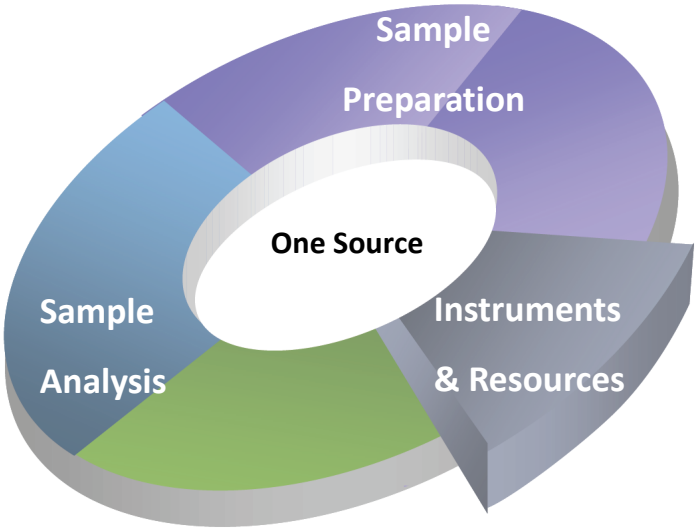
RubyPro™

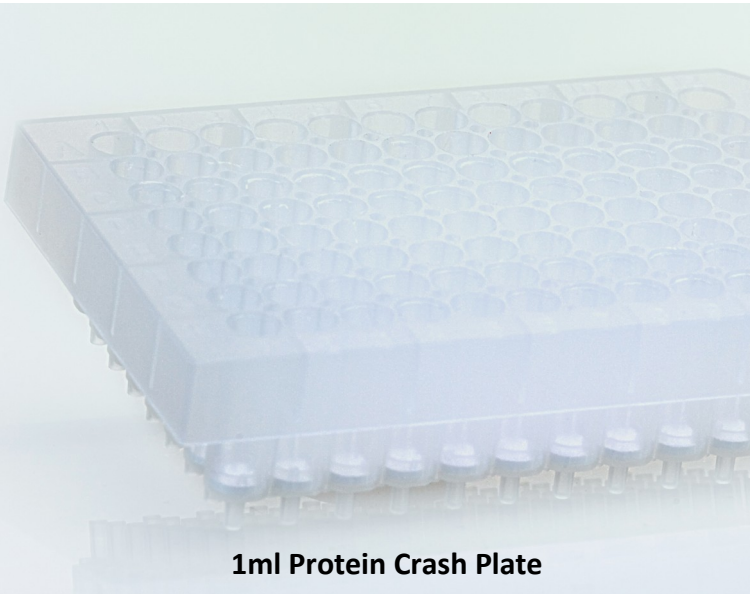
Protein Crash Plates

2ml Protein Crash Plate

Protein precipitation is a sample preparation technique for removing proteins from fluid biological samples before analysis. Orochem's Ruby Pro plates are optimized for high throughput automation of Protein precipitation in the 96-well format, using automated liquid handling and vacuum manifold systems.

- **Rapid**
- **Ease of Automation**
- **High Throughput**
- **No solvent leakage**
- **No crosstalk during incubation**





1ml Protein Crash Plate

General Protocol:

1. Condition the plates by adding Acetonitrile/Methanol
2. Load the sample
3. Incubate and hold the plate
4. Filter the sample
5. Cleanest extract ready for LCMS injection

OROCHEM
Molecular Purification by Design

Protein Precipitation Demonstrated in Microwell Filter Plate Format for LC-MS/MS Bioanalytical Sample Preparation

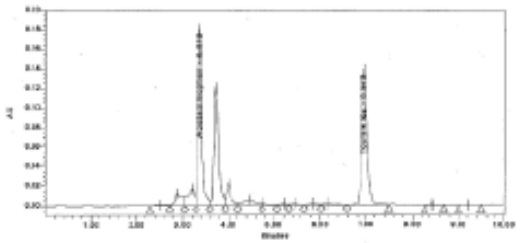
Introduction:
Protein precipitation is often used as a sample preparation technique when an analytical method needs to be developed quickly. Removal of the protein in the sample matrix via denaturation with acetonitrile affords a simple, generic extraction approach. As the numbers of samples continue to increase, and as timelines shorten, the need for a high throughput approach to performing protein precipitation is clearly evident. The purpose of this study was to compare the performance, ease of use and throughput of flow-through filter microplates with traditional techniques.

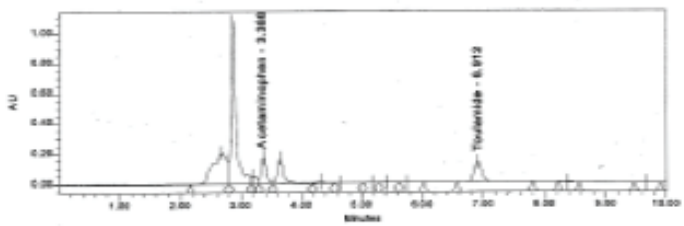
Methods:
Protein precipitation in a flow-through filtration microplate, centrifugation, SPE, and dilution of serum were compared. The Orochem protein precipitation plates OC21PP20 and OC21PPT20, were used. For the first three techniques, the acetonitrile/porcine serum ratio used was 3:1 (v/v), and the serum was spiked with acetaminophen and toluamide. During the filtration microplate method acetonitrile was added first followed by the serum. The plate was sealed and shaken followed by application of positive pressure. This enabled rapid flow of filtrate. Centrifugation of the acetonitrile and spiked serum was followed by aspiration of the supernatant. A known SPE protocol was used to process the sample with a hydrophilic DVB sorbent cartridge. Dilution of the spiked serum was made 1:10 with 5% methanol in buffer.

Preliminary Data:
RP-HPLC-UV was used to assess recovery and cleanliness of the as is dilute serum and of the evaporated eluent for the other three techniques. Percent recoveries (n=2) of acetaminophen and toluamide using the filtration plate were 106% and 90% respectively, compared with 94% and 77% using traditional centrifugation. Performance was comparable using both techniques, however, the filtration microplate (rated at 0.2 mm) affords much higher throughput capabilities than centrifugation, especially when used with liquid handling stations, as it eliminates sample transfer and centrifugation. SPE (n=2) recoveries were 97% and 87% for acetaminophen and toluamide, respectively, and the chromatogram showed less interference as it produced cleaner eluent. Using dilute serum sample preparation, percent recoveries (n=2) were 116% and 96%. The high value for acetaminophen in this approach can be attributed to interference due to UV measurement. However, the MS detector can tolerate such interference by selective analysis.

Overall, cleanliness was identical between the two precipitation techniques. The diluted serum approach requires only dilution so recovery is excellent but can suppress ionization. SPE recoveries were comparable to the other approaches while offering a cleaner eluent. Orochem OCNPT20 filter plate demonstrated no leakage and crosstalk during incubation and mixing, eliminating the need for bottom sealing. The Orochem filtration microplate offers a generic sample preparation technique, which is rapid, affords a higher throughput and ease of automation.

Sample Prep Method	Analyte	Expected Concentration, mg/ml	Actual Concentration recovered, mg/ml	% Recovery
OC21PP20	Acetaminophen Toluamide	0.0031 0.0031	0.0033 0.0028	106 90
OC21PPT20	Acetaminophen Toluamide	0.0031 0.0031	0.0033 0.0028	106 90
Dilute Serum	Acetaminophen Toluamide	5.10E-04 5.10E-04	5.90E-04 4.90E-04	116 96
SPE	Acetaminophen Toluamide	0.0031 0.0031	0.003 0.0027	97 87
Centrifuge	Acetaminophen Toluamide	0.0031 0.0031	0.0029 0.0024	94 77





Based on the above results, the protein precipitation filter plates provide better recovery of the analytes compared with SPE and centrifugation. Diluting the serum does provide better recovery of the analytes in this case. However the chromatogram shows the peak area percentages of the analyte peaks are very small. For low levels of analyte, a concentration procedure would allow for better detection and quantitation. Also, analyte recovery is the same for PTFE coated plates and non-coated plates.

Total Drug analysis of Biological Samples using an Automated High

Orochem Protein Precipitation plate has been developed and optimized for use in a wide range of aqueous and organic sample preparation applications including total drug analysis. The total drug analysis in the Orochem plate with supporting methodology produces 96 samples for the determination of (total) drug in a biological sample, typically serum, following protein precipitation and filtration in the well without using a capmat or seal.

The OroFlex - 96 Personal Pipettor has a flexible design for liquid transfers and pipetting operations for 96 -well microplates and can also be used for serial dilutions by row or column. It features an intuitive graphical user interface via serialinterface control

Materials and Methods

Reagents

• Organic Solvent - (9:1 ACN:H2O)

• Serum, FBS Fetal Bovine Serum

Materials

• Orochem Protein Precipitation Plate, OC21PPT20

• Deep Well collection plate Orochem OT-850356

• Orochem HPLC Orpheus C18 column Equipment

Equipment

• OroFlex - 96 Personal Pipettor

Protocol

1) 800 µL of 90 % Acetonitrile spiked with the analyte was added to the to all wells of the plate.

2) 200 mL of serum was added (FBS-Fetal Bovine Serum) drop wise above the solvent to all wells of the plate.

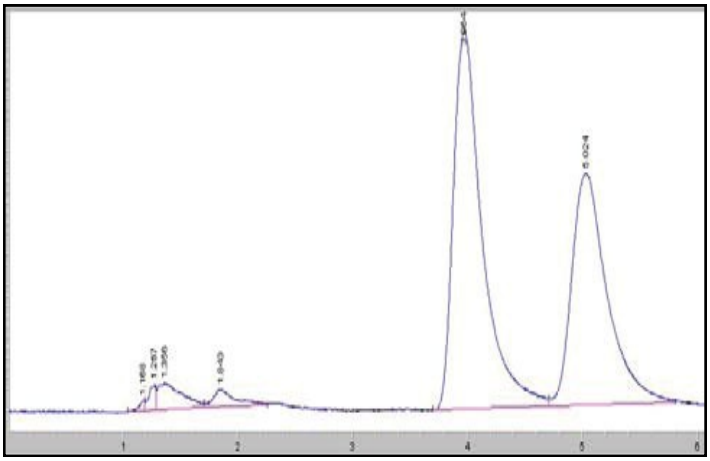
3) This was followed by mixing for 5 minutes on a plate shaker and then filtered at max (>18" Hg), leaving the vacuum on for approximately an additional 45-60 seconds upon complete filtration.

Results and Conclusion:

The Solvent First Technique afforded no leakage. No capmat or seal was required. The Protein Crash method is fast and simple. The total recoveries of Valerophenone and Hexanophenone were 85.20% and 82.89% respectively. The total sample preparation time was only 20 minutes. Preparing serum or plasma samples for total drug analysis using the Protein Crash Plate offers precise and accurate results.

#	Time	Area	Height	Width	Symmetry
1	1.168	7.1	1.9	0.0507	2.978
2	1.267	21.4	5	0.0545	3.555
3	1.356	76.7	5.2	0.1809	0.343
4	1.843	47.5	3.4	0.1828	0.476
5	3.964	1282.5	72.8	0.2545	0.548
6	5.024	967.4	44.8	0.2789	0.605

Table 1: Data in rows 5&6 represents the analytes Valerophenone and Hexanophenone



Chromatogram of analytes Valerophenone and Hexanophenone following protein crash

Evaluation of Acetylsalicylic acid (acidic) drug in serum samples with the RubyPro protein precipitation plates

Analysis of serum samples for endogenous substances and drugs presents special problems because of the high and variable components of proteins and other ions. Deproteinization of serum samples with acetonitrile is a useful and rapid technique in analysis of drugs in serum samples.

Acetylsalicylic acid (acidic drug) was analyzed in Serum by an Automated Protein Crash Methodology using the Orochem Protein Crash Filtration device and the OroFlex-96 Personal Pipettor. The Orochem Protein Precipitation 96-well plate is designed to hold acetonitrile or methanol in the -lter well without a cap mat or seal. The ability to interface the 96-well Protein Crash plate with robotic workstation, allows high throughput solvent dispensing, followed by sample transfer and mixing for complete precipitation of proteins in high throughput format. Following protein precipitation in these “reactor” wells, sample -ltration is accomplished with the Orochem Positive pressure processor (ORPSP-96). The resulting extract was analyzed by HPLC. The samples were prepared in duplicate, along with 2 controls (that contained only the solvent mixture). High Performance Liquid Chromatography A Symmetry C18 column (3.5, 4.6 HPLC150 mm) was (Water/ Methanol/Acetonitrile, 20/40/40) was pumped at 0.5mL/min. A 20-L injections were made, and the peaks were monitored at Absorbance 220.

Reagents

Organic Solvent - 100% aqueous acetonitrile.

Serum, FBS Fetal Bovine Serum

Acetylsalicylic acid and Acetaminophen drugs

Materials

Orochem Protein Precipitation Plate, OC21PPT20

Deep Well collection plate Orochem OT-850356

HPLC ,C18 column equipment

OroFlex-96 Personal Pipettor

Orochem Positive pressure processor (ORPSP-96)

Protocol Overview

Serum Spiking

For Acetylsalicylic acid drug, 0.1 ml of the concentrated drug solution (0.1mg/ml) was mixed with 0.9 ml bovine serum to obtain a nal concentration of 0.01mg/ml of drug in serum.

Serum Deproteinization

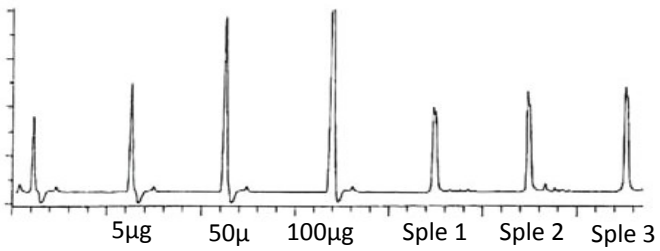
1) 800 mL of 100 % acetonitrile was added to all the wells of the plate.

2) 200 mL of spiked (FBS-Fetal Bovine Serum) drop wise was added to all wells of the plate.

3) This was followed by mixing for 5 minutes on a plate shaker and then ltered at max leaving the Positive pressure on for an additional 45-60 seconds upon complete

Results and Conclusions

The Protein Crash method is fast and simple. Recovery of both acidic and basic drugs from spiked serum is satisfactory (100% for Acetylsalicylic acid). The total sample preparation time was only 20 minutes. Orochem Protein Crash Plate along with Positive Processor offers a rapid and accurate method for analysis of drugs in Serum or Plasma samples. The ability to interface the 96-well Protein Crash plate with robotic workstation, allows high throughput solvent dispensing, followed by sample transfer and mixing for complete



Chromatogram of analyte Acetyl Salicylic Acid