Straightforward SPE Method Development: Avoiding Common Pitfalls with a Simple Approach

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ABSTRACT

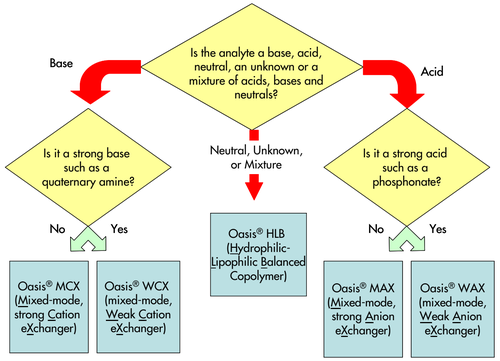
In today's highly competitive chemical markets, high sample throughput has become the norm. Faster analyses require a high level of automation and fast method development. And, as formulations become more potent, additional emphasis has been directed on sample preparation for sub ng/mL levels of components. Classical extraction techniques, such as liquid-liquid extraction (LLE) or protein precipitation (PPT), are not often suitable procedures for high throughput. More importantly, the extracts from LLE and PPT are often not clean enough to be able to achieve the low limits of detection now required. Therefore, solid phase extraction (SPE) has become the sample preparation tool of choice. However, many chemists feel that SPE method development is too time consuming or too difficult. To address these issues, we took a systematic approach to SPE method development and outlined all the steps including sample pretreatment, sample solubility, SPE sorbents, wash steps and solvents, elution solvents, calculation of recovery and method reproducibility. We used a variety of sample probes to cover acids, bases and neutrals, as well as the range from polar to non-polar analytes. We used a variety of sorbents in the 96-well plate format and LC/MS/MS for analysis at ng/mL levels. In this presentation, we outline all the important steps for SPE method development, highlighting the pitfalls and how to avoid them. Based on these data, we then outline a straightforward approach to SPE method development.

INTRODUCTION

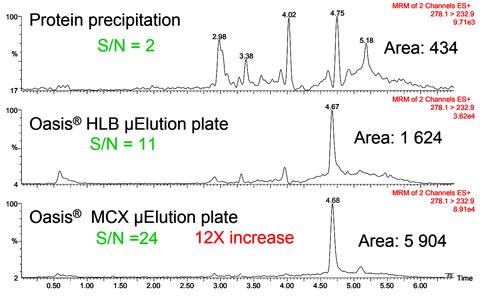
Sample preparation techniques utilizing SPE are often perceived as being tricky, tedious and time-consuming. However, if one has the right tools in hand and an organized approach to method development, SPE does not need to be a chore. For instance, the Oasis® family of copolymeric SPE sorbents offers five chemistries to satisfy all sample clean up needs. Oasis® HLB is based on a hydrophilic-lipophilic balanced copolymer that offers superior retention of both polar and non-polar analytes. In addition, four mixed-mode chemistries offer additional retention and clean-up benefits when analytes are ionizable. What we offer in this presentation is an outline of what sorbent to start with and then once that choice has been made, some handy tips to help with SPE method development.

CHOOSING THE SORBENT

The key point in understanding what sorbent to use is determining the nature of the analytes. The following flow chart outlines the sorbent selection process.



Whenever the analytes are ionizable (i.e. acids or bases), it is an advantage to select a mixed-mode ion exchange sorbent. This type of sorbent offers the best selectivity and the cleanest extracts. In the figure shown below, we can see the differences in signal-to-noise for rat plasma spiked with amitriptyline at 0.1 ng/mL taken through protein precipitation, reversed-phase and mixed-mode ion exchange clean-up steps. Clearly, the samples prepared using Oasis® MCX are the cleanest and offer the best sensitivity.



STARTING SPE METHODS

These are the recommended starting conditions for the various sorbents.
For all sorbents:
Prepare sample
Condition/Equilibrate with MeOH and Water
Load Sample Solution

Oasis® HLB:
Wash with 5% MeOH in water
Elute with 100% MeOH

Oasis® MCX:
Wash 1 with 2% FA
Wash 2 100% MeOH
Elute with 2% NH_4OH in MeOH

Oasis® MAX:
Wash 1 with 2% NH_4OH
Wash 2 with MeOH
Elute with 2% FA in MeOH

Oasis® WCX:
Wash 1 with 25 mM Citrate Buffer in water, pH 5
Wash 2 with 100% MeOH
Elute with 2% FA in 20:80 MeOH:ACN

TIPS AND TECHNIQUES FOR TROUBLESHOOTING

Definition of Recovery

Because the term "recovery" can be confusing, it is important to state a simple definition for the overall recovery of the SPE extraction procedure.
Recovery of the Extraction Procedure (RE)[1] is as follows:

$$\%\,RE = \frac{Response_{Extracted\ Sample}}{Response_{Post\text{-}Extracted\ Spiked\ Sample}} \times 100$$

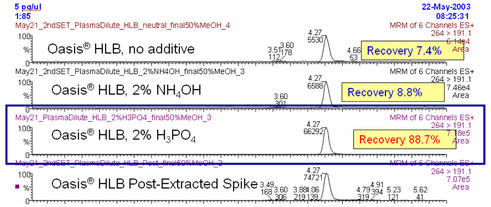
Additionally, in LC/MS/MS analyses, the matrix can cause the suppression or enhancement of the signal. Therefore, it is important to measure this effect.
Matrix Effect (ME)[1] is as follows:

$$\%\ Matrix\ Effect = \frac{Response_{Post\text{-}Extracted\ Spiked\ Sample}}{Response_{Non\text{-}extracted\ Sample}} \times 100$$

Plasma Sample Pre-Treatment

To optimize the contact time with the sorbent and to prevent the plugging of wells, we recommend the following steps:
1. Dilute 1:1 with water
2. Disrupt protein binding by adding H_3PO_4 to a final concentration of 2% (use base if compound is acid labile).
   Note: Steps 1 & 2 can be combined into one.
3. Add the internal standard — the amount of organic solvent should be less than 10% of the final volume — this will help to prevent protein precipitation and well plugging.
4. If possible, mix the solution before loading onto the sorbent.

Many times analytes will bind strongly to proteins in biological samples. If this binding is not disrupted, the analyte will pass through the SPE device and result in low SPE extraction recoveries. Therefore, it is important to determine whether or not binding is occurring. In this example, protriptyline binds strongly to rat plasma proteins. Only H_3PO_4 is strong enough to disrupt this binding. Note that the addition of NH_4OH does not disrupt the binding.



Breakthrough in the Loading Step

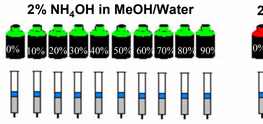
If a sample is in too high an organic solvent, for example, the supernatant from an extraction step, the sorbent may not retain the analyte and breakthrough may occur.

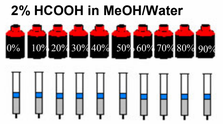
If the analyte is bound to proteins in the matrix, the analyte may pass through the sorbent with the proteins.

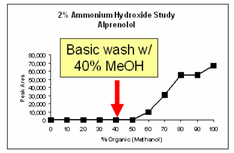
The flow rate during this step is also crucial — make sure to observe the drops and adjust the vacuum so discrete droplets (i.e. not a stream) are flowing from the device.

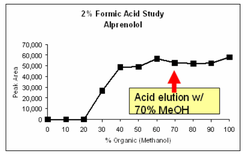
Wash Steps

The wash steps in a method help to remove the interfering sample components and are crucial in reducing matrix effects. A good methodology for determining the best wash and elution steps is to run a 2D Optimization. Let's walk through the Oasis® HLB 2D Optimization. Solutions of base and acid are made in methanol. The analyte is spiked onto the SPE device and then eluted with each of the solutions. These eluents are analyzed and the data plotted as in the next figure.





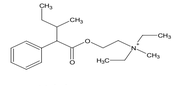




From these plots, an optimized method can be developed:
Wash 1: Base with 5% MeOH (remove proteins to prevent clogging of wells; bases are in neutral state for more retention)
Wash 2: Base with 40% MeOH (removes hydrophilic bases and neutrals and all acids)
Wash 3: 100% Water (removes residual ammonium hydroxide)
Elute: Acid with 70% MeOH (101% recovery from rat plasma)

METHOD DEVELOPMENT EXAMPLE — OASIS® WCX

The analyte is valethamate, a quaternary amine. Following the sorbent selection flow chart, the Oasis® WCX material should be used.



Valethamate

Prior to SPE method development, we determined that valethamate does not bind to plasma proteins. This is done by spiking a known amount of valethamate into rat plasma, treating aliquots with or without acid, and taking the samples through the SPE method. The same results were obtained for both sets of samples. Therefore, we did not use acid in the pretreatment step. We utilized the suggested starting SPE protocol for the Oasis® WCX µElution Plate:
Condition: 200 µL MeOH
Equilibrate: 200 µL H_2O
Load: 100 µL Spiked rat plasma (diluted 1:1 with H_2O, 1 ng/µL valethamate)
Wash 1: 200 µL 25 mM Citrate Buffer, pH 5
Wash 2: 200 µL MeOH
Elute: 50 µL 20:80 MeOH:ACN with 2% formic acid
SPE Extraction Recovery: **102%**

CONCLUSIONS

- SPE Method development can be simplified by first correctly selecting the sorbent
- Once the sorbent is selected, following the outlined tips for the various SPE steps will result in a robust, reproducible SPE method.

REFERENCES

[1]Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M. *Anal. Chem.* **2003**, *75*, 3019-3030.