Simplified Protein Precipitation: A New Technology for Precipitation and Filtration

Kimvan Tran[1], Diane M. Diehl[1], Ziling Lu[1], Tim Schultz[2], Wilf Wixwat[2], Brian P. Murphy[1], Jeff Kane[2], Emily Berlin[2], Kevin Seeley[2], Jeffrey R. Mazzeo[1]
[1]Waters Corporation, Milford, MA; [2]Pall Life Sciences, Ann Arbor, MI

OVERVIEW

To address performance issues on commercially available protein precipitation plates, we developed a new 96-well protein precipitation and filtration plate that offers a dripless technology with a gradient of purpose-selected filtration media, together with a vented closure to allow for rapid 'in-well' sample preparation. We ran a series of ion suppression and extraction studies to ensure the cleanliness of the plate materials. We validated the plate performance for a variety of polar and non-polar acids, bases and neutrals spiked into porcine and rat plasmas. We compared the results to traditional centrifugation methods and other commercially available products. In our studies, we did not observe liquid flow until vacuum was applied. Additionally, less than 1% of the wells plugged — and in most plates we did not see any wells plug. The time for filtration ranges from 30 seconds to 3 minutes, depending on the type and amount of plasma, and the time to process an entire plate of 96 samples is approximately 15 minutes. There is no observable cross talk between the wells. The eluents are free of observable particulates and offer a cleaner eluent than the traditional centrifugation method. This new Sirocco™ Protein Precipitation Plate out-performs current commercially available products.

INTRODUCTION

Pharmaceutical chemists require fast and efficient bioanalytical procedures. Sample preparation is often the rate limiting step and much research and product development has been devoted to increasing the speed of sample preparation. In recent years, sample preparation in the 96-well format has risen in popularity. However, protein precipitation has not been as successfully developed in this format. Many of the commercially available protein precipitation filter plates suffer from poor performance. Depending on the procedure and the plate, plasma often leaks into the filter before a vacuum is applied, leading to a cloudy filtrate. Other plates suffer from a high percentage of blocked wells. To address these performance issues, we developed the new Sirocco™ Protein Precipitation Plate that offers a dripless technology, a gradient of purpose-selected filtration media, together with a vented closure to allow a rapid 'in-well' sample preparation.

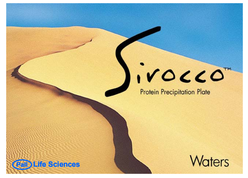
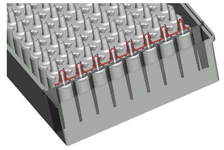
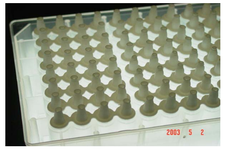


PLATE DESIGN

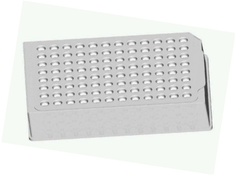
The 96-well plate was designed with 1-mL reservoirs. Each well contains a stack of chemically resistant and low extractible membrane filters. The selection of filtration media and the design of the flow path was customized for the protein precipitation purification solvents so that the wells do not empty until vacuum is applied. This allows the precipitation reaction to occur in the filtration plate without the need of a transfer step.



Specially designed and patent-pending vented closures prevent solvent flow until vacuum is applied.



Sirocco™ Protein Precipitation Plate showing the vented closures.

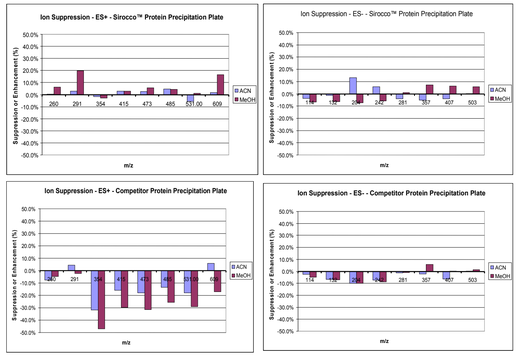


Schematic of the capmat design. Vents in the capmat allow for vortexing and filtration without removal of the capmat — eliminating crosstalk.

MATERIAL SELECTION

An integral part of the design process was the selection of materials for the body of the plate, the vented closures, the capmat and the filtration media. Our requirements were to have ultra-low ion suppression and/or enhancement when using a mass spectrometer (MS) for analysis, as well as ultra-low amounts of extractables as detected by UV or MS. These requirements are crucial for scientists using protein precipitation in the pharmaceutical industry. We developed a series of experiments to test these potential materials.

To examine the ion suppression/enhancement effects of the materials, we either soaked the material in acetonitrile or methanol (typical PPT crash solvents), or passed the solutions through prototype plates. We set up an HPLC system with a mobile phase containing a series of basic (m/z 260-609) or acidic (m/z 114-503) analytes (1). The mobile phase was 50:50 methanol:water with the basic analytes or acidic analytes. With the HPLC flowing mobile phase into the mass spectrometer, we infused 50 µL of the solutions directly into this flow with an infusion pump. Blank solutions (i.e. soak solutions without material) were infused first to establish a baseline value for the ion count for each analytes. Then the soak solution itself was infused.



The top two plots show the low degree of ion suppression and enhancement in both the ES+ and ES– modes of ionization for the Sirocco™ Protein Precipitation Plate, indicating the cleanliness of these plates. The bottom two plots show the high level of ion suppression, mainly in the ES+ mode, for a competitive protein precipitation plate, which would result in difficulties in the analysis of samples run on this plate.

For the extractables studies, two experiments were performed. In the first experiment, we made an injection of the soak solution onto an HPLC column and used a photodiode array detector. In the second experiment, the soak solutions were infused into the MS without the HPLC flow on. We ran a full scan to determine what ions were present in the solution.

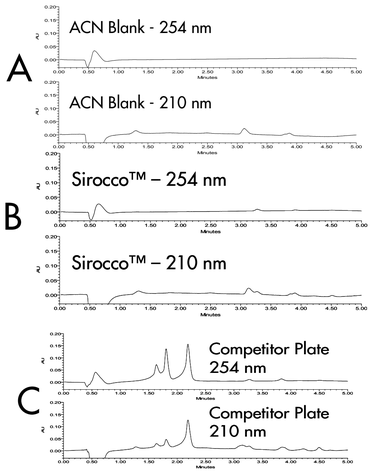
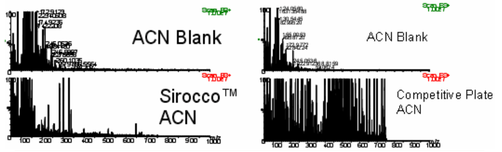


Figure A is an injection of ACN onto an HPLC column. Detection is UV at 210 and 254 nm. There are two small peaks in the 210 nm trace. Figure B is an injection of ACN eluent from the Sirocco™ PPT plate. These chromatograms are nearly identical to the ACN blanks. Figure C is an injection of ACN eluent from a commercially available PPT plate. Notice the significant amount of contamination from this plate at both wavelengths.



The above spectra are the results for the direct infusion of ACN eluents from the Sirocco™ PPT plate and a competitive PPT plate. In each figure, the top spectra is for the infusion of an ACN blank and the bottom is for the plate eluent. There are a few species from the Sirocco™ plate that are present in low amounts. Clearly, the data on the right for the Competitive plate indicate high levels of contaminants.

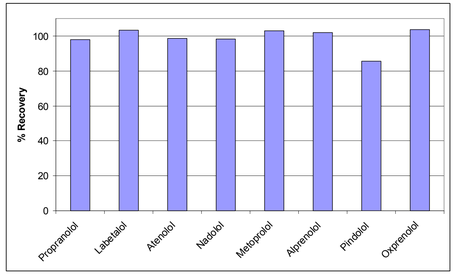
PROTOCOL

1. Set the Sirocco™ plate on a collection plate. This keeps the valve tips suspended in wells of collection plate during processing.
2. Add crash solvent (typically acetonitrile with ISTD)
3. Rapidly inject plasma into wells
4. Apply vented cap mat (use a roller for good seal)
5. Vortex PPT plate/collection plate stack at a medium speed – ensures complete mixing and precipitation – for 1 minute.
6. Filter on vacuum manifold at 8-10″ Hg. Alternatively, centrifuge for 5 min at 2000 rpm.

Note: Never remove cap mat or valve tips – this eliminates cross talk between wells. The entire assembly is disposed after filtration.

RESULTS

We tested the Sirocco™ plate with 100 µL of rat plasma spiked with 8 analytes at a concentration of 10 pg/µL. 300 µL ACN with internal standard added to plate (N = 6), followed by the spiked rat plasma. The precipitation protocol was then followed. The excellent recoveries are shown in the plot below.



CONCLUSIONS

- State-of-the-art design ends unpredictable leaking
- Maximizes mass spectrometry sensitivity and uptime by eliminating cloudy filtrates
- All materials used in manufacturing the plate are highly clean, eliminating ion suppression and enhancement
- Plate design allows for minimized sample cross-talk and maximum sample recoveries

(1) Mallet, C. R., Lu, Z., Mazzeo, J. R. Rapid Commun. Mass Spectrom. 2004; 18, 49-58.