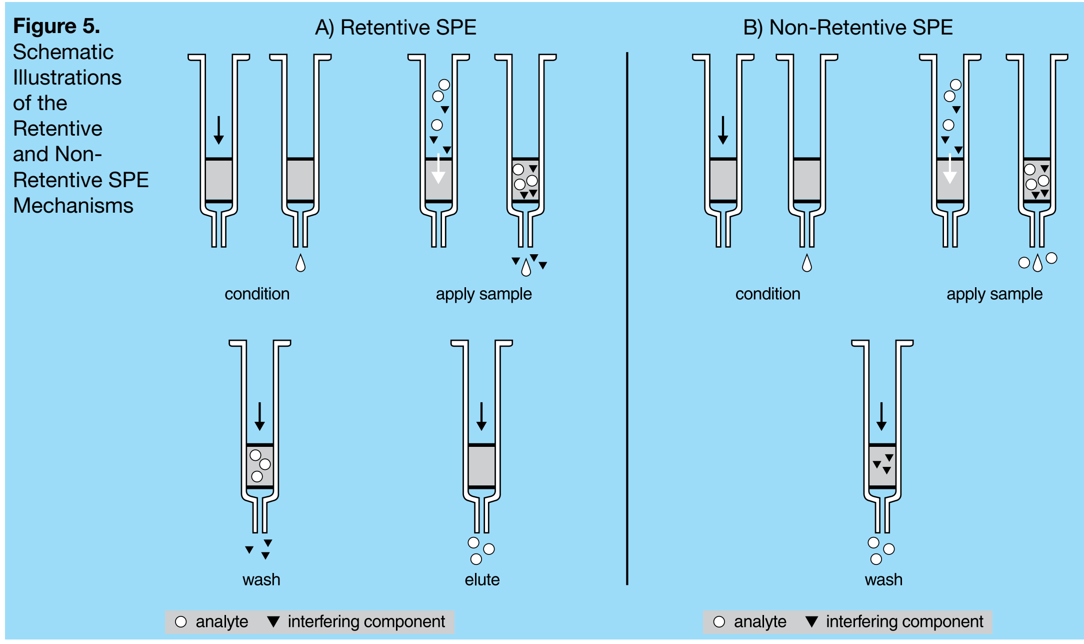
Prior to loading the sample/matrix, the SPE column is typically washed and "wetted" with methanol, isopropanol or another organic solvent(s) of intermediate polarity. This conditioning step removes trapped air, and solvates or activates the ligands on the chromatographic surface, enabling them to interact more effectively with the target analyte(s).

The conditioning step often consists of two substeps: an initial "solvation" step (described above), followed directly by an "equilibration" step.

The equilibration step typically employs a solvent with a composition that is similar to the sample/matrix in terms of the solvent ratio, ionic strength and pH. This solvent helps remove residual methanol remaining from the solvation step, and equilibrates the sorbent in a solvent that will maximize the interactions with the target analyte(s) in order to promote retention. The solvation step is typically followed directly by the equilibration solvent (and then, by the actual sample/matrix) in order to prevent the sorbent bed from drying out or losing solvation due to evaporation.

Specific details on the conditioning solvents used for each type of extraction mechanism (or sorbent) are provided in Section 10. In general, methanol has traditionally been the solvation/conditioning solvent of choice for *reversed phase* sorbents and *ion-exchangers*. However, isopropanol is now recognized to be a bit more effective in this regard, owing to its slightly longer alkyl chain which interacts more strongly with the hydrocarbon chains on these sorbents.

Figure 5. Schematic Illustrations of the Retentive and Non-Retentive SPE Mechanisms



See glossary *(p.45)* for definition

Table 2. Four Basic Steps for Sample Preparation with SPE

Step	Purpose
Conditioning	To prepare the sorbent for effective interactions with the analyte(s) by solvation or activation of the ligands on the chromatographic surface, followed by equilibration in a solvent similar to the sample/matrix
Sample/Matrix Pre-treatment and Loading	To adjust the sample/matrix composition (via dilution, etc.) such that the analyte(s) is quantitatively retained on the sorbent while the amount of bound impurities is minimized
Wash	To remove impurities that are bound to the sorbent less strongly than the analyte(s)
Elution	To selectively desorb and recover the analyte(s) by disrupting the analyte-sorbent interactions

For *normal phase* or adsorption chromatography, the conditioning step often consists of a single equilibration step with a solvent system composed of mostly a non-polar organic solvent (such as hexane, toluene, methylene chloride or chloroform) containing a small percentage of a miscible polar organic solvent (such as an alcohol). In many cases, the initial solvation step with methanol or isopropanol is omitted, since these solvents are able to remove some of the absorbed water from the surface of the sorbent, and this can have a dramatic effect on selectivity (*see Section 10-B-2*).

In general, solvent volumes that are between 2 to 4 times the sorbent bed volume are typically necessary to ensure proper conditioning, washing and elution. Conditioning with less than 2 to 4 bed volumes increases the risk of incomplete solvation of the bed and low or irreproducible recoveries, while more than 4 bed volumes is typically unnecessary.

Conventional packed-bed SPE products typically have a bed volume of approximately 150 µL per 100 mg of sorbent. The densities and void volumes of SPE membranes and discs are much more variable, depending upon the loading density and the physical nature of the membrane support material (*see Section 9-C*).

B. Sample Loading Step

The sample loading step includes any sample/matrix pre-treatment and/or dilution that may be required, as well as the actual application and introduction of the sample/matrix onto the SPE column.

Sample pre-treatment often consists of a dilution step in which the sample/matrix is mixed with a “weak” solvent which promotes analyte retention, as described in Section 10.

See glossary *(p.45)* for definition

The major goal of the sample loading step is to ensure that the target analyte(s) is quantitatively retained by the sorbent. As a result, it is imperative that the sample/matrix composition is adjusted in a manner which facilitates the binding of the analyte(s). This may require the dilution of the sample in a “weaker” solvent; one with a higher polarity (water) for *reversed phase*, a non-polar solvent such as hexane for *normal phase*, or a low ionic strength, pH-adjusted buffer for ion-exchange. For details, consult Technical Note TN-0013.

The second (often overlooked) goal of the sample loading step is to adjust the solvent conditions in order to minimize the number of impurities that are bound. This will often enhance the capacity of the sorbent for the target analyte, and improve purity. However, accomplishing this goal typically requires the loading conditions to be adjusted in the opposite direction of those required to facilitate the retention and recovery of the analyte (as described above). As a result, this method optimization tool is often omitted altogether, and is typically used only as a last resort in order to remove strongly retained or tenacious contaminants. Details on the exact conditions required for loading samples on various sorbents are given in Section 10.

The degree of separation achieved during the sample loading step, and chromatographic specificity in general, are highly dependent upon the solvent composition of the sample/matrix (and the conditioning solvent). Changes in the polarity, aqueous:organic composition, ionic strength and/or pH of the sample/matrix as well as the conditioning solvent can each have a dramatic effect on analyte recovery as well as the level and the nature of the contaminants that are retained. As noted in Section 7-D the loading conditions can actually determine the chromatographic retention mechanism that predominates on any given sorbent.

C. Wash Step

The wash step(s) is designed to elute impurities that are retained on the sorbent less strongly than the target analyte(s), and to rinse or “push out” residual, unretained sample/matrix components that may remain from the sample loading step. The ideal wash solvent removes all of these impurities without affecting the retention and subsequent recovery of the target analyte(s). Clearly, the wash step must contain a solvent of intermediate strength, not as “weak” as the loading solvent and not as “strong” as the elution solvent. Another restriction placed on the wash solvent is that it must be miscible with the diluted sample/matrix as well as the elution solvent; otherwise, the column must be thoroughly dried in between steps.

The wash step typically receives the greatest attention during method development and optimization studies. This is probably because it often provides the most dramatic improvements in purity, and the effects of systematic changes in the wash solvent composition can readily be assessed.

In situations in which the surface chemistry of the SPE and analytical HPLC columns are similar (e.g., both are C18), the HPLC mobile phase composition approximates the optimal SPE wash solvent.

For *reversed phased* extractions, the wash solvent typically consists of an aqueous mixture of either acetonitrile or methanol with an organic composition between 5 and 50 %. For *normal phase* extractions, the wash solvent typically contains a small amount of polar organic solvent added to the same non-polar organic solvent that was used to load the sample. For *ion-exchangers*, the wash step typically consists of a buffer with a low to intermediate ionic strength.

Optimal volumes for the wash step are typically around 1 mL for every 100 mg of sorbent, or about 7 sorbent bed volumes. This volume should be sufficient to provide the consistent and effective removal of the residual, unretained impurities and to elute some of the bound contaminants. Smaller elution volumes may yield inconsistent results, while significantly larger volumes are often a waste of solvent and indicate that the wash solvent is too "weak" to efficiently remove the contaminants in a timely manner.

D. Elution Step

Once the more weakly retained contaminants have been washed from the sample, a "strong" solvent designed to disrupt the analyte-sorbent interactions is used to selectively desorb or elute the analyte from the sorbent.

In many cases, the elution solvent must contain a mixture of several different solvents and/or chemicals (acid, base, etc.) in order to effectively break the primary interactions as well as any secondary retention mechanisms that may also be responsible for analyte binding. For example, in *reversed phase*, a combination of methanol, acetonitrile and a strong acid is often required in order to obtain quantitative recoveries for polar analytes. In *normal phase*, an acid may be added to the polar organic solvent. For *ion-exchangers*, a polar organic solvent may be used in combination with a strong acid or base and/or a salt.

In general, it is highly desirable to choose an elution solvent that is compatible with the final analytical method, or with any sample manipulation steps that may be required prior to the actual analysis (such as concentration, evaporation, and/or derivatization). For analytes that are destined to be analyzed by GC, volatile elution solvents are typically the most desirable. For HPLC analysis, the SPE elution solvent should be miscible with the initial mobile phase and typically must be diluted with a "weaker" solvent prior to injection. For details, see Section 10.

The elution volume is typically kept to a minimum in order to improve the detection limits and analytical sensitivity. In general, at least 0.3 mL of elution solvent are typically required for every 100 mg of sorbent bed mass. In general, larger volumes (0.5 mL) are required in order to maximize recovery and reproducibility. It should also be noted that two elution steps composed of smaller elution volumes are typically more effective than one, particularly when the first aliquot is allowed to remain or "soak" in the sorbent bed prior to loading the second.

Flow rates during the elution step should also be kept to a minimum in order to promote the elution process and enhance recovery. As noted above, a "soak" step may help provide the highest recoveries.

The solubility rule of "like dissolves like" holds special significance in terms of which solvent systems can be successfully used for sample dilution and analyte elution, and which retention mechanisms and sorbents can be utilized effectively. This old adage can also be embellished upon for SPE elution solvents and sorbents, since in SPE, "like elutes like", and "like retains like". For example, a polar solvent will elute a polar analyte from a polar *normal phase* column. A hydrophobic, organic solvent will bring about the elution of a hydrophobic analyte from a hydrophobic *reversed phase* column. And mobile phases with a high ionic strength or charge will desorb ionic analytes from *ion-exchangers*.

Tables 4 and **5** contain detailed information on the eluotropic (eluting) strength, polarity index, miscibility, and solubility of all the common chromatography solvents. This information can be used to help pick one of the more appropriate elution solvents for *normal phase* or *reversed phase* SPE applications. **Table 11** lists the relative strength of all common displacer counter ions for the elution of analytes from *ion-exchangers*. In addition, Section 10-C provides valuable hints on the optimal elution conditions for each type of *ion-exchanger*.

9. METHOD DEVELOPMENT AND OPTIMIZATION

The major goal of method development is to optimize the extraction efficiency (maximize the recovery of the target analyte(s) and minimize the amount of co-eluted contaminants) under conditions that provide reproducible results in a simple, economic manner.

Table 3 summarizes the 12 basic steps required to optimize any SPE method. Details are provided in the remainder of this section.

See glossary *(p.45)* for definition

Choosing the proper SPE product involves making four basic decisions, including the mode of analyte retention, the sorbent chemistry, the sorbent mass, and the physical configuration and design of the SPE product.

A. Sorbent Chemistry

The first step in developing an effective SPE method involves the determination of the most appropriate retention mechanism and the judicious choice of a sorbent. The most important considerations in the sorbent selection process are the physical nature of the sample/matrix, the chemical structure of the analyte, and the unique properties of each individual sorbent in terms of selectivity and specificity.

Table 3. Systematic Steps for SPE Method Development: Critical Factors and Points to Consider

1. **Classify the Analyte(s)**
   - A. Chemical class: inorganic, organic macromolecule, or organic small molecule
   - B. Solubility/Insolubility: aqueous, polar organic, and/or non-polar organic solvents
   - C. Polarity and hydrophobicity: polar, moderately polar, or non-polar
   - D. Charge: neutral, ionic-neutralizable, or ionic-permanent
   - E. Stability: reactive, acid or base labile, and/or prone to precipitation/aggregation
   - F. Concentration: affects recovery and capacity
2. **Classify the Sample/Matrix**
   - A. Solid: dissolution and pre-treatment requirements, and type (powder, solid, or semi-soft)
   - B. Gas: capture and solubilization requirements, and type (gaseous or aerosol)
   - C. Liquid: dilution requirements, and type (aqueous, polar organic, or non-polar organic)
   - D. Impurities: concentration, and type (particulates, salts, surfactants, fats, proteins, etc.)
3. **Determine Analytical Technique**
   - A. Detection limits: peak purity, signal to noise ratio and resolution of co-extracted solutes
   - B. SPE elution solvent: requirements or restrictions on analytical method and visa versa
   - C. Purity requirements for SPE step: resolution and specificity of analytical method
   - D. Analyte concentration: sample load (volume) required in SPE method
4. **Determine Extraction Mechanism**
   - A. Analyte class and properties (see 1, above)
   - B. Sample/matrix class and properties (see 2, above)
   - C. Post-extraction sample manipulation requirements: evaporation, derivatization, etc.
   - D. Analytical technique: GC or LC (reversed phase, normal phase, or ion-exchange)
5. **Choose Sorbent Chemistry**
   - A. Selectivity: purity of the target analyte(s) and overall specificity
   - B. Recovery: retention without breakthrough and/or irreversible binding
6. **Consider Sample/Matrix Volume**
   - A. Analyte concentration
   - B. Sorbent capacity
   - C. Elution volume
   - D. Analytical detection limit
7. **Choose Sorbent Mass**
   - A. Capacity and sample load
   - B. Recovery
   - C. Final elution volume and detection limits
8. **Choose Product Configuration/Column Size**
   - A. Type (tube, 96-well plate, on-line cartridge)
   - B. Tube size (sample reservoir)
   - C. Sample volume
9. **Optimize Sample/Matrix Preparation**
   - A. Dilution (see 2, above)
   - B. Pre-treatment to remove impurities (see 2D, above)
   - C. Hydrolysis (for conjugates)
10. **Optimize Wash Conditions**
11. **Optimize Elution**
12. **Analyze and Quantitate**

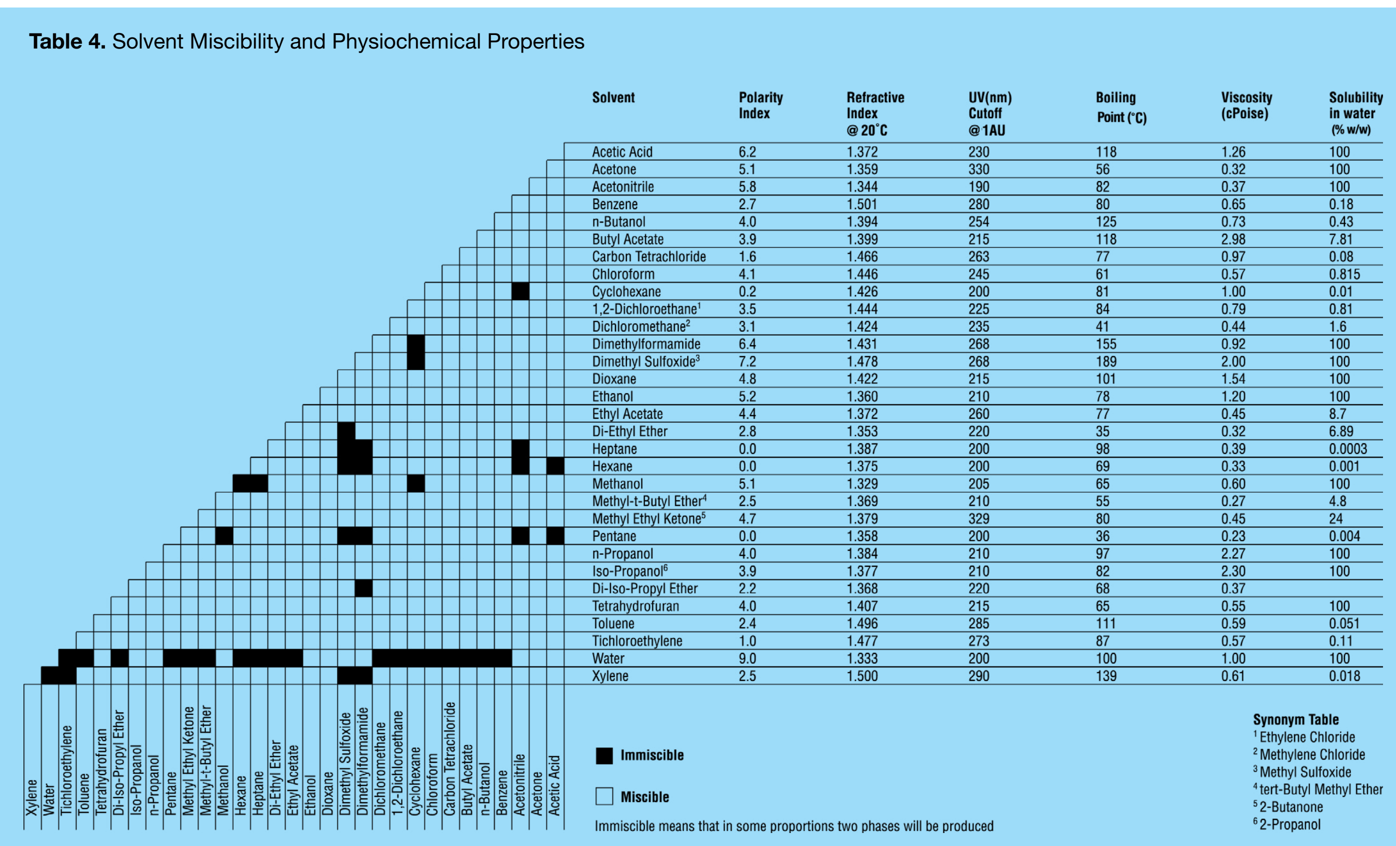


Table 4. Solvent Miscibility and Physiochemical Properties

Solvent	Polarity Index	Refractive Index @ 20˚C	UV(nm) Cutoff @1AU	Boiling Point (˚C)	Viscosity (cPoise)	Solubility in water (% w/w)
Acetic Acid	6.2	1.372	230	118	1.26	100
Acetone	5.1	1.359	330	56	0.32	100
Acetonitrile	5.8	1.344	190	82	0.37	100
Benzene	2.7	1.501	280	80	0.65	0.18
n-Butanol	4.0	1.394	254	125	0.73	0.43
Butyl Acetate	3.9	1.399	215	118	2.98	7.81
Carbon Tetrachloride	1.6	1.466	263	77	0.97	0.08
Chloroform	4.1	1.446	245	61	0.57	0.815
Cyclohexane	0.2	1.426	200	81	1.00	0.01
1,2-Dichloroethane[1]	3.5	1.444	225	84	0.79	0.81
Dichloromethane[2]	3.1	1.424	235	41	0.44	1.6
Dimethylformamide	6.4	1.431	268	155	0.92	100
Dimethyl Sulfoxide[3]	7.2	1.478	268	189	2.00	100
Dioxane	4.8	1.422	215	101	1.54	100
Ethanol	5.2	1.360	210	78	1.20	100
Ethyl Acetate	4.4	1.372	260	77	0.45	8.7
Di-Ethyl Ether	2.8	1.353	220	35	0.32	6.89
Heptane	0.0	1.387	200	98	0.39	0.0003
Hexane	0.0	1.375	200	69	0.33	0.001
Methanol	5.1	1.329	205	65	0.60	100
Methyl-t-Butyl Ether[4]	2.5	1.369	210	55	0.27	4.8
Methyl Ethyl Ketone[5]	4.7	1.379	329	80	0.45	24
Pentane	0.0	1.358	200	36	0.23	0.004
n-Propanol	4.0	1.384	210	97	2.27	100
Iso-Propanol[6]	3.9	1.377	210	82	2.30	100
Di-Iso-Propyl Ether	2.2	1.368	220	68	0.37	
Tetrahydrofuran	4.0	1.407	215	65	0.55	100
Toluene	2.4	1.496	285	111	0.59	0.051
Tichloroethylene	1.0	1.477	273	87	0.57	0.11
Water	9.0	1.333	200	100	1.00	100
Xylene	2.5	1.500	290	139	0.61	0.018

■ Immiscible

□ Miscible

Immiscible means that in some proportions two phases will be produced

Synonym Table
[1] Ethylene Chloride
[2] Methylene Chloride
[3] Methyl Sulfoxide
[4] tert-Butyl Methyl Ether
[5] 2-Butanone
[6] 2-Propanol

Table 5. Solvent and Sorbent Polarity Chart

Relative Polarity	Compound Formula	Chemical Group	Representative Solvents	Eluting Strength[1] (ε_0)	Polarity Index[2] (P')	Water Miscible	Water Solubility (% W/W)	Relative Polarity	Sorbent Polarity
Nonpolar	R-H	Alkanes	Petroleum ether	0.0		No		**Nonpolar**	SDB Polymers
			Ligroin	0.0		No			
			Hexane	0.0	0.06	No	0.001		C18 (EC)
			Heptane	0.0	0.2	No	0.0003		
			Isooctane	0.01		No			C18 (Non-EC)
			Cyclohexane	0.03	0.0	No	0.01		
	Ar-H	Aromatics	Toluene	0.22	2.4	No	0.051		C8/Octyl
			Benzene	0.27	3.0	No	0.18		
	R-X	Alkyl halides	Carbon tetrachloride	0.11	1.6	No	0.08		PH/Phenyl
			Chloroform	0.31	4.4	No	0.815		
			Methylene chloride	0.32	3.4	No	1.6		CN/Cyano
	R-O-R	Ethers	Tetrahydrofuran	0.35	4.2	Yes	100		
			Diethyl ether	0.38	2.9	Slight	6.89		Si/Silica
			Dioxane	0.49		Yes	100		
	R-CO-R	Ketones	Methyl ethyl ketone	0.39	4.5	Slight	24		NH_2/Amino
			Acetone	0.43	5.4	Yes	100		
	R-CO-OR	Esters	Ethyl acetate	0.45	4.3	Slight	8.7		Fl/Florisil
	R-CN	Nitriles	Acetonitrile	0.50	6.2	Yes	100		
	$R-NR_2$	Amines	Pyridine	0.55	5.3	No			Al/Alumina
			Triethylamine	0.73		Yes	100		
	R-OH	Alcohols	Isopropanol	0.63	4.3	Yes	100		SCX/Aromatic
			Ethanol	0.70		Yes	100		Sulfonic acid
			Methanol	0.73	6.6	Yes	100		
	$R-CO-NR_2$	Amides	Dimethylformamide	0.73		Yes	100		SAX/Quaternary
	R-COOH	Carboxylic acids	Acetic acid	>0.73	6.2	Yes	100		amine
Polar	H_2O	Water	Water	>0.73	10.2	Yes	100	**Polar**	

[1]Eluotropic strength (ε_0) = *Strength as an eluting solvent on silica*
[2]Polarity index (P') = *Measure of ability of solvent to interact as a proton-donor, proton acceptor, or dipole.*

Table 5 contains detailed information on the relative polarity and hydrophobicity of most common SPE sorbents. This information can be used to select several likely sorbent candidates for the initial screening experiments in situations in which a *reversed phase* or a *normal phase* mechanism is appropriate. For *ion-exchange* applications, Section 10-C provides details on the correct *ion-exchanger* for each particular target analyte(s).

Normal Phase Specificity: The relative polarity of the most common SPE sorbents is listed in **Table 5**. In general, CN is the least polar of all the sorbents used for *normal phase* chromatography. As a result, it is one of the most useful, since it typically provides good resolution and recoveries, even for extremely polar analytes, and it can also be used for *reversed phase* extractions. In contrast, SCX and SAX are the most polar sorbents and probably the least popular for *normal phase* SPE. Their extreme polarity in conjunction with their permanent charge makes recovery problematic under *normal phase* conditions, particularly for charged, polar analytes.

Prior to the development of silica-based bonded phases, bare silica, alumina, Keiselgur and Florisil® were the most popular sorbents for *normal phase* chromatography. Each one of these sorbents is moderately polar (**Table 5**). Florisil is still quite popular for the extraction of pesticides, and basic sorbents such as alumina are particularly useful for the retention of acidic compounds.

In recent years, silica and NH_2 have become the most commonly used *normal phase* sorbents. Both have moderate to low polarities and provide good resolution and recoveries. Silica is particularly useful for basic compounds which are strongly retained on acidic (acid-treated) silicas.

Critical factors for *normal phase* SPE sorbents are their relative polarity, surface area, surface coverage, surface pH and water content (LOD or loss on drying); each of these must be fairly consistent from tube-to-tube and lot-to-lot in order to obtain reproducible results.

Reversed Phase Specificity: The relative polarity of the most common reversed phase SPE sorbents is listed in **Table 5**. In general, SDB-based polymers are the most hydrophobic of all SPE stationary phases, followed closely by endcapped C18. Among the silica-based sorbents for reversed phase, hydrophobicity decreases in conjunction with the alkyl chain length. Differences in selectivity are typically observed on aliphatic and aromatic ligands of similar hydrophobicity. For example, aromatic solutes have a higher affinity for aromatic sorbents such as Phenyl (relative to C8), and SDB (relative to C18). Similar differences in selectivity can be observed as a result of endcapping. In most cases, non-endcapped phases have a higher affinity for more polar organic analytes/solutes. Even among the different C18 products that are commercially available for SPE, enormous differences in selectivity and hydrophobicity exist.

Critical factors for the chromatographic performance of reversed phase SPE sorbents include surface area, carbon load, surface coverage, endcapping efficiency, LOD and pore size. Each of these properties must be fairly consistent from lot-to-lot in order to obtain reproducible results.

Ion-Exchange Specificity: Positively charged analytes containing amines are retained on negatively charged *"cation-exchangers"* such as sulfonic acids (SCX, PRS) or carboxylic acids (WCX, CBA). In contrast, negatively charged analytes containing sulfonic acid or carboxylic acid groups are retained on positively charged *"anion-exchangers"*. These sorbents include, 1° amines (WAX, NH_2), 2° amines (PSA or PEI), 3° amines (DEAE), or 4° amines (SAX, Q, or Quat). Details on the relative specificity of each type of *ion-exchange* sorbent are provided in Section 10-C.

Ion-exchange retention is based on specific, high-energy coulombic interactions between the sorbent and the analyte. Only species of the proper charge are retained by the column, so most matrix contaminants are simply rinsed away to waste during the loading and the wash steps. For this reason, *cation-exchange* SPE is commonly used for the extraction of basic compounds (drugs and other amines) from complex biological samples. The target analytes are typically eluted with high ionic strength salts and buffers or with strong acids or bases.

Critical factors for *ion-exchangers* include their titration behavior and pK_a, total *ion-exchange* capacity, surface chemistry, and counter ion content.

See glossary *(p.45)* for definition

1. **Sample/Matrix Effects**

As noted in Sections 7-D and 8-B, the solvent composition of the sample/matrix determines the extraction mechanism and is an important consideration in the sorbent selection process. As outlined in **Tables 7**, **8** and **9**, each of the three major extraction mechanisms requires a specific set of solvent conditions for analyte retention (and elution). Polar, aqueous environments facilitate retention on reversed phase sorbents. Non-polar organic solvents enhance binding on normal phase sorbents. Low ionic strength, pH-adjusted, aqueous conditions promote retention on *ion-exchangers.*

Phenomenex Technical Note TN-0013 guides the user through a series of simple decisions based on the physiochemical nature of the analyte and the composition of the sample/matrix, ultimately leading to the identification of likely SPE mechanisms and sorbents. Although rather imposing at first glance, careful study reveals that it represents a simple, straightforward tool for choosing the proper SPE column. This user-friendly approach to the sorbent selection process can be applied to any sample/matrix, including solids, liquids, or gases, and analytes with a wide range of chemical structures and properties.

2. **Analyte Structure and Properties**

The chemical structure of the target analyte(s) is another critical factor used in the selection of an SPE sorbent. Chemical structure typically determines polarity, solubility and charge, and each of these are important factors that should be used as a guide for determining the most appropriate SPE mechanism for the separation problem at hand.

Although detailed information on the solubility, polarity, and charge characteristics of any particular analyte can often be obtained from reference literature such as the Merck Index, the CRC Handbook of Chemistry, and the like (see References 5-9 in Appendix III), it can easily be determined experimentally, or even approximated by simple examination of the chemical structure.

For example, visual examination of the chemical structure of a given analyte will generally allow one to make a rough estimate of its relative hydrophobicity or polarity. This information, in turn, can be used to choose the appropriate retention mechanism and then predict retention behavior on a particular sorbent. For example, hydrophobic (hydrocarbonaceous) analytes have a high proportion of unsubstituted alkyl chains and/or aromatic rings and a low level of polar functional groups (hydroxyl, amino, carbonyl, etc.) and tend to bind strongly to *reversed phase* sorbents, but interact weakly with *normal phase* sorbents. In contrast, polar analytes have a higher proportion of polar functional groups on their hydrocarbon skeletons and a high affinity for *normal phase* sorbents, but are weakly retained on *reversed phase* columns.

In a similar manner, solubility information about the target analyte(s) can also be useful in terms of determining the most appropriate extraction mechanism and sorbent, and the most appropriate loading and elution conditions. Although analyte solubility can also be determined quite easily through simple experimental means, it can also be approximated from chemical structure. In general, the standard solubility rule of "Like Dissolves Like" holds true. **Tables 4** and **5** contain detailed information on the polarity, miscibility, solubility, and eluting strength of most common solvents for chromatography.

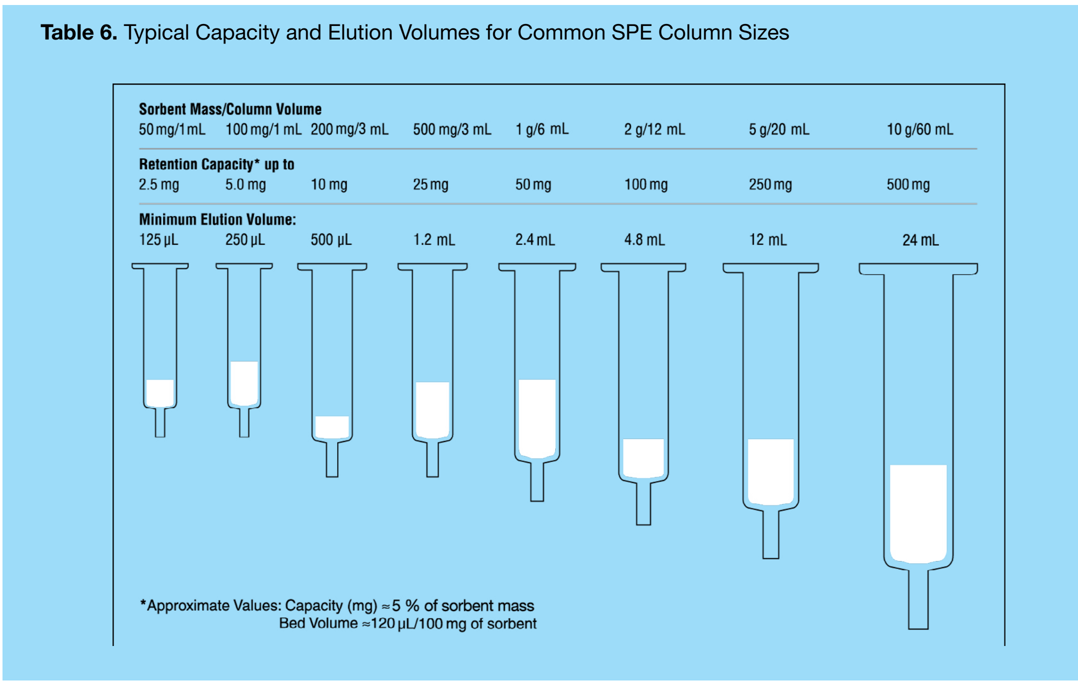
Numerous examples of how solubility, charge, and polarity/hydrophobicity information can be used to select the appropriate retention/extraction mechanism, sorbent, and solvent systems are provided in Technical Note TN-0013.

3. **Sorbent Specificity**

Even sorbents that rely on the same retention mechanism (*reversed phase*, *normal phase*, or *ion-exchange*) often provide dramatically different extraction results. Subtle differences in the chemical structure and polarity amongst the sorbents within each of these individual groups can lead to major changes in selectivity, retention, and recovery for a given analyte. For example, C18 and Phenyl are both *reversed phase* sorbents, but C18 sorbents exhibit stronger affinities for analytes containing alkyl chains, while Phenyl sorbents interact more strongly with aromatic analytes. Different selectivities can also be expected with any two *normal phase* sorbents (for example, CN vs. NH_2 vs. Si), and to a lesser extent, amongst *cation-exchangers* or *anion-exchangers* with differing functional groups.

Even relatively minor differences in the chemical structure or other physiochemical properties of two otherwise similar sorbents can often translate into dramatic differences in extraction efficiency. For this reason, it is important to screen several similar sorbents when developing a new method. In fact, early optimization work during the sorbent screening and method development stages can often lead to major long term benefits with respect to method reproducibility, recovery, and cleanliness.

Table 6. Typical Capacity and Elution Volumes for Common SPE Column Sizes



Sorbent Mass/Column Volume	50 mg/1 mL	100 mg/1 mL	200 mg/3 mL	500 mg/3 mL	1 g/6 mL	2 g/12 mL	5 g/20 mL	10 g/60 mL
Retention Capacity* up to	2.5 mg	5.0 mg	10 mg	25 mg	50 mg	100 mg	250 mg	500 mg
Minimum Elution Volume:	125 µL	250 µL	500 µL	1.2 mL	2.4 mL	4.8 mL	12 mL	24 mL

*Approximate Values: Capacity (mg) ≈ 5 % of sorbent mass
Bed Volume ≈ 120 µL/100 mg of sorbent

4. **SPE Method Development with HPLC**

   In most cases, effective SPE method development cannot be conducted without a quantitative analytical method that is capable of discriminating between the target analyte(s) and other endogenous contaminants in the sample/matrix. In many cases, this analytical method can provide valuable insights into the chromatographic retention behavior of the target analyte(s) and potential contaminants that can be useful when attempting to determine the initial extraction conditions. HPLC methods that are conducted on a column with the same surface chemistry as the SPE column (e.g., both are C18) typically provide valuable information about the conditions required during the extraction, and can be used to optimize the extraction method. In general, the mobile phase conditions required for isocratic elution from the HPLC column approximates the optimal wash solvent conditions for the SPE column.

B. Sorbent Mass

After the extraction mechanism and a specific sorbent chemistry have been identified, the next logical step is to choose the sorbent mass. The proper sorbent mass for a given extraction is one that provides sufficient capacity to retain both the analyte and any contaminants that may also be retained during the loading step. Choosing the proper sorbent mass is critical because insufficient sorbent leads to column overload and low or irreproducible recoveries, while an excess of sorbent increases solvent requirements and may also reduce recovery and purity.

A good rule of thumb is that SPE cartridges retain a mass of solute (analyte plus retained contaminants) that is equivalent to 5 % of the sorbent mass. Therefore, a 100 mg cartridge can retain approximately 5 mg of total solute mass (**Table 6**). Since the majority of SPE is performed on relativity "dirty" samples that contain a complex milieu of contaminants, it is common practice to err on the side of safety and choose a slightly larger sorbent mass.

In a situation in which the analyte is at extremely low concentrations and/or the detection signal is relatively weak, it may be advantageous to push the limits of the column capacity and load relatively large volumes of sample. For example, as much as 2 to 3 mL of serum, plasma or urine can typically be loaded on 100 mg of sorbent without adversely affecting recovery. In order to further improve detection limits, large sorbent masses (and sample volumes) should be employed in combination with the lowest possible volume of elution solvent.

A practical technique for determining sorbent capacity is to conduct a single extraction using this general rule of thumb, and then determine recovery. For more detailed information on capacity and sorbent mass requirements, it is advisable to perform several "breakthrough" experiments in which either the sorbent mass is varied (e.g., 50, 100, 200, 500 mg), or, the sample/matrix volume is increased (e.g., 0.5, 1, 2, 4 mL). Analysis of the eluant for analyte recovery (and purity) will provide information on the optimal loading capacity and sorbent mass.

See glossary *(p.45)* for definition

C. Product Configuration and Size

SPE manufacturers typically pack the same sorbent mass into a wide range of tube sizes (**Table 6**) and/or product configurations including tubes, cartridges, discs and 96-well plates (**Fig. 6**). Selection of a specific product configuration often has more to do with user preference, hardware and product availability, or the manner of sample processing, than any actual differences in extraction performance. The major types of commercially available product configurations are described below.

1. **Syringe Barrel Columns**

   The most popular SPE configuration is the luer-tipped syringe barrel packed with sorbent (**Figs. 1** and **6**). The sorbent bed is held in place by porous polyethylene frits and the syringe barrel is typically manufactured from high purity, medical-grade polypropylene. The frits are typically manufactured from high-purity polyethylene with low levels of leachables, and are often laser cut to ensure purity and consistent fit. SPE tubes are available in 6 different sizes (1, 3, 6, 12, 20 and 60 mL) with sorbent masses ranging from 10 mg to 10 grams (**Table 6**). The smaller tube sizes are amenable to batch processing and automation and are available in straight barrel or large reservoir configurations. Adapters are also available that allow columns to be stacked in series, or to provide additional reservoir capacity for large sample volumes.

2. **Cartridges**

   SPE cartridges represent another popular style of packed bed SPE product (**Fig. 6**). These have no reservoir capacity and are fitted with both male and female luer lock fittings.

3. **96-Well Plates**

   SPE sorbents are also available in the 96-well (microtiter) plate format for high-throughput manual or automated SPE. 96-well plates for SPE are equivalent to 96 individual SPE cartridges combined in a single, compact, convenient product (**Fig. 6**). When combined with a fully automated robotic SPE processor, the 96-well plate provides the highest sample throughput currently available. Typical sizes include 10, 25, 50 and 100 mg of sorbent per well.

4. **Disc Membranes**

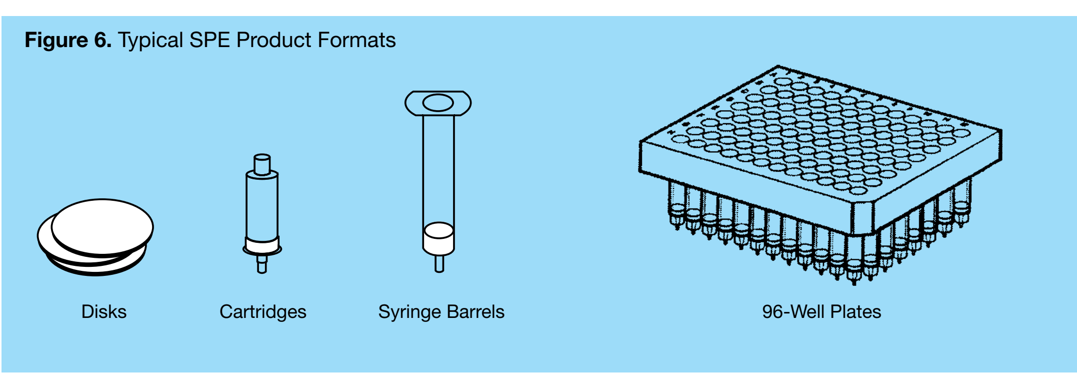
   SPE disc membranes consist of sorbent particles enmeshed in a porous solid support. The most common solid supports for these SPE discs include PTFE®/Teflon®, glass-fiber and paper. Upon manufacture, the membrane is then cut into discs of various sizes; 25-, 47-, and 90-mm diameter discs can be inserted into a disc manifold, while smaller 4-, 8-, and 12-mm diameter membranes are available in traditional SPE columns and 96-well plates. SPE discs in 96-well plates are popular in the pharmaceutical industry due to their low bed volume, which allows for the efficient extraction of smaller volume biological samples.

   Historically, SPE membranes have contained sorbents of a smaller particle size (10 µm). However, the recent trend has been towards larger (50 µm) particle sizes due to the poor flow characteristics of some of the original products.

5. **SPE Manifolds**

   SPE columns have traditionally been processed with the use of vacuum or positive pressure manifolds, syringes, centrifugation, or even simple gravity flow. Commercially available SPE vacuum manifolds come in a variety of sizes and can be used to simultaneously process up to 10, 12, 20 or 24 SPE tubes (**Fig. 7**). Luer-tipped SPE tubes or cartridges form an air-tight seal with each port of the manifold. Each port contains a stopcock valve, which can be individually adjusted in order to regulate the flow through each column or to totally divert the flow to individual columns. Since the vacuum pulls liquids through the column from the luer-tipped outlet, the column inlet and reservoir are open at all times. This allows the sample or solvents to be added directly to the column reservoir, drawn through the sorbent by vacuum and routed either to waste or to individual collection tubes inside the manifold.

Figure 6. Typical SPE Product Formats



See glossary *(p.45)* for definition

6. **Batch Processing and Automation**

Simultaneous batch processing of multiple samples with traditional SPE manifolds, 96-well plate vacuum manifolds, or via automation dramatically reduces labor costs and provides enormous increases in sample throughput and productivity. Traditional SPE columns facilitate the manual batch processing of 20 or more samples in an average of 15 minutes, with little more investment than a vacuum manifold and a vacuum pump.

For a dramatic increase in productivity, SPE methods can be fully automated using one of the commercially available robotic extraction devices in combination with either standard SPE tubes or 96-well plates. Experience has shown that manually-developed methods can be readily transferred to robotic systems with little or no change in extraction efficiency.

Robotic SPE processors are capable of handling hundreds of samples per day with minimal user intervention. As a result, automation with traditional tubes or 96-well plates is becoming increasingly popular in the pharmaceutical industry for high volume combinatorial chemistry and drug screening applications. In fact, it is becoming essential in order to keep pace with these enormous sample demands, and the rapid analytical run times of only a few minutes that are inherent to the use of extremely short HPLC columns for LC-MS.

10. STANDARD SPE PROTOCOLS

As noted in Section 8, a typical SPE procedure consists of 4 basic steps: conditioning, sample loading, wash and elution.

Conditioning: The sorbent bed is rinsed with polar organic solvent in order to remove trapped air and prepare the chromatographic ligands for interaction with the sample. This "activation" or "solvation" solvent is then displaced by an "equilibration" solvent with a composition similar to the diluted sample/matrix.

Sample Loading: The sample/matrix is diluted in a "weak" solvent designed to enhance the interactions between the analyte and the sorbent, and is then passed through the column.

Wash: Bound contaminants are removed with a relatively "weak" solvent that is not strong enough to disrupt the sorbent-analyte interactions. To insure that all traces of wash solvent and contaminants are removed, the cartridge is often dried under full vacuum for several minutes.

Elution: A "strong" solvent designed to disrupt the interactions between the sorbent and the analyte is passed through the cartridge and the eluant is collected for analysis.

Details on each of these steps are provided in Section 8.

Figure 7.
Schematic Illustration of an SPE Vacuum Manifold (A) and Manual Sample Processing with the Use of a Syringe (B)

A) Vacuum Manifold

B) Syringe

Outlined below are standard extraction procedures for each of the major sorbent categories. Consider these simple guidelines as starting points for developing methods. Please refer back to the previous text for more detailed information on the three basic retention mechanisms (Section 7), and method development and optimization, in general (Section 9).

A. Reversed Phase Extraction Procedure

In *reversed phase* extractions, hydrophobic analytes containing aromatic rings and/or alkyl chains are extracted from aqueous solutions (water, biological fluids, etc.) with non-polar sorbents such as C18, C8, Phenyl, CN, and SDB (**Table 7**). Elution occurs upon the addition of an organic solvent that effectively disrupts the interactions between the sorbent and the target analyte(s).

1. Sample/Matrix Preparation

Although each individual sample/matrix and target analyte(s) may require slightly different pre-treatment prior to the actual extraction, the final sample must be in a mostly aqueous environment. Some general guidelines are provided below.

Notes:

1. Sample/Matrix Composition: Optimal retention occurs with an aqueous sample/matrix and with analytes that are uncharged/neutralized and relatively non-polar/hydrophobic. In general, aqueous samples and biological fluids can be loaded directly onto the column. However, samples containing organic solvents typically need to be diluted with water or buffer. The actual concentration of organic solvent that can be tolerated without affecting the recovery and/or capacity depends upon the hydrophobicity of the target analyte(s), the eluotropic strength of the solvent, and the sample volume. In general, the organic content of the sample/matrix must be less than 5 %, or lower than that required to elute the analyte(s) from a reversed phase column of similar surface chemistry in 10 column volumes or less.
2. Sample pH: The pH of the sample/matrix (and the mobile phases) may require adjustment with buffer so the net charge on the target analyte(s) is zero.
3. Viscosity: Viscous samples should be diluted further with water or buffer in order to improve the flow rate and prevent the column from clogging.
4. Particulates: In most cases, particulates and other insoluble material and/or debris do not have to be removed prior to the actual extraction since the frits act as an effective filter.
5. Solids: Details on the sample preparation requirements for soils, powders, plant and animal tissues, ointments, and other solids are provided in Technical Note TN-0013.

Table 7. Reversed Phase SPE Protocol

Sorbent Polarity	Low	SDB, C18, C8, PH, CN
Analyte Properties	Low to moderate polarity (or non-polar) Hydrophobic Hydrocarbonaceous Neutralized/uncharged	Pharmaceuticals Pesticides, herbicides
Sample/Matrix	Aqueous, diluted with buffer	Biological fluids River water
Conditioning Step	1. Solvation – polar organic solvents 2. Equilibration – aqueous, buffers	1. MeOH 2. H_2O or buffer
Wash Step	Aqueous buffers with 5 to 50 % polar organic solvent	MeOH:H_2O (1:9)
Elution Step	Polar or non-polar organic solvent(s) with or without water, buffer and/or strong acid or base	MeOH:ACN:HCl (4:4:2)

2. Sorbent Conditioning

Solvation: Rinse the cartridge with one column reservoir or several sorbent bed volumes of a polar organic solvent such as methanol, isopropanol, acetonitrile, or ethanol. See Section 8-A for details.

Equilibration: Rinse the cartridge with one column reservoir volume or several sorbent bed volumes of an equilibration solvent such as water or buffer (the same as that used to dilute the sample).

Notes:

1. Equilibration Solvents: Low ionic strength (<30 mM) buffers are preferred. In theory, the pH, ionic strength, and ionic composition should be identical to that of the diluted sample/matrix.
2. Drying: In general, there is absolutely no need to dry the column in between the two conditioning solvents or prior to the sample loading step. Do not allow the sorbent to dry under full vacuum for more than 1 minute before applying the sample since this can lead to the total evaporation and loss of the solvating solvent. If the cartridges are inadvertantley dried for too long, simply repeat the conditioning step(s) described above.
3. Flow Rate: Flow rate is not particularly critical during conditioning; 3 to 5 mL per minute is typical.

3. Sample Loading

Apply the sample to the cartridge with the vacuum or pressure turned off, and then, either aspirate the sample through the cartridge at a flow rate of approximately 1 mL per minute, or allow it to flow by gravity.

Notes:

1. Flow Rate: A slow rate of sample application is generally preferable since the kinetics of sorbent-analyte interactions is highly variable and strongly influenced by the composition of the sample/matrix and the nature of the target analyte(s).
2. Throughput: In order to maximize sample throughput, perform a series of experiments investigating the effects of flow rate on analyte recovery and "split peak" behavior for the target analyte(s).

4. Wash

Rinse the cartridge with 1 to 2 column reservoir volumes (or 5 to 10 sorbent bed volumes) of an aqueous wash solution containing a low concentration of organic solvent.

Notes:

1. Common Solvents: Common wash solvents are water (or the sample dilution buffer) containing small amounts of water-miscible organic solvents (e.g., 5 to 50 % methanol). The optimum organic content of the wash solvent must be determined empirically for each particular target analyte.
2. Flow Rate: The flow rate should be approximately 1 to 2 mL per minute.

5. Dry

After the wash solvent has been passed through the column, continue to apply vacuum or positive pressure. Dry the cartridge under full vacuum (or pressure) for 2 to 5 minutes or until dry. Turn off the vacuum, and wipe the tips of the manifold needles in order to remove any residual sample/ matrix or wash solvent.

Notes:

1. Reproducibility: Drying removes all traces of residual sample/matrix and wash solvent from the sorbent. This produces a more concentrated final extract with a constant volume and composition, and ensures better reproducibility and higher recoveries.
2. Immiscibility: Drying is especially critical when using elution solvents that are immiscible with water (e.g. hexane, ethyl acetate, and methylene chloride). Residual water on the sorbent bed will prevent proper elution, resulting in low recoveries and irreproducibility.
3. Overdrying: Avoid overdrying the column, since in some cases, analyte recovery may decrease with excessive vacuum or drying times above 5 minutes. Dry cartridges look distinctly different from moist ones. Make a note of the cartridge appearance before and after conditioning.

6. Elution

Insert collection tubes into the manifold rack. Pass a minimum of 2 to 4 sorbent bed volumes of elution solvent slowly through the cartridge at 1 to 2 mL per minute and collect the analyte.

Notes:

1. Elution Solvents: Typical reversed phase elution solvents include moderately polar organic solvents such as methanol, isopropanol, acetonitrile, THF, acetone, and ethyl acetate or non-polar organic solvents such as hexane and methylene chloride. These should be used either alone or in combination with each other, often with a small percentage of water or buffer containing a strong acid or a strong base. Hexane mixtures are often very effective at eluting relatively non-polar target analyte(s) from non-endcapped reversed phase sorbents while driving more polar contaminants back onto the chromatographic surface. Details on the relative polarity index, eluotropic strength and miscibility of common reversed phase elution solvents are provided in ***Tables 4*** and ***5***.
2. Silanols: In many situations, amines and other polar solutes may also be retained by silanol interactions. These may be disrupted by adding a low concentration acid or base, such as 0.1 to 1 % concentrated HCl or ammonia in methanol. In many cases, a weak organic acid may be preferred (e.g., 1 to 10 % HOAc), since it may enhance analyte solubility and often provides good recoveries without eluting an excess number of contaminants.
3. Generic Recipes: The most effective elution solvent for polar analytes is often a combination of methanol:acetonitrile:HCl. In general, the total organic content should be high (60-90 %) and the HCl concentration in the aqueous portion should be between 0.1 to 1.0 N. Additional acid may be required for highly polar analytes such as amines that can interact strongly with the free silanols, particularly on non-endcapped sorbents. The relative proportion of acetonitrile to methanol should be adjusted according to the chemical structures of the target analyte(s) and the sorbent (as described below).
4. Solvent vs Sorbent: For aromatic analytes on aromatic sorbents (e.g. Phenyl or SDB), acetonitrile is a stronger elution solvent and is preferred over methanol, particularly for analytes that provide low recoveries.
5. Sorbent Polarity: The relative polarity of the most common SPE sorbents is listed in ***Table 5*** and is discussed in Section 9-A. Critical factors for the reproducible chromatographic performance of reversed phase SPE sorbents are their relative polarity, surface area, surface coverage, endcapping efficiency, LOD, and pore size.
6. Compatibility: As noted in Section 8-D, it is often advantageous or even imperative that the composition of the elution solvent be adjusted in order to be compatible with subsequent sample manipulation or analytical steps. For example, volatile "non-polar" organic elution solvents such as hexane and methylene chloride should be used prior to concentration/evaporation, derivatization and/or direct GC analysis. For HPLC analysis on a reversed phase column, the SPE elution solvent should be water-miscible and as similar as possible to the initial HPLC mobile phase so that the SPE eluant can simply be diluted in buffer. This will facilitate binding on the HPLC column and help to minimize peak fronting and/or split peak behavior.

See glossary *(p.45)* for definition

Ideally, non-polar organic SPE elution solvents such as methylene chloride or hexane can be loaded directly onto a normal phase HPLC column with little or no sample manipulation.

7. Soak Time: Allow the elution solvent to remain and "soak" in the sorbent bed (without vacuum) for approximately one minute in order to optimize recovery.
8. Multiple Extractions: In order to improve recovery, conduct multiple extractions. Use two or more aliquots containing a smaller volume of the elution solvent rather than a single, larger volume.

B. Normal Phase Extraction Procedure

In normal phase extractions, target analytes and other compounds with a large proportion of polar functional groups (amines, amides, hydroxyls, carbonyls, heteroatoms) are extracted from non-polar organic solvents (hexane, chloroform) using polar sorbents such as Si, NH_2, CN, Fl and Al (**Table 8**). Retention is facilitated by loading under non-polar solvent conditions. Elution occurs in the presence of polar solvents.

1. Sample/Matrix Preparation

Dissolve or dilute the sample/matrix in a non-polar organic solvent, unless the concentration of polar organic solvents is already less than 5 %.

Notes:

1. Sample/Matrix Composition:

A. Non-Polar Solvents: In general, the retention of polar analytes occurs only when the sample/matrix is composed almost entirely of a non-polar, water-immisible solvent(s). As a result, the orginal sample/matrix should ideally be composed of solvents such as "alkanes" (hexane(s) or petroleum ether), aromatics (toluene or benzene) or alkyl halides (carbon tetrachloride, chloroform or methylene chloride). These solvents have a low polarity index and a low eluotropic strength; that is, a low strength as an eluting solvent on bare silica. In general, moderately polar analytes will be adsorbed on bare silica SPE columns in the presence of solvents with an eluotropic strength (ε_0) of 0.4 or less. These include the solvents listed in the top half of ***Table 5***.

B. Polar Organic Solvents: Sample/matrices containing moderately polar organic solvents (such as alcohols, that have ε_0 values well above 0.4) must be diluted extensively in non-polar organic solvents prior to sample loading. In fact, alcohols are extremely potent elution solvents for normal phase SPE. For particularly polar analytes that are strongly retained, the dilution factor can be reduced slightly in order to improve capacity and purity by selectively binding the analyte(s) while less polar contaminants flow through the column.

C. Aqueous Samples: Aqueous sample/matrices need to be diluted first in a water-miscible, polar organic solvent (such as THF, acetone, or isopropanol) and then, diluted extensively in a non-polar organic solvent. Tetrahydrofuran (THF) is probably the best intermediate solvent for this purpose, since it is fully miscible with water as well as with non-polar organic solvents, and, it has a relatively low eluotropic strength (ε_0 = 0.35). Since aqueous sample/matrices require such extensive preparation and dilution prior to normal phase SPE, they are more often processed on reversed phase (or ion-exchange) columns.

2. Sample pH: The pH of the sample/matrix (and the mobile phases) may need to be adjusted with acid or base, or buffered so the net charge on the analyte is zero.
3. Viscosity: Viscous samples should be diluted further with solvent in order to improve the flow rate and prevent the column from clogging.
4. Particulates: In most cases, particulates and other insoluble material and/or debris do not have to be removed prior to the actual extraction since the frits act as an effective filter.
5. Solids: Details on soils, powders, tissues, ointments, and other solids are provided in Technical Note TN-0013.

2. Sorbent Conditioning

Solvation: For normal phase SPE, this step is optional and usually omitted (see note 1, below).

Equilibration: Add one column volume or several sorbent bed volumes of the same non-polar organic solvent that was used to dilute the sample, and aspirate it slowly through the cartridge.

Notes:

1. Solvation: The choice of the conditioning solvent is critical in normal phase SPE. In most circumstances, it is not necessary to solvate the sorbent bed prior to adding an equilibration solvent. However, when developing new methods, an initial solvation step (which includes isopropanol and methylene chloride (1:1), either alone or in combination with hexane or another non-polar organic solvent) may be beneficial, particularly on bonded silicas such as CN and NH_2. This will help remove trapped air and help fully solvate the entire chromatographic surface and the upper frit, allowing the non-polar organic solvent in the equilibration solvent and the actual sample/matrix to gain full access to the entire sorbent bed.

Table 8. Normal Phase SPE Protocol

Sorbent Polarity	High	Silica, Florisil, Alumina, Amino, Cyano
Analyte Properties	Moderate to high polarity compounds (neutralized/uncharged)	Pesticides
Sample/Matrix	Non-polar organic solvents or moderately polar organic solvents	Hexane, chloroform, petroleum ether, toluene or methylene chloride
Conditioning Step	1. Solvation – polar organic solvents (optional) 2. Equilibration – sample/matrix solvent	1. MeOH (optional) 2. Hexane or chloroform
Wash Step	Non-polar organic solvents with a low concentration (1 to 5 %) of moderate to low polarity organic solvents	Hexane with 1 % THF, ethyl acetate, acetone, acetonitrile or IPA
Elution Step	Non-polar organic solvents containing higher concentrations (5 to 50 %) of moderate to high polarity organic solvents	Hexane with 10 % THF, ethyl acetate, acetone, acetonitrile or IPA

2. Equilibration: The equilibration solvent should be similar in composition to the diluted sample/matrix, but should contain as little polar solvent as possible, particularly water.
3. Drying: In general, there is absolutely no need to dry the column in between the two conditioning solvents or prior to the sample loading step. Do not allow the sorbent to dry under full vacuum for more than 1 minute before applying the sample since this can lead to the evaporation and total loss of the solvating solvent. If the cartridges are inadvertently dried for too long, simply repeat the conditioning step(s) described above.
4. Flow Rate: Flow rate is not particularly critical during conditioning; 3 to 5 mL per minute is typical.

3. Sample Loading

Apply the sample to the cartridge with the vacuum or pressure shut off, and then either aspirate the sample through the cartridge at a flow rate of approximately 1 mL per minute, or alternatively, allow it to continue to flow by gravity.

Notes:

1. Flow Rate: A slow rate of sample application is generally preferable since the kinetics of sorbent-analyte interaction is highly variable and strongly influenced by the composition of the sample/matrix.
2. Throughput: In order to maximize throughput, perform a series of experiments investigating the effects of flow rate on analyte recovery.

4. Wash

Rinse the cartridge with 1 to 2 column reservoir volumes (or 5 to 10 sorbent bed volumes) of wash solvent. This wash should contain the same non-polar organic solvent that was used to dilute and load the sample, plus a small percentage (1 to 2 %) of a moderately polar organic solvent.

Notes:

1. Common solvents: Common wash solvents are non-polar organic solvents such as hexane containing either a small amount of polar organic solvent (e.g. 1 to 2 % isopropanol or methanol), or preferably, a slightly higher concentration of a moderately polar solvent (e.g., 2-8 % methylene chloride, acetone, or THF), alone or in combination. The optimum composition of the wash solvent must be determined empirically for each particular analyte.
2. Flow Rate: The flow rate should be approximately 1 to 2 mL per minute.

5. Dry

After the wash solvent has been passed through the sorbent bed, continue to apply vacuum or positive pressure. Dry the cartridge under full vacuum (or pressure) for 2 to 5 minutes or until dry. Turn off the vacuum, and wipe the tips of the manifold needles in order to remove any residual sample/matrix or wash solvent. For details, see the notes in the Reversed Phase extraction procedure in Section 10-A.

6. Elution

Insert the collection tubes into the manifold rack, and pass a minimum of 2 to 4 sorbent bed volumes of elution solvent slowly through the cartridge (1 to 2 mL/min). Collect the analyte. Elution is accomplished by the addition of polar organic solvents.

Notes:

1. Elution Solvents: Typical elution solvents include low levels of polar organic solvents (such as isopropanol, acetonitrile or methanol) either alone or in combination with moderately polar organic solvents (such as methylene chloride, THF and acetone), all dissolved in non-polar organic solvents (such as hexane, toluene or petroleum ether). Elution generally takes place in the presence of relatively low concentrations of any given polar organic solvent (typically less than 20 %), or in slightly higher concentrations of moderately polar solvents (typically less than 50 %).
2. Sorbent Polarity: The relative polarity of the most common SPE sorbents is listed in ***Table 5*** and is discussed in Section 9-A. Critical factors for the reproducible chromatographic performance of normal phase SPE sorbents are their relative polarity, surface area, surface coverage, surface pH and water content (LOD); each of these must be fairly consistent from tube-to-tube and lot-to-lot in order to obtain reproducible results.
3. Compatibility: As noted in Section 8-D, the composition of the SPE elution solvent should be compatible with subsequent sample manipulation or analytical steps. Volatile, non-polar organic elution solvents should be used prior to concentration/evaporation, derivatization and/or direct GC analysis. For HPLC analysis on a reversed phase column, the SPE elution solvents should be as similar as possible to the initial HPLC mobile phase so that the eluant can simply be diluted in water, buffer or mobile phase. Alternatively, the use of a normal phase HPLC column typically allows the SPE eluent to be diluted in a non-polar organic solvent and injected directly for analysis. Since most target analytes can be eluted with an extremely low concentration of a polar organic solvent, the SPE eluent will require only minor dilution, particularly relative to that required for reversed phase analysis.
4. Soak Time: Allow the elution solvent to remain or “soak” in the sorbent bed (without vacuum) for approximately one minute in order to optimize recovery.
5. Multiple Extractions: In order to improve recovery, use two or more aliquots containing a smaller volume of elution solvent rather than a single, larger volume.

C. Ion-Exchange Extraction Procedures

Ion-exchange extractions are performed with charged sorbents on target analytes that contain ionizable functional groups (**Table 9**). The sample/matrix composition is typically aqueous, although strong anions and cations may also be extracted from organic samples. *Ion-exchange* sorbents include weak and strong *anion-exchangers*, and *weak* and *strong cation-exchangers*. Detailed protocols for each of these four types of ion-exchangers are provided below, following some general guidelines for their use.

Mobile Phase

In *ion-exchange*, the ionic strength must be kept relatively low at all times prior to the elution step in order to facilitate analyte retention. In addition, the mobile phase pH must be controlled with buffers in order to ensure that the analyte(s) and the sorbent are oppositely charged prior to the elution step.

pH and pK_a

Optimal control of retention behavior in *ion-exchange* SPE is facilitated by a sound understanding of acid-base chemistry and accurate pK_a data on the sorbent, the target analyte(s), and if possible, the contaminating solutes (**Table 10**). A good working knowledge of the acid-base properties of the typical functional groups found on *ion-exchange* sorbents as well as the target analyte(s) when used in conjunction with careful experimental design and method optimization strategies can have a profound effect on the quality of the final extraction.

The most important factors in *ion-exchange* chromatography are the pK_a of the analyte and the sorbent, and the pH and ionic strength of the mobile phase. The pK_a is the pH at which half of the molecules are protonated and the other half are deprotonated. The quantitative ionization or neutralization (99.5 %) of a functional group occurs at a pH value 2.0 units above or below the pK_a. Detailed information on the pK_a values of a variety of common chemicals and specific functional groups are provided in **Table 10**, and in References 5-9 in Appendix III.

Carboxylic Acids: The pK_a of a typical carboxylic acid is 4, so these weak acids are charged at pH 6 and neutralized at pH 2. At a pH of 4, half of the molecules are protonated and neutral, and the other half are ionized/charged. Consequently, low recovery values would be expected for carboxylic acid analytes on *anion-exchangers* at pH 4 or below. In contrast, at pH 6, approximately 99.5 % of the analytes would be ionized, and retained. The target analyte(s) can typically be eluted with buffers at pH of 2 or less, at which point, the carboxylic acid groups become protonated and neutralized, effectively disrupting the ionic interactions between the two.

Similar pK_a values are found on simple aliphatic and aromatic carboxylic acids (**Table 10**). Dicarboxylic acids, however, tend to retain their charge at lower pH values, since the pK_a values are considerably lower (~2) as a result of the inductive effect and hydrogen bonding between the protonated acid and its charged counterpart (**Table 10**). Lower pK_a values are also found on amino acids and other monocarboxylic acids with electron withdrawing groups that are in close proximity to the acid (**Table 10**).

Consequently, unsubstituted monocarboxylic acids can typically be separated from dicarboxylic acids, amino acids or other monocarboxylic acids containing electron withdrawing groups with the use of buffers between pH 2 and 4. In a similar manner, most weak organic (carboxylic) acids can be separated from strong organic acids (sulfonic or phosphoric acids) at pH values between 1 and 2.

Weak cation-exchangers tend to exhibit an extremely broad range of pK_a values. Like dicarboxylic acids, the inductive effects and hydrogen-bonding forces between the enormous number of adjacent carboxylic acid groups produces a chromatographic surface that titrates over a wide range of pH values. As a result, elution from *weak cation-exchangers* is best accomplished with strong acids that are able to neutralize all of the carboxylic acids on the sorbent, or alternatively, with strong bases that can titrate the amines on the analytes.

Table 9. Ion-Exchange SPE Protocol

Sorbent Polarity	High; Positively or negatively charged	SCX, SAX, WAX, WCX
Analyte Properties	Ionized/charged compounds	Amino acids
Sample/Matrix	Aqueous; Low ionic strength buffers (<30 mM), pH adjusted	Biological fluids plus buffer
Conditioning Step	1. Conditioning – polar organic solvents 2. Equilibration – low ionic strength buffers, pH adjusted	1. MeOH 2. 25 mM Tris-OAc, pH 7.0
Wash Step	Aqueous buffers of low to intermediate salt concentrations with or without organic solvent	50 mM Tris-OAc pH 7.0 plus 50 mM NH_4Cl plus 20 % methanol
Elution Step	•Neutralize the charge on the weak anion or cation •Increase the ionic strength and counter ion concentration •Add a strong displacer	50 mM Tris-OAc plus 200 mM NH_4Cl, pH 2 plus 20 % methanol

See glossary *(p.45)* for definition

Sulfonic Acids: Organic sulfonic acids such as those found on most *strong cation-exchangers* have pK_a values well below 1, and are essentially charged at all pH values (**Table 10**). As a result, weak organic amine analytes are best eluted from SCX by neutralization with base, since attempts to elute the analytes with strong acids are usually unsuccessful.

Quaternary Amines: Quaternary (4°) amines such as those found on *strong anion-exchangers* do not have pK_a values, since they contain covalently bound alkyl (or aromatic) groups which cannot be dissociated or titrated (**Table 10**). As a result, analytes containing carboxylic acids should be neutralized and eluted from SAX with strong acids, since the sorbent cannot be neutralized with base.

Weak Amines: Weak aliphatic amines all tend to have fairly similar acid-base properties, even though minor differences do exist between secondary (2°) amines relative to primary (1°) and tertiary (3°) amines (**Table 10**). In general, weak amines have pK_a values of approximately 10, and are charged (and retained on *cation-exchangers*) at pH 8, and neutralized (and eluted) at pH 12.

N-alkyl-substituted aromatic compounds such as N-benzyl and higher alkyl homologs have pK_a values similar to alkyl amines (~10). In contrast, aniline derivatives (aromatic amines) and heterocyclic amines have pK_a values of 5 or less due to the fact that the amino group is either contained within or attached directly to the aromatic ring (**Table 10**). As a result, they are only fully charged at pH 3 or less and can only be extracted efficiently on *strong-cation exchangers*, since weak *cation-exchangers* have lost most of their charge below pH 4.

In general, simple N-alkyl substituted 1°, 2°, and 3° amines each exhibit slightly different pK_a ranges (**Table 10**). Compounds that are otherwise identical in structure have slightly different pK_a values depending on the number of alkyl chains on the amine. In general, the pK_a ranges for typical primary (9.5-10.5), secondary (10.8-11.3) or tertiary (8.5-10.5) amines are often different enough to obtain a good separation by carefully adjusting the pH of the loading, wash and/or elution solvents. For example, target analytes containing 2° amine groups with a pK_a of 11 can be retained on a *strong-cation exchanger* at pH 10, while a majority of the 1° and 3° amines will fail to bind (or be washed off in the wash step) at that same pH.

Counter Ion Effects

In *ion-exchange*, counter ions in the sample/matrix compete with the analyte for charged binding sites on the sorbent surface. Low recoveries can occur in the presence of counter ions that have a greater affinity for the sorbent than does the analyte, or, in the presence of high concentrations (ionic strengths) of virtually any counter ion.

Anions can be ranked according to their affinity for anion-exchange matrices, with shorter solute retention times being observed in the presence of higher affinity displacers such as iodide ($I^- > Br^- > NO_3^- > Cl^- > H_2PO_4^- > CH_3COO^- > F^-$). In a similar manner, cations can be ranked in terms of their affinity for cation-exchangers ($Ca^{+2} > Mg^{+2} > K^+ > NH_4^+ > Na^+ > Li^+$), with calcium exhibiting the strongest displacer properties and inhibiting analyte retention the most (**Table 11**).

The retention of analytes is facilitated by loading the sample in a low ionic strength buffer comprised of low affinity or weak displacer counter ions (e.g., 20 mM NaOAc), and at the proper pH.

Elution is promoted by high ionic strength salts and buffers containing strong displacer counter ions (e.g., 500 mM KI), and/or by adjusting the pH with strong acids or bases in order to fully neutralize the charged groups on either the sorbent or the analyte.

Ionic Strength

The ionic strength must be kept low (<30 mM) during the sample loading step. Conductivity is also an important factor as are the actual counter ion species that are present. In general, the sample/matrix should be diluted in a low ionic strength buffer (~10 mM) at pH 7.0. Physiological fluids (other than urine) contain approximately 120 mM NaCl, so most biological fluids (blood, serum, plasma) should be diluted at least four-fold prior to loading. Urine specimens require more extensive dilution.

Wash solvents containing an intermediate (~ 50 to 100 mM) salt concentration can be a particularly effective means to remove contaminants. This can also be further facilitated by the addition of low levels of a water-miscible polar organic solvent such as methanol. The use of acid or base can also be a particularly effective means to remove titratable contaminants in situations in which the target analyte(s) is a sulfonic acid or a quaternary amine, respectively. Elution can be accomplished with virtually any salt species, provided the ionic strength is high (>200 mM). In general, the most effective elution techniques involve the addition of strong acid or base in combination with an intermediate salt concentration and a small percentage of a polar organic solvent, since this can be used to desorb virtually all of the bound solutes, including the target analyte(s). However, it is often more advantageous to use pH alone in order to selectively separate the target analyte(s) from the contaminants bound to the sorbent.

See glossary *(p.45)* for definition

Table 10. Typical pK_a Ranges for Important Functional Groups

Functional Group	Class	Type	pK_a Range	Example	Structure
Carboxylic Acid	Mono-Carboxylic Acid	Aliphatic	4 – 5	Acetic acid pK_a = 4.8	$H_3C-C(=O)-O-H$
		Aromatic	4 – 5	Benzoic acid pK_a = 4.2	
		Electron Withdrawing Groups		Trichloroacetic acid pK_a= 0.7	$Cl_3C-C(=O)-O-H$
		Amino Acid	1.7 – 2.6 9 – 10.8	Alanine pK_a = 2.4 pK_a = 9.7	
	Di-Carboxylic	Aliphatic Acid	1.3 – 6.2	Malonic acid pK_a = 2.4 pK_a = 5.7	
Sulfonic Acid		Aromatic	< 0	Benzenesulfonic acid pK_a = - 0.6	
Amine	Primary	Aliphatic	9.5 – 11	Cyclohexylamine pK_a = 10.6 (protonated)	NH_3^+
	Secondary	Aliphatic	10.8 – 11.5	Diethylamine pK_a = 11.0 (protonated)	$Et_2NH_2^+$
	Tertiary	Aliphatic	8.5 – 11	Triethylamine pK_a = 10.7 (protonated)	Et_3NH^+
	Quaternary	Aliphatic		Tetramethylammonium chloride	$Me_4N^+ Cl^-$
	Primary	Aromatic	3.9 – 6.6	Aniline pK_a = 4.6 (protonated)	NH_3^+
	Heterocycle		1 – 7.5	Pyridine pK_a = 5.2 (protonated)	NH^+
				Imidazole pK_a = 7.5 (protonated)	HN NH^+
Phenol			9 – 11	Phenol pK_a = 10.0	OH
		Electron Withdrawing Groups	0 – 10	Picric acid pK_a = 0.25	O_2N, NO_2, NO_2, OH

Table 10. Typical pK_a Ranges for Important Functional Groups (*continued*)

Functional Group	Class	Type	pK_a Range	Example	Structure
Thiol		Aliphatic	10 – 11	Ethanethiol pK_a = 10.5	EtSH
↓		Aromatic	6 –8	Benzenethiol pK_a = 7.8 (Thiophenol)	SH
Amide		Aliphatic		Acetamide pK_a = 15.1	O, NH_2
Imide		Aliphatic		Succinimide pK_a = 9.6	O, NH, O
↓		Aromatic		Phthalimide pK_a = 7.4	O, NH, O
Sulfonamide		Aromatic		Benzenesulfonamide pK_a = 10.0	SO_2NH_2
Nitro				Nitromethane pK_a = 11.0	CH_3NO_2
1,3-Di-carbonyl	Diketone			2,4-Pentanedione pK_a = 9.0 (Acetylacetone)	O O
	Ketone-Ester			Ethyl acetoacetate pK_a = 11.0	O O OEt
↓	Diester			Diethyl malonate pK_a = 13.0	

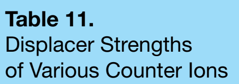


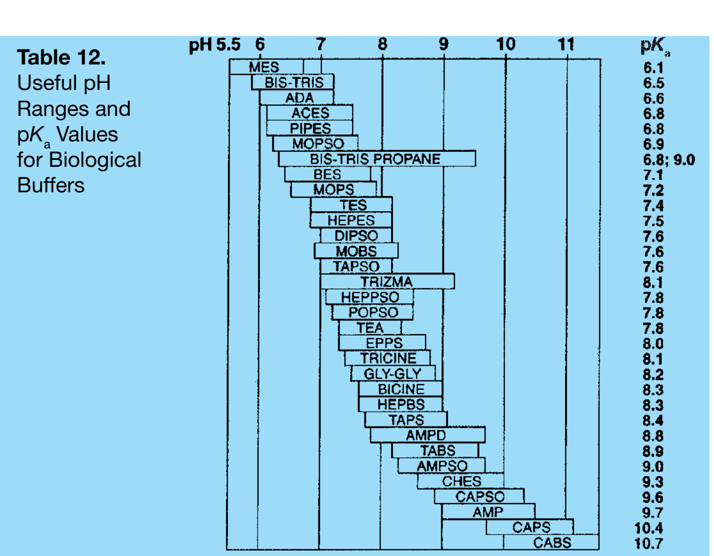
Table 11.
Displacer Strengths of Various Counter Ions

Listed from weak to strong; the ions on the right will displace ions to the left.

Cations
Li<H<Na<NH_4<K<Mg
<Fe<Ca<Cu<Pb<Ag<Ba

Anions
OH<F<Acetate<Formate
<HPO_4<HCO_3<Cl<Br
<Citrate<Benzene Sulfonate

Table 12.
Useful pH Ranges and pK_a Values for Biological Buffers



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Buffers

The traditional buffers used in SPE have typically included simple phosphate, acetate and carbonate salts. However, organic zwitterions such as the "biological" or "good" buffers tend to have better buffer capacities, provide lower conductivities, and are available in a wide range of pK_a values. **Table 12** lists the most popular commercially available biological buffers, their pK_a values, and the pH ranges at which they are most effective.

Tris (TRIZMA®) tends to be an excellent buffer for *anion-exchange*, and MES or MOPSO (alone or in combination with Tris) are excellent buffers for *cation-exchange* applications. For higher pH applications (~10), CAPS is recommended.

Solvent: Salt Mixtures

Mixtures of salts and polar organic solvents make excellent elution solvents for *ion-exchangers.* Some salts such as a NH_4Cl and NH_4OAc are readily and highly soluble in methanol and are ideal for the preparation of elution solvents that are high in both salt and organic solvent.

Viscosity, Particulates and Solids

Viscous samples should be diluted further with water or buffer in order to improve the flow rate and prevent the column from clogging. In most cases, particulates and other insoluble material and/or debris do not have to be removed prior to the actual extraction since the frits act as an effective filter. Details on the sample preparation requirements for soils, powders, tissues, ointments, and other solids are provided in Technical Note TN-0013 .

Sorbent Selection

As a general rule, only *weak ion-exchangers* should be utilized to extract analytes containing strong cations or strong anions (sulfonic acids or quaternary amines). This is because the recovery of these analytes is often low on *strong ion-exchangers* due to the inability to titrate/neutralize the quaternary amines or the sulfonic acids on either the sorbent or the analyte. The use of weak *ion-exchangers* facilitates the extraction of organic sulfonic acids or quaternary amines since *weak cation-exchangers* can be neutralized at pH 2 following the addition of a strong acid, while *weak anion-exchangers* become neutralized at pH 12 in the presence of basic elution solvents.

On the other hand, analytes containing weak amines or carboxylic acid moieties can be extracted on either weak or strong *ion-exchangers*, since pH adjustment can be used to neutralize the charge on the analyte in order to bring about elution. However, the use of *strong ion-exchangers* is generally preferred for the extraction of analytes containing weak ions, since elution can be accomplished at acidic or basic pH values (2 or 12), and under these conditions, contaminants containing sulfonic acids or quaternary amines will remain bound to the sorbent (SAX and SCX, respectively).

1. Weak Cation-Exchange

Weak *cation-exchangers* contain carboxylic acid groups and are typically used to extract quaternary (4°) amines. Weak cations such as primary, secondary, and tertiary (1°, 2°, 3°) amines may also be retained, although the pH range at which quantitative retention will occur is typically quite narrow and specific (pH 6-8). The elution of the target analyte(s) occurs in the presence of acidic conditions at pH 2 or less, at which point the charge on the carboxylic acid groups on the sorbent is fully neutralized.

1. **Sample/Matrix Preparation**

   Dilute the sample with buffer, ensuring that the salt concentration is below 30 mM and the pH is approximately 7.0. Typical buffers include 20 mM Tris-OAc, pH 6 to 8, and in some cases, 20 mM CAPS, pH 10.0, 20 mM CAPS-NH_3, pH 12, or 20 mM MES, pH 3 to 6.

   Notes:

   1. Quaternary Amines: For target analytes that contain quaternary amine groups, the pH should be at least 6 (or 2 full pH units above the pK_a of the weak cation-exchanger). However, pH values as high as 12 may be used successfully for these analytes, since 4° amines will remain charged, while most potential contaminants (1°, 2°, 3°) amines will be neutralized and fail to bind.
   2. Weak Aliphatic Amines: For target analytes containing 1°, 2° and/or 3° amine groups, the pH of the sample/matrix should be approximately 7.0. In particular, at least 2 pH units below the pK_a of the analytes (pH<8), and 2 pH units above the pK_a of the weak cation-exchanger (pH>6). For target analytes like 2° amines, that have a relatively high pK_a value, a higher pH can be used in order to prevent the retention of other (1° or 3°) amines that may be potential contaminants. In general, however, it may be "safer" to bind all these solutes and then selectively desorb the contaminants during the wash step.
   3. Weak Aromatic Amines: Adjust the pH to 7 and pre-treat the sample/matrix the same as for weak aliphatic amines (above).
   4. Heterocyclic Amines: For analine derivatives or heterocyclic amines that have pK_a values of 5 or less, retention on weak cation-exchangers is problematic at any pH value. Use a strong cation-exchanger, and adjust the pH to 3 or less.
   5. Inorganic Cations: For the extraction of simple inorganic cations and metal ions, the sample/matrix pH is relatively inconsequential, since these analytes remain charged at relatively high pH values. Load at a pH above 7.

2. **Sorbent Conditioning**

Solvation: Pass one column volume of water-miscible organic solvent through the cartridge.

Equilibration: Follow this directly with one column volume of the sample/matrix dilution buffer at a low ionic strength and pH equal to that of the sample/matrix. The flow rate should be 2 to 4 mL per minute.

Notes:

1. Solvents: Typical water-miscible conditioning solvents are methanol, isopropanol and acetonitrile. The equilibration solvent should be the same buffer used for the sample dilution and pH adjustment. Low ionic strength (10 to 20 mM) buffers are typical.
2. Drying: Do not allow the sorbent to dry under full vacuum for more than 1 minute before applying the sample, since this can cause the sorbent bed to dry out. If the cartridge dries out, simply repeat the conditioning steps.

3. **Sample Loading**

Aspirate the sample/matrix through the conditioned SPE cartridge at about 1 mL per minute.

Notes:

1. Flow Rate: Maintain a slow flow rate through the cartridge because the kinetics of ion-exchange process is relatively slow. Use gravity flow if the recoveries are low.

4. **Wash**

Rinse the cartridge with 1 to 2 column reservoir volumes of wash solution. The flow rate should be 1 to 2 mL per minute.

Notes:

1. Aqueous Solvents: Wash solvents must not disrupt the ionic interaction between the sorbent and the analyte, so it is critical to maintain a relatively low ionic strength, a low proportion of strong displacer counter ions, and the proper pH. Typical buffers include 20 mM Tris-OAc, pH 6 to 8, 20 mM CAPS, pH 10.0, 20 mM CAPS-NH_3, pH 12 and 20 mM MES, pH 3 to 6.
2. Organic Solvents: Ion-exchange wash solutions often contain organic solvents, since they are able to remove non-polar and moderately polar contaminants and have no acid-base properties which may cause the premature elution of the target analyte(s).
3. Quaternary Amines: For target analytes that contain quaternary amine groups, the most effective wash will include a basic buffer at a pH 11 or higher, since this will effectively neutralize/deprotonate 1°, 2° and 3° amine contaminants that may have been retained, and elute them from the sorbent. This may be followed by a second wash at a more neutral pH (and then, elution of the 4° amines with a mixture containing a strong acid and a polar organic solvent).
4. Weak Amines: For target analytes containing 1°, 2°, and/or 3° aliphatic or aromatic amine groups, the wash solvent should consist of a low salt buffer at pH values around 7. This should remove any weakly retained heterocyclic amines or aniline derivatives. For 2° amines or other target analytes with high pK_a values, a higher pH (~9 or 10) may be useful in removing bound contaminants such as 1° or 3° amines.

5. **Dry**

After the wash solvent has been passed through the sorbent bed, continue to apply vacuum or positive pressure. Dry the cartridge under full vacuum (or pressure) for 2 to 5 minutes or until dry. Turn off the vacuum, and wipe the tips of the manifold needles in order to remove any residual sample/matrix or wash solvent. For details, see the notes in the *Reversed Phase* procedure in Section 10-A.

6. **Elution**

Insert the collection tubes into the manifold rack, and pass a minimum of 2 to 4 sorbent bed volumes of elution solvent slowly (1 to 2 mL/min) through the cartridge. Collect the analyte. Elution is accomplished by the addition of strong acids in combination with polar organic solvents and/or high concentrations of a strong displacer counter ion.

Notes:

1. Elution Solvents: In general, the optimal elution solvent composition depends on the structure and charge characteristics of the target analyte, and the analytical technique (see Note 5). In many cases, a weak organic acid such as 1 to 10 % HOAc may be preferable to HCl, since it is able to neutralize the sorbent and solubilize target analytes without eluting an excess number of contaminants.
2. Quaternary Amines: Elute with a strong acid (1N HCl) or an organic acid (10 % HOAc) and/or a high concentration of a strong displacer counter ion (e.g., 200 mM KI, NH_4OAc or NH_4Cl) with a low to moderate concentration of a polar organic solvent (e.g., 20 to 50 % methanol).
3. Weak Amines: Elute with the same combination of acid:salt:organic solvent that was recommended above. To minimize the amount of quaternary amine contaminants, elute the weak target amines with base at pH 12. To minimize the amount of 2° and 4° amine contaminants, elute 1° or 3° target analytes at pH 9 (or 10) with a low to moderate concentration of a strong buffer (e.g., 50 mM CAPS).
4. Inorganic Cations: Elute with a strong acid and/or a stronger displacer counter ion.

5. Compatibility: The composition of the SPE elution solvent should be adjusted in order to be compatible with subsequent sample manipulation or analytical steps. For example, volatile elution solvents should be used prior to concentration/evaporation, derivatization and/or direct GC analysis. These volatile components include water-miscible, polar organic solvents (methanol, isopropanol, etc.), salts (NH_4OAc, NH_4COO, etc.), acids (HCl, TFA, HOAc, etc.), and/or bases (NH_4OH, TEA, etc.). Following evaporation, this type of sample is amenable to analysis by either GC or HPLC. For HPLC analysis on a reversed phase column, the SPE elution solvent should be mostly aqueous and as similar as possible to the HPLC mobile phase so that the eluant can simply be diluted in water, buffer, or mobile phase (the eluant salt concentration is not an issue). Prior to analysis on a normal phase HPLC column, the SPE eluant should be diluted in a water-miscible organic solvent and then in a non-polar organic solvent, provided that the analyte(s) is soluble and the salt concentration is relatively low. HPLC on an ion-exchange column usually requires that the SPE eluant be pH adjusted, buffered, and/or diluted in order to lower the salt concentration. As a result, elution with salt is not recommended prior to HPLC analysis on an ion-exchanger.
6. Soak Time: Allow the elution solvent to remain or "soak" in the sorbent bed (without vacuum) for approximately one minute in order to optimize recovery.
7. Multiple Extractions: In order to improve recovery, use two or more aliquots containing a smaller volume of elution solvent rather than a single, larger volume.

2. Strong-Cation Exchange

Strong cation-exchangers typically contain aromatic sulfonic acid groups and are typically used to extract 1°, 2° and 3° amines. Although quaternary amines will also be retained, *strong cation-exchangers* are not recommended for these target analytes, since elution requires the use of a polar organic solvent in conjunction with a high salt concentration or an extremely strong acid, and is often problematic. Elution of 1°, 2° and 3° amine target analytes occurs in the presence of basic conditions at pH 12 or 13, at which point they are fully neutralized.

1. Sample/Matrix Preparation

Dilute the sample with buffer, ensuring that the salt concentration is below 30 mM and the pH is less than or equal to 7.0. Typical buffers include 20 mM Tris-OAc, pH 6 to 8, 20 mM CAPS, pH 10.0, 20 mM CAPS-NH_3, pH 12, 25 mM MES-OAc, pH 5.0, and 25 mM MES plus 1 mM HCl, pH 3.0.

Notes:

1. Quaternary Amines: For target analytes that contain quaternary amine groups, a weak cation-exchanger should be used. However, on either type of cation-exchanger, the sample/matrix should be loaded at a pH of 11 or greater in order to prevent the retention of 1°, 2° and/or 3° amines.
2. Weak Aliphatic Amines: For target analytes containing 1°, 2° and/or 3° amine groups, the pH of the sample/matrix should be less than 8.0, or at least 2 pH units below the pK_a of the analytes. For target analytes like 2° amines, that have a relatively high pK_a value, a higher pH (9) can be used in order to prevent the retention of other (1° or 3°) amines that may be potential contaminants. In general, however, it may be safer to bind all these solutes and then selectively desorb the contaminants during the wash step.
3. Weak Aromatic Amines: Pre-treat the sample/matrix the same as that described for weak aliphatic amines, above.
4. Heterocyclic Amines: For analine derivatives or heterocyclic amines that have pK_a values of 5 or less, retention occurs at a pH of 3 or less.
5. Inorganic Cations: For the extraction of simple inorganic cations and metal ions, the sample/matrix pH is relatively inconsequential since these analytes remain charged at relatively high pH values.

2. Conditioning

Solvation: Pass one column volume of water-miscible organic solvent through the cartridge (e.g., methanol, isopropanol or acetonitrile).

Equilibration: Follow this directly with one column volume of the sample/matrix dilution buffer. The flow rate should be 2 to 4 mL per minute. Do not allow the sorbent to dry under full vacuum for more than 1 minute before applying the sample.

3. Load

Aspirate the sample/matrix through the conditioned SPE cartridge at a rate of 1 mL per minute. Use gravity flow if the recoveries are low.

4. Wash

Rinse the cartridge with 1 to 2 column reservoir volumes of wash solution. The flow rate should be 1 to 2 mL per minute.

Notes:

1. Aqueous Wash Solvents: The wash solvent must not disrupt the ionic interactions between the sorbent and the analyte so it is critical to maintain a low ionic strength, a low proportion of strong displacer counter ions, and the proper pH. Typical buffers were listed in number 1, Sample/Matrix Preparation.

2. Organic Solvents: Ion-exchange wash solvents often contain a low concentration of organic solvents since they are able to remove non-polar contaminants that may be retained on the aromatic sulfonic acid ligands.
3. Quaternary Amines: For target analytes that contain quaternary amine groups, the most effective wash will include a basic buffer at a pH of 12 or greater, since this will effectively neutralize/deprotonate 1º, 2º and 3º amine contaminants and elute them from the sorbent. This may be followed by a second wash at a more neutral pH (and then, elution of the 4º amines with a mixture containing a strong displacer counter ion and a polar organic solvent).
4. Weak Amines: For target analytes containing 1º, 2º, and/or 3º aliphatic amine groups, the wash solvent should consist of a relatively low salt buffer at pH values around 7. For 2º amines or other target analytes with a high pK_a value, a higher pH (~9) may be useful in removing bound contaminants such as 1º or 3º amines.
5. Aromatic vs. Aliphatic Amines: Due to the presence of the aromatic ring on most strong cation-exchangers, N-alkyl-aromatic amines may be more strongly retained than simple aliphatic amines of similar structure. In addition, more hydrophobic amines should also be more strongly retained than polar amines with a similar pK_a. As a result, some selectivity may be obtained by using higher pH values in the wash and avoiding the addition of even low concentrations of acetonitrile or methanol in order to wash off aliphatic or more polar weak amine contaminants.
6. Heterocyclic Amines: For aniline derivatives and heterocyclic amines, the wash solvent should contain a low concentration of salt at pH 4 or less. Under these conditions, all other anions will also be retained.

5. Dry

After the wash solvent has been passed through the sorbent bed, continue to apply vacuum or positive pressure. Dry the cartridge under full vacuum (or pressure) for 2 to 5 minutes or until dry. Turn off the vacuum, and wipe the tips of the manifold needles in order to remove any residual sample/matrix or wash solvent. For details, see the *Reversed Phase* protocol in Section 10-A.

6. Elution

Insert the collection tubes into the manifold, and pass a minimum of 2 to 4 sorbent bed volumes of elution solvent slowly (1 mL/min) through the cartridge. Collect the analyte. Elution is accomplished by the addition of strong base in combination with polar organic solvents and/or high concentrations of a strong displacer counter ion.

Notes:

1. Elution Solvents: In general, the optimal elution solvent composition depends on the charge characteristics and structure of the target analyte, and the analytical technique (see Note 7).
2. Quaternary Amines: Elute with a high concentration of a strong displacer counter ion (e.g. 200 mM NH_4OAc or NH_4Cl) with a moderate concentration of a polar organic solvent (methanol).
3. Weak Amines: Elute with a combination of base:salt:organic solvent. To minimize the amount of quaternary amine contaminants, elute the weak amine target analyte(s) with base at pH 12 without added salt. To minimize the amount of 2º and 4º amine contaminants, elute 1º or 3º target analytes at pH 9 with a low to moderate concentration of a strong buffer (e.g., 50 mM CAPS).
4. Aromatic vs. Aliphatic Amines: The addition of polar organic solvents such as acetonitrile or methanol may be required for the elution of hydrophobic analytes due to the presence of aromatic sulfonic acid groups on the sorbent. Some selectivity may be obtained by adjusting the solvent species and organic content in order to selectively remove the aliphatic and the more polar amines, while the N-alkyl-aromatic amines as well as the more hydrophobic amines remain bound. Alternatively, the wash solvent pH (see above) can be increased in the absence of organic solvent in order to promote the desorption of aliphatic and/or polar amines, followed by the elution of the N-alkyl-aromatic and more hydrophobic amines with a high pH and a high organic solvent concentration.
5. Heterocyclic Amines: Elute aniline derivatives and heterocyclic amines with a low salt concentration and a strong buffer at pH 7 or 8 (e.g., 25 mM Tris-HCl, pH 7.5). This will remove all the target analyte(s), while the 1º, 2º, 3º and 4º amine contaminants will remain on the sorbent.
6. Inorganic Cations: Elute with a strong acid and/or a stronger displacer counter ion.
7. Compatibility: Adjust the elution conditions so that the SPE elution solvent is compatible with subsequent sample manipulation or analytical steps. Details are provided in Section 10-C-1 in the Weak Cation-Exchange protocol.
8. Soak Time: Allow the elution solvent to remain or "soak" in the sorbent bed (without vacuum) for approximately one minute in order to optimize recovery.
9. Multiple Extractions: In order to improve recovery, use two or more aliquots containing a smaller volume of elution solvent rather than a single, larger volume.

3. Weak Anion-Exchange

Weak anion-exchangers contain titratable 1°, 2°, and/or 3° amine groups and are typically used to extract anionic target analytes such as carboxylic acids, sulfonic acids and organophosphates. Strong anions such as sulfonic acids or organophosphates are typically retained at pH values between 1 and 8. In contrast, weak acids are only retained within a narrow range of pH values (6 to 8); as a result, target analytes containing carboxylic acids are typically extracted with strong anion-exchangers, as detailed in the next section. Elution of all analytes and solutes occurs at pH values of 12 or above (via the neutralization of the sorbent), and/or in the presence of high concentrations of strong displacer counter ions such as Ca^{+2}, K^{+} or NH_4^{+}. Analytes containing carboxylic acids can be selectively eluted at pH 2 or less.

1. Sample/Matrix Preparation

Dilute the sample with buffer, ensuring that the salt concentration is below 30 mM and the pH is approximately 7.0 (see details below).

Notes:

1. Monocarboxylic Acids: Simple carboxylic acids must be loaded at approximately pH 7. Under these conditions, however, most of the other acidic components in the sample/matrix will also be retained. A typical buffer is 25 mM Tris-OAc at pH 7.5.
2. Dicarboxylic Acids: The sample should be loaded at pH 4 or 5, so most of the monocarboxylic acids will fail to bind. A typical buffer is 25 mM MES, pH 4.5.
3. Amino Acids: Load the sample/matrix at pH 4 or 5 in order to retain amino acids or weak acids containing adjacent electron withdrawing groups. Under these conditions, simple monocarboxylic acids will fail to bind.
4. Sulfonic Acids: Load the sample/matrix at pH 2 or 3, so that the sulfonic acid target analyte(s) will be retained, while most carboxylic acids will fail to bind. A typical "buffer" is 25 mM MES-HCl, pH 2.5.
5. Organophosphates: Load the sample/matrix at pH 2 or 3. Under these conditions, organophosphate target analytes and sulfonic acid contaminants will bind, while most carboxylic acids will not be retained. A typical "buffer" is 25 mM MES-HCl, pH 2.5.
6. Inorganic Anions: For the extraction of simple inorganic anions, the sample/matrix pH is relatively inconsequential, since these analytes remain charged, even at the lowest pH values.

2. Conditioning

Solvation: Pass one column volume of water-miscible organic solvent through the cartridge (e.g., methanol, isopropanol or acetonitrile).

Equilibration: Follow this directly with one column volume of the sample/matrix dilution buffer at a low ionic strength and pH equal to that of the sample/matrix. The flow rate should be 2 to 4 mL per minute. Do not allow the sorbent to dry under full vacuum for more than 1 minute before applying the sample.

3. Load

Aspirate the sample/matrix through the conditioned SPE cartridge at about 1 mL per minute. Use gravity flow if the recoveries are low.

4. Wash

Rinse the cartridge with 1 to 2 column reservoir volumes of wash solution. The flow rate should be 1 to 2 mL per minute.

Notes:

1. Aqueous Solvents: Wash solvents must not disrupt the ionic interaction between the sorbent and the analyte, so it is critical to maintain a low ionic strength, a low proportion of strong displacer counter ions, and the proper pH.
2. Organic Solvents: Ion-exchange wash solvents often contain organic solvents, since they are able to remove non-polar and moderately polar contaminants.
3. Monocarboxylic Acids: The wash solvent should be adjusted to pH 7 with a strong buffer such as the one used to equilibrate the column during conditioning step and dilute the sample/matrix (e.g., 25 mM Tris-OAc, pH 7.5). It may also contain a low concentration of a polar organic solvent (e.g., 10 to 20 % methanol), and possibly, an intermediate salt concentration (e.g., 50 to 100 mM NH_4Cl or NH_4OAc).
4. Dicarboxylic Acids: The wash solvent should be at pH 4 or 5 in order to remove weakly bound monocarboxylic acids.
5. Amino Acids: Wash the column with a strong buffer at pH 4 or 5, as described above.
6. Sulfonic Acids: The wash solvent pH should be approximately 2 in order to remove any weakly retained carboxylic acids.
7. Organophosphates: Wash with a combination of strong "buffers" and acid at pH 2.
8. Inorganic Anions: Wash at pH 7.0.

5. Dry

After the wash solvent has been passed through the sorbent bed, continue to apply vacuum or positive pressure. Dry the cartridge under full vacuum (or pressure) for 2 to 5 minutes or until dry. Turn off the vacuum, and wipe the tips of the manifold needles in order to remove any residual sample/matrix or wash solvent. For details, see the *Reversed Phase* procedure in Section 10-A.

6. **Elution**

Insert the collection tubes into the manifold rack, and pass a minimum of 2 to 4 sorbent bed volumes of elution solvent slowly (1 mL/min) through the cartridge. Collect the analyte. Elution is accomplished by the addition of strong base in combination with polar organic solvents and/or high concentrations of a strong displacer counter ion.

Notes:

1. Elution solvents: In general, the optimal elution solvent composition depends on the structure and charge characteristics of the target analyte, and the analytical technique (see Note 8).
2. Monocarboxylic Acids: The elution solvent should be adjusted to pH 2 or 3 with a combination of strong buffers and a strong acid (e.g., 25 mM MES plus 0.01 M HCl, pH 2.5). Under these conditions, the monocarboxylic acids will be eluted, while most of the other weak acids and all of the strong acids will remain bound. An organic solvent can be added in order to facilitate the elution process. A lower pH value or a high concentration of a strong displacer counter ion (e.g., 200 mM NH_4Cl or KCl) can be used, but these may decrease selectivity and bring about the elution of other carboxylic acids and some of the strong acids. A similar loss of selectivity will occur at pH 12, since the weak anion-exchange groups will be titrated and all of the bound solutes will be eluted.
3. Dicarboxylic Acids: Elute with a strong buffer plus a strong acid (0.1N HCl) at pH 1.
4. Amino Acids: The elution solvent should contain a strong acid at pH 1.
5. Sulfonic Acids: Strong acids should be eluted by titrating the weak anion-exchange groups on the sorbent surface with a strong base at pH 12 or above. Any weak acids that still remain bound to the sorbent following the acidic wash steps (see above) will also be eluted along with any other strong acids. As a result, the elution solvent may as well also contain a high concentration of strong (organic-soluble) displacer counter ions, along with some organic solvent (e.g., 200 mM NH_4Cl or NH_4OAc plus NH_4OH, pH 12, in 20 % methanol). Other common elution solvents include organic solvents such as methanol or methylene chloride:isopropanol containing 1 % NH_4OH.
6. Organophosphates: The elution solvent composition should be similar to that used for sulfonic acid target analytes.
7. Inorganic Anions: Elute with a strong acid, strong base, or a stronger displacer counter ion.
8. Compatibility: Adjust the elution conditions so the SPE elution solvent is compatible with subsequent sample manipulation or analytical steps. Details are provided in Section 10-C-1 in the Weak Cation-Exchange protocol.
9. Soak Time: Allow the elution solvent to remain or “soak” in the sorbent bed (without vacuum) for approximately one minute in order to optimize recovery.
10. Multiple Extractions: In order to improve recovery, use two or more aliquots containing a smaller volume of elution solvent rather than a single, larger volume.

4. Strong Anion-Exchange

Strong anion-exchangers contain quaternary amine groups and are typically used to extract target analytes that contain carboxylic acids. Although strong anions such as sulfonic acids or organophosphates are retained at virtually any pH value, they are difficult to elute with only acid or base, since neither the sorbent nor the solute is easily neutralized. In contrast, most carboxylic acids are only retained at pH values above 6, so elution is possible with strong acids or buffers at pH values of 4 or below. Elution of all analytes and solutes occurs in the presence of high concentrations of strong displacer counter ions such as KCl or NH_4Cl.

1. **Sample/Matrix Preparation**

Dilute the sample with buffer, ensuring that the salt concentration is below 30 mM and the pH is 7.0 or above. Typical buffers include those described in the previous section on *Weak Anion-Exchange*.

Notes:

1. Monocarboxylic Acids: Simple carboxylic acids must be loaded at pH 7 or above. Under these conditions, however, most of the other acidic components in the sample/matrix will also be retained. A typical buffer is 25 mM Tris-OAc, pH 7.5.
2. Dicarboxylic Acids: The sample should be loaded at pH 4 or 5, so that most of the monocarboxylic acids will fail to bind. A typical buffer is 25 mM MES-HCl, pH 4.0.
3. Amino Acids: Load the sample/matrix at pH 4 in order to retain target analytes such as amino acids or weak acids containing adjacent electron withdrawing groups. Under these conditions, simple monocarboxylic acids will fail to bind.
4. Sulfonic Acids: Load the sample/matrix at pH 2, so that the sulfonic acid target analyte(s) will be retained, while most carboxylic acids will fail to bind. A typical buffer is 25 mM MES-HCl, pH 2.5.
5. Organophosphates: Load the sample/matrix at pH 2. Under these conditions, organophosphate target analytes will bind, while most carboxylic acids will not be retained. A typical buffer is 25 mM MES-HCl, pH 2.0.
6. Inorganic Anions: For the extraction of simple inorganic anions, the sample/matrix pH is relatively inconsequential, since these analytes and the sorbent both remain charged at virtually all pH values. Load at pH 7.0.

2. **Conditioning**

Solvation: Pass one column volume of water-miscible organic solvent through the cartridge (e.g., methanol, isopropanol or acetonitrile).

Equilibration: Follow this directly with one column volume of the sample/matrix dilution buffer at a low ionic strength and pH equal to that of the sample/matrix. The flow rate should be 2 to 4 mL per minute. Do not allow the sorbent to dry under full vacuum for more than 1 minute before applying the sample.

3. **Load**

Aspirate the sample/matrix through the conditioned SPE cartridge at about 1mL per minute. Use gravity flow if the recoveries are low.

4. **Wash**

Rinse the cartridge with 1 to 2 column reservoir volumes of wash solution. The flow rate should be 1 to 2 mL per minute.

Notes:

1. Aqueous Solvents: Wash solvents must not disrupt the ionic interactions between sorbent and analyte, so it is critical to maintain a low ionic strength, a low proportion of strong displacer counter ions, and the proper pH.
2. Organic Solvents: Ion-exchange wash solvents often contain organic solvents, since they are able to remove non-polar and moderately polar contaminants.
3. Monocarboxylic Acids: The wash solvent should be adjusted to pH 7 or 8 with a strong buffer such as the one used to equilibrate the column and dilute the sample/matrix (e.g., 25 mM Tris-OAc pH 7.5). It should also contain a low concentration of a polar organic solvent (e.g.,10 to 20 % methanol), and possibly, an intermediate salt concentration (e.g., 50 to 100 mM NH_4Cl or NH_4OAc).
4. Dicarboxylic Acids: The wash solvent should be at pH 4 or 5 in order to remove weakly bound monocarboxylic acids.
5. Amino Acids: Wash the column with a strong buffer at pH 4 or 5, as above.
6. Sulfonic Acids: The wash solvent pH should be approximately 2 or 3 in order to remove any weakly retained carboxylic acids.
7. Organophosphates: Wash with a combination of strong "buffers" at pH 2 or 3.
8. Inorganic Anions: Wash at pH 7.0.

5. **Dry**

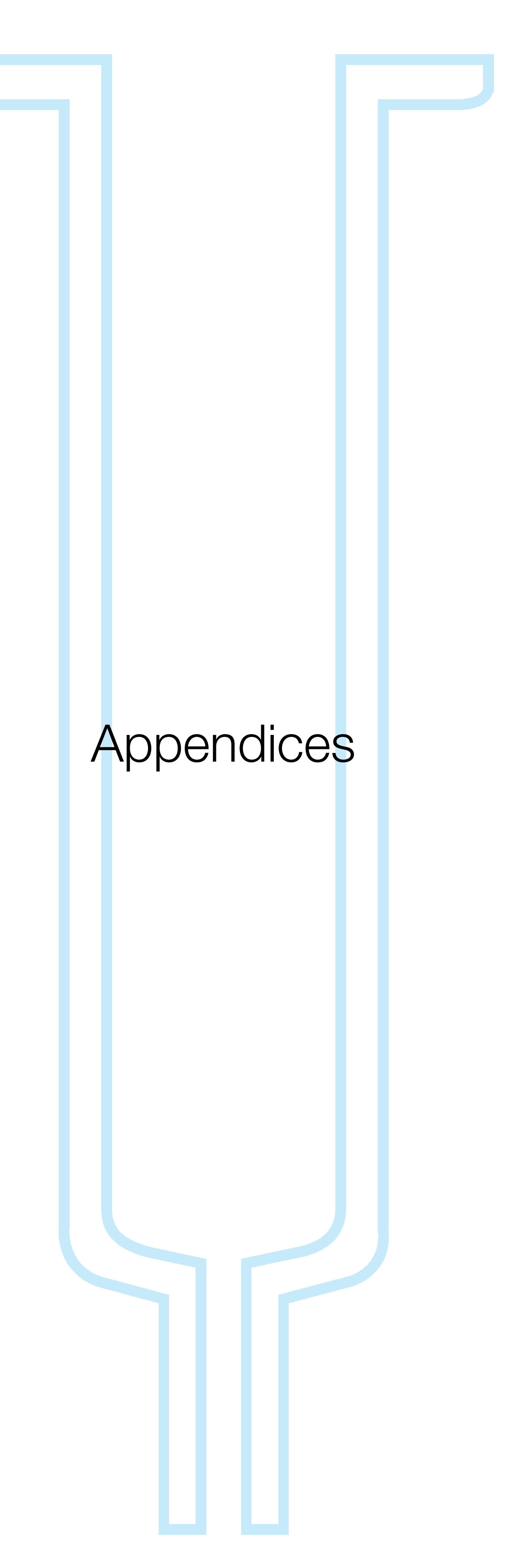
After the wash solvent has been passed through the sorbent bed, continue to apply vacuum or positive pressure. Dry the cartridge under full vacuum (or pressure) for 2 to 5 minutes or until dry. Turn off the vacuum, and wipe the tips of the manifold needles in order to remove any residual sample/matrix or wash solvent. For details, see the *Reversed Phase* protocol in Section 10-A.

6. **Elution**

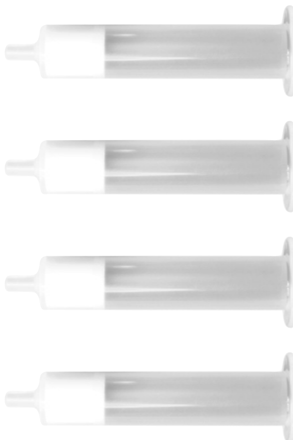
Insert the collection tubes into the manifold rack, and pass a minimum of 2 to 4 sorbent bed volumes of elution solvent slowly (1 mL/min) through the cartridge. Collect the analyte. Elution is accomplished by the addition of strong acid in combination with polar organic solvents and/or high concentrations of a strong displacer counter ion.

Notes:

1. Elution Solvents: In general, the optimal elution solvent composition depends on the structure and charge characteristics of the target analyte, and the analytical technique (see Note 8).
2. Monocarboxylic Acids: The elution solvent should be adjusted to pH 2 or 3 with a combination of strong buffers and a strong acid (e.g., 25 mM MES-OAc plus 0.01N HCl, pH 2.0). Under these conditions, the monocarboxylic acids will be eluted, while some of the other weak acids and all of the strong acids will remain bound. An organic solvent can be added in order to facilitate the elution process. A lower pH value or a high concentration of a strong displacer counter ion (e.g., 200 mM $CaCl_2$, KCl or NH_4Cl) can be used, but these may decrease selectivity and bring about the elution of other carboxylic acids and some of the strong acids.
3. Dicarboxylic Acids: Elute with a strong buffer plus a strong acid at pH 1.
4. Amino Acids: The elution solvent should contain a strong acid at pH 1.
5. Sulfonic Acids: Strong acids should be eluted with an elution solvent containing high concentrations of strong (organic-soluble) displacer counter ions along with some organic solvent (e.g., 200 mM NH_4Cl plus 0.1N HCl, pH 1, in 20 % methanol). Any weak acids that still remain bound to the sorbent following the acidic wash steps (see above) will also be eluted, along with any other strong acids.
6. Organophosphates: The elution solvent composition should be similar to that used for sulfonic acid target analytes.
7. Inorganic Anions: Elute with a strong acid or a stronger displacer counter ion.
8. Compatibility: Adjust the elution conditions so the SPE elution solvent is compatible with subsequent sample manipulation or analytical steps. Details are in Section 10-C-1 in the Weak Cation-Exchange protocol.
9. Soak Time: Allow the elution solvent to remain or "soak" in the sorbent bed (without vacuum) for approximately one minute in order to optimize recovery.
10. Multiple Extractions: In order to improve recovery, use two or more aliquots containing a smaller volume of elution solvent rather than a single, larger volume.



Appendices



Appendix I

Critical Factors in SPE: Guidelines for Product Selection

Although SPE provides enormous benefits, there are a number of critical factors that need to be considered prior to choosing a product. Each of these is discussed below.

1. Reproducibility

Product consistency continues to be a major dilemma for chromatographers around the world. Problems with lot-to-lot and/or column-to-column reproducibility continue to plague customers, even on well-established products. Reproducibility problems with sample preparation products (LLE, SPE, etc.) are known to account for a major loss of productivity and throughput in analytical laboratories worldwide.

In a recent survey of chromatographers, the sample-processing step was said to be the number one source of error in sample preparation and analysis (for details, see Reference 12 in Appendix III). Over 30 % of those surveyed claimed to have experienced problems with product reproducibility. More than 90 % rated batch-to-batch reproducibility as their major concern, and as the most important criteria used to select an SPE product or vendor.

Immeasurable amounts of time, labor, and money are wasted every day as methods often need to be modified and re-validated, and samples need to be re-extracted and re-analyzed. Clearly, lot-to-lot sorbent reproducibility, as well as tube-to-tube consistency, are critical factors that need to be considered prior to choosing an SPE product.

2. Performance and Extraction Efficiency

Achieving consistently high extraction efficiencies (with the reproducible, quantitative recovery of target analyte(s) and the effective removal of contaminants) is the number one goal of most sample preparation procedures. Consequently, it is also the major focus during method development and optimization studies.

Low extraction efficiency (poor sample recoveries and/or contamination with interferences) is the most frequently encountered problem in sample preparation today (Reference 12 in Appendix III). Although these problems are often associated with a lack of sorbent and/or column reproducibility, they most frequently result from sub-optimal extraction conditions (Reference 12 in Appendix III).

In a series of recent side-by-side comparisons conducted in our laboratories, we found that even published SPE methods often provide extremely low extraction efficiencies, regardless of the brand of the SPE column that was used. Subsequent method optimization experiments allowed us to optimize these methods and obtain excellent extraction efficiencies. This groundwork allowed us to develop an excellent set of generic extraction protocols that can be successfully applied to other samples in order to achieve high extraction efficiencies. Details on these protocols are provided in Section 10 of the main text of this guide.

3. Quality

SPE products from several of the major vendors undergo an extensive array of quality control test procedures. All raw materials, the bonded phase, and the final SPE tubes are lot-tested to ensure product identity, purity, performance, and conformance to strict quality specifications. This QC data is provided to customers in the form of a detailed Certificate of Analysis and comes with every box of product. These ensure lot-to-lot reproducibility and column-to-column consistency, and allow customers to verify that each new lot will perform according to their particular needs, and provide accurate, reliable results.

4. Technical Support

Most major SPE vendors provide technical support. Phenomenex provides timely and effective assistance in addressing your technical support needs. Please contact the Technical Department at the office nearest you. (See back of guide)

5. Delivery/Availability

Slow delivery and back-order problems with SPE products continue to be a major source of frustration for laboratory personnel. Phenomenex is committed to providing rapid delivery of SPE products virtually anywhere in the world.

6. Price/Economy

SPE products are extremely economical relative to LLE. In addition, volume discounts are available from most major vendors. Contact your Phenomenex representative for details.

7. Product Diversity

Several vendors offer a diverse line of products for SPE. The breadth of these product lines allows the end-users to choose from a variety of sorbent chemistries and product configurations. These products are each described and illustrated in Sections 5 and 9C. Visit www.phenomenex.com for a complete listing of available phases and formats.

APPENDIX II

SPE TROUBLESHOOTING GUIDE

This appendix contains a comprehensive SPE troubleshooting guide that can be used to resolve virtually any potential difficulty that may arise with any particular sample, analyte(s), column, or method. When used in conjunction with other guidebooks from Phenomenex for HPLC and GC (see Appendix III), these sections will allow the chromatographer to develop, optimize and solve any separation problem, regardless of its complexity, and streamline the entire analytical procedure from sample preparation through the final analysis.

The initial part of this SPE Troubleshooting Guide is separated into four separate subsections that cover the major categories of problems or "symptoms" that can be encountered in SPE. These include problems with the following:

1. *Recovery (pages 35-36),*
2. *Impurities (pages 37-38),*
3. *Reproducibility (pages 39-42), and*
4. *Flow (page 43).*

Virtually every conceivable problem encountered in SPE is covered in one of these four major subsections.

This SPE Troubleshooting Guide is laid out in the form of a table that contains information on specific dilemmas or **symptoms**, various **clues** from the experimental data, potential **problems**, potential **causes**, and finally, a series of **suggestions** for solving the problem. Virtually any SPE problem can be solved methodically by analyzing the available data for clues, identifying the problem, and consulting the SPE Troubleshooting Guide for potential causes and solutions.

Recovery

SYMPTOM	CLUE	PROBLEM	CAUSE	SOLUTIONS/SUGGESTIONS
1. Analyte recovery is less than 100 %	A. Analysis of the unretained fraction (void volume) indicates that the analyte is present in the "flow through"	1. Analyte binding is not quantitative during the sample loading step	A. Column conditioning is improper, incorrect, or not optimized	1. Condition column with methanol or isopropanol 2. Use sufficient methanol or isopropanol to wet the entire sorbent bed; allow more than two column volumes to percolate slowly under low vacuum 3. Follow the methanol or isopropanol directly with one column volume of a solution with a composition similar to the actual sample (pH adjusted) but without the analyte(s) 4. Do not use too much of this second "conditioning solvent" in (3) above, or allow it to remain in the column for too long 5. Do not over-dry the column during or after conditioning (use low vacuum for ~1 minute)
			B. Sample/matrix is in (or contains) a solvent which is "too strong"	1. Dilute the sample in a "weaker" solvent/solution to promote binding (less polar for Normal Phase, more polar for Reversed Phase, less salt, buffered and/or pH adjusted for Ion-Exchange. 2. Increase the sample dilution with a "weak" solvent 3. Load less sample 4. Increase the sorbent mass 5. Decrease the flow rate during sample loading 6. Use a "stronger" sorbent with a higher affinity for the analyte 7. Add an organic modifier (or adjust the pH) to enhance binding affinity for the analyte(s) 8. Adjust the sample pH so that the analyte is neutral for Reversed Phase or charged for Ion-Exchange 9. Add salt (5 to 10 % NaCl) to increase the solvent polarity and to enhance the retention of highly polar analytes in Reversed Phase 10. Add an ion-pair reagent to enhance binding of charged analytes in Reversed Phase
			C. Column "mass overload" (column is too small and/or the total mass of bound solutes/components is too large)	1. Decrease the volume of sample loaded 2. Increase the sorbent mass 3. Use a sorbent with higher surface area 4. Use a "stronger" sorbent 5. Decrease the flow rate during loading (to enhance "diffusion") 6. Decrease the column inner diameter but use the same sorbent mass (to increase the pressure drop, reduce flow, and enhance "diffusion") 7. Dilute the sample in a "weaker" solvent to improve capacity
			D. Flow rate is too high during the sample loading step	1. Decrease the flow rate during loading 2. Increase the sorbent mass 3. Decrease the column inner diameter to reduce flow 4. Use a sorbent with a higher surface area
			E. Sorbent is "too weak" (has poor or low affinity for the analyte relative to the sample/matrix and/or the dilution solvent	1. Switch to a "stronger" sorbent, to one with a higher ligand density, from a non-endcapped to an endcapped sorbent (or vice versa) 2. See "Solutions/Suggestions" for Cause C ("Column mass overload…"), above 3. See Solutions/Suggestions for Cause B ("Sample/matrix is in (or contains) a solvent which is "too strong"), above
			F. Conditioning solvent is "washed away" with large sample volumes	1. Add 2 % methanol or isopropanol to the sample to prevent alkyl chain collapse during the sample loading step

Recovery *(cont'd)*

SYMPTOM	CLUE	PROBLEM	CAUSE	SOLUTIONS/SUGGESTIONS
1. Analyte recovery is less than 100 %	B. Analysis of the wash fraction indicates that the missing analyte is present	1. Analyte is partially eluted during the wash step	A. Column wash step is incorrect and not optimized	1. Decrease the wash solvent "strength" 2. Decrease the wash solvent volume 3. Balance the wash solvent strength and volume (try to use 2 column volumes or more to maximize reproducibility) 4. Switch to a "stronger" sorbent with higher affinity for the analyte 5. Increase the sorbent mass 6. Dry the column thoroughly prior to the wash step
	C. Analysis of the wash and the void fractions does not reveal any analyte, but a second elution step does recover the missing analyte	1. Analyte was not fully eluted in the first elution step	A. Elution step is not optimized and the elution solvent is "too weak" and/or insufficient volume is being used	1. Increase the volume of the elution solvent, or use half the original elution volume and elute twice 2. Increase the "strength" of the elution solvent 3. Change the pH of the elution solvent, or add strong acid or base 4. Mix two or more miscible elution solvents (e.g., methanol plus acetonitrile), and/or add acid 5. Decrease the flow rate during the elution step and allow the solvent to percolate through the column 6. Decrease the sorbent mass
	D. Analysis of the wash and the un-retained fractions and the second elution/eluate does not reveal the missing analyte	1. Analyte may be irreversibly bound to the sorbent	A. Sorbent chemistry is not compatible with this analyte	1. Switch to a "weaker" sorbent or from end-capped to non-endcapped (or vice versa) 2. See "Solutions/Suggestions" 2,3,4 & 6 for Recovery Section 1C1A directly above ("Elution step is not optimized and "too weak…") 3. "Back elute" the column since the analyte may be strongly absorbed on the top of the sorbent
			B. Column drying was excessive	1. Decrease the column drying time prior to elution 2. Decrease the vacuum strength during drying
		2. Analyte may have precipitated	A. Sample was diluted in the wrong solvent	1. Centrifuge the sample prior to SPE, and analyze the supernatant for the target analyte(s); use an alternative diluent or back elute the column 2. Always warm the sample to room temperature (after storage in a refrigerator) prior to any use
		3. Entire sample was not loaded or the entire eluant was not collected	A. Non-quantitative or incomplete transfer, loss or splattering of analyte-containing sample or solvent	1. Avoid non-quantitative or incomplete transfer of the sample or eluant, pipetting errors, residual sample/solvent left in pipettes, and tubes, spills, splattering and the like (see also Reproducibility Sections 1A3E and 1A11A and B, below)
	E. The missing analyte cannot be found; all four clues above (A,B,C, and D) have been ruled out, and the method is well-established. Also a clue: HPLC peaks may be asymmetrical or fronting	1. Quantitation errors or the analytical method	A. Calculation errors	1. Check math and account for all dilution factors
			B. Integration errors	1. Reintegrate peaks, or use peak height and/or manual calculation methods
			C. Standards were not treated exactly the same as the extracts	1. Treat both standard and extract the same with respect to dilution factors, final solvent composition/mix, final volume, reconstitution, hydrolysis, derivatization, and so on
			D. Analytical method may be irreproducible or inconsistent (see also "Symptom" 3)	1. Dilute all extracts and standards in the same manner prior to analysis; for HPLC injection, dilute with a "weaker" solvent than the initial HPLC conditions, and do not overload the HPLC column 2. Troubleshoot HPLC method (or GC method) for factors that may cause poor reproducibility/quantitation (HPLC column, injection volume, detector problems, flow rate, gradient reproducibility, and check fittings or connections). Consult the Phenomenex HPLC and GC Troubleshooting Guides 3. Recovery of the analyte(s) or the internal standard is not reproducible on the HPLC or GC column or system; switch to another analytical method or column

Impurity

SYMPTOM	CLUE	PROBLEM	CAUSE	SOLUTIONS/SUGGESTIONS
1. Analyte recovery is greater than 100 % (relative & absolute recovery)	A. Analysis of the blank extracts reveals co-eluting peaks (control experiments may reveal that the true source of these impurities is the sample itself, or that they are artifacts from the sorbent, solvents, or reagents in the SPE method or from the analytical method)	1. Potential interferences are not being removed in the wash step, the loading step, or the elution step (interferences are present in the sample itself and are not artifacts from external sources)	A. Wash step is not fully optimized to remove all the impurities that were present in the sample	1. Increase the "strength" of the wash solvent. Use a series of SPE tubes in which only the blank is extracted, and in each tube, increase the "strength" of the wash until the blank is clean; check for analyte recovery 2. Increase the volume of the wash solvent (use a series of tubes as in (1), directly above) 3. Try different wash solvents or combinations of these species (e.g., isopropanol or acetonitrile vs. methanol) to selectively remove impurities 4. Modify the pH of the wash solvent to selectively remove impurities 5. Balance wash solvent "strength" and volume in order to enhance purity (see Recovery Section 1B1A3) 6. Switch to a sorbent that is more selective towards the analyte or the impurity (e.g., from C18 to PH or CN) or switch to a "weaker" sorbent 7. Decrease the sorbent mass 8. Use a second SPE column (preferably with a different chromatographic mechanism) to selectively remove the interference(s) 9. Modify the analytical method in order to resolve the impurity (e.g., change mobile phase or switch the HPLC column from C18 to PH or CN) 10. Modify the detection method or analytical wavelength to reduce the observed level of interference; use MS or MS-MS 11. Run blanks with every extraction and "subtract out" the interfering peak in order to quantitate the analyte
			B. Sample loading step is not fully optimized to prevent the retention of the impurities	1. Carefully reduce the sample/matrix dilution factor (or add a "stronger solvent") to prevent the impurities from binding in the first place, but without affecting the target analyte(s) recovery (see Impurity "Solutions/Suggestions" 1A1A1, above) 2. See Impurity "Solutions/Suggestions" 1A1A6 through 11
			C. Elution solvent is "too strong" and is also desorbing the impurities, causing their co-elution with the target analyte(s)	1. Reduce the "strength" or the volume of the elution solvent in order to selectively elute the target analyte(s) without eluting the impurities, but without affecting analyte(s) recovery (see Impurity "Solutions/Suggestions" 1A1A1) 2. See Impurity "Solutions/Suggestions" 1A1A6 through 11
		2. Potential interferences are being added by the SPE column itself (sorbent, tube, or frit)	A. SPE column (sorbent, tube or frits) is not clean	1. Wash (clean) the column with the elution solvent prior to the conditioning step 2. Switch lots or sorbent type 3. Switch elution solvents and avoid solvents and pH extremes that may generate leachates 4. Use glass tubes/columns, Teflon® frits, and/or presoaked sorbent (or presoaked polypropylene tubes and polyethylene frits)
		3. Potential interferences are being added by the SPE elution solvent and/or reconstitution solvent	A. Solvents are not clean	1. Switch to another solvent vendor, another lot or a better grade of solvent, use an alternative elution/reconstitution solvent species, or redistill the solvents 2. Prior to use, pass the impure solvent through a different SPE column that has a strong(er) affinity for the impurity

Impurity *(cont'd)*

SYMPTOM	CLUE	PROBLEM	CAUSE	SOLUTIONS/SUGGESTIONS
1. Analyte recovery is greater than 100 % (relative & absolute recovery)	A. Analysis of the blank extracts reveals co-eluting peaks (control experiments may reveal that the true source of these impurities is the sample itself, or that they are artifacts from the sorbent, solvents, or reagents in the SPE method or from the analytical method)	4. Potential interferences are being added during the hydrolysis or derivatization steps	A. Reagents are not clean and/or the reaction is not optimized	1. Optimize the hydrolysis step 2. Optimize the derivatization step 3. Determine the actual need for these steps 4. Use clean reagents 5. See "Solutions/Suggestions" under Reproducibility Sections 1A4 and 1A5
		5. Potential interferences are coming from the analytical method (the blank gradient contains the impurities)	A. HPLC or GC "column bleed" or concentration of impurities present in the initial HPLC mobile phase (solvent A)	1. Switch columns, mobile phase, gradient profile, detection method, or wavelength
2. Analyte recovery is greater than 100 % (relative recovery only)	A. Absolute recovery of the internal standard is less than 100 %, and the blanks do not contain any potential co-eluting impurities	1. Potential interferences come from other samples	A. Cross-contamination from sample/solvent splattering within the manifold, residual sample/solvent left in pipettes, or pipetting errors and the like	1. See Reproducibility Sections 1A3E and 1A11A and B
		2. Internal standard is either not bound quantitatively during sample loading, being washed off during the wash, or not fully eluted during the elution step	A. Wrong internal standard is being used	1. Switch to an internal standard with a chemical structure (or with a chromatographic behavior) similar to the analyte, or one which provides quantitative absolute recovery 2. Use an external (chromatographic) standard which is added to the elution solvent (or the eluate) and/or the dilution solvent/solution, and calculate absolute recovery
		3. Internal standard may have precipitated	A. Wrong internal standard is being used	1. Switch to an internal standard with a chemical structure (or with a chromatographic behavior) similar to the analyte, or one which provides quantitative absolute recovery 2. Use an external (chromatographic) standard which is added to the elution solvent (or the eluate) and/or the dilution solvent/solution, and calculate absolute recovery
		4. Internal standard may be irreversibly bound to the sorbent	A. Wrong internal standard is being used	1. Switch to an internal standard with a chemical structure (or with a chromatographic behavior) similar to the analyte, or one which provides quantitative absolute recovery 2. Use an external (chromatographic) standard which is added to the elution solvent (or the eluate) and/or the dilution solvent/solution, and calculate absolute recovery
		5. Quantitation errors or the analytical method	A. Analytical method is not reproducible, or there are calculation or integration errors	1. See Recovery Section 1E1 2. Consult the Phenomenex HPLC and GC Troubleshooting Guides

Reproducibility

SYMPTOM	CLUE	PROBLEM	CAUSE	SOLUTIONS/SUGGESTIONS
1. Analyte relative recovery is not reproducible/ consistent (may be less than or greater than 100 %)	A. Absolute recovery of the internal standard and/or the analyte may be less than 100 %	1. Any of those (listed under Impurity “Symptoms” 1 and 2) that are related to the SPE method	A. Any of those (listed under Impurity “Symptoms” 1 and 2); and in particular, any extraction con-ditions that are “too strong” or “on the border-line” or are not run consistently so as to cause partial or variable binding, washing, or elution of the analyte or the internal standard	1. Any of those listed under Impurity “Symptoms” 1 and 2, including those related to the quality and/or consistency of execution of the SPE method; in particular, problems with the following: • the conditioning step • the binding affinity is low • failure to dilute the sample properly/ sufficiently • excessively rapid flow rates • incorrect sorbent • column overload • the wash is “too strong” • the elution solvent is “too weak” • precipitation • irreversible binding • cross-contamination
		2. Any of those (listed in the Recovery Section 1E1) that are related to the analytical method	A. Any of those (listed in the Recovery Section 1E1) that are related to the analytical method	1. See Recovery Section 1E1 (A, B, C and D); in particular, the inconsistent treatment of the sample and the standards in terms of dilution and solvent composition prior to analysis, any quantitation, integration, or calculation errors or any of the problems listed in the Phenomenex GC or HPLC Troubleshooting Guides
		3. Eluant dry down (evaporation step) and reconstitution	A. Reconstitution volume is too small	1. Add more reconstitution solvent 2. Vortex the tube
			B. Reconstitution solvent does not solubilize/dissolve all of the analyte(s) or the internal standard (IS)	1. Use a reconstitution solvent in which the analyte(s) and the internal standard(s) are fully soluble 2. Use a larger volume of reconstitution solvent and vortex the tube 3. Add the reconstitution solvent down the sides of the test tube
			C. Reconstitution volume is incon-sistent, and the analytical method is not linear for the IS or the analyte(s)	1. Use a more consistent (and/or larger) volume of reconstitution solvent and graduated col-lection tubes, and use a standard curve(s) to calculate recovery(s)
			D. Analyte or internal standard (IS) volatility	1. Avoid any dry-down/evaporation step alto-gether 2. Use a more volatile reconstitution solvent 3. Reduce the temperature and/or gas flow 4. Use He instead of N_2 or air 5. Add water and/or a non-volatile acid or base to the eluent prior to dry down in order to reduce volatility and loss
			E. Splattering and drying of the sample on the walls of the tube and incomplete reconstitution	1. Turn down the gas flow during the dry down step 2. Increase the volume of reconstitution solvent 3. Vortex well following reconstitution 4. Add reconstitution solvent down the sides of the test tube
		4. Sample hydrolysis step	A. Hydrolysis is incomplete or irreproducible, may decompose the native analyte or the internal standard (IS), or adds impurities	1. Determine the need for a hydrolysis step in the first place using a conjugated standard or using side-by-side analyses of authentic (real life metabolic) samples with and without the hydrolysis step 2. Consult standard protocols and troubleshoot-ing guides for these reagents/protocols 3. Reagent may have degraded – order fresh chemicals 4. Optimize the reaction conditions (time, tem-perature, reagent concentration, pH, solvent composition, and so on) 5. Adjust sample/reaction solvent composition prior to SPE 6. Reagent being used may be unsuitable for this reaction and/or analyte/conjugate (or the IS)

Reproducibility *(cont'd)*

SYMPTOM	CLUE	PROBLEM	CAUSE	SOLUTIONS/SUGGESTIONS
1. Analyte relative recovery is not reproducible/ consistent (may be less than or greater than 100 %)	A. Absolute recovery of the internal standard and/or the analyte may be less than 100 %	5. Derivatization step	A. Derivatization is incomplete or ir-reproducible either for the analyte or the internal standard (IS)	1. Determine the need for a derivatization step in the first place; compare the linearity and the sensitivity of the analytical method with and without derivatization (also compare the levels of co-eluted impurities) 2. Consult standard protocols (from manufactur-ers of the reagents) and/or troubleshooting guides for these procedures 3. Reagent may have degraded – order fresh chemicals 4. Optimize the reaction conditions – time, tem-perature, reagent concentration, pH, solvent composition, etc. 5. Adjust sample/reaction solvent composition after the SPE step or reconstitute in the proper solvent for the reaction 6. Make sure to remove trace amounts of water prior to the reaction 7. Reagent being used may be unsuitable for this reaction and/or analyte or IS; switch reagents 8. Too many impurities may be present for the reaction to provide quantitative derivatiza-tion and a clean baseline; optimize the SPE method 9. Reagent is being lost due to evaporation; keep the reaction mix closed in a reaction vial
		6. Analyte or IS degradation or reaction	A. Analyte and/or internal standard (IS) are reactive un-der the extraction conditions being used	1. Modify the extraction conditions 2. Switch to another sorbent 3. Reduce the temperature or extraction time or take other steps to prevent analyte degrada-tion or reaction 4. Switch to another internal standard (IS)
		7. Operator technical error or other prob-lems not related with the extraction method itself	A. Human error or poor technique, poor calibration of instruments, calculation errors, or improper preparation of buf-fers, solvent and/or reagents	1. Repeat the procedure in the presence of an SPE expert or someone well-versed in the procedure, particularly since the finer points of the method may not have been spelled out precisely in the published procedure 2. Check pipettes, balances, and pH meters for calibration, consistency, and accuracy 3. Check all calculations 4. Make fresh buffers, reagents and solvent mixtures 5. Avoid mixing up samples, and prelabel all tubes, vials, and columns; maintain sample identity 6. Avoid the cross-contamination of samples (see Reproducibility Section 1A11) 7. Avoid the use of cold samples or hastily mixed/diluted samples in which the analyte or internal standard may have precipitated
		8. SPE column or sorbent	A. Poor lot-to-lot sor-bent reproducibility (bonding)	1. Obtain more of the "good lot" of sorbent from the manufacturer 2. "Lot-select" to find another "good lot" 3. Demand reproducibility
			B. Poor lot-to-lot col-umn reproducibility (packing density, sorbent mass, flow rate, etc.)	1. Obtain additional columns from the same production lot (manufacturing lot) of columns 2. "Lot select" to find another good lot 3. Demand reproducibility
		9. Solvent immiscibility	A. Column is not be-ing dried properly prior to using an-other (immiscible) solvent	1. Dry column thoroughly prior to adding an immiscible solvent 2. Use an alternative solvent that is miscible

Reproducibility *(cont'd)*

SYMPTOM	CLUE	PROBLEM	CAUSE	SOLUTIONS/SUGGESTIONS
1. Analyte relative recovery is not reproducible/ consistent (may be less than or greater than 100 %)	A. Absolute recovery of the internal standard and/or the analyte may be less than 100 %	10. Flow Rate	A. Excessive flow rate used during load, wash or elution, or erratic flow from experiment to experiment	1. Reduce the vacuum (or pressure) on the SPE manifold in order to reduce the flow rate 2. Analyze the void, wash and elution fractions (as well as a second eluant) in order to identify which steps require attention with regards to the flow rate 3. During the elution step, allow the solvent to slowly percolate through the column before the vacuum is applied 4. In ion-exchange applications, use particularly slow flow rates during the loading step in order to avoid "split peaks"/"flow through" 5. Always use the same vacuum or pressure setting and the same number of SPE columns on the manifold during each run/extraction/ experiment in order to ensure that the flow rate is constant from run to run 6. Check to make sure that some columns are not plugged or flowing more slowly than others; take the preventive actions described in Section Flow Rate 1B
		11. Cross-contamination from one sample or standard to another	A. Non-SPE related cross-contamination of samples due to insufficient cleaning of pipettes, pipette tips, syringes, glassware, and so on	1. Use clean pipettes and pipette tips for each sample or wash, flush and evacuate pipettes and syringes in between samples with a solvent or buffer which is of similar composition to the sample 2. Avoid using standards at concentrations that are more than double (twice) those that are expected to be found within the actual samples 3. Keep all samples, blanks, and standards somewhat segregated and separate from one another 4. Attempt to arrange samples in order of lowest to highest predicted analyte concentration
			B. Excessive vacuum or rapid loss, interruption or release of vacuum causes sample splattering, or the ejection of the eluant from the collection tube	1. Reduce the vacuum 2. Use positive pressure, particularly for biological samples or those containing proteins or surfactants in order to avoid or minimize froathing/foaming and splattering from the manifold needles 3. Release the vacuum slowly following the elution step using the vacuum gauge controller 4. Prior to releasing the vacuum, do not remove any columns from the manifold, break any fittings, detach the vacuum hose/line, or turn off the pump 5. Use long/tall eluant collection tubes in order to avoid splattering and loss of the eluate and cross-contamination 6. Use long manifold port needles in order to avoid splattering, loss of eluate, and cross-contamination 7. Clean the manifold port needles prior to the elution step in order to avoid contaminating the eluate with any of the original sample or wash solvent that may remain behind (particularly for proteinaceous samples or samples/solvents that contain surfactants or ion-pair reagents) 8. Wipe or clean the manifold port needles in solvent following the elution step in order to avoid the cross-contamination of future samples 9. Clean, flush and dry the manifold ports with solvent to avoid cross-contamination of future samples 10. Vortex the collection tube in order to dissolve any splattered eluant, and/or add (slowly down the sides of the tube) additional solvent or any diluant required to make the eluant

Reproducibility *(cont'd)*

SYMPTOM	CLUE	PROBLEM	CAUSE	SOLUTIONS/SUGGESTIONS
1. Analyte relative recovery is not reproducible/ consistent (may be less than or greater than 100 %)	A. Absolute recovery of the internal standard and/or the analyte may be less than 100 %	12. Inconsistent volume of final eluate	A. Inconsistent volume of elution solvent added to column	1. Add a consistent volume of elution solvent to each column 2. Use a larger volume of elution solvent than is required for full recovery of the analyte(s) 3. Use graduated eluant collection vials 4. Bring all samples up to a consistent final volume with elution solvent 5. Add an external (chromatographic) standard to the elution solvent and the diluant, and calculate absolute analyte recovery
			B. Incomplete drying of the column prior to the elution step increases the volume of the final eluate and causes the elution solvent to be “less strong”	1. Dry the column fully following the wash step 2. Use an excess volume of elution solvent, use graduated eluate collection vials, bring all samples to a consistent final volume and use an external standard (as in section 1A12A directly above)

Flow Rate

SYMPTOM	CLUE	PROBLEM	CAUSE	SOLUTIONS/SUGGESTIONS
1. Flow rate is slow, stopped or inconsistent	A. Little or no flow out of the bottom of the tubes, and even the conditioning solvent enters the sorbent bed slowly or does not flow at all	1. Vacuum is inadequate	A. Vacuum settings are wrong, pump is inadequate, or lines are clogged or collapsed	1. Increase the vacuum settings on the vacuum gauge/controller 2. Use a stronger vacuum pump or switch to a positive pressure manifold 3. Check for leaks in the manifold or the tubing and look for collapsed vacuum tubing 4. Sample/solvent waste line or the exit valve may be clogged (or simply closed) causing liquid waste to fill the manifold and back up into the vacuum line and the pump; remove the liquid waste from the manifold, insert a trap (erlenmeyer flask) in between the manifold and the vacuum pump, and clean out the vacuum pump and line
			B. Manifold settings or seals and vacuum integrity	1. Close all of the ports that do not have columns or that are not being used 2. Check the integrity of the manifold and whether it is able to hold a vacuum; seal any leaks or replace the manifold
		2. SPE column packing	A. Tubes are packed too tightly	1. Switch to a wider column size with a larger frit and a lower pressure drop 2. Use tubes from another production lot 3. Hand pack or repack all of the tubes 4. Check the flow of each column during the conditioning step; discard bad columns 5. Use fewer columns on the manifold and close any unused ports
	B. Little or no flow out of the bottom of the tubes, but only after the sample has been loaded	1. SPE column is clogged	A. Sample contains too many particulates	1. Remove particulates by centrifugation or filtration (on an inert filter) 2. Sonicate the sample in a warm water bath 3. Dilute the sample in a "weak" solvent in which the particulates are soluble 4. Load less sample
			B. Sample viscosity is too high	1. Dilute the sample in a "weak" solvent/solution that has a low viscosity when combined with the sample/matrix 2. Increase the sample dilution factor 3. Warm the sample in a water bath 4. Avoid solvent combinations that increase viscosity (e.g. methanol:water)
			C. Sample-solvent immiscibility and/or salt precipitation	1. Avoid diluting the sample in a solvent in which it is not miscible, or using immiscible solvents in consecutive loadings/washes in particular, avoid mixing aqueous samples/solutions of high ionic strength directly with water-immiscible organic solvents 2. First dilute the sample in an intermediate solvent in which it and the final diluant are fully miscible before adding the final diluant (e.g., aqueous samples should be diluted in THF and then in hexane prior to normal phase SPE) 3. Increase the sample dilution factor and load less sample 4. Warm the sample in a water bath 5. Dry the column thoroughly before adding an immiscible solvent
			D. Solute or protein precipitation	1. Avoid diluting the sample in a solvent/solution in which it is not soluble 2. Avoid solvents or pH extremes that denature proteins and cause protein precipitation (e.g., strong acid, acetonitrile, extremely low ionic strength solutions [<0.01 M], high concentrations of $ZnCl_2$ or Na_2SO_4, etc) 3. Dry the column thoroughly after the conditioning step and after the loading step 4. Use all samples, buffers and solvents at room temperature 5. "Wash" the column with an initial low ionic strength buffer (e.g., 0.1 M) prior to loading the actual wash solvent

APPENDIX III

Literature References and Suggested Reading

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APPENDIX IV

SPE USERS GUIDE GLOSSARY

Absolute Recovery	Quantitative method for the determination of the actual amount of analyte captured and retrieved during the extraction process. Analyte recovery is based on an external standard that is added to the eluant at the end of the extraction procedure (rather than being based on an internal standard that is co-extracted with the analyte).
Analyte	The target compound(s) that needs to be extracted, analyzed and quantitated. Often referred to as the target analyte(s).
Anion-Exchanger	A positively charged sorbent that retains anions.
Bed Volume	The sum of the interstitial volume plus the pore volume of the sorbent within the column. Roughly synonymous with the terms void volume and dead volume.
Bonded Phase	A chromatographic support or sorbent in which an organic functional group is covalently bonded to the silica surface.
Breakthrough	Lack of analyte retention that occurs when the total mass of the solutes (analyte plus interferences) that are present in the sample loading step exceeds the capacity of the sorbent, or in situations in which the loading conditions are not properly optimized.
Buffer	Aqueous solutions that efficiently resist and prevent major changes in pH following the addition of acid or base; this is due to the presence of either a weak acid and its conjugate base or a weak base and its conjugate acid that have pK_a values near the target pH.
Capacity	The total mass of solutes (target analytes plus interferences) that a given sorbent mass can retain under a specific set of loading conditions. More generally, the maximum amount of sample/matrix that can be loaded without decreasing the recovery of the target analyte(s).
Cation-Exchanger	A negatively charged sorbent that retains cations.
Chromatography	Techniques in which sample components (analytes and contaminants) are separated as a result of differential intermolecular interactions with a stationary phase in the presence of a mobile phase that flows through that sorbent.
Counter Ion	The ionic species that pairs or associates with the ionic functional group of opposite charge which is covalently attached to the surface of an ion-exchange sorbent. In order to be retained, a charged analyte must displace the counter ion associated with the ion-exchange group.
Dipole-Dipole Interactions	The interaction between polar molecules with a permanent dipole in which the positively charged portion of one dipole molecule is electrostatically attracted to the negatively charged portion of another dipole molecule. A molecule has a dipole moment when the bonding electron pair is displaced toward the more electronegative atom in the bond; molecules with this asymmetric distribution of charge are considered to be polar. The dipole moment is a measure of this polarity and is the sum of all the individual bond polarities. The magnitude of these forces is relatively strong (~5 kcal/mole).
Dispersion (London, van der Waals) Forces	Short range intermolecular interactions which occur when two molecules are so close that the temporary dipoles caused by electronic vibrations in one molecule induce dipoles in neighboring molecules. These interactions are relatively weak (~1 kcal/mole) and increase in proportion to the molecular weight.

Term	Definition
Eluate	The elution solvent plus any analytes and interference which are eluted from the column. The eluent after it passes through the column.
Eluent	The solvent used for elution. Synonymous with the terms elution solvent and mobile phase.
Eluotropic Series	Arrangement of common solvents in order of increasing strength for the elution of analytes from a particular sorbent. The eluotropic strength of a solvent is represented by ε_0.
Elution Volume	The volume of solvent used to elute the analyte or the volume of elution solvent required to elute the analyte with quantitative (~100 %) recovery.
Elution	The desorption of the target analyte from the sorbent which can be brought about by changing the solvent composition in order to disrupt the interactions between the solutes and the stationary phase.
Emulsion	A suspension of globules or micelles of one liquid in a second liquid, which will not mix.
Endcapping	The reaction of small organosilanes such as TMCS with unreacted, free silanols that is designed to reduce polar and ionic secondary interactions.
Extraction	The transfer of the analyte from one phase to another. Includes SPE, LLE and other related techniques.
Free Silanols	Unreacted silanol groups that remain on the silica surface after the primary bonding reaction.
Frit	Porous materials (typically polyethylene) used to retain and confine the sorbent in the SPE column.
Hydrogen Bonding	Intermolecular interactions between a hydrogen atom covalently bonded to an electronegative atom and a nonbonding electron pair on an electronegative atom (such as F, Cl, N, O, or S) in another molecule. Hydrogen attached to a carbon atom such as in HCN, acetylene or chloroform may have hydrogen bonds also. The magnitude of the hydrogen bond is relatively strong (~ 5 kcal/mole).
Hydrophilic	Molecules that are rich in polar functional groups, and that tend to be extremely soluble in water. Literally, “water-loving” and synonymous with the terms polar, lipophobic, and to some extent, normal phase.
Hydrophobic	Moderately to highly non-polar molecules that have a large proportion of unsubstituted alkyl or aromatic groups (hydrocarbonaceous) with a low portion of polar functional groups, which tend to be highly soluble in organic solvents. Literally, “water-hating” and synonymous and interchangeable with the terms non-polar, lipophilic, and to some extent, reversed phase.
Immiscible	Solvents that are insoluble in one another, (except at extremely low concentrations) which form a two phase system when mixed.
Interference	A substance in the sample or separation system which may influence the retention of the analyte on the sorbent or may be co-eluted with the analyte and influence the process of analysis and quantitation.
Intermolecular Forces	The attraction or repulsion that occurs between two molecules in close proximity as a result of positive and negative regions in the molecules. Examples include dipole-dipole interactions, dispersion forces, and hydrogen bonding.

Term	Definition
Ion-Exchange	A chromatographic mode in which ions or charged molecules are retained by oppositely charged groups that are covalently bonded to the stationary phase. Analyte ions are retained by displacing the counter ions associated with the bonded functional group.
Ionic Strength	The total concentration of a particular ion or salt in a solution. Typically expressed in units of molarity (M) or millimolarity (mM).
Liquid-Liquid Extraction/LLE	Sample preparation technique in which an immiscible liquid is mixed with the sample/matrix in order to selectively partition the target analyte(s) into one phase, while the contaminants partition into the other phase.
Luer Tip	A standardized adapter such as that on the tip of a syringe barrel that is available in both male and female configurations in order to allow the connection of one device with another.
Miscible	Solvents that are soluble in one another, and can be mixed in any ratio.
Non-polar Molecule	A molecule with a symmetric distribution of charge. Typically used as a more generic term to include even moderately polar molecules and sorbents that may, in fact, not be totally symmetrical in terms of their charge distribution.
Normal Phase	Extraction mode relying upon intermolecular hydrogen bonding and polar dipole-dipole interactions for retention in which moderately polar to polar molecules are extracted from a non-polar solvent using a polar sorbent. Synonymous with polar phase and hydrophilic phase.
pH	Negative log of the hydrogen ion concentration in an aqeuous solution. SPE extractions are normally performed in a pH range of 1 to 12.
pK_a	The pH at which fifty percent of the ionizable groups on a molecule are ionized and fifty percent are uncharged and neutralized. Adjustment of the pH of a solution containing an acid to two pH units above the pK_a results in approximately 99 % ionization.
Polar Molecule	Molecules with an unsymmetric distribution of charge.
Polarity Index	Classification of solvents based on their ability to solvate analytes and act as a proton donor, proton acceptor, or a dipole.
Pore Size	The average diameter of the porous openings on the surface of a sorbent particle.
Relative Recovery	Indirect determination of analyte capture and retrieval during the extraction process based on the recovery of an internal standard (which is a compound with a structure similar to the target analyte) that is added to the crude sample/matrix prior to the actual extraction procedure.
Resin	Common terminology used for polymer-based sorbents such as SDB.
Reversed Phase	Extraction mode relying upon intermolecular van der Waals, London, or dispersion forces for retention in which non-polar to moderately polar analytes are extracted from a polar (aqueous) solution using a non-polar sorbent. Synonymous and used interchangeably with the terms non-polar phase and hydrophobic phase.

Sample Preparation	A broad and diverse group of laboratory techniques performed on a sample that are designed to remove matrix interferences, concentrate and/or solubilize the analyte, and make the sample more amenable to chromatographic analysis by HPLC or GC. Often synonymous with SPE.
Sample/Matrix	The crude solution or material that contains the target analyte(s) which is to be analyzed or extracted, or more specifically, the actual composition of the sample.
Selectivity	The degree of chromatographic discrimination obtained between one chemical species (usually the target analyte) and others (usually contaminants). The selectivity of the sorbent can refer to the ability to retain only the target analyte(s), or the degree of purity achieved as a result of the extraction.
Solid Phase Extraction/SPE	Sample preparation technique in which specific intermolecular interactions between a solid stationary phase and the analyte results in the removal of contaminants and the concentration of the analyte.
Sorbent	Silica-based bonded phases, polymers or adsorbents used as a stationary phase in solid phase extraction.
Specificity	Selective interactions between a specific analyte and the sorbent, or the differential retention behavior provided by different sorbents of the same class.
Split Peak	Breakthrough or failure of some or all of the target analyte(s) to bind to the column (SPE or HPLC), resulting in a retained peak as well as one in the void or unretained fraction.
Stationary Phase	The immobilized sorbent through which the sample solution, mobile phase, or solvent passes.
Surface Area	The sum of the external surface area of a sorbent particle plus the accessible surface area within the pores (m^2/g).
Surface Coverage	A measure of the extent of bonding of the available silanols on a sorbent surface ($\mu g/m^2$).

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