

# Ammonium Sulfate Precipitation as a Novel First Dimension in the Multidimensional Analysis of a Complex Proteome

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## Overview

We have applied the classic protein purification technique of ammonium sulfate precipitation to the first dimension of a 2-D analysis of the human plasma proteome. This approach has a number of advantages:

- Simple offline technique, accessible to all labs; avoids complicated column formats and switching schemes
- Unlimited by pI, hydrophobicity of analyte components
- Easily scaleable
- Minimizes the number of steps to avoid sample loss
- Allows for multiple subsequent analyses
- Captures extremely hydrophobic proteins as well as small peptides
- Operates at the protein level: peptides from the same protein remain together for the subsequent dimension of reverse-phase LC-MS/MS analysis

## Introduction

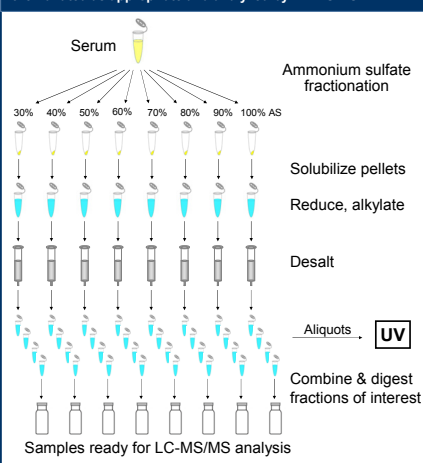
Multidimensional separation techniques<sup>1</sup>, e.g. cation exchange followed by reverse phase chromatography, are often employed in order to obtain increased depth and dynamic range relative to 2-D gels in the analysis of complex proteomes. In this study, the use of ammonium sulfate fractionation<sup>2</sup> in place of cation exchange as the 1<sup>st</sup> dimension separation method is investigated. The wide dynamic range of the serum proteome made it an attractive subject for this study.

## Methods

Ammonium sulfate (AS) precipitation was used to fractionate a human serum sample. 0.1 mL of 1M Tris pH 7 was added to 0.9 mL serum to maintain pH during the fractionation process. Protein pellets produced from stepwise 10% increases in AS concentration from 30% to 100% were solubilized in 0.5 mL of 6 M guanidine HCl, 500 mM Tris pH 7.8. The proteins were then reduced with DTT and S-carboxymethylated. Desalting was performed on a small G25 size exclusion column, exchanging the proteins into 2 mL of 10 mM ammonium bicarbonate which is both an ideal buffer for trypsin digestion and a friendly matrix for subsequent reverse phase separation.

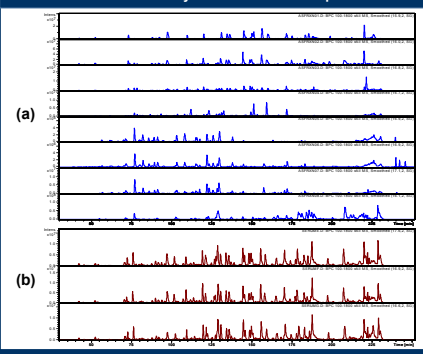
The peptide mixtures were analyzed by LC-MS/MS using the Agilent "proteomics solution" LC-MS system including 1100 series capillary and nano HPLC pumps and XCT ion trap. After online pre-concentration onto a 5 x 0.3 mm Zorbax peptide trap, separations were performed on a 600 x 0.075 mm capillary column packed with 5 µm Vydac C18 material and fitted with an 8 µm orifice PicoTip. Spectrum Mill software was used for the data analysis; results shown here were validated using the recommended default parameters. For comparison, a parallel but unfractionated plasma sample was analyzed 8 times under identical conditions.

**SCHEME 1. Experimental protocol.** Serum proteins are precipitated by means of stepwise increases in ammonium sulfate concentration. Pellets are resolubilized in denaturing buffer, then reduced, alkylated, and desalted. UV spectroscopy is used to identify the protein-containing fractions; these are combined and digested with trypsin. The solution digests are then diluted as appropriate and analyzed by LC-MS/MS.

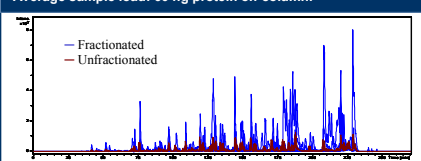


## Results

**FIGURE 1. Fractionated & unfractionated samples: base peak chromatograms.** (a) 8 ammonium sulfate fractions, (b) duplicate analyses of the unfractionated mixture (3 of 8 shown). 70% more proteins were identified in the 8 AS fractions than in the combined 8 analyses of the mixed sample.



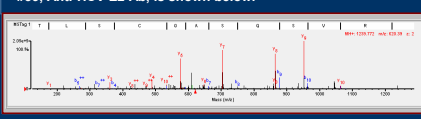
**FIGURE 2. Fractionation increases peptide intensity observed in each reverse-phase separation.** Base peak plots of the fractionated vs. unfractionated samples are shown. Average sample load: 60 ng protein on-column.



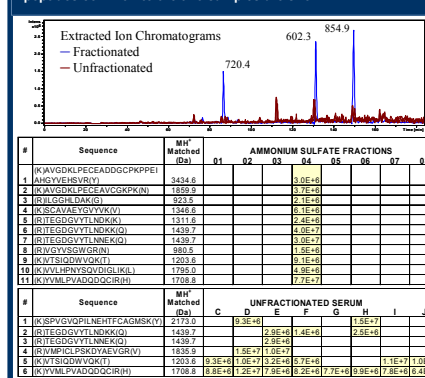
**TABLE 1. Distribution of proteins across 8 AS fractions**  
Average load 12.5 µg protein on column; increased loading on a 600 nm column improved protein coverage. Spectrum Mill searching with default parameters identified 68 proteins. Highly abundant proteins segregated over 24 fractions, while low abundance proteins were found mainly in 1 to 3 fractions. Confidence in proteins identified on the basis of one peptide is increased by location of the peptide in adjacent AS fractions.

F1	F2	F3	F4	F5	F6	F7	F8	%AA	Sumo	atlas	Group	Protein Name
								coverage	Search	Score		
0	0	0	0	0	0	0	0	84	1293.75	1	Serum albumin precursor	
0	17	17	31	0	1	0	0	45	647.93	2	Transferrin precursor	
0	1	0	0	0	0	0	0	45	636.47	3	Complement C3 precursor	
2	4	13	9	0	1	0	0	40	551.78	4	alpha-2-macroglobulin precursor	
0	0	0	0	2	0	0	0	34	446.75	5	Apolipoprotein B100 precursor	
0	0	1	1	1	0	0	0	24	338.1	6	Immunoglobulin heavy chain	
0	1	1	10	4	6	4	0	20	270.98	7	Ceruloplasmin	
0	1	0	0	0	4	1	0	15	214.54	8	Alpha-1-Antitrypsin	
2	4	13	9	0	1	0	0	14	206.87	9	Immunoglobulin M heavy chain	
2	2	7	13	3	0	0	0	15	204.93	10	Ig heavy chain V(L)UJ region	
0	0	0	0	6	2	1	0	13	198.76	11	Ig kappa light chain V(L)J region	
2	4	13	9	0	1	0	0	12	180.75	12	Apolipoprotein A1 precursor	
0	0	0	1	4	7	10	0	12	180.59	13	Vitamin D-binding protein precursor	
0	1	0	0	0	1	0	0	13	175.19	14	Haptoglobin 2 precursor	
0	0	0	0	0	0	0	0	12	160	15	Complement component C4	
0	0	1	5	0	1	0	0	11	150.08	16	PK-120 precursor	
0	0	0	0	0	0	0	0	11	148.23	17	Fibrinectin precursor	
1	5	4	4	14	12	7	4	10	140.53	18	Trypsin	
1	4	14	4	1	1	0	0	9	129.59	19	Ig lambda chain	
0	0	0	0	0	0	0	0	9	129.27	20	Hemopexin precursor	
0	0	0	0	0	0	0	0	8	119.44	21	Inter-alpha (globulin) inhibitor	
0	0	0	0	0	0	0	0	7	101.4	22	Inter-alpha trypsin inhibitor	
0	0	0	0	0	0	0	0	1	17.34	51	Fibrinectin	
0	0	0	0	0	1	1	0	1	15.8	52	Nitrophenol binding protein	
0	0	0	0	0	0	0	0	1	15.59	53	Carboxypeptidase N E3 KDa chain	
0	0	0	0	0	0	0	0	1	15.58	54	Ig kappa chain V region	
0	0	0	0	0	0	0	0	1	15.53	55	Complement subcomponent C	
0	0	0	0	0	0	0	0	1	14.55	56	Protein tyrosine kinase Tec1c1	
0	0	0	0	0	0	0	0	1	14.42	57	Ig heavy chain variable region	
0	0	0	0	0	0	0	0	1	14.31	58	Heterodisulfide reductase	
0	0	0	0	0	0	0	0	1	14.27	59	Anti-HCV E2 Ab, VK segment	

**FIGURE 3. MS/MS data.** One-peptide identifications which pass the default Spectrum Mill validation thresholds are of high quality. The match for the single peptide from hit #59, Anti-HCV E2 Ab, is shown below.



**FIGURE 4. Haptoglobin peptides observed in fractionated vs. unfractionated serum.** 11 peptides are found exclusively in AS fraction 04; between 1 and 5 peptides are found in individual analyses of the unfractionated mixture. EIC plots for 3 of the peptides common to the two samples are shown.



## Conclusions

Ammonium sulfate precipitation is an effective method for increasing proteome coverage. Although highly abundant proteins are not confined to single fractions, proteins of reasonable and low abundance segregate and are enriched. Long reverse phase capillary HPLC columns are invaluable in providing the high loading capacities and exacting separations required for these types of complex mixtures.

### Further studies:

- Optimization of ammonium sulfate fractionation protocol
- A more accurate estimate of protein amount per fraction to assist in calculating sample loadings
- Range-finding experiments to determine the optimal amount of sample to load on-column for RP LC-MS
- Effect of immunoglobulin and/or albumin depletion

## References

<sup>1</sup> Wolters, D.A., et al. *Analytical Chemistry* **2001**, *73*, 5682-5690. Adkins, J.N., et al. *Molecular and Cellular Proteomics* **2002**, *1*, 947-955.  
<sup>2</sup> *Data for Biochemical Research*, 2<sup>nd</sup> ed.; Dawson, R.M.C., et al., Eds.; Oxford University Press: New York, 1969.  
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