Sample Preparation and Protein: A Comparison of Protein Removal with Various Sample Preparation Techniques using Gel Electrophoresis

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Introduction

Sample extract cleanliness is very important when using analytical methodologies such as liquid chromatography and hyphenated techniques such as LC-MS. Residual protein contained in extracts can not only lead to ion suppression/enhancement effects in the LC/MS system but also result in increases in HPLC column pressure and premature degradation. This poster evaluates the effectiveness of various sample preparation techniques for the removal of serum protein. By performing 1-D-gel electrophoresis on the extracts it was possible to obtain protein profiles for the various techniques.

The techniques investigated were:

1. Protein Precipitation.
2. Supported Liquid Extraction (SLE+).
3. Non-polar silica-based SPE.
4. Non-polar polymer-based SPE.
5. Silica-based mixed-mode SPE (anion and cation exchange).
6. Polymer-based mixed-mode cation exchange SPE.

Experimental Procedure

Reagents

All solvents required for sample preparation were HPLC grade and Milli-Q water used throughout. Blank rat serum was obtained from the NCTR animal colony. All materials for the gel electrophoresis work were obtained from Invitrogen (Carlsbad, CA. USA).

Sample Preparation

In all cases 100 µL of pooled rat serum was extracted using generic methodology and modified protocols for each technique.

Protein Precipitation: ISOLUTE® Array PPT+ Protein Precipitation Plate**.** A range of serum/crash solvent ratios were evaluated.

Supported Liquid Extraction (SLE): ISOLUTE® SLE+ 200 µL Supported Liquid Extraction Plate.

Non-polar SPE (silica based): ISOLUTE® Array C2, C8, C18 25 mg/1 mL, ISOLUTE MFC18 25 mg/1 mL.

Non-polar SPE (polymer-based): EVOLUTE® Array ABN 25 mg/1 mL versus three major competitors.

Mixed-mode cation exchange SPE (silica-based): ISOLUTE® Array HCX, HCX-3 and HCX-5 25 mg/1 mL.

Mixed-mode anion exchange SPE (silica-based): ISOLUTE® Array HAX 25 mg/1 mL.

Mixed-mode cation exchange SPE (polymer-based): EVOLUTE® CX Array 25 mg/1 mL versus two competitor products.

Post Extraction:

The extracts were evaporated to dryness using a centrifugal vacuum concentrator and transferred for gel electrophoresis work-up.

Gel Electrophoresis

Samples were reconstituted in 10 µL H_2O. LDS sample buffer (4 µL) and reducing agent (2 µL) were added to each sample and boiled at ~100°C for 5 minutes, spun to pull down volume, and allowed to cool to room temperature. Electrophoresis was performed using a NuPAGE NOVEX 12% Bis/tris mini gel with MOPS SDS running buffer. Gels were run at 200V, 120 mA and 12.5 W for aprroximately 65 minutes to ensure complere protein migration.

Results

Protein Precipitation (PPT)

This experiment investigated the optimal MeCN crash ratio for protein removal. **Figure 1** shows a comparison of 1:1, 1:2, 1:3 and 1:6 (v/v) serum/MeCN crash ratios compared to raw serum. The results demonstrate that 1:3 (v/v) ratios gave more protein removal than either the 1:1 or 1:2 (v/v) ratios. However, there was no improvement when increasing to a 1:6 (v/v) crash ratio.

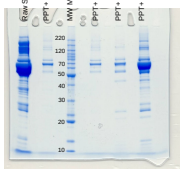

Figure 1. *Gel electrophoresis protein profile comparing various crash ratios.*

Supported Liquid Extraction (SLE)

SLE was performed using 1:1 serum:H_2O pre-treatment and extraction with a commonly accepted extraction solvent, MTBE. As demonstrated in **Figure 2**, no protein was observed in the final extract.

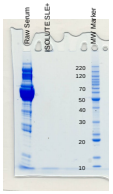

Figure 2. *Gel electrophoresis protein profile using ISOLUTE® SLE+.*

Non-polar SPE (silica-based)

This experiment was aimed at comparing the chain length of silica based sorbents and the effect of pore size on protein retention. **Figure 3.** demonstrates the protein profile observed for the four silica sorbents tested. The C2 sorbent shows slightly more protein than either the C8 or C18 but the larger pore size MFC18 (100 Å compared to standard C18 of 60 Å) demonstrated highest protein content in the extracts.

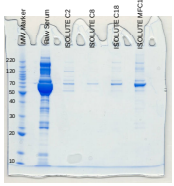

Figure 3. *Gel electrophoresis protein profile comparing various silica-based SPE sorbents.*

Non-polar SPE (polymer-based)

This experiment compared EVOLUTE® ABN with three other manufacturer's polymer-based SPE sorbents. As demonstrated in **Figure 4**. EVOLUTE® ABN gives the cleanest protein profile. On the other hand competitor A demonstrated the highest amount of protein in the extract showing far greater protein retention.

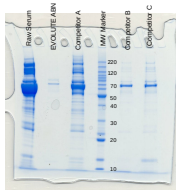

Figure 4. *Gel electrophoresis protein profile comparing various polymer- based SPE sorbents.*

Mixed-mode cation/anion exchange SPE (silica-based)

This experiment compared three silica based mixed-mode strong cation exchange sorbents with different carbon chain lengths (HCX (C8); HCX-3 (C4); HCX-5 (C18)). As an additional experiment the silica-based mixed-mode strong anion exchange sorbent, HAX, was tested. **Figure 5.** shows the profiles obtained for the four sorbents for both the final interference wash step and the elution solvent. Some protein was retained on the cation exchange sorbents until the final MeOH interference wash step. However, no protein was observed in the final elutions for any of the HCX sorbents. The HAX showed very little protein in either the wash step or the final elution step.

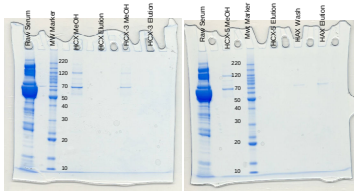

Figure 5. *Gel electrophoresis protein profile comparing silica-based mixed-mode SPE sorbents (strong cation, HCX family; and strong anion, HAX).*

Mixed-mode cation exchange SPE (polymer-based)

EVOLUTE® CX was compared with two other manufacturer's polymer-based mixed-mode strong cation exchange SPE sorbents. **Figure 6.** demonstrates the protein profiles of the MeOH interference wash step and the analyte elution steps. Some protein was observed in the MeOH wash steps for all three sorbents. However, as with the silica-based mixed-mode cation exchange very little protein was observed in the final elution.

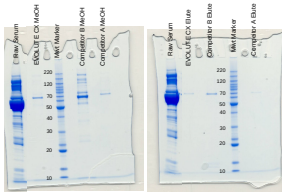

Figure 6. *Gel electrophoresis protein profile comparing polymer-based mixed-mode cation exchange SPE sorbents.*

Conclusions

- **Protein precipitation** demonstrated higher protein levels for crash ratios below the suggested 1:3 (v/v) ratio. Increasing the crash ratio to 1:6 did not improve protein removal. A rough protein quantitation (n=1) showed greater than 99% of total protein removed using ISOLUTE® PPT+ with a 1:3 crash ratio.
- **Supported Liquid Extraction**, demonstrated no protein in the final extract. This is due to the low solubility of proteins in the water immiscible extraction solvent, in this case MTBE.
- **Silica-based non-polar SPE.** C8 gave the lowest protein levels out of the various chain lengths. This could be due to a better balance between the hydrophobic and steric effects of the C8 compared to the others. The MFC18 shows considerably more protein retention than the standard C18, indicating that pore size has a substantial effect on protein retention.
- **Polymer-based non-polar SPE.** EVOLUTE® ABN gave the lowest amount of protein retention compared to the three competitor resin-based SPE sorbents. This is due to the optimized pore size, structure and distribution of EVOLUTE® ABN compared to the other products
- **Silica-based mixed-mode cation exchange SPE.** Some protein was observed after the MeOH interference wash step, but the final eluate was free from protein.
- **Silica-based mixed-mode anion exchange SPE.** Very little protein was observed in either the interference wash or the elution solvent.
- **Polymer-based mixed-mode cation exchange SPE.** All three sorbents demonstrated very little protein in the final extracts. This is due to the rugged interference elution regime afforded by mixed-mode cation exchange sorbents.
- **Rough protein quantitation** (n=1) on the extracts tested showed greater than 99% removal of serum proteins for all but a few techniques.
  - PPT extracts using 1:1 and 1:2 crash ratios only gave 94.5% and 98.4% removal the larger pore size MFC18 gave 98.5% removal
  - Non-polar resin-based SPE competitor sorbents A and B only gave 95.7 and 98.8% removal.

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