A Rapid Method to Desalt and Concentrate Proteomic Samples Using the Agilent mRP-C18 Column

Application

Proteomics

Authors

William Barrett, James Martosella, Barry Boyes,
Cory Szafranski, and Gordon Nicol
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

The Agilent mRP-C18 column is shown to provide optimized desalting and concentration for human serum after depletion of the high-abundance proteins using the Agilent Multiple Affinity Removal System. This combined workflow enables high recovery for further downstream processing in biomarker research. Additionally, the elimination of a spin concentration step with a molecular weight cutoff (MWCO) allows the safe recovery of peptides and polypeptides less than the typical 5 kDa MWCO. Fast processing time with reproducible, high recovery allows for confident sample desalting and concentration without the typical losses associated with conventional reversed-phase (RP) techniques. This technology can be coupled, allowing for orthoganol separations without interference from salts or other additives removable by the mRP-C18 column.

Introduction

Automation of the steps required to reduce the complexity of proteomic samples would fulfill a critical need for biomarker discovery. Many researchers employ multidimensional separations for their samples, which often involve salts or chaotropic agents such as urea. The deleterious effects of these reagents in sample analysis by liquid chromatography/mass spectrometry (LC/MS) often makes it necessary to remove salts and denaturing agents prior to MS studies. Many methods to perform sample cleanup exist, but often recovery levels are poor in the range of 30%–80% and often are not reproducible. A RP HPLC method for desalting and concentrating immunodepleted protein samples was developed using a novel macroporous RP column (mRP-C18). The optimized method and special column material combine to provide high protein recoveries, reproducible desalting/concentration, and higher column loads than conventional RP LC columns. Samples of human serum, depleted of the six most abundant proteins, were desalted/concentrated on the mRP-C18 column with >98% recovery of total protein. The ability to use the Agilent Multiple Affinity Removal System to deplete the top six high-abundance proteins in serum followed by a RP system to concentrate and desalt proteins allows researchers to simplify the sample workflow for additional downstream studies.

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. 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. Depleted serum samples containing low-abundance proteins in the flow-through fractions were collected and stored at –20 °C until analysis.

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

Flow-through fractions from the immunodepleted serum were collected, and protein concentrations were determined via a BCA Protein Assay (Pierce, Rockford, IL). Approximately 300 µg total protein from each flow-through fraction, in about 1 mL of Buffer A from immunodepletion, was prepared for direct-loading onto the mRP-C18 column. Two sample sets were analyzed. The first was prepared by adding 0.48 g of urea pellets and 13 µL of neat glacial acetic acid to the sample. The approximate final concentration of urea is 6M with 1.0% acetic acid. The second set of samples was used directly from the immunodepletion column without alteration.

Protein Sample Desalting

The immunodepleted serum samples were desalted/concentrated under RP conditions using a linear multi-segment gradient (Table 1). Desalting/Concentrating of the flow-through fraction from immunodepletion was performed on the mRP-C18 column (4.6-mm × 50-mm; part number 5188-5231). Fraction collection was performed by time, collecting 1.5-minute time slices starting at 1 minute and continuing to 13 minutes.

Table 1. Protein Desalting/Concentration Gradient

Flow	0.75 mL/min
Stoptime	13 min
Posttime	15 min
Column temp	80 °C
Starting solvent composition	
Solvent A	Water/0.1% TFA
Solvent B	ACN/0.08% TFA
Detection UV	280 nm
Pressure limit	250 bar
Gradient	
Time (min)	%B
0	10
3	10
5	70
6	100
8	100
10	10

Recovery Conditions

To measure protein recovery, several injections of sera (approximately 47 µg with urea and 35 µg without urea) were performed without the column installed inline using the gradient conditions shown in Table 1. Fractions were collected and pooled for each sample and then dried using a SpeedVac. The column was then placed inline and several injections were performed for each serum sample and the fractions were collected, pooled and dried as above. The proteins 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 accurately determine the absolute protein recoveries.

Results and Discussion

The need to desalt/concentrate proteomic samples typically arises prior to performing orthogonal techniques such as isoelectric focusing or gel electrophoresis. The Agilent mRP-C18 column provides a tool to automate processing of immunodepleted samples for further downstream analysis by desalting and concentrating. The mRP-C18 column is a RP column with several advantages over standard-RP columns including high recovery, reproducibility, and loadability. The recovery from a column is

critical since many downstream applications result in protein losses. The recovery data for samples obtained with and without the mRP-C18 column inline show >99% recovery when prepared with urea and acetic acid (Table 2). The samples used directly from the immunodepletion column show >92% recovery indicating that urea enhances overall column recovery.

Table 2. Recovery of Serum from the Agilent mRP-C18 Column as Determined by a BCA Assay

Protein conc. (µg) no column (+ urea)	Protein conc. (µg) mRP-C18 recovery (+ urea)	% Recovery	Protein conc. (µg) no column (– urea)	Protein conc. (µg) mRP-C18 (– urea)	% Recovery
49.8	49.3	99	34.8	32.1	92.2

Analysis of the chromatograms shows some separation, which can be reduced with either an increase in the flow rate or reducing the gradient from a 2-minute gradient to a 1-minute gradient (Figure 1). The difference in recovery between sample preparation methods suggests that sample preparation is required as shown by the >98% recovery when prepared with urea and glacial acetic acid and the 92% recovery without using urea and glacial acetic acid. The excellent recovery obtained using the mRP-C18 column enables rapid desalting and concentration for downstream analysis.

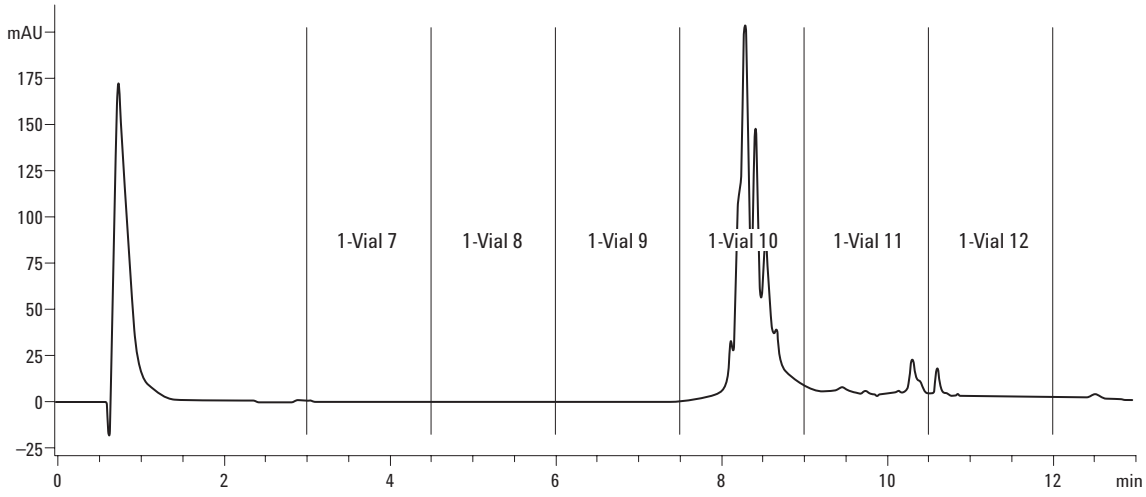

Figure 1. Typical desalting chromatogram for about 47 µg of protein after sample preparation including the addition of urea and acetic acid. Fractions pooled for protein assay were in vials 10–12.

Conclusions

The Agilent mRP-C18 column provides optimized desalting and concentrating for human serum after depletion of high-abundance proteins using the Agilent Multiple Affinity Removal System. This combined workflow enables high recovery for further downstream processing in biomarker research. In addition, the elimination of a spin concentration step with a MWCO filter allows the safe recovery of peptides and polypeptides less than the typical 5 kDa MWCO. The fast processing time with reproducible, high recovery allows for confident sample desalting and concentration without the typical losses associated with conventional RP techniques. This technology can be coupled, allowing for orthoganol separations without interference from salts or other additives that are removable by the mRP-C18 column.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem/bioreagents.

E-mail 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.

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

Barry Boyes is R&D Manager for BioReagents in the Integrated Biology Solutions Group with Agilent Technologies

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

Correspondence: Dr. William Barrett
E-mail: bill_barrett@agilent.com

Printed in the USA
March 29, 2005
5989-2506EN

Agilent Technologies