Empore™

96-Well Solid Phase Extraction Plates

Method Optimization Guide

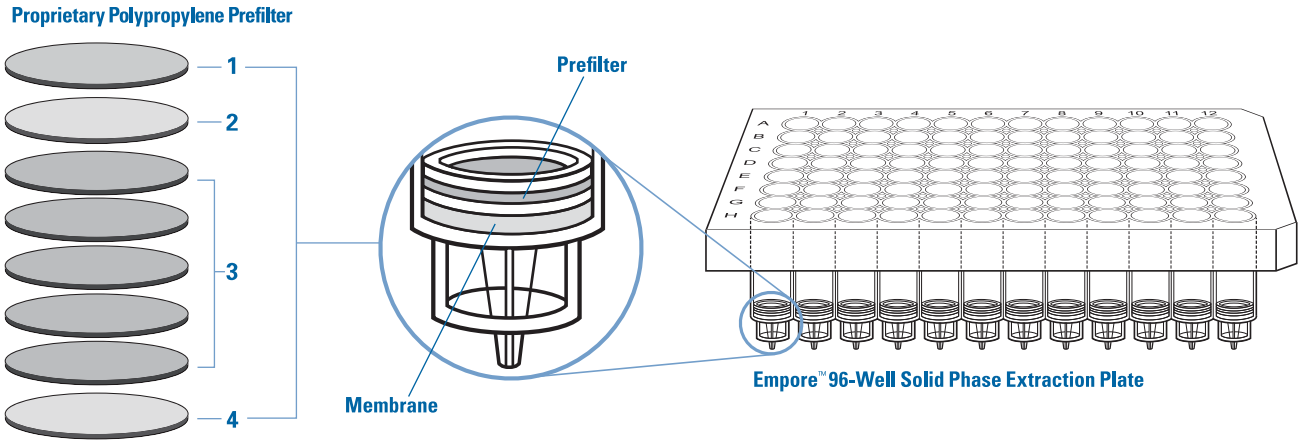
Introduction

A reversed phase solid phase extraction (SPE) method can be designed using Empore™ 96-Well Solid Phase Extraction Plates by following the recommendation in the accompanying example. The small bed mass of sorbent in Empore 96-Well Solid Phase Extraction Plates allows for the use of smaller solvent volumes than with traditional packed bed SPE products. However, note that each assay may benefit from further optimization and the guidelines in the example are meant to be only general in nature. The specific physical and chemical characteristics of the analyte, sorbent and solvents need to be considered. This document will address each step of the extraction process (condition, sample load, wash and elution steps) and provide conceptual approaches for optimization. The 96-well plate format is ideal for examining multiple parameters within a single plate and can reduce the time required for method optimization.

Example of SPE Method Using C18 or C8 Disk Plates

Condition 100 µL Methanol 200 µL Water
Load 96 Samples into Disk Plate
Wash 500 µL Water 500 µL Methanol/Water (10/90)
Elute 100-150 µL Organic Solvent Dilute with Aqueous Component* (Commonly 1-3 times volume of organic used)
Direct Inject Aliquot LC/MS/MS

* Resulting mixture is compatible with mobile phase for direct injection



CDS Analytical

Condition Step

Solvents

Conditioning the disk before use can be effectively performed with methanol, isopropanol, or acetonitrile. It may be more convenient to condition with the elution solvent (if 100% organic) to eliminate a reagent reservoir. Water is used as the second conditioning reagent to displace the organic solvent from the disk prior to loading the sample.

Conditioning Tips

- Wet disk with 75-100 µL methanol to avoid using a vacuum step before adding water
- Allow methanol to soak into disk for 15 sec
- Remove excess methanol with 200 µL water or greater
- Activate vacuum briefly to process water through disk

Volumes and Vacuum Steps

Optimization of the conditioning step involves using the smallest effective volumes of methanol and water, and examining the effects on analyte recovery and precision. A volume of 100 µL methanol is suggested for effective conditioning. Use of greater than 100 µL is not detrimental in any way and only affects throughput, since it takes slightly longer to process 500 µL than 100 µL. Vacuum is traditionally used to process the methanol through the disk without letting the disk dry out. A volume of water approximately twice the volume of methanol used (200 µL water in this example) is suggested to displace the methanol and adjust the sorbent bed to an aqueous environment. Vacuum is used to process the water through the disk. In some cases, a buffer solution can be used after water addition, or instead of water, to prepare the sorbent bed at the optimal pH for sample loading.

Note that vacuum is not always necessary when only 100 µL conditioning solvent is used, since the methanol soaks into the prefilter and sorbent bed, and does not adversely mix with the subsequent water addition. However, vacuum is suggested when volumes greater than 100 µL are used.

Advanced Technique

An advanced conditioning step that has been successfully used in some analyte extractions involves conditioning the sorbent bed with 75 to 100 µL methanol (without vacuum), allowing the solvent to soak into the disk for at least 15 sec, then adding the sample directly to the disk. At this point, vacuum is applied for the first time and the sample is processed through the disk. Proper optimization, if this technique is tried, will involve examining the effect of this advanced technique on analyte recovery and precision in comparison with a traditional conditioning technique. ***Extreme caution must be taken to ensure that the sample does not contact the organic conditioning solvent above the membrane as this may result in precipitation of protein and challenges to flow.***

Sample Load Step

Pretreatment of Sample

Traditionally, solid phase extraction methods have included a full range of sample pretreatment options, e.g., buffer addition for pH adjustment or disruption of protein-bound analyte. Within a 96-well format, any sample pretreatment resulting in a "cleaner" matrix will yield improved performance in terms of flow properties through the disk. When developing automated methods for large numbers of samples, pretreatment consideration may yield significant gains in throughput.

Choice of Anticoagulant

Some users have reported that the choice of anticoagulant used may influence sample flow properties during the extraction process. Through repeated freeze-thaw cycles, samples containing heparin may more readily produce precipitation that adversely affects flow properties through the sorbent bed. An alternative choice, such as EDTA, may provide improved flow results. Note that the choice of anticoagulant must be made prior to sample collection.

Dilution

Undiluted plasma or serum may be processed through the plate depending on volume. It is generally suggested that a sample be diluted with an aqueous solution (water or buffer) to reduce the viscosity of the sample matrix and improve extraction flow characteristics. When buffer is used as the diluting solution, it also helps maintain the optimal pH of the sample analyte for efficient adsorption to the sorbent. Common dilution ratios reported by users range from 10% to 300% of sample volume, but will ultimately depend on the sample volume required to meet assay detection limits.

Note that subject variability of plasma and serum makes it impossible to ensure that flow may be visibly improved in all cases by using this technique.

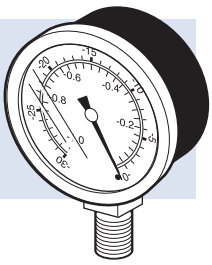
Vacuum Considerations

Optimization of the sample load step should include evaluation of different vacuum pressures (corresponding sample flow rates) and their effect on analyte recovery. Load the sample matrix at both a low vacuum (5-7 in Hg; 0.17-0.24 bar) and a high vacuum (about 17-20 in Hg; 0.58-0.68 bar) and examine the effect on analyte recovery. Greater throughput will be achieved when loading sample at a high vacuum or pressure.

Note that if an analyte has a low affinity for the sorbent, it may need to pass through the sorbent bed more slowly during the load step for sufficient attraction to occur. In this case, perhaps another sorbent chemistry may be more optimal.

Sample Load Tip

Load the sample matrix at both a low vacuum (5-7 in Hg; 0.17-0.24 bar) and a high vacuum (about 17-20 in Hg; 0.58-0.68 bar) and examine the effect on analyte recovery.



Wash Step

Objective

The goal of the wash step after sample loading is to remove co-extracted substances that could potentially interfere with the subsequent analysis.

Aqueous Wash

Water and buffers are commonly used as wash solvents for reversed-phase extractions. They are effective at removing adsorbed proteins and salts remaining on the surface of the sorbent bed. **It is recommended that an aqueous wash step always be used after sample loading**, rather than using only an organic/aqueous mixture.

Secondary Wash

Water or buffer alone may not provide sufficient clean-up in each assay. The second wash should contain a small percentage of organic solvent (5 to 50% in aqueous) to more efficiently remove potential interfering substances. Note that the secondary wash should be chosen so that it removes as many interfering substances as possible without adversely affecting retention of the analyte(s) of interest.

Two Wash Steps Are Recommended

500 µL Water
500 µL Organic/Water Mixture

These two wash steps together are of critical importance in ensuring the performance of the membrane and the usability of the final eluate.

Importance of Two Wash Steps

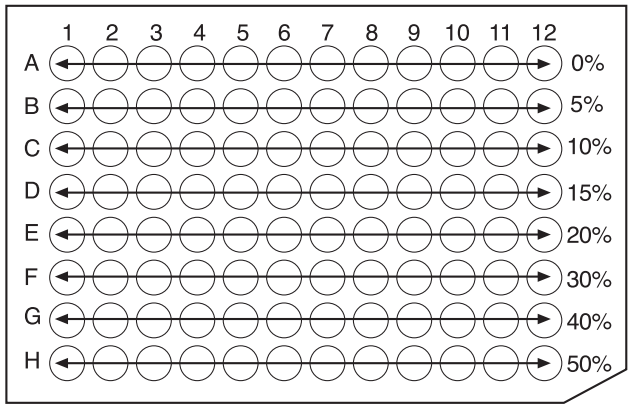
These two wash steps together are of critical importance in ensuring the performance of the membrane and the usability of the final eluate. If an aqueous wash step is not used, residual proteins may not be fully removed. These proteins may precipitate during a low percent organic wash and reduce flow through the disk. Also, if protein is not adequately removed before elution, the eluate may be contaminated with residual protein that may precipitate with organic. These proteins can gradually build up on the LC column over time and raise the operating pressure.

Optimization

The varying percentages of organic content in the second wash can influence analyte recovery. This effect can be evaluated using one 96-well disk plate. Multiple wash solutions can be processed simultaneously through **different** columns or rows of the plate. The analyte recoveries are determined for each variable.

Optimize the Secondary Wash Step

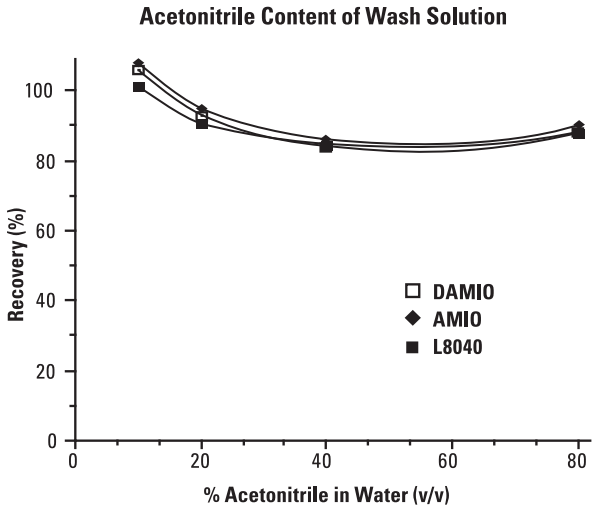
1. Use varying percentages of organic in water, delivered at a constant volume (e.g., 0, 5, 10, 15, 20, 30, 40, 50% organic in water delivered at 500 µL)
2. Determine recovery from each replicate
3. Plot mean analyte recovery vs. percent organic in water wash solution



Example

An example of wash solution optimization is shown below for the antiarrhythmic drug amiodarone (AMIO), its desmethyl metabolite (DAMIO), and internal standard (L8040), extracted from serum using a C8-SD Empore disk. The wash volume was delivered at 500 µL for each determination.

In this example, all three drugs could tolerate 10% acetonitrile in the wash solution without adversely affecting recovery. At 20% organic, minimal drug loss occurred which was acceptable because the resulting chromatography was much cleaner than at 10% organic. However, at 40% organic, the recovery of all analytes dropped off significantly. Thus, for this assay, 20% acetonitrile in water was considered optimal as it balanced minimal drug loss with improved chromatograph cleanliness.



Elution Step

General Considerations

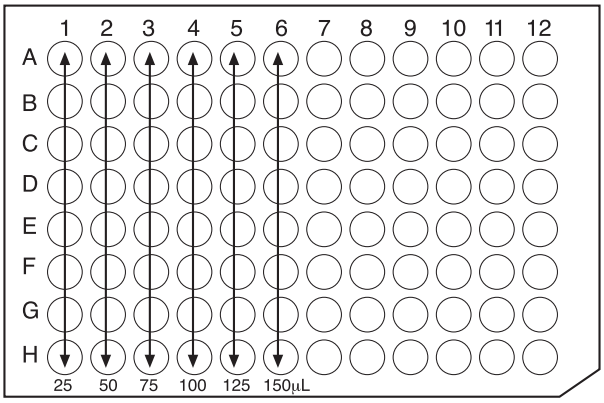
The small bed volume of the disk allows for efficient desorption, or removal, of retained analytes in a small volume of elution solvent. Optimization of the volume required for elution is important to ensure that an analyte is efficiently removed from the sorbent bed using the smallest volume that will yield reproducible recoveries. *The volume required for elution will depend on the particular analyte, its affinity for the sorbent, and the strength of the eluting solvent used to remove it from the sorbent bed.* Thus, it is important to optimize this volume for each individual assay.

Optimization

The 96-well microtiter plate format allows different elution volumes to be examined across rows or columns within the same plate, making it convenient to optimize the elution step for an assay in a single experiment. A graph of drug recovery vs. elution volume provides the necessary information to define the optimal elution conditions (solvent volume, concentration of analyte eluted in each fraction, and the required number of aliquots). Ultimately, it is the decision of the analyst to prioritize these aspects of the assay to be optimized.

Example

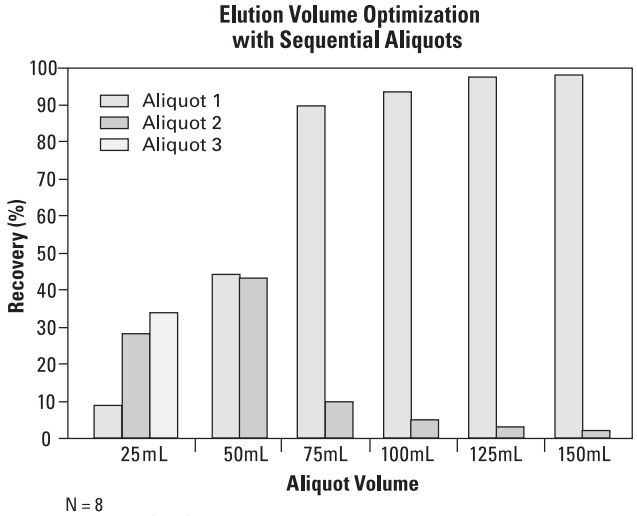
As an example, consider the elution of diazepam from C18-SD disk plates using methanol as the elution solvent. The following volumes of methanol are added to the plate in columns one through six: 25, 50, 75, 100, 125 and 150 µL, and vacuum is applied. The replicate samples in a column (n=8) are collected and analyzed for recovery. To determine if analyte remains on the disk following a given elution volume, a second elution from the same wells of the same plate is performed using the scheme above. If desired, a third elution can be performed. Note that separate collection plates are used for each of the elution steps.



Elution Step (continued)

Interpreting Results

The elution data show that recovery of diazepam can be achieved with as little as 25 µL, though working with such small volumes may prove difficult. This small volume also requires three sequential elutions to obtain maximal recovery, creating additional processing time. Elution using 50 µL or 75 µL aliquots is more practical, both yielding nearly complete recovery after only two aliquots. While total recovery is similar between 2 x 50 µL and 2 x 75 µL, a comparison of the first aliquots from each approach shows that elution with 75 µL yields a more concentrated fraction than does elution with 50 µL.

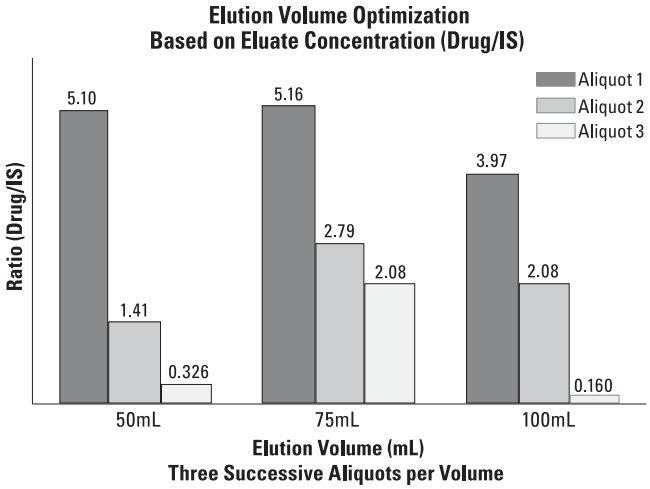


N = 8
Diazepam; C18-SD Disk Plate; Methanol Elution

Achieving Assay Objectives

In some cases it may be preferable to use only the first fraction (75 µL), the most concentrated one, and dilute that volume with aqueous solution for direct injection. Such an approach allows more mass injected on column for a given injection volume, achieving greater sensitivity. Additional consideration needs to be given to the concentration of analyte in a given volume as well as the importance of internal standard (IS) recoveries and ratios. While this approach can be assay and analyte specific, in some cases a 50% recovery may be sufficient for a successful extraction when LC/MS is used for analytical detection.

In the example below, a single 75 µL elution yields the greatest ratio (drug/IS) of 5.16, while elution volumes of 50 µL and 100 µL give lower ratios of 5.10 and 3.97, respectively. The 75 µL elution volume best meets the objectives for this assay, and is diluted with aqueous solution for direct injection. By performing such experiments early in the method development process, elution optimization can improve performance for each assay.



Automation Considerations

While all of these experiments may be effectively evaluated manually, Empore Extraction Disk Plates are most commonly utilized with a variety of automated liquid handling equipment. All of the designed experiments discussed here may be stored as templates and applied to future assays for rapid method optimization studies.

Multiple Dispensing

A consideration of multiple dispensing options should be made. Use of multiple pipetting features can reduce the time required to process a plate using an automated workstation.

It is important to ensure that volumes utilized are properly configured for the maximum volume of liquid pipettable on the system. For example, use of a 1 mL syringe on the system to deliver 200 µL volumes of water during the conditioning step allows for four aliquots of 200 µL to be delivered to sample wells before being refilled from the reagent reservoir. In this example, the number of cycles is limited to four (instead of five) due to required volumes necessary for aspiration and transfer air gaps.

Unique features of individual workstations (i.e., stacking reagents with air gaps between them) may also be considered for their impact on throughput. For example, some users have reported a water conditioning step may be omitted by loading methanol and a small sample volume directly onto the sorbent bed in rapid succession. The vacuum is turned on prior to this solvent delivery scheme.

Conclusions

The Empore™ 96-Well Extraction Disk Plate is a tool to achieve high throughput SPE. This document demonstrates the versatility of examining multiple parameters within a single plate for the goal of optimizing a specific method. Each individual step of the method should be critically reviewed for potential gains in throughput and performance (recovery, precision, and cleanliness). While each throughput and performance gain provides a benefit, the potential drawback of each gain should be considered. For example:

1. While a cleaner chromatogram may be achieved, there may be slightly lower recovery.
2. While a greater concentration of the drug/IS ratio for direct injection may be achieved, slightly lower total mass may be recovered.
3. While more effective removal of proteins and/or interfering substances may be achieved by the use of two sequential wash steps, the total extraction time may be slightly increased.
4. While you may develop a fully automated method for processing 96 samples, additional time may be required for a sample pretreatment step.

It remains the responsibility of the analyst to prioritize the assay objectives and identify the best conditions that generate the fully optimized method.

Note: Empore Solid Phase Extraction Products are intended for solid phase extraction during scientific research only. These products are not intended for use in medical devices or in assessment and treatment of clinical patients.

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