Comparative Analysis of Serum Samples with the Agilent mRP-C18 Column - A New Technology for Protein Fractionation

Application

Proteomics

Authors

William Barrett, James Martosella, Nina Zolotarjova, Liang-Sheng Yang, Cory Szafranski, and Gordon Nicol
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

Proteomic samples such as human serum are an extreme challenge to study due to the large dynamic range of the proteins of interest. The proteins in human serum can range from those considered "high-abundance" proteins, present at 2%–50% of the total protein mass (typically the µg/mL–mg/mL range) to those considered tissue biomarkers present in the pg/mL–ng/mL range. Multidimensional separation techniques have provided one way to address the complexity of these samples. A reversed-phase (RP) HPLC method for the separation and fractionation of immunodepleted protein samples was developed using a novel macroporous reversed-phase (mRP) column. The optimized method and special column material combine to provide high-protein recoveries, reproducible fractionation, and higher column loads. Samples of human serum, depleted of the six most abundant proteins, were separated on a novel mRP-C18 column resulting in high protein recovery, good protein resolution, and excellent reproducibility. This allowed for a direct comparison of several immunodepleted human sera samples, which showed differences in chromatographic UV profiles, providing a quick screen of protein differences in fractions that can then be analyzed by liquid chromatography/mass spectrometry.

Introduction

The drive to discover novel protein serum biomarkers in either drug development or disease models is not only vital to further research, but is also a major challenge. The dynamic range of proteins in human serum spans greater than 10 orders of magnitude with six proteins representing 85% of the total protein mass, including albumin, IgG, IgA, transferrin, haptoglobin, and alpha-1-antitrypsin. Depletion of high-abundance proteins with the Agilent Multiple Affinity Removal System can provide access to previously undetectable proteins. Typically, immunodepleted samples are further fractionated with techniques such as 2-dimensional gel electrophoresis (2DGE), multidimensional liquid chromatography/mass spectrometry (LC/MS), or offline methods employing orthogonal separation techniques. The fractionation strategies continue to develop as scientists test for optimized separations of proteins, which provide high recoveries, efficient separations, and reproducibility, all of which are required for the eventual validation of protein biomarkers. Currently, multi-dimensional separations suffer from poor sample recoveries and reproducibility. Lack of reproducibility associated with 2DGE often makes it difficult to compare samples directly. In addition, protein recoveries from RP chromatography typically range from 30%–80% and are often not reproducible. The

ability to couple the Agilent Multiple Affinity Removal System to deplete the top six high-abundance proteins in serum with a RP system to fractionate proteins would enable researchers to simplify serum samples and identify potential biomarkers. In this study, the protein fractionations from three immunodepleted serum samples, a control, a cortisol-deficient sample, and a high rheumatoid factor sample were compared for differences. The high recovery and increased resolution enabled an improved comparative analysis of serum samples. This technique allows one to rapidly screen differences that can then be identified by LC/MS.

Experimental

The Multiple Affinity Removal System for removing albumin, transferrin, IgG, IgA, haptoglobin and antitrypsin from human serum is a product from Agilent Technologies (Wilmington, DE). A 4.6-mm × 100-mm Multiple Affinity Removal column (part number 5185-5985) was used with a mobile phase reagent kit (Agilent Technologies, part number 5185-5986). Sample loading, washing, and column regeneration is done using Buffer A, and for bound protein elution Buffer B is used according to manufacturer protocols. Injections of diluted serum were performed according to manufacturer protocols for a 4.6-mm × 100-mm column. The flow-through fractions were automatically collected by time into 1.5-mL plastic tubes (part number 5188-5251) using an Agilent 1100 HPLC equipped with a thermostatted analytical-scale fraction collector. The depleted serum samples containing low-abundance proteins in the flow-through fractions were collected and stored at –20 °C until analysis. A sample of serum from a healthy control subject, cortisol-deficient sera (Sigma number 7269), and sera with high rheumatoid factor (Sigma number 3145) were all immunodepleted to remove six high-abundance proteins as per manufacturer's protocol.

Sample Preparation of Immunodepleted Serum for Loading onto the mRP-C18 Column

The flow-through fractions from each immunodepleted serum were collected, and protein concentrations determined via the BCA Protein Assay (Pierce, Rockford, IL). Total protein (300 µg) from each flow-through fraction in approximately 1 mL was prepared for direct-loading onto the mRP-C18 column. Into each sample, 0.48 g of urea pellets was dissolved and 13 µL of neat glacial acetic acid added to acidify the samples and prevent carbamylation at the elevated temperature required for the separation. Approximate final concentrations were 6M urea and 1.0% acetic acid.

Samples of serum were depleted of the high-abundance proteins using the Agilent Multiple Affinity Removal Column as stated. Protein fractionation of the flow-through fraction from immunodepletion was performed on the 4.6 mm ID × 50-mm mRP-C18 column (part number 5188-5231). Sample preparation prior to loading on the mRP-C18 column is necessary for resolution and reproducibility and contributes to the high recovery of low-abundance proteins.

LC Fractionation System

An Agilent 1100 HPLC equipped with a binary pump, thermostatted autosampler, thermostatted column compartment, and a thermostatted analytical-scale fraction collector was used for fractionating the samples. Due to the larger volume required for complete injection of the samples, the standard 100-µL needle and sample loop from the autosampler was replaced with a 900-µL needle and metering device (Agilent part number G1363A).

Note: The use of multidraw is not recommended or supported due to decreased peak shape and large delay volume.

Repeat Inject Method for Large Volume Loading

For loading sample volumes greater than 900 µL, an isocratic method (Figure 1) and sequence table is required. The method involves the injection of a desired volume based on total sample volume followed by an isocratic run of 3 minutes to load the mRP-C18 column. This can be repeated several times for larger volumes using the sequence table until the final injection, which would be done using the optimized separation method (Figure 2).

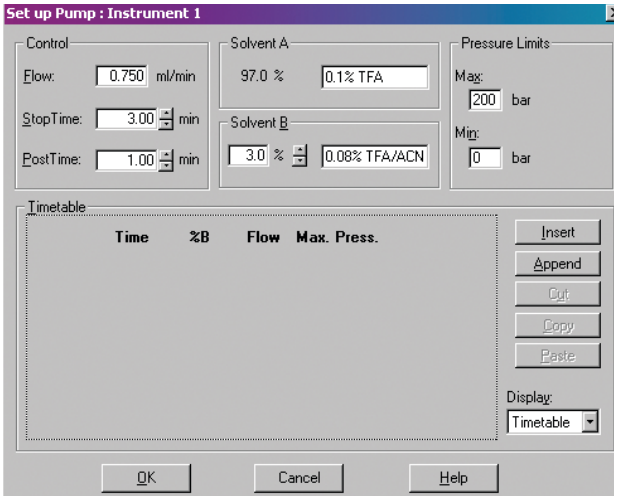

Figure 1. Example Agilent 1100 set-up screen for isocratic loading of large sample volumes.

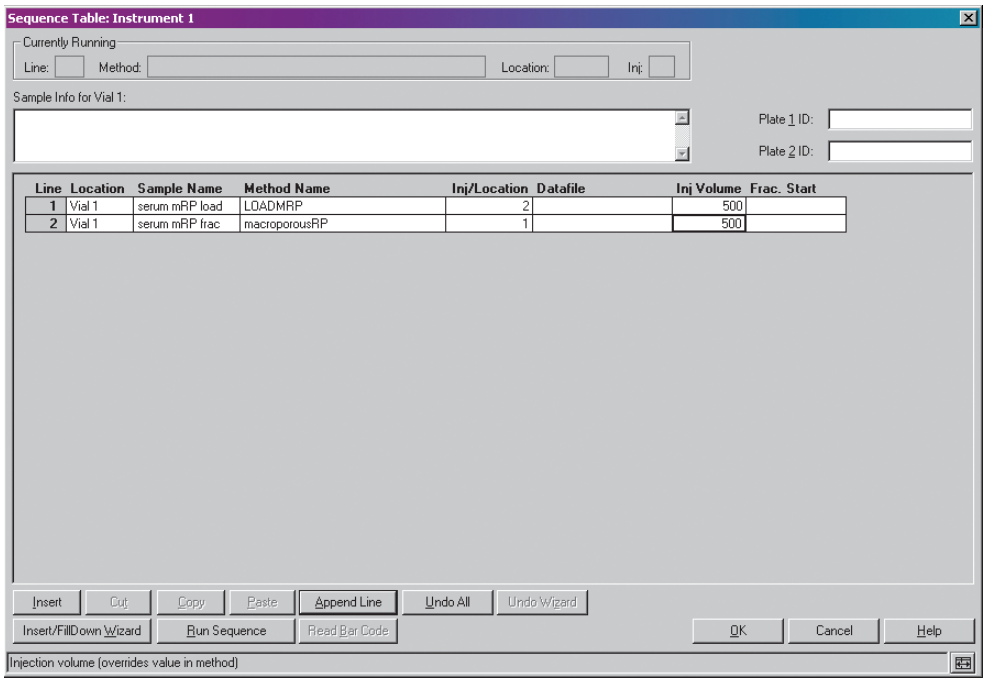

Figure 2. Sequence table to load large sample volumes; the example demonstrates two isocratic injections of 500 µL followed by the last injection that runs the gradient elution.

Protein Fractionation

After sample preparation with urea and glacial acetic acid, the immunodepleted serum was separated under RP conditions using a linear multisegment gradient (Table 1). Fraction collection was performed by time, collecting 1-minute time slices starting at 1 minute and continuing to 53 minutes.

Table 1. Protein Fractionation Linear Multisegment Gradient

Flow	0.75 mL/min	
Stoptime	58 min	
Posttime	20 min	
Column temperature	80 °C	
Detection	UV 280 nm	
Starting Solvent Composition		
Solvent A	97% (Water/0.1% TFA)	
Solvent B	3% (ACN/0.08% TFA)	
Pressure limit	200 bar	
Gradient	Time (min)	% B
	0	3
	1	3
	6	30
	39	55
	49	100
	53	100
	58	3

Recovery Conditions

To measure protein recovery, several injections of sera (approximately 47 µg) were performed without the column installed inline using a fast gradient typically employed for desalting and concentrating the flow-through fraction (Table 2). Fractions were collected and pooled and the sample dried using a SpeedVac. The column was then placed inline and several injections were performed for each serum sample and the fractions collected, pooled and dried as above. The pellets were resolubilized in 3M urea/1% Triton X-100 detergent solution. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). Recovery data for the sample with and without the column installed were used to determine accurately the absolute protein recoveries.

Table 2. Protein Desalting/Concentration Gradient

Flow	0.75 mL/min	
Stoptime	13 min	
Posttime	10 min	
Column temperature	80 °C	
Detection	UV 280 nm	
Starting Solvent Composition		
Solvent A	97% (Water/0.1% TFA)	
Solvent B	3% (ACN/0.08% TFA)	
Pressure limit	250 bar	
Gradient	Time (min)	% B
	0	10
	3	10
	5	70
	6	100
	8	100
	10	10

Tryptic Digest of Protein Fractions

The selected mRP-C18 column fractions were resuspended in 25 µL of 50-mM NH_4HCO_3 and 25 µL of trifluoroethylene (TFE). Then 3 µL of 200-mM dithiothreitol (DTT) was added and the sample was mixed briefly on a vortex mixer. The solution was incubated at 60 °C for 1 hour. The reduced proteins were alkylated with iodoacetamide (IAM) by adding 3 µL of a 200 mM IAM solution followed by incubation in the dark at room temperature for 1 hour. After incubation, 3 µL of 200 mM DTT was added to react with the excess IAM.

Prior to trypsin digestion, the TFE concentration in the solution was adjusted with 50 mM NH_4HCO_3 to less than 5%. Trypsin was added at a ratio of approximately 50:1 protein to enzyme and proteins were digested overnight at 37 °C. An aliquot was used for LC/MS analysis.

LC/MS Analysis

Digested fractions from the mRP-C18 column were analyzed by LC/MS on an Agilent 1100 MSD Trap SL. A trapping column (Agilent 300SB-C18, 5 µm, 0.5 mm × 35-mm column) was used to capture and concentrate the sample peptides prior to separation on a RP column (ZORBAX 300SB-C18 5 µm, 0.3 mm × 150 mm, Agilent Technologies). The flow rate for the RP separation was 5 µL/min. Peptides were trapped on the trapping column, which was then switched inline with the flow for the analytical RP column, and the peptides were eluted off the trap column and onto the RP column. The gradient for the RP and salt slices is as follows:

Time (min)	0	5	50	55	58	60
%B	5	5	40	70	70	5

Spectrum Mill software was used for data analysis. The following filters were used for peptide and protein identification: peptide score 9, protein score 11 and %SPI: 70%. Only fully tryptic peptides were considered with one tryptic missed cleavage allowed.

Results and Discussion

The ability to reduce the complexity of proteomic samples typically involves orthogonal techniques such as 2D-chromatography or the use of gel electrophoresis. The Agilent mRP-C18 column provides a tool to reduce complexity at the protein level prior to downstream analysis. The mRP-C18 column offers several advantages over standard reversed phase columns including high recovery, reproducibility, and resolution. The recovery from a column is critical in the analysis of samples to prevent cross-contamination due to carryover since typical recoveries in RP HPLC methods can range from 30%–80%. Recovery is also important in the validation of biomarkers by allowing for a direct quantification experiment between control and treated/ diseased samples, where inefficient recovery from typical RP HPLC methods prohibits its use in biomarker validation.

Recovery data for samples that were obtained with and without the mRP-C18 column inline show 99% recovery (Table 3).

Table 3. Recovery of Serum Proteins from the Agilent mRP-C18 Column as Determined by the BCA Assay

Protein conc. no column (µg)	Protein conc. mRP-C18 recovery (µg)	% Recovery
49.8	49.3	99

*N=5. Percent recovery is calculated from the mRP-C18 column recovery versus the fraction collected with no column.

A further examination of protein recovery was performed by injecting and separating immunodepleted serum on the mRP-C18 column, followed by a blank injection. Analysis of the chromatograms shows no significant UV absorbance for the blank sample suggesting greater than 99% recovery of the serum from the first injection (Figure 3). The excellent recovery from the mRP-C18 column provides the ability to compare a diseased and control sample without carryover concerns.

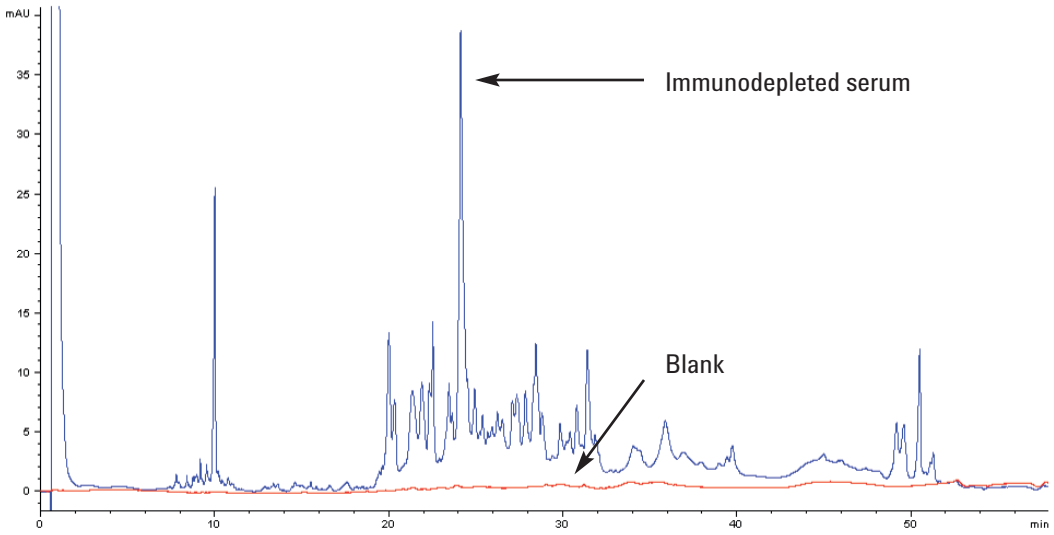

Figure 3. **Two chromatograms show the separation of immunodepleted serum followed by a blank injection. The high recovery from the mRP-C18 column is demonstrated by the absence of significant absorbance in the blank run.**

Reproducibility would provide a measure of confidence to compare and validate potential protein biomarkers identified in serum. The reproducibility of five immunodepleted serum injections is attained with the optimized method and temperature for protein fractionation as shown in Figure 4. The inset demonstrates the robust reproducibility of the Agilent mRP-C18 column for two immunodepleted serum samples that were injected 1 week apart. The column was used extensively during the week for many different serum samples.

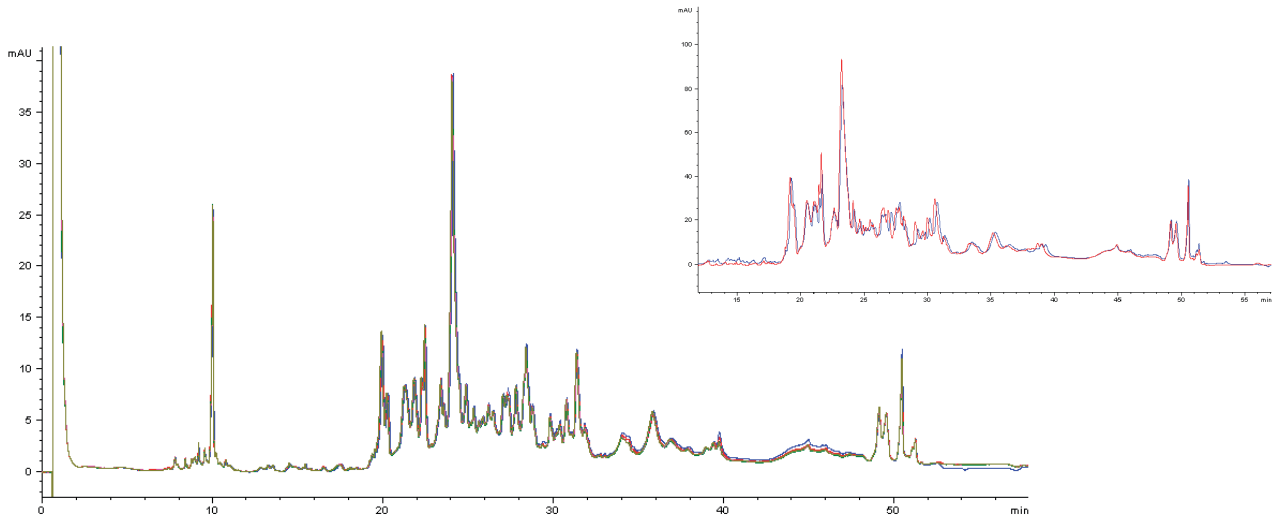

Figure 4. **Reproducibility of the mRP-C18 column as shown in five consecutive injections. Inset, shows two injections of serum performed 1 week apart.**

Reproducibility, high recovery, and efficient resolution allow for the comparison between a control serum sample (blue), a cortisol-deficient serum sample (red), and an elevated Rheumatoid factor serum sample (green; Figure 5). Each serum sample was immunodepleted of their high-abundance proteins using the Agilent Multiple Affinity Removal column. Samples were then injected and separated on the mRP-C18 column. Chromatograms were overlaid and analyzed for differences in the UV absorbance profiles. The mRP-C18 column provides a way to fractionate at the protein level and focus on specific fractions, with obvious differences, as shown in the inset of Figure 5 as a potential method to identify biomarkers.

Fractions from the regions indicated in Figure 5 were collected, dried, and resuspended in appropriate buffer. A trypsin digestion in TFE was performed on each sample. An aliquot was removed from each sample and analyzed by LC/MS. The LC/MS results were processed with Spectrum Mill software. The results for the time range between 19–21 minutes are shown in Table 4. The highlighted regions show obvious differences in proteins between the samples. The cortisol-deficient serum sample had no complement H or apolipoprotein H, while the high rheumatoid factor serum showed a relative loss in complement H as noted by the lower number of spectra found compared to the control serum.

A similar analysis on the fraction from 29–32 minutes was also performed on the three samples as noted by the differences in UV absorbance (Table 5).

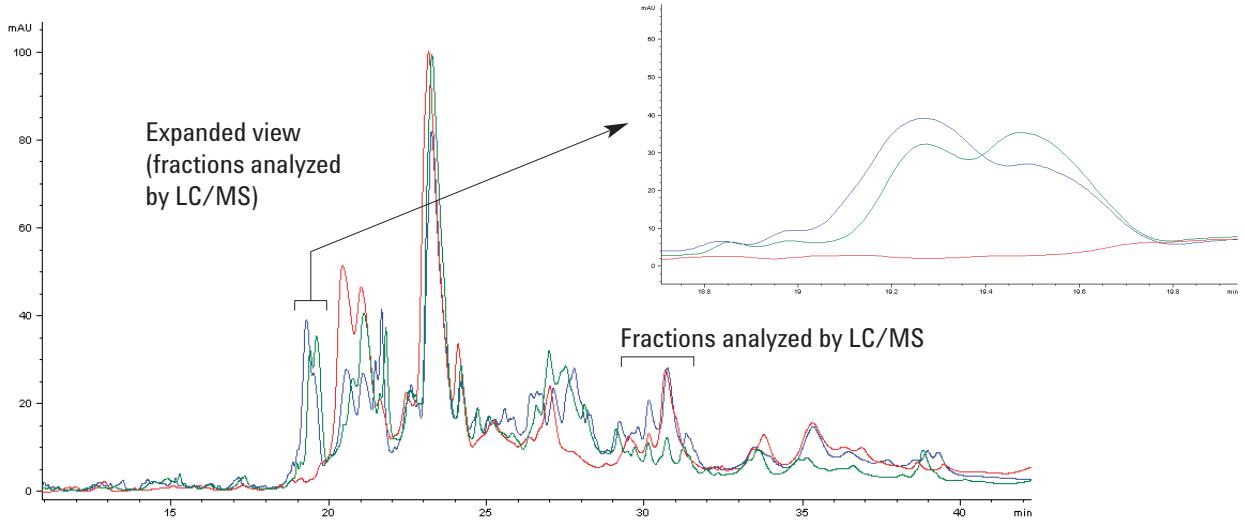

Figure 5. Immunodepleted serum samples of control serum (blue), cortisol-deficient serum (red), and high rheumatoid factor serum (green) as fractionated on the mRP-C18 column. Inset shows the expanded range of chromatograms with obvious differences in UV absorbance. Protein loads for each sample were 300 µg.

Table 4. Protein Identifications from Fractions of each Serum in the Time Range of 19–21 Minutes

Serum no. spectra total intensity	Def. cort. no. spectra total intensity	High rheumatoid no. spectra total intensity	No. unique peptides	Score	Protein
14	0	4			
3.09E+07	0.00E+00	8.15E+06	12	178.37	H factor 1 (complement)
8	0	9			
1.94E+07	0.00E+00	2.26E+06	8	115	Apolipoprotein H (beta-2-glycoprotein I)
0	3	1			
0.00E+00	4.65E+06	1.60E+06	3	39.47	Ceruloplasmin
0	0	2			
0.00E+00	0.00E+00	3.54E+06	2	30.34	Complement component 1 inhibitor precursor
2	0	0			
8.65E+06	0.00E+00	0.00E+00	2	28.61	Apolipoprotein C-III precursor
1	0	2			
1.84E+06	0.00E+00	3.13E+06	2	27.34	Complement factor B preproprotein
0	0	2			
0.00E+00	0.00E+00	3.40E+06	2	24.99	Hemopexin
0	0	2			
0.00E+00	0.00E+00	4.13E+06	2	24.77	Alpha-1-acid glycoprotein 2 precursor

Table 5. Protein Identifications from Fractions of each Serum in the Time Range of 29–32 Minutes

Serum no. spectra total intensity	Def. cort. no. spectra total intensity	High rheumatoid no. spectra total intensity	No. unique peptides	Score	Protein
37	26	11			
2.04E+08	1.13E+08	2.27E+07	34	555.56	Complement component 3
15	2	2			
4.43E+07	3.58E+06	2.16E+06	15	235.16	Complement component 4A preproprotein
12	1	11			
7.48E+07	2.30E+06	3.84E+07	11	178.25	Apolipoprotein A-I precursor
4	7	8			
1.13E+07	2.55E+07	2.87E+07	9	132.42	Antithrombin III variant
4	0	2			
7.05E+07	0.00E+00	2.77E+07	4	54.58	Apolipoprotein A-II precursor
0	2	2			
0.00E+00	1.94E+06	2.53E+06	3	36.72	Serine (or cysteine) proteinase inhibitor, clade A, member 3, precursor
1	0	0			
1.56E+06	0.00E+00	0.00E+00	1	18.22	Inter-alpha-trypsin inhibitor C-terminal

The highlighted boxes clearly show a difference in protein levels between the control serum and abnormal sera as indicated on the chromatograms. The high rheumatoid serum shows a lower level of complement component 3 compared to the control or cortisol-deficient serum. For both cortisol-deficient serum and high-rheumatoid-factor serum there is a decrease in complement component 4A, while cortisol-deficient serum has a clear decrease in apolipoprotein A-1 protein levels. Several of those proteins have shown a reduction in patients with systemic lupus erythematosus (SLE), rheumatoid arthritis, and scleroderma [1]. These proteins were easily identified by protein fractionation after immunodepletion and reduction of sample complexity with the mRP-C18 column while comparing UV traces of suspect serum to control serum.

Conclusions

The Agilent mRP-C18 column provides optimized separation for human serum after depletion of the high-abundance proteins using the Agilent Multiple Affinity Removal column. This combined workflow enables high recovery, excellent resolution and reproducibility in reducing sample complexity for biomarker research. The ability to recover >98% with immunodepleted serum also provides the opportunity to validate biomarkers that are differentially expressed in control versus disease/treated serums. The ability to reproducibly fractionate at the protein level with high resolution enables efficient separation and reduced overlap of proteins in collected RP fractions. Coupling of immunodepletion with the mRP-C18 column methods allowed for rapid screening for UV absorbance differences from which differences in proteins could be determined via LC/MS analysis of those selected fractions. Researchers may be able to further optimize separation methods for use on other samples such as membrane proteins or yeast protein samples.

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Reference

1. Grant et al., (2000) *J. Immunol Methods.*, **244**, 41–7.

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Email technical inquiries to affinity_removal@agilent.com

William Barrett is a Proteomics Application Scientist for Proteomic BioReagents in the Integrated Biology Solutions Group with Agilent Technologies.

Nina Zolotarjova, James Martosella, Gordon Nicol and Hongbin Liu are R&D Scientists for Proteomic BioReagents in the Integrated Biology Solutions Group with Agilent Technologies.

Liang-Sheng Yang is a Manufacturing/Quality Engineer for Proteomic BioReagents in the Worldwide Order Fulfillment Group with Agilent Technologies.

Cory Szafranski is the Product Manager for Proteomics BioReagents in the Integrated Biology Solutions Group with Agilent Technologies

Correspondance: Dr. William Barrett

Email: bill_barrett@agilent.com

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