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

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Introduction

Extract cleanliness is very important in LC-MS/MS analysis. When analyzing serum or plasma the very nature of the matrix can cause problems with the analytical methodology. Endogenous components such as salts, proteins and phospholipids are all present and can have a marked effect on instrument response in terms of ion suppression or enhancement effects. This variation in signal can lead to quantitation issues and method reliability problems.

This poster deals with the specific problem of serum protein removal and evaluates the effectiveness of various sample preparation techniques. By performing 1-D-gel electrophoresis on the sample extracts it was possible to obtain protein profiles for the various techniques. The techniques investigated in this study were; Protein Precipitation using a solvent first protein precipitation plate (ISOLUTE® PPT+); Supported Liquid Extraction (ISOLUTE SLE+); non-polar silica-based SPE comparing the effect of carbon chain length and pore size (ISOLUTE C2, C8, C18 and MFC18); non-polar resin-based SPE, comparing EVOLUTE® ABN with three other commercially available products; silica-based mixed-mode SPE using both anion and cation exchange sorbents (ISOLUTE HCX, HCX-3, HCX-5 and HAX); resin-based mixed-mode cation exchange SPE, comparing EVOLUTE CX with two other commercially available products.

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

100 µL of pooled rat serum was extracted using standard methodology for each technique (details below). Following sample preparation, the final extracts were evaporated to dryness using a centrifugal vacuum concentrator and transferred for gel electrophoresis work-up. In the case of the mixed-mode sorbents both the final methanol wash and the elution solvents were subjected to gel electrophoresis testing.

a) Protein Precipitation

ISOLUTE Array PPT+ Protein Precipitation Plate

1. Add 100, 200, 300 or 600 µL acetonitrile to each well (serum crash ratios:- 1:1, 1:2, 1:3 and 1:6 (all v/v)).
2. Add 100 µL serum to each well.
3. Allow to stand for 5 minutes.
4. Apply vacuum at -20 "Hg and collect filtrate.

b) Supported Liquid Extraction (SLE).
ISOLUTE SLE+ 200 mg Supported Liquid Extraction Plate
1. Mix 100 µL of serum with 100 µL H_2O and apply to each well.
2. Apply a short pulse of vacuum to initiate sample load.
3. Allow to stand for 5 minutes.
4. Apply MTBE (1 mL) and elute under gravity for 5 minutes. Apply vacuum (2 minutes) to completely remove extraction solvent.

c) Non-polar SPE (silica based sorbents).
ISOLUTE Array C2, C8, C18 25 mg/1 mL, ISOLUTE MFC18 25 mg/1 mL (buffer volumes adjusted to 1 mL).
1. Condition each well with methanol (1 mL).
2. Equilibrate with ammonium acetate buffer (0.05 M, pH 6, 250 µL).
3. Load serum sample (100 µL diluted 1:1 (v/v) with ammonium acetate buffer (0.05 M, pH 6)).
4. Wash with buffer/methanol (95:5 (v/v), 250 µL).
5. Elute with methanol / 1 M ammonium acetate (99.5:0.5 (v/v), 250 µL).

d) Non-polar SPE (resin-based sorbents).
EVOLUTE Array ABN 25 mg/1 mL vs. three major competitors. Generic methods shown in **Table 1**.

Table 1. Resin-based Generic SPE methods

Step	EVOLUTE ABN Generic Method	Competitor A Generic Method	Competitor B Generic Method	Competitor C Generic Method
Condition	1 mL MeOH	1 mL MeOH	1 mL MeOH	1 mL MeOH
Equilibration	1 mL 0.1% Formic acid aq	1 mL H_2O	1 mL H_2O	500 µL H_2O
Load	400 µL 1:3 (v/v) serum/1% formic acid aq	200 µL 1:1 (v/v) serum/H_2O	200 µL 1:1 (v/v) serum/2% H_3PO_4	400 µL 1:3 (v/v) serum/1% formic acid aq
Interference Wash	1 mL 95:5 (v/v) H_2O/MeOH	1 mL 95:5 (v/v) H_2O/MeOH	1 mL 95:5 (v/v) H_2O/MeOH	1 mL 95:5 (v/v) H_2O/MeOH
Elution	500 µL MeOH	1 mL MeOH	1 mL MeOH	1 mL MeOH

e) Mixed-mode cation exchange SPE (silica-based sorbents)
ISOLUTE Array HCX, HCX-3 and HCX-5 25 mg/1 mL
1. Condition each well with methanol (1 mL) .
2. Equilibrate with ammonium acetate buffer (0.05 M, pH 6, 250 µL).
3. Load serum sample: (100 µL diluted 1:1 (v/v) with ammonium acetate buffer (0.05 M, pH 6)).
4. Wash with: (i) ammonium acetate buffer (0.05 M, pH 6, 250 µL),
   (ii) acetic acid (1 M, 250 µL), dry sorbent with 30 sec pulse of vacuum,
   (iii) methanol (250 µL).
5. Elute with methanol/NH_4OH (95:5 (v/v), 2 x 100 µL).

f) Mixed-mode anion exchange SPE (silica-based sorbent)
ISOLUTE Array HAX 25 mg/1 mL
1. Condition each well with methanol (1 mL) .
2. Equilibrate with 2% formic acid aq (250 µL).
3. Load serum sample: (100 µL diluted 1:1 (v/v) with 2% formic acid aq).
4. Wash with: (i) ammonium acetate buffer (0.1 M, pH 7, 250 µL),
   (ii) methanol/water (50:50 (v/v), 250 µL).
5. Elute with methanol/acetic acid (98:2 (v/v), 2 x 100 µL).

g) Mixed-mode cation exchange SPE (resin-based sorbent)
EVOLUTE CX Array 25 mg/1 mL versus two competitor products.

Table 2. Resin-based Mixed-mode cation exchange Generic SPE methods

Step	EVOLUTE CX Generic Method	Competitor A Generic Method	Competitor B Generic Method
Condition	1 mL MeOH	1 mL MeOH	1 mL MeOH
Equilibration	1 mL 50 mM NH_4OAc, pH 6	1 mL H_2O	1 mL H_2O
Load	400 µL 1:3 (v/v) serum/50 mM NH_4OAc, pH 6	200 µL 1:1 (v/v) serum/2% H_3PO_4	200 µl 1:1 (v/v) serum/2% H_3PO_4
Interference Wash 1	1 mL 50 mM NH_4OAc, pH 6	1 mL 2% Formic acid	1 mL 0.1M Formic acid
Interference Wash 2	1 mL MeOH	1 mL MeOH	1 mL MeOH
Elution	1 mL 5% (v/v) NH_4OH/MeOH	1 mL 5% (v/v) NH_4OH/MeOH	1 mL 5% (v/v) NH_4OH/MeOH

Gel Electrophoresis Procedure

Sample extracts were reconstituted in water and 5 µL serum equivalents removed. LDS sample buffer (4 µL) and reducing agent (2 µL) were added to each aliquot and boiled at ~100°C for 5 minutes, spun to pull down volume, and allowed to cool to room temperature. Electrophoresis was performed on two cells (running four gels simultaneously) using NuPAGE Novex 12% Bis-Tris mini gels with MOPS SDS running buffer at 200 V, 120 mA and 12.5 W. The total method time was set to 65 minutes to allow complete migration of the protein in each gel. 5 µL equivalent of each serum extract was compared to 0.5 µL equivalent of raw serum and 4 µL of the Benchmark Protein Ladder molecular weight marker in each gel.

Results

Protein Precipitation (PPT)

This experiment was designed to investigate the amount of protein removed from serum using ISOLUTE PPT+ (solvent first protein precipitation plate) and to see which serum:acetonitrile crash ratio peformed best using this filter plate. **Figure 1** shows a comparison of 1:1, 1:2, 1:3 and 1:6 (v/v) serum/acetonitrile crash ratios compared to raw serum.
The results showed that 1:3 ratios gave more protein removal than either the 1:1 or 1:2 ratios, however, there was no difference increasing from 1:3 to 1:6 (v/v) crash solvent ratios.

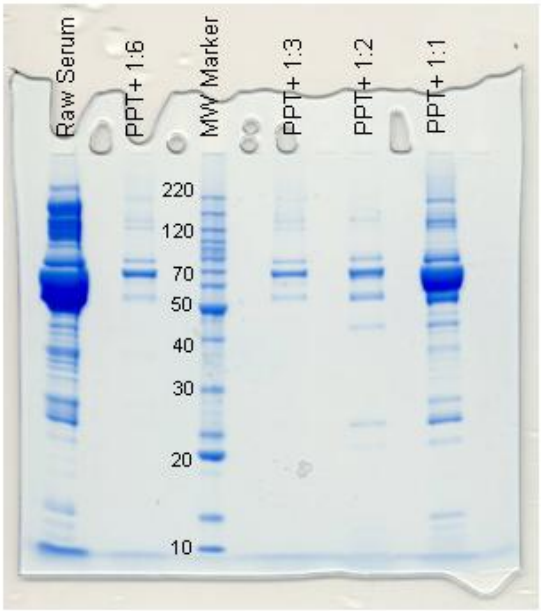

Figure 1. Gel electrophoresis protein profile comparing various crash ratios.

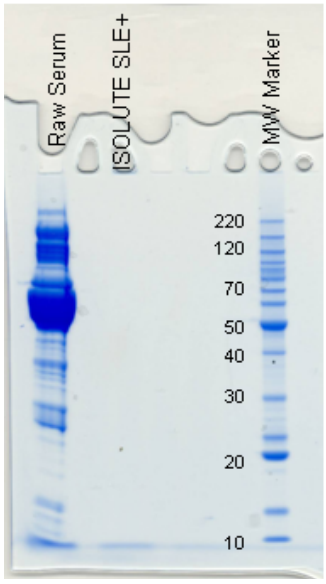

Figure 2. Gel electrophoresis protein profile using SLE.

Supported Liquid Extraction (SLE)

A basic SLE procedure was performed using a commonly accepted extraction solvent (MTBE). As can be seen in **Figure 2** no protein was observed in the final SLE extract.

Non-polar SPE (silica-based)

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

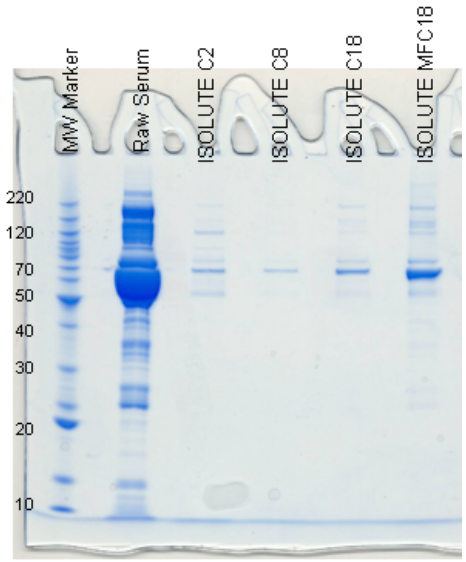

Figure 3. Gel electrophoresis protein profile comparing various silica-based SPE sorbents.

Non-polar SPE (resin-based)

This experiment compared EVOLUTE ABN with three other manufacturer's resin-based SPE sorbents. As can be seen in **Figure 4**, EVOLUTE ABN gives the cleanest protein profile by far, showing the lowest retention of proteins. On the other hand competitor A gives the highest amount of protein in the extract showing far greater protein retention.

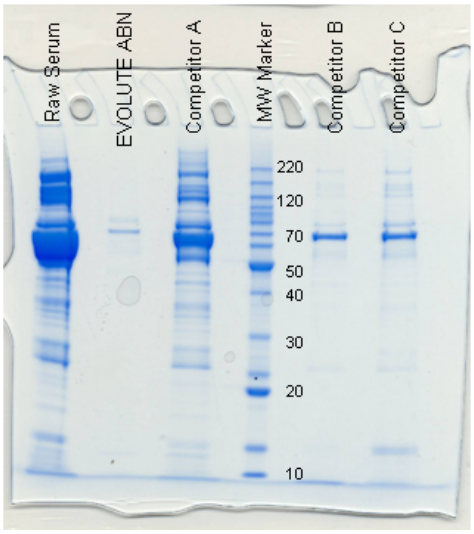

Figure 4. Gel electrophoresis protein profile comparing various resin-based SPE sorbents.

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

This experiment compared three silica based strong cation exchange mixed-mode sorbents with different carbon chain lengths (HCX (C8); HCX-3 (C4); HCX-5 (C18)) for protein removal. As an additional experiment the silica-based mixed-mode strong anion exchange sorbent, HAX, was tested. **Figure 5 (page 5)** shows the gel electrophoresis 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.

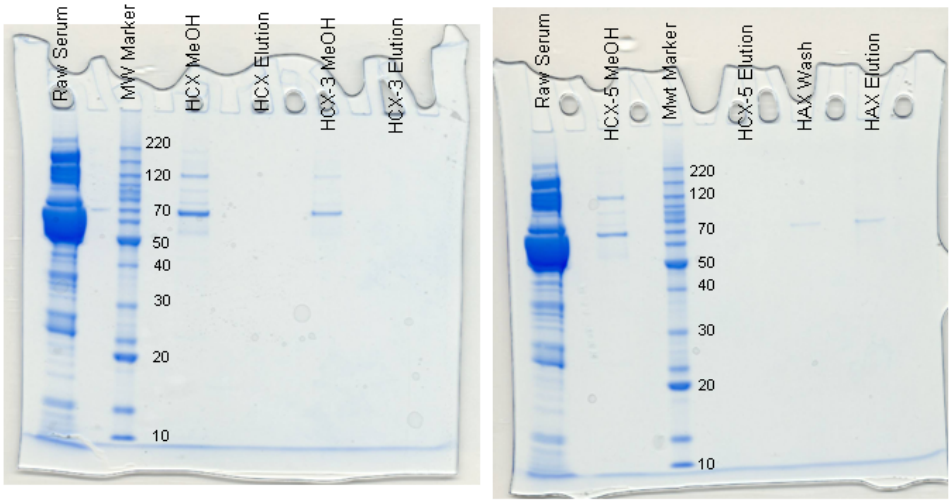

Figure 5. Gel electrophoresis protein profile comparing silica-based mixed-mode SPE sorbents (strong cation, HCX family; and strong anion, HAX).

Mixed-mode cation exchange SPE (resin-based sorbents)

This experiment compared EVOLUTE CX with two other manufacturer's resin-based mixed-mode cation exchange SPE sorbents. **Figure 6** shows the protein profiles for both the final MeOH wash step and the elution steps. Some protein was observed in the MeOH interference wash steps for all three sorbents, Competitor B showing the most retention. However, as with the silica-based mixed-mode cation exchange very little protein was observed in the elution solvents.

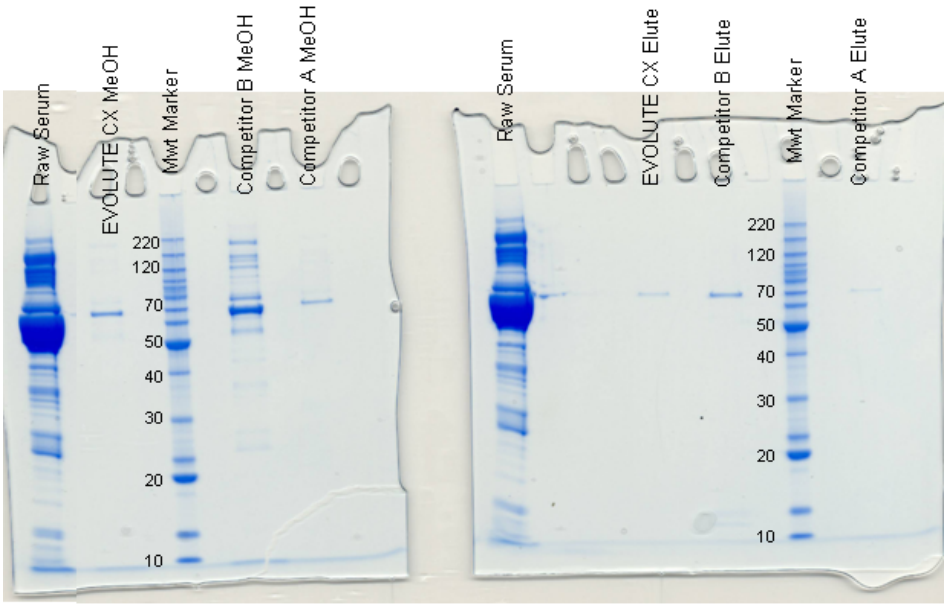

Figure 6. Gel electrophoresis protein profile comparing resin-based mixed-mode cation exchange SPE sorbents.

Conclusions

- PPT shows far greater protein levels for crash ratios below the suggested 1:3 ratio. Increasing the crash ratio to 1:6 did not show increased protein removal. A rough protein quantitation (n=1) showed greater than 99% of total protein removed using PPT with the generic method.
- SLE as expected showed no protein in the final extract. This is due to the low solubility of proteins in the water immiscible extraction solvent (MTBE).
- Non-polar SPE (silica-based sorbents). 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 chain compared to the others. The larger pore size of the MFC18 shows considerably more protein retention than the standard C18 product indicating that pore size has a substantial effect on protein retention.
- Non-polar SPE (resin-based sorbents). EVOLUTE ABN showed the lowest amount of protein retention compared to the three other manufacturer's resin-based SPE sorbents. This is due to the optimized pore size, structure and distribution of the material compared to the various competitors.
- Mixed-mode cation exchange SPE (silica-based HCX family sorbents). Some protein was observed in the MeOH interference wash steps, however, no protein was observed in the final elution solvent.
- Mixed-mode anion exchange SPE (silica-based HAX sorbent). Very little protein was observed in either the interference wash or the elution solvent.
- Mixed-mode cation exchange SPE (resin-based sorbents). All three sorbents showed 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 (v/v) 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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