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Precipitation of Large, High-Abundance Proteins from Serum with Organic Solvents

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INTRODUCTION

In serum and other complex biological samples, a few proteins often constitute a high percentage of the total protein. These high-abundance proteins can mask the numerous proteins of lower abundance in proteomics analyses, whether intact or in tryptic digests. Affinity materials can remove specific proteins such as albumin or immunoglobulins, but it would be tedious and expensive to develop such materials for the various high-abundance proteins in every biological sample. If the proteins and peptides of interest are smaller than ~ 20 KDa, then a simple alternative is to add organic solvent sufficient to precipitate proteins larger than this size. This would include the six most abundant proteins in serum, which represent > 90% of the total protein. The supernatant could then be subjected to proteomics analysis.

We demonstrate this approach with serum. Size-Exclusion Chromatography (SEC) was used to estimate the molecular weights of the proteins and peptides in the samples.
Precipitated proteins were removed via two alternative methods:

1) Centrifugation;

2) Passage through a solid-phase extraction (SPE) cartridge, here acting as a depth filter.

MATERIALS AND METHODS

SAMPLES

Serum was collected from a single individual. The peptide corresponding to prion protein residues 218-232, with fluorescein attached to the N-terminus via a γ-aminobutyric acid link [FITC-GABA-RESQAYYQRGASVIL-NH2] was a product of PolyLC. All other SEC standards were obtained from Sigma Chemical Co. (St. Louis, MO).

ALTERNATIVE SERUM TREATMENTS

1) **Centrifugation:** To 200 µl of serum was added 200, 400 or 600 µl of acetonitrile (ACN), with gentle mixing. The mixtures were left at room temp. for 30' and were then spun 4' at 12K rpm. The supernatants were removed via pipet (except for the lowest 10-20 µl) and the volumes reduced to 50 µl in a SpeedVac. The resulting samples were reconstituted to the original concentration in serum by addition of 150 µl of water apiece.

2) **Depth Filtration:** To 50 µl serum was added 50, 100 or 150 µl of ACN, with gentle mixing. The mixtures were left at room temp. for 30'. A TopTip™ disposable SPE pipet tip packed with C-18- coated silica (bed volume 25 µl) [Glygen Corp. item# TT2C18] was conditioned with two washes of 50 µl ACN. Each mixture was then applied via pipettor to the open end of a TopTip, which was then attached to a syringe. Air displacement was used to force the samples through the packed bed. The filtrates were collected and taken to dryness in a SpeedVac. 50 µl of water were added to reconstitute each to the original concentration in serum.

SIZE EXCLUSION CHROMATOGRAPHY

Two columns of PolyHYDROXYETHYL Aspartamide™ (PolyLC item# 209HY0503)[1,2] were connected in series. Each column was 200x9.4-mm, packed with 5-µm, 300-Å pore material.
Flow rate: 0.7 ml/min. Sample volume: 10 µl (except for Fig. 1).
Mobile phase: 100 mM KH_2PO_4, pH 6.6.
Detection: 220 nm (0.64 AUFS [Fig. 1] or 0.08 AUFS [Figs. 2 and 3]).

PROTEIN CONTENT OF SERUM[3]

Protein	Mol. Wt. (Da)	G. per 100 g. serum proteins
Albumin	66,000	52 - 68
IgG	150,000	13.5 - 23
Transferrin	80,000	3 - 7
α1-Antitrypsin	52,000	2 - 4
IgA	180,000 - 500,000	0.8 - 2.9
IgM	950,000	0.7 - 1.8

TABLE 1. These six proteins represent ~ 90% of the total protein in serum (disregarding lipoproteins). All have mol. wt. > 50,000 Da.

SEC of Crude Serum

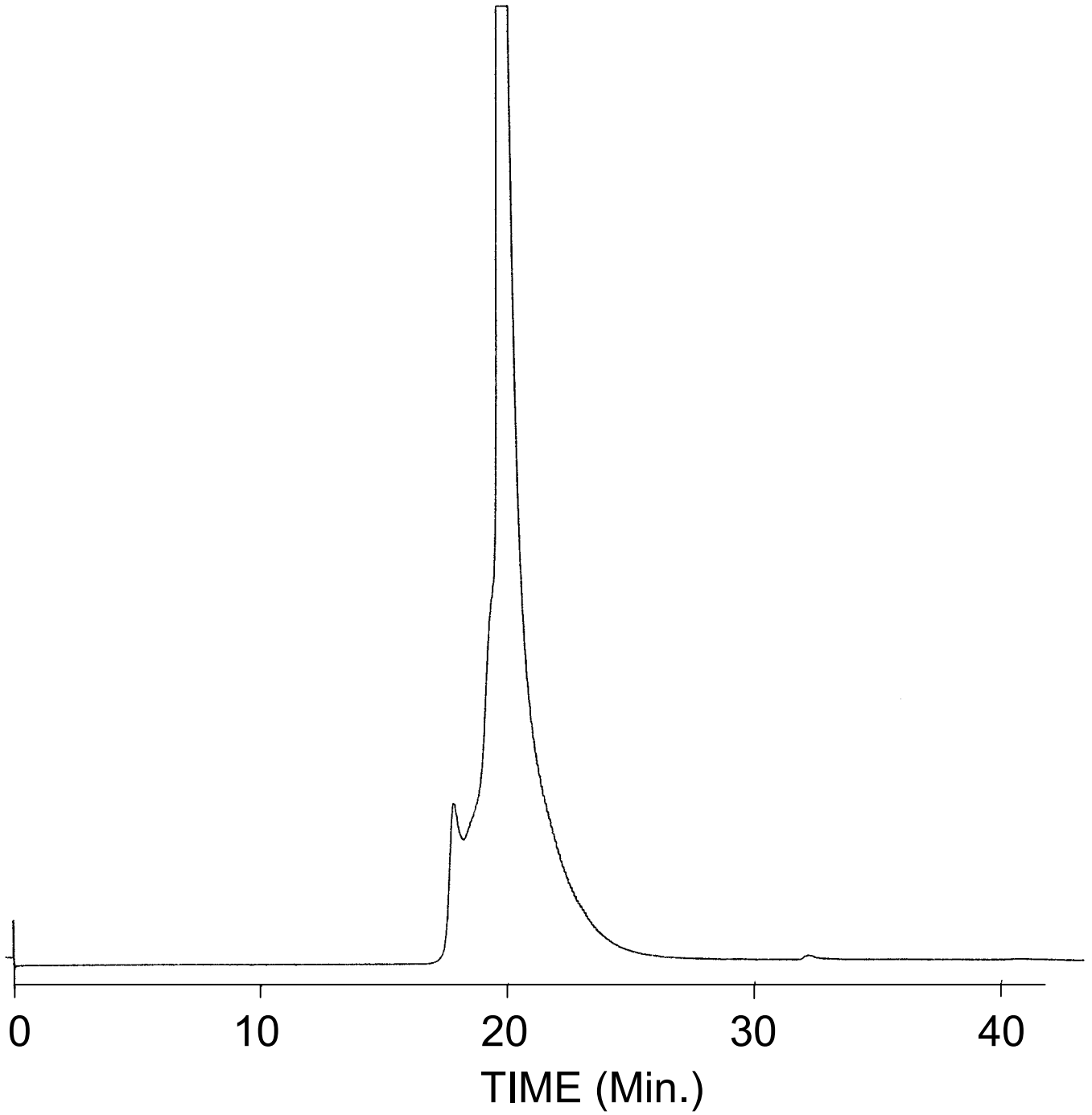


Figure 1

Serum + Acetonitrile

- SEC of Supernatants Post-Centrifugation -

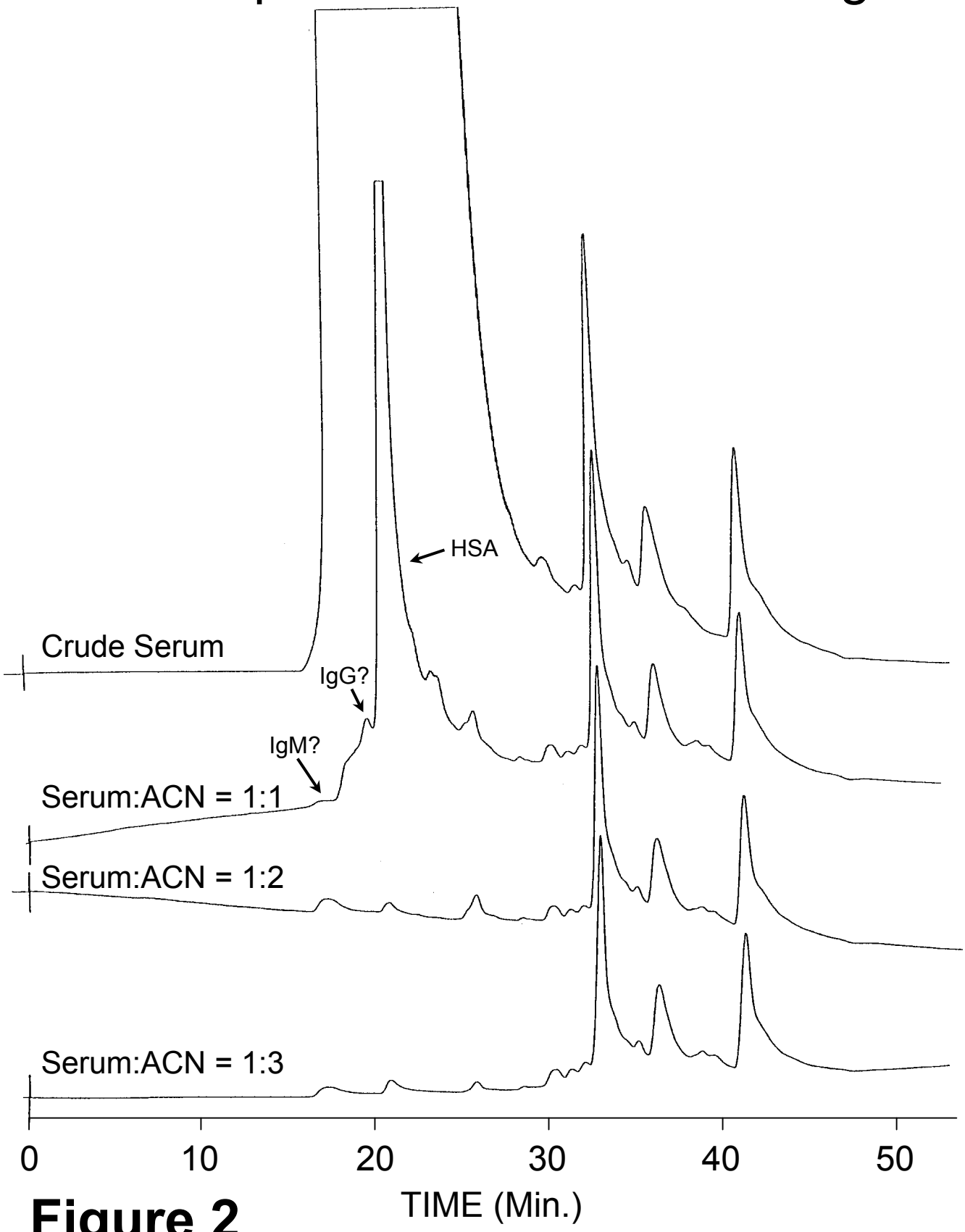


Figure 2

Serum + Acetonitrile

- SEC of TopTip™ Filtrates -

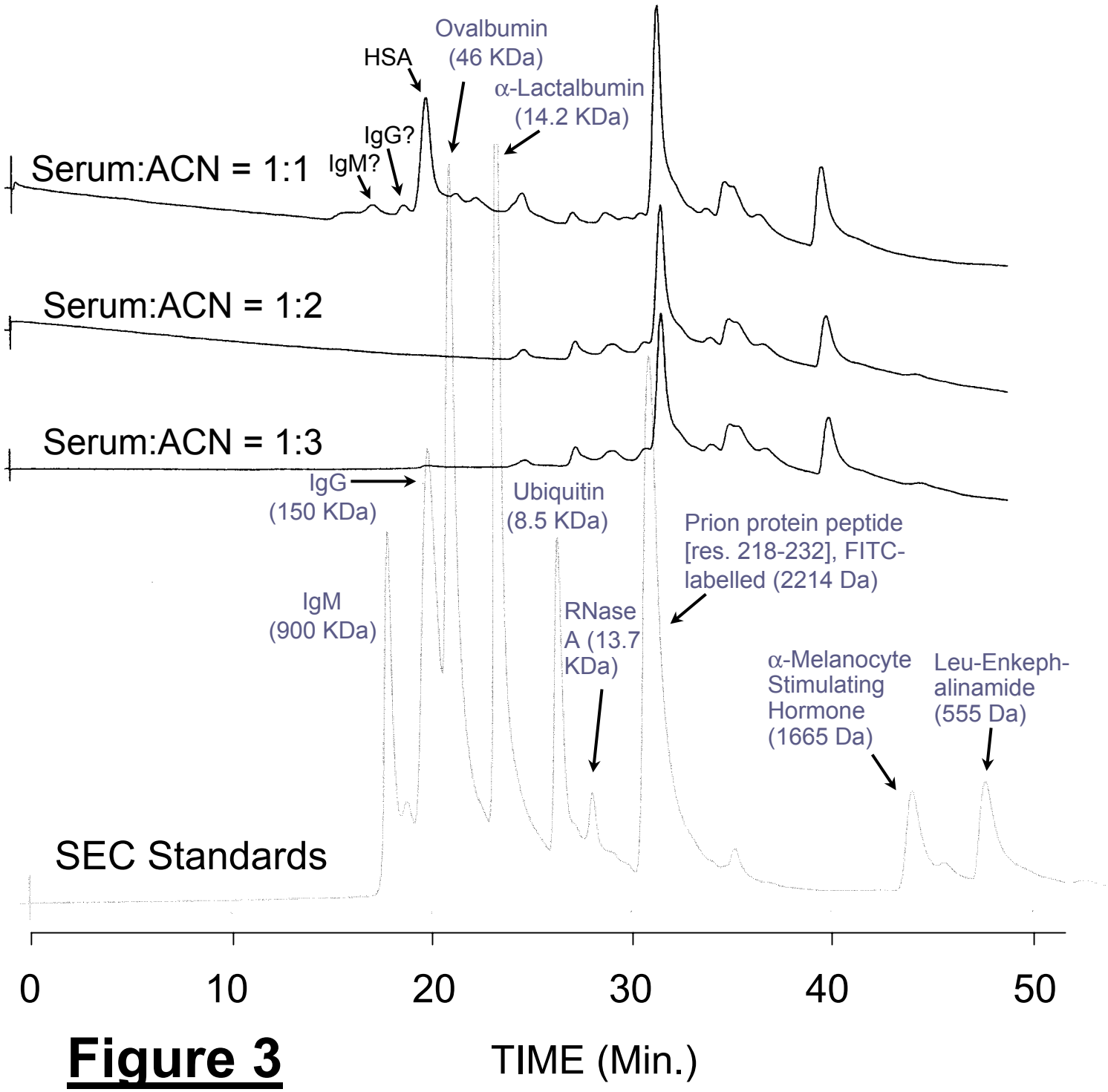


Figure 3

FIGURE 1: Crude serum (low sensitivity run).

Sample: 2 µl of serum dil. 1:3 with Mobile Phase.
Detection: 220nm (0.64 AUFS)

FIGURE 2: Centrifuged Samples.

Components > 40 KDa (see chromatogram of mol. wt. standards in Fig. 2): Addition of 1 vol. ACN eliminated 97-98% of the albumin and the other high-abundance proteins. However, albumin is still far more abundant than any other component of the mixture. Addition of 2 vol. ACN removed all but trace amounts of albumin and other proteins > 20 KDa. The sample that received 3 vol. ACN does not look significantly different than the sample that received 2 vol.

Components 15-40 KDa: These were not as thoroughly precipitated by 1 vol. ACN as were the larger proteins. With less albumin to mask them, their peaks are more prominent in the chromatogram; compare to Fig. 1.

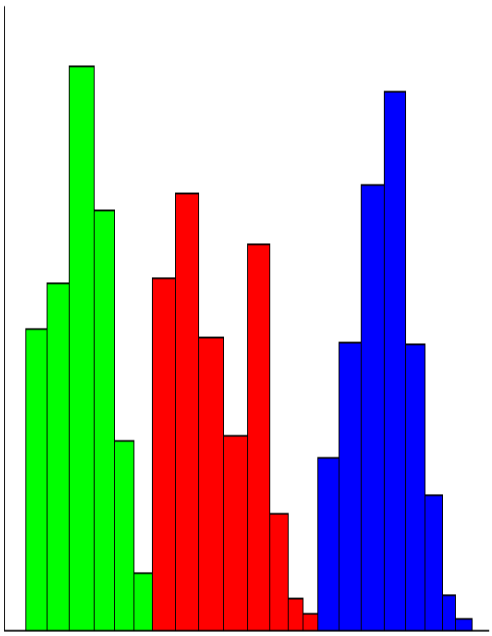
Components < 20 KDa: Addition of either 1, 2 or 3 vol. ACN did not significantly change the composition of the sample in this range.

FIGURE 3: TopTip™-Filtered Samples.

Components > 20 KDa: Filtration was significantly more effective at removing precipitated proteins than was centrifugation. With only 1 vol. of ACN added, albumin is no more abundant than several other components of the sample. Albumin and other large proteins are all but undetectable after addition of 2 or 3 vol. ACN.

Components < 20 KDa: Again, these were not particularly affected by addition of the ACN.

Figure 4. FRACTIONATION OF SERUM WITH ORGANIC SOLVENTS



Idealized SEC chromatogram of serum proteins

Column: PolyHYDROXYETHYL A (300 Å)

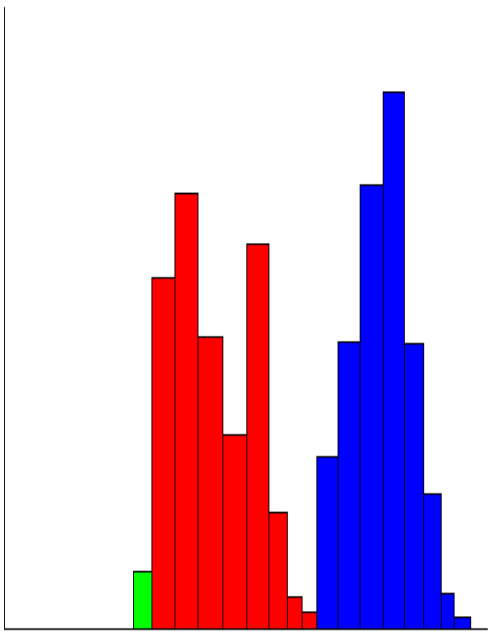
= Big proteins

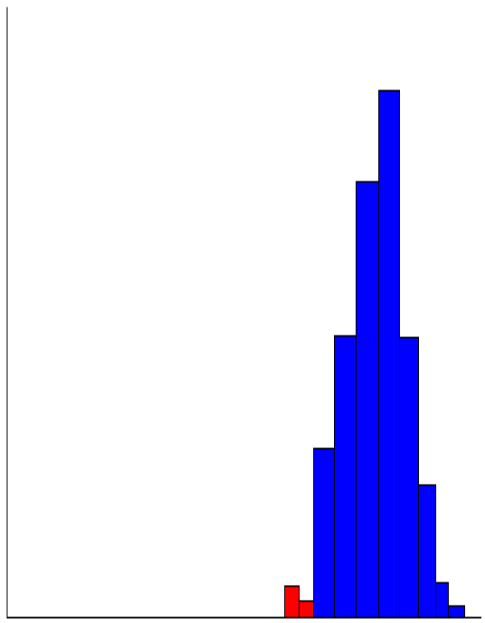
= Medium proteins

= Small proteins

+ 1 vol ACN

+ 2 vol ACN





DISCUSSION

2 vol. ACN precipitates virtually all serum proteins larger than ~ 20 KDa. 1 vol. ACN may suffice if the precipitated proteins are removed efficiently or if the proteins 15-40 KDa are of interest. Filtration proved to be significantly more effective at removal of precipitated proteins than did centrifugation.

Further study will involve more quantitative comparison of the two methods with regard to removal of large proteins and retention of small ones. The two methods will also be compared with a combination of both centrifugation and filtration, which has already been used in the analysis of folates and methotrexate in plasma[4].

ACKNOWLEDGEMENTS

TopTips™ (patent pending) are a trademark of Glygen Corp.
PolyHYDROXYETHYL Aspartamide™ is a trademark of PolyLC Inc.

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ABSTRACT

The proteins of high abundance in serum are larger than 40 KDa in mol. wt. Addition of 1 vol. of acetonitrile (ACN) leads them to precipitate. Subsequent centrifugation removes 97-98% of the large proteins. Depth filtration with a C-18 SPE cartridge is more effective than centrifugation. Addition of 2 vol. ACN removes virtually all large proteins using either method. Components smaller than 15 KDa are little affected by addition of up to 3 vol. ACN. Removal of proteins between 15-40 KDa varies with the method used. If the components of interest in a biological sample are smaller than 15 KDa, then this selective precipitation is a convenient way to remove large proteins of high abundance.