Poster P1

A Novel Desalting Column With High Mechanical Strength for Faster Desalting of Proteins

Atis Chakrabarti, Roy Eksteen
Technical Service
Tosoh Bioscience LLC
3604 Horizon Drive, Suite 100
King of Prussia PA 19406
atis.chakrabarti@tosoh.com

Desalting on the principle of size exclusion chromatography – a widely used method in separation science, is the removal of high concentration of salt from the sample. Proteins and other biopolymers elute at high or elevated salt concentration in various chromatographic modes, including hydrophobic interaction (HIC), ion exchange (IEC) and size exclusion chromatography (SEC). When proteins are analyzed by SEC under denaturing conditions, for instance in the presence of guanidine hydrochloride or urea, such salts need to be removed or diluted for subsequent investigations.

Dextran, cellulose and polyacrylamide, commonly used as porous gel matrices in desalting columns can withstand only minimal pressure. As a result, desalting is either time consuming or a rather inefficient process. The use of a high-performance desalting column packed with a robust small particle size packing material to speed up the separation has not yet been reported in the literature.

Using a continued cross-linking process we have been able to increase the mechanical strength of polyacrylamide gel by four-fold over that of conventional gels. The increase in physical strength allowed the use of a particle size down to 13µm. Further improvements in the manufacturing process substantially narrowed the particle size distribution. In this poster we report on the properties and application of two TSKgel BioAssist DS columns, each comprised of PEEK hardware with the following dimensions: 4.6 mm I.D. x 15 cm and 10 mm I.D. x 15 cm and designed for fast desalting of proteins and polynucleotides at semi-preparative scale. The results of our study show that excellent desalting can be obtained within 5minutes and with high recovery down to ng protein injected.

Poster P2

Stability of a silica-based size-exclusion column for antibody analysis

<u>Atis Chakrabarti</u>, Roy Eksteen
Technical Service
Tosoh Bioscience LLC
3604 Horizon Drive, Suite 100
King of Prussia PA 19406
atis.chakrabarti@tosoh.com

There are many answers to the question of how long a column lasts. The most trivial reply is still the most appropriate - it all depends on what you do with the column. Particle structure, bonded phase composition, number of injections, mobile phase, pH, flow rate, sample type/mass/volume, temperature, pressure, storage, cleaning protocol, et cetera - all play a more or less important role in determining when to write the column's inevitable obituary.

When it comes to gel filtration chromatography, particle and thus column stability is even more of an issue than in reversed phase chromatography for the simple reason that the pore volume of SEC columns is maximized to provide optimal mass resolution, and the higher the pore volume the more fragile is the particle.

From a user's perspective, it would be most desirable if no precautions would be required to obtain a long column lifetime. From a manufacturer's perspective, such a column would be impossible to make and even if it could be made, such a column would be too costly to sell.

In this initial report of our ongoing study about the stability of the TSKgel G3000SWxl column for quality control of monoclonal antibodies, we evaluated three columns each of three bonding lots. Preliminary indications are that retention times and efficiency did not change over at least 1000 injections of a 10μL mixture of protein standards into a 100μL injection loop. During the lifetime studies the analytical column was neither protected by a guard column nor by inline filters other than the ones that are a standard fixture in the Agilent 1100 HPLC system.

Poster P3

Affinity Purification and Comparative Analysis of Human NGAL from Urine, Leukocytes, Neutrophils, Neutrophil Granules

Svetoslava Gregory, Tracey Rae, Ryan Bonn, Carol Ramsay, Cheng Zhao, Jeffrey Fishpaugh,
Kevin Rupprecht
Analytical Research
Abbott Diagnostic Division
100 Abbott Park Rd
Abbott Park, Illinois 60064
svetoslava.gregory@abbott.com

Neutrophil Gelatinase-Associated Lipocalin (NGAL) is a new 25 kDa protein biomarker for renal injuries. NGAL is a glycoprotein expressed primarily in neutrophils and various epithelial cells. It is also present in serum and urine.

Urine huNGAL exists primarily in a monomeric state though oligomers (homodimers) occur as well. To further investigate the role of NGAL in inflammatory disorders, a reliable method for purification and concentration of this low abundance urine protein is needed.

We have developed an affinity purification method that utilizes a monoclonal anti-huNGAL antibody covalently coupled to cyanogen bromide activated beads. Elution of huNGAL is accomplished with a low pH buffer. Applying this method, we purified huNGAL from various human source matrices: urine, leukocytes, neutrophils and neutrophil granules. The characteristics of affinity purified huNGAL was analyzed using a wide variety of analytical techniques. The isolated material was >98% pure by SDS-PAGE and reactive to two monoclonal antibodies having known epitopes and a polyclonal anti-NGAL antibody. Size exclusion chromatography as well as NativePAGE ^{TM} gel system of the affinity purified protein revealed the existence of monomer, dimmer, trimer and even higher molecular weight forms.

Affinity-purified huNGAL protein from human leukocytes and normal human urine was subjected to further analysis toward apparent substrate content. UV-Vis spectroscopy and ICP-MS analysis revealed the presence of an iron and spectra similar to those previously published for a siderophore-like substrate. This apparent substrate is shown to be tightly bound to the protein, as the protein retains the visible spectrum absorption through GPC-HPLC separation.

In summary, we have developed a robust and reproducible method for concentration and purification of huNGAL from various human matrices which facilitates tremendously the study of expression, post-translational modification and function of huNGAL as well as further investigation of the biological significance of its substrate.

Poster P4

Two-Dimensional Chromatography for Quantitation and MS Analysis of Monoclonal Antibodies in complex samples

Sean McCarthy, Thomas E. Wheat, Ying Qing Yu, Jeff R. Mazzeo
Biopharmaceuticals
Waters
34 Maple Street
Milford MA 01520
sean_m_mccarthy@waters.com

Identification, characterization, and quantitation of monoclonal antibodies (mAbs) is required at many stages of biopharmaceutical research and development. The primary analytical tools are liquid chromatography coupled with UV or mass spectrometry (MS). Both techniques can be compromised by interferences in the sample matrix, including high salt concentrations, other proteins, or the components of cell culture media. A high throughput analytical technique should combine sample preparation and chromatographic techniques to ensure accurate and robust quantitiation. Affinity chromatography on immobilized Protein A can be used to isolate the antibody from a complex matrix while reversed phase LC is useful for introducing a salt free, concentrated sample into an MS ion-source.

Affinity chromatography is often used to determine protein titer by UV absorbance using peak area due to its high specificity, linearity, and reproducibility. Mass spectrometric analysis allows the practitioner to determine the mass, glycoprofile, and other mass based attributes of the analyte. While each of these methods can be performed very rapidly, they are generally accomplished by using separate chromatographic systems since the eluents used for affinity chromatography are incompatible with mass spectrometry. In addition, each analysis requires different sample preparation protocols which can increase the time necessary for analysis.

In this presentation we describe the use of an ACQUITY UPLC system with 2D technology. The 2D system allows for simultaneous purification and quantitation of monoclonal antibodies by Protein A affinity chromatography and determination of mass profile by MS analysis after desalting on a short reversed phase column. The peak of interest is eluted from the first dimension affinity separation to a second dimension reverse phase separation. Our data demonstrates the linearity and reproducibility of response for mAbs from complex matricies. We also discuss carryover and chromatographic reproducibility observed when performing protein A affinity chromatography with the 2D system. 2D UPLC system optimization for peak collection, desalting, and MS detection of antibody samples is also discussed.

Poster P5

A Novel LC-UV/MS Based Approach for Quantitating Intact Lipid Transfer Protein, A Food Allergen in Maize Kernels

Krishnamoorthy Kuppannan, Scott Young, Dave Albers, Barry Schafer, Demetrius Dielman
Analytical Sciences
The Dow Chemical Company
1897 Building
Midland MI 48667
kkuppannan@dow.com

Maize (Zea mays) is not considered as a major allergenic food, but for those individuals allergic to maize, Lipid Transfer Protein (LTP) is predominantly responsible for food-induced allergenic and immunologic reactions. LTP is an extremely stable protein that is resistant to both proteolytic attack and food processing, which permits the allergen to reach the gastrointestinal immune system in an immunogenic and allergenic conformation, allowing sensitization and induction of systemic symptoms. During the development of a method to quantitate LTP present in maize kernels, multiple approaches including immunological and signature peptide methodologies were considered. When using immunological approaches, there exists a potential for inconsistent results. The methodology of protein quantitation by analyzing a signature peptide may pose several challenges including identification of a suitable peptide where results would be reflective of molecular heterogenity. Due to the presence of different variants and isoforms of LTP in maize resulting from molecular heterogeneity, a liquid chromatography with ultraviolet and mass spectrometric detection (LC-UV/MS) method was developed for quantitation of LTP. The UV-based intact protein quantitation method allowed minimal sample handling, automated analysis, and relatively short analysis cycles for rapid data collection. Differentiation between LTP and its variants was achieved by this method, enabling for the quantification of three different forms of LTP simultaneously. A quantitation method for the determination of LTP over a concentration range from 29 to 1030 µg/g in maize kernel samples was developed. Assay recovery (% Relative Error) was measured at 11 different concentrations ranging from 4 to 147 µg/mL and did not exceed 5.1%. The precision (%CV) was measured at 3 concentrations on each of 4 days and did not exceed 14.4%. The assay was successfully used to determine and compare the levels of LTP in various maize lines and was sufficient to measure concentrations at physiologically relevant levels. Characterization of the LTP and its variants was performed by LC-MS/MS and MALDI-TOF MS.

Poster P6

Chromatographic Separations of Proteins using a System for Automated Control of Mobile Phase pH and Ionic Strength

Thomas Wheat, Daniel Root, Patricia McConville
Waters Corporation
34 Maple Street
Milford MA 01757
tom_wheat@waters.com

The properties of proteins in solutions are directly affected by the pH and ionic strength of the solution. These parameters can, therefore, form the basis of a strategy for developing separation methods. This principle applies most directly to ion exchange separations, but it can still guide development of any separation technique where preservation of native structure and biological activity is desirable. While screening these parameters is recognized as one of the most useful strategies for an optimizing chromatographic methods, the preparation of the several buffers required for experimental evaluation is time-consuming, labor-intensive, and subject to error. To simplify this process, quaternary HPLC pumps have been used to blend buffer stocks. This approach is derived from the common practice of volumetric mixing of concentrates according to buffer preparation tables. Programming the desired methods as a percentage flow from each bottle has been successfully used. A more straightforward system permitting programming directly in units of pH and salt concentration is now available. The operating software uses a defined buffer system to calculate the required percentage from each reservoir for a given pH and ionic strength. In its most direct form, the algorithm is based on the Henderson-Hasselbalch equation. A second type of calibration is available for accurate proportioning of buffers across the range of ionic strengths commonly used for protein separations. The performance of the system was tested by executing methods and buffer systems typical of protein separations in anion exchange, cation exchange, and size exclusion chromatography. Fractions of the system effluent were collected during these experiments and the pH measured with a pH meter. These tests included pH gradients which proved to be very accurate and predictable because the algorithms recalculate the required proportions at each pump stroke during the separation. A formal validation procedure was developed based on these tests and used to demonstrate equivalent performance across several systems. The use of this system permits systematic evaluation of the parameters that most directly affect the quality of a protein separation. The process is automated so that small increments of composition can be examined without incurring extra labor and errors. And a true optimum can be approached because the experiments do not require continuous operator attention. This enhanced chromatographic system will produce more robust and reliable protein analytical methods.

Poster P7

Ion Exchange Analysis of Proteins with Automated pH Gradients

<u>Thomas Wheat</u>, Daniel Root, Patricia McConville
Waters Corporation
34 Maple Street
Milford MA 01757
tom_wheat@waters.com

Complete analysis of proteins requires the combination of several analytical techniques. Where chromatographic separations are part of the analytical suite, it is often desirable to use multiple modes of separation and to preserve the biologically active structure of the protein. Among the chromatographic techniques that meet these objectives, ion exchange can be the most discriminating. Obtaining the best resolution with this technique requires optimization of both the column and the mobile phase. Among the options for adjusting the separation, the pH of the chromatographic eluents has the largest effect on selectivity. Efficient protocols have been developed for screening separations with a selection of columns over a series of buffers that differ in pH. This process has been automated in a system that combines the chromatographic instrument and the control software. In addition to these experiments, many investigators have tested pH gradients for protein separations. While useful separations have often been reported, such pH gradients have not been widely applied because they are difficult to control. Simple buffer mixing does not give the desired pH profile because pH is a logarithmic function. When the intended pH is further from the pK, pH changes become very large with small changes in buffer proportions. It is also complicated to control the pH profile when changing column dimensions or flow rate. We have developed and evaluated algorithms and a software user interface to simplify this process. The technology is implemented on a four solvent pump and the gradient table is programmed directly in units of pH and salt concentration. The system uses a "buffer system" that is freely defined by the user to calculate the required proportions of the four mobile phase reservoirs and to recalculate those proportions at each pump stroke. This recalculation permits accurate and precise pH gradients over the range of the buffer system, and it generates both linear and non-linear gradients of pH as programmed by the user. With this control over the buffer properties as delivered to the column, it is possible to identify the ways in which the ion exchange media alters the pH of the buffer as it passes through the column. Several examples of protein separations, including monoclonal antibodies, will also be shown. With the use of this accurate and precise control of pH gradients, new selectivity becomes available for optimizing selectivity in protein analysis methods.

Poster P8

High Throughput Proteomic Analysis Using Fast Gradient Reverse Phase HPLC Separations

Darryl Johnson, Barry Boyes, Taylor Fields, Rachel Kopkin, Ron Orlando
Department of Chemistry
University of Georgia, CCRC
315 Riverbend Road
Athens GA 30602
15djohnson@gmail.com

The increasing demand on instrument time for the analysis of proteomic samples via LC-MS/MS has created a need for improved high throughput methodologies. Recently developed high efficiency column packing materials, such as sub-2 um diameter particles and superficially porous particles, offer significantly improved peptide separations. Narrow peak widths, often only a few seconds at half-height can permit a 15 minute LC-MS run to have a similar peak capacity as a 60 minute separation using traditional HPLC materials. In theory, greater peak capacity separations should provide higher protein coverage and/or more protein identifications during reduced time when included in a typical proteomic workflow. The fast analysis can be achieved by increasing flow rate as well as optimizing gradient conditions and using appropriate columns. By the use of a superficially porous particle designed for peptide separations using a Halo Peptide ES-C18 column, we are able to increase flow rate while maintaining peak capacity and operating at backpressures feasible with a standard capillary LC system (<350 bar). During the process of developing fast gradients for RP-LC-MS/MS, we systematically addressed aspects of hardware and used conditions that decrease experiment time, in particular conditions required for loading, washing and re-equilibrating the analytical column. This required simple plumbing changes to the factory-supplied instrument setup as well as defining gradient delay volume and other sources of dwell volume. Tryptic peptides from simple protein digests as well as synthetic peptides were employed to measure separation features for the method development approach.   We were able to reduce experimental run time from 90 minutes to 20 minutes using a 0.2 x 50 mm Halo Peptide ES-C18 column at a flow rate 9 uL/min, while obtaining a peak capacity greater than 50. While flow rates could be further increased, current instrumentation limits, including scan speed of our mass spectrometer and a significant gradient delay volume, will not allow us to reasonably reduce experiment time further without sacrificing the quality of LC-MS data.   The high throughput utility of the improved RP-HPLC separation and LC-MS analysis conditions are demonstrated in proteomics experiments using a whole cell lysate of *trypanosome brucei*. We were able to show highly informative peptide and protein identifications using fast gradient and flow rate conditions with Fused-core columns.

Poster P9

Applied MS-Based Protein Analysis In Biotechnological Industry

M. Akeroyd[1], Nicolas Abello[1], R.A.M. van der Hoeven[1], P. Klaassen[1], M.W.E.M. van Tilborg[1], M.M.A. Olsthoorn[1], Qing-Yuan Yin[2]

[1] *DSM Biotechnology Center, Delft, The Netherlands*

[2] *DSM Biotechnology Center China, Shanghai, China*

DSM Biotechnology Center
Alexander Fleminglaan 1
Delft 2613 AX
The Netherlands
Nicolas.Abello@dsm.com

MS-based analysis tools for detection of metabolites and proteins are applied within DSM from the early lead discovery all the way to product quality control.

We have developed a method for fast identification and quantification of the top ~800 proteins in microbial samples. The method includes a generic sample preparation for LC-MS/MS followed by protein identification and label-free quantification. The label-free quantification is based on spectral counting and machine-learning with a program called APEX (Absolute Protein Expression, Lu *et al.* Nature Biotech 25, 1, 2007).

Total protein content or cell numbers are determined, followed by BSA spiking as an internal control and cell lysis in case of intracellular protein analysis. Next, proteins are isolated by precipitation and subsequently digested with trypsin. The resulting peptides are analyzed with LC-MS/MS using multiple injections per sample. Database searches are performed in dedicated databases using the Sorcerer 2 search engine. Search results are processed using the Trans-Proteome Pipeline (TPP) and APEX.

This workflow has been applied in several of DSM's white-biotechnology projects. These projects are aiming for the development and optimisation of strains producing renewable alternatives to crude oil. The strength of the method becomes clear when rational genetic engineering is applied for strain improvement, since cloned genes and gene knock-outs can directly be confirmed at the protein level using our method. Monitoring of random mutagenesis strain improvement can also be performed using our method in order to identify potential knock-out or over-expression genes for further improvements. Due to the observed reproducibility of relative quantification, possibilities for absolute protein quantification are being exploited.

Furthermore, in order to monitor closely some molecular pathways for e.g. yield improvement, we are developing an SRM approach for the quantification of dozens of specific intracellular enzymes.

Poster P10

MAbPac SCX small particle phases for monoclonal antibody (MAb) variant analysis

Srinivasa Rao, Yuanxue Hou, Hongmin Zhang, Agroskin Yury, Chris Pohl
R&D
Thermo Fisher Scientific
445 Lakeside Drive
Sunnyvale CA 94085 Srinivasa.rao@thermofisher.com

Monoclonal antibodies (MAbs) undergo several post-translational modifications including oxidation, deamidation, and truncation. Manufacturing of MAbs and subsequent stability testing procedures involve routine analysis and monitoring of the impurities resulting from these modifications.

The MAbPac Strong Cation Exchange Phase (MAbPac SCX^{TM}) was recently introduced for the characterization of heterogeneity of MAbs. It was a complimentary addition to the existing Thermo Scientific Dionex ProPac™ WCX-10 column providing high resolution and orthogonal selectivity for MAb charge variant analysis. Both the ProPac WCX and MAbPac SCX stationary phases are based on nonporous 10 µm, highly cross-linked styrenic type of polymeric media with different proprietary hydrophilic coatings.

Currently, we are developing 5- and 3-µm MAbPac SCX small particle phases for MAb variant analysis. The main objective is to develop high-resolution columns comparable to the MAbPac SCX-10 columns (4x250 mm) but with a much faster analysis time. The longer length advantage from a 10 µm particle column for producing high resolution is compensated by short smaller particle size columns. Since the column is shorter, analysis time can be reduced and throughput can be increased. This study presents various fast applications including acidic, basic variant analysis, and lysine truncation variant characterization of a MAb using these small particle columns. Both salt and pH gradients are used in these experiments. Ruggedness of 3 µm and 5 µm small particle phase columns is also demonstrated.

Poster P11

A fast screening method for identification of multiple classes of metabolites in serum using UPLC-ESI-MS/ MS

Jun Feng Xiao, Habtom W. Ressom
Oncology Georgetown University Medical Center
Lombardi Cancer Center
3970 reservior RD NW
Washington DC 20007
drjunfengxiao@gmail.com corresponding author: hwr@georgetown.edu

Quadrupole linear ion trap mass spectrometer (QTRAP) provides multiple acquisition modes including neutral loss (NL) scanning, precursor ion (PI) scanning, and selected reaction monitoring (SRM) for unknown metabolite screening and target quantitation. These acquisition modes can work as survey scans to trigger the acquisition of enhanced product ion (EPI) spectra. We have developed a rapid and sensitive method using AB Sciex QTRAP 4000 for screening and identifying several classes of metabolites that have common structural motifs. These include bile acid adducts (glycine, taurine, glucuronide and sulfated conjugates), bilirubin adducts (glucuronide conjugates), sphingosine-1-phosphate (S-1-P) and lyso phosphatidylcholine (LPC). Precursor ion scanning was performed to confirm target analytes in serum extract using characteristic product ions of m/z 74 ($H_2NCH_2CO^{2\,-}$) for glycine conjugate, m/z 80 ($SO3^-$), 107($CH_2CHSO3^{\,-}$) and 124 ($H_2NC_2H_4SO3^{\,-}$) for taurine adduct, glucuronide-derived fragment of 176 Da loss for glucuronide adduct, m/z 97 ($HSO_4^{\,-}$) and 80 Da ($SO3$) for sulfated adduct, fragment ion of m/z 184 (+) for phosphatidylcholine. SRM transitions of each adduct were determined (S-1-P: 378→79; taurocholic acid (TCA): 514 →80; glycochenodeoxycholic acid 3-sulfate: 528→97; glycocholic acid (GCA): 464 →74; taurochenodeoxycholic acid (TCDCA): 498 →80; glycodeoxycholic acid (GDCA): 448 →74; CDCA 3-glucuronide (567 → 391); bilirubin glucuronide (761 → 585) ; lysoPC(17:0): 510 → 184; lysoPC(16:0): 498 → 184). Then SRM-EPI was performed for identification of target metabolites by comparing the MS/MS spectra and retention time with those of authentic standards. The isotope labeled internal standard analogues were spiked in serum samples (D_4_GCA, D_4_GDCA, D_5_TCA, D_{31}_lysoPC (16:0), D_{35}_ lysoPC(18:0) and D_7_S-1-P) for absolute quantitation. Preliminary quantitative results show that this method is fast in analysis (10 min run), sensitive in detection (LOD of 2 fmol) and has a wide dynamic range of four orders of magnitude and linearity of 0.999 in correlation coefficients.

Poster P12

Relative quantitation of N-glycans by labeling the reducing end with H$_2$^{18}O

Shujuan Tao, Ron Orlando
Department of Chemistry
CCRC, UGA
315 Riverbend Rd.
Athens GA 30602
ShujuanTao@GMail.com

Quantitation of glycans through stable isotopic labeling coupled with mass spectrometry analysis is a promising method for the comprehensive study of glycomics. Several isotopic labeling strategies have been developed via permethylation for quantitative analysis. However, with all the benefits of permethylation, a unique issue caused by those strategies is that large errors can be introduced by small variation in labeling efficiency due to the large number of methylation sites. To address the problem, a novel isotopic labeling method is proposed which can incorporate ^{18}O labeling into the reducing end prior to permethylation. It can be simply realized by using H$_2$^{18}O in place of H$_2$^{16}O during N-glycan release with PNGase F without altering the general workflow for glycomics.

To investigate the labeling efficiency, labeling stability and linearity dynamic range of this labeling method, several standard glycoproteins were used for the initial studies. The results showed that the labeling efficiency was >95% and that the labeled species did not undergo significant loss of the isotope for 24 hours in the standard laboratory solutions, which would be used for a typical glycomics experiment. For linearity study, serial dilutions were applied, which gave a linear dynamic range that extended over two orders of magnitude with an average RSD below 10%.

Also, in order to demonstrate the applicability of this labeling technique on complex samples, relative quantitation of N-glycans released from human serum were performed. With analyzing the identified 25 N-glycan peaks from the sample, the maximum observed error was 7.5%, and the average error was only 2.7%. Furthermore, the comparison of the ^{18}O labeling approach to other methods showed that this new approach can provide more precise and more accurate results for relative quantitation of N-glycans.

Poster P13

Peptide Separations Using Size Exclusion Chromatography

Haiying Chen, Katherine Mclaughlin
Marketing
Sepax Technologies, Inc.
5 Innovation Way
Newark DE 19711
hchen@sepax-tech.com

Size exclusion chromatography (SEC) has been applied successfully to separate different sizes of proteins under native conditions. Different pore sizes have been developed to accommodate different ranges of molecular weight of biological samples. In order to apply the size exclusion chromatography to peptides under 10,000 Da, a few limitations have to be overcome. Even very small peptides can exist in different conformations and exhibit secondary structures. Therefore, peptides tend to adsorb to column matrices by ionic and hydrophobic interactions. High salt concentrations, denaturing agents, and organic additives will minimize such interactions, thus enabling the separation of peptides according to their molecular weights. Sepax Zenix SEC columns are based on uniform, hydrophilic, and neutral nanometer thick films chemically bonded on high purity and mechanically stabilized silica.   Zenix SEC-80 is specifically designed for small protein and peptide separations.   Its phase is similar to the other Zenix SEC with the same particle size 3µm, but different pore size at 80 Å.   Here we present separation of four peptides bradykinin (1,060 Da), angiotensin I (1,297 Da), glucagon (3,483 Da) and insulin (5,778 Da) under different separation conditions. Separation of *E. coli* tryptic digest on Zenix-80 is also investigated with different mobile phase conditions.

Poster P14

Direct Protein Analysis Using Size Exclusion Chromatography Zenix-SEC and Mass Spectrometry

Haiying Chen, Katherine Mclaughlin
Marketing
Sepax Technologies, Inc.
5 Innovation Way
Newark DE 19711
hchen@sepax-tech.com

Size exclusion chromatography has been widely used in protein analysis. Aggregates, monomers and degradation products can be separated on size exclusion columns based on their molecular weights under native conditions. In general protein native buffer conditions such as salts pH are not mass spectrometry friendly. In this study, we investigated monoclonal antibody subunits with SEC separation coupled with mass spectrometry. An intact monoclonal antibody was reduced to generate heavy and light chain subunits. Then the heavy and light chains were separated on Zenix-SEC, and the mass of each chain was obtained with mass spectrometry under an optimized mobile phase condition. The combination of Zenix-SEC and mass spectrometry achieves the separation of proteins and protein subunits on SEC according to their sizes and the determination of their molecular weight by mass spectrometry.

Poster P15

Characterization of a Novel High- Capacity Weak Cation Exchange Resin

Chinlun Huang, R. Christopher Manzari, J. Kevin O'Donnell
Technical Service
Tosoh Bioscience LLC
3604 Horizon Dr
King of Prussia PA 19406
chinlun.huang@tosoh.com

There is an increasing demand for ion exchange resins with increased capacity and improved selectivity. Tosoh Corporation created the Toyopearl GigaCap family of resins to meet this demand. This poster will focus on Toyopearl GigaCap CM-650M as a novel high-capacity weak cation exchange resin for the purification of proteins and other biomolecules.

Toyopearl GigaCap CM-650M utilizes the well-known Toyopearl HW-65 size-exclusion resin as the base bead. The base bead is chemically modified with carboxymethyl groups in the 1000Å pores of the base bead which result in greater than 100 mg/mL dynamic binding capacity for IgG-type molecules. The binding capacity for other proteins is also significantly increased. Toyopearl GigaCap CM-650M is stable at high linear velocities with good pressure flow characteristics. It has a pressure rating of 3bar and is stable in the pH range of 3-13. The higher capacity and stability of Toyopearl GigaCap CM-650M allows for increased throughput in downstream purification steps while still maintaining excellent binding and elution kinetics.

Toyopearl GigaCap CM-650M binds mAbs under a variety of conditions. Even at pH 3.6, Toyopearl GigaCap CM-650M bound a humanized mAb in a buffer containing greater than 300mmol/L NaCl. As part of this study we wanted to further explore the operating range of this resin. Experiments were performed to determine the upper and lower pH boundaries for adsorption, as well as the optimal pH for binding of a specific humanized monoclonal antibody. Using these values, we determined the maximal conductivity for mAb binding. In addition, experiments were performed to determine the impact of increasing protein loads on selectivity.

Characterization of Protein Glycoforms by Hydrophilic Interaction Chromatography, nanoReverse Phase LC-MS/MS and Mass Spectrometry in Patients with Liver Diseases

Petr Pompach, Zuzana Brnakova, Kevin Chandler, Nathan Edwards, Miloslav Sanda, Radoslav Goldman
Oncology
Georgetown University
3970 Reservoir Rd NW
Washington DC 20057
pp282@georgetown.edu

Glycoproteins influence many physiological processes including cell proliferation and differentiation. The severity of congenital disorders of glycosylation (CDGs), which result from mutations of enzymes involved in N-glycosylation, underlie the importance of N-glycosylation in pathophysiological processes. Modern analytical tools allow the study of this common posttranslation modification in increasing detail. In this study we present a workflow for the analysis of glycan microheterogeneity of haptoglobin and hemopexin by using two dimensional chromatography consisting of hydrophilic interaction chromatography and nano reverse phase C18 chromatography couplet to a Q-TOF mass spectrometer. Big separation potential and high sensitivity enable to observe glycopeptide forms that were not described before. In house glycan database search program, GlycoPeptideSearch, was used to match N-glycopeptide MS/MS spectra to peptide-glycan pairs in digests of isolated proteins, with glycans drawn from the GlycomeDB database. In our study we have focused on differences between haptoglobin and hemopexin glycopeptides isolated from sera of patients with HCC and controls with/out chronic liver disease. The results show that site-specific glycosylation of the proteins is altered in association with liver damage and the presence of HCC.

Twenty five years of the Houghten & DeGraw puzzle: a quantitative description of the interactions of amphipathic helixes with reversed-phase sorbents as a key for correct peptide retention prediction

Oleg Krokhin, Janice Reimer, Vic Spicer
Internal Medicine
University of Manitoba
799 JBRC, 715 McDermot Avenue
Winnipeg Manitoba R3E 3P4 Canada
krokhino@cc.umanitoba.ca

Development of simultaneous multiple-peptide synthesis has provided chromatographers with extensive sets synthetic peptides and real chance to look deeper into peptide retention mechanisms. 25 years ago the study of the RP-HPLC retention of 298 peptide analogues, including 260 peptides representing positional substitution in the 13-mer molecule YPYDVPDYASLRS with all 20 naturally occurring amino acids was published in Journal of Chromatography [1]. This represented a significant breakthrough in the complexity of retention data sets used to develop retention prediction algorithms. In this paper the authors challenged state-of-the-art assumption that peptide retention can be presented as a sum of individual hydrophobicities of constituent amino acids, and suggested that prediction models must be sequence specific. Their results showed that "each position within the peptide would require a different set of retention coefficients to accurately predict peptide retention times". While the reason for such behavior wasn't clear, the authors suggested taking into account the influence of "individual sequence domains" for more accurate retention prediction.

Accelerated developments in proteomics renewed interest in peptide retention prediction, as it can be used as additional constraint in peptide's identification. A number of novel prediction algorithms appeared, each claiming significant improvement over existing models [2-5] and taking into account both peptide composition and sequence. We have investigated the performance of these models on the Houghten & DeGraw collection of peptides as a method for evaluating how far we have progressed in both our fundamental understanding and quantitative description of RP-HPLC separation mechanisms in 25 years.

We re-created the experimental data from the Houghten & DeGraw set by analyzing several peptides corresponding to the YPYDVPDYASLRS substituted analogs, then using these to re-align the retention values of all 260 species from the literature data. Surprisingly, all modern retention prediction models evaluated performed below their claimed prediction accuracies, giving 0.82-0.9 R^2-values vs. 0.96-0.98 reported for their training datasets. Moreover, a simple additive model exhibits comparable performance. Clearly, the question raised 25 years ago remains unanswered, despite recent significant progress in the field. All predictive models exhibit the largest magnitude sign-correlating deviations from the observed retention times on species where the amino acid substitution occurs within the hydrophobic or hydrophilic faces of the framework peptide. This indicates that a better understanding and quantitative description of the stabilization of amphipathic helical structures upon interaction with the C18 phase is necessary for future progress in this field of chromatographic science.

1. R.A. Houghten, S.T. DeGraw, J. Chromatogr. 386 (1987) 223.
2. O.V. Krokhin, Anal Chem 78 (2006) 7785.
3. K. Petritis, et al., Anal Chem 75 (2003) 1039.
4. A.V. Gorshkov, et al., Anal Chem 78 (2006), 7770.
5. L. Moruz, et al., J. Proteome Res. 9 (2010) 5209.

New High Throughput Polymer-Based Chromatography Media for Process-Scale Protein Separation

Kazuhiko Tokunaga, Yoshito Fukuda, Noriyuki Yasuda, Ali Ozhan Aytekin, Ryousuke Tsuhara, Katsuaki Takahashi, Tadashi Adachi
Separation Materials Laboratory
Mitsubishi Chemical Corporation
1000 Kamoshida-cho, Aoba-ku,
Yokohama, 227-8502, Japan 227-8502
4407404@cc.m-kagaku.co.jp

New high throughput protein chromatography media have been developed for process-scale separation. The base matrix is highly rigid porous polymethacrylate, which enables higher velocity operation in combination with taller packed bed height. Based on such polymeric chemistry, the dynamic binding capacity (DBC) of the cation-exchange media showed more than 100 g-IgG/L at higher flow rate. The DBC for the *r*Protein-A media also showed a small dependence on the flow rate over the higher velocity.

Quantitative Analysis of Hemagglutinin in Influenza Vaccine Preparations by Reversed-Phase HPLC: Method Optimization, Validation and Comparative Evaluation with in vitro biochemical assays

Michel Girard, Barry Lorbetskie, Jun Wang, Caroline Gravel, Cynthia Allen, Aline Rinfret, Mike Walsh, Xuguang Li
Centre for Vaccine Evaluation
Health Canada
251 – Sir Frederick Banting Driveway
Ottawa Ontario K1Z 7W8
Canada
Michel.Girard@hc-sc.gc.ca

A previously described reversed-phase HPLC (RP-HPLC) method based on fast separations on a non-porous silica stationary phase was optimized and qualified for the quantitative determination of hemagglutinin (HA) in influenza vaccine preparations. Optimization of the gradient elution conditions led to improved separation of the HA1 subunit from other vaccine constituents. The sensitivity of the method was significantly increased by using native fluorescence detection, resulting in an approximately 10-fold increase as compared to UV-VIS detection. The method was qualified for linearity, range, limit of detection, limit of quantitation and precision. Overall, it was found to be linear over the range of 2.5 to 100 μg HA / mL for all subtypes examined. This range covered 50 -150% of the concentration found for individual strains in seasonal influenza vaccines and in the pandemic H1N1 vaccine. The limit of detection and limit of quantitation for each subtype were found to be suitable for the method's intended purpose and compared well to values found by the single radial immunodiffusion (SRID).

Triton X was found in some preparations and interfered with the quantification of the HA1 subunits. In such cases modifications to the gradient elution enabled the complete separation of the Triton peak.

The RP-HPLC results were compared to amounts detected by Western blot analysis using a hemagglutinin universal antibody for a set of 15 monovalent A/California H1N1 preparations and showed good correlation. Similarly, the quantitative nature of the RP-HPLC method was assessed in relation to the SRID assay currently used for the determination of the HA content in bulk antigen and final vaccine preparations. Thus, for a series of 23 monovalent A/Brisbane/59/2007 H1N1 bulks, ranging between 12.7 and 15.9 μg HA/mL by SRID, the RP-HPLC values were found to be in very good agreement, ranging between 11.9 and 14.1 μg HA/mL (n=5) for five determinations carried out on 5 different days.

During the 2009-2010 H1N1 influenza pandemic the quantitative RP-HPLC method was used alongside several other test methods for the analysis of pandemic H1N1 vaccine preparations that included bulk antigen and final vaccines. The HA content of vaccines formulated at 15 or 30 μg/mL was measured by RP-HPLC and SRID and results showed that the HA content determined by RP-HPLC correlated well to that determined by SRID and observed by Western blot. Overall, the results provided further evidence of the usefulness of RP-HPLC for the detection and quantitation of the HA content in monovalent vaccine bulks once a reference standard has been established.

Evaluation of Ultra Performance Size Exclusion Chromatography for the Analysis of Proteins and Higher Order Aggregates

Paula Hong, Tom Wheat, Kenneth Fountain
Chemistry Applications
Waters Corp
34 Maple Street
Milford MA 01757
paula_hong@waters.com

Complete characterization and analysis of biopharmaceuticals includes size exclusion chromatography (SEC) to measure protein aggregates and other size variants. Current size-based HPLC separations (HP-SEC) have traditionally been under low pressure conditions with silica-based material. These methods can be unreliable due to changes in retention time, peak shape, resolution as well as irreproducibility between columns. New advances in both packing materials and instrumentation have allowed for improvements in these isocratic separations, including improved resolution, higher throughput and sensitivity.

In this presentation, we will discuss the factors that can influence quantitation of biomolecules on size exclusion packing materials. The effect of pore size and particle size will be demonstrated for the analysis of aggregates and lower molecular weight species. Other variables such as mobile phase composition and formulation excipients will also be evaluated. Evaluation criteria will include resolution and protein purity. Due to the increasing growth in the production of biopharmaceuticals, these studies will help establish guidelines for reliable assessment of protein purity.

Spiral Countercurrent Chromatography for Bio-molecules

Martha Knight
CC Biotech LLC
9700 Great Seneca Highway
Rockville, MD 20850
marthaknight@ccbiotech.us

The liquid-liquid partitioning process for separation of molecules has been embodied in many instruments over the years. The instruments and apparatus include countercurrent distribution, droplet countercurrent chromatography and more recent ones, such as centrifugal partition chromatographs and the multi-layer coils in the planetary centrifuges. These are all methods of countercurrent chromatography (CCC). In CCC carried out in the open flow-tubing coils rotated in planetary centrifuges, there is a new design where spirals of tubing are more spread out. Recently, Yoichiro Ito, at the National Institutes of Health, invented a spiral plate assembly with widely spaced flow channels that could hold more viscous solvent systems which are optimal for peptides. This was the first rotor for spiral CCC. Now the spiral flow has been made possible in continuous tubing held in a frame. The wide spacing between the tubing significantly increases the stationary phase retention. Now all types of two-phase solvent systems can be used for chromatography. A spiral tubing support (STS) frame with circular channels was constructed by laser sintering technology into which FEP tubing was placed in 4 spiral loops per layer from the bottom to the top and a cover affixed allowing the tubing to connect to flow-tubing of the planetary centrifuge. The total volume of the coil is 135 ml. The instrumentation will be shown.

Applications of this new separation rotor were developed. Synthetic peptides including a very hydrophobic peptide were purified to a very high purity level in a sec-butanol solvent system in preparative purification experiments. Samples of up to 100 mg were loaded in these experiments. In the STS rotor, high stationary phase retention was possible with the aqueous sec-butanol solvent system at a normal flow rate. Two-phase solvent systems with this alcohol are not well retained in multi-layer coils available in instruments of other manufacturers. Finally, the two-phase aqueous polyethylene glycol-potassium phosphate solvent system was applied successfully to separate a protein from a lysate of an *E. coli* expression system. These experiments demonstrate the versatility of spiral CCC to purify bio-molecules.

Antibody Purification Via Affinity Chromatography That Utilizes the Nucleotide Binding Site

Nathan Alves, Michael W. Handlogten, Jonathan D. Ashley, Samuel Stimple, Tanyel Kiziltepe, Başar Bilgiçer,
Department of Chemical and Biomolecular Engineering
Notre Dame University, South Bend, IN 46556
nalves@nd.edu

Described here is a novel method of small molecule affinity chromatography for purifying antibodies through a highly conserved domain in the antibody Fab fragment. Based on antibody crystal structure overlays and taking into account antibody symmetry there are at least two conserved nucleotide binding sites per antibody. An in silico screening of small molecules was carried out to select for a nucleotide analog with relatively high affinity for the nucleotide binding sites, monovalent K_d = 1-10 µM based on various IgG and IgE antibodies tested. These sites allow for a selective multivalent interaction with the surface immobilized nucleotide analog to purify antibodies from complex protein mixtures such as ascites fluid and cell lysate. Antibody capture was accomplished by injection of samples while running equilibration buffer (50 mM sodium phosphate pH 7.0) and elution of the antibody was achieved by running a gradient of mild elution buffer (2M NaCl in 50 mM phosphate pH 7.0). The column exhibited a capture efficiency of >96%, with an antibody purity of >93% using Rituximab as a proof of concept pharmaceutical antibody. This small molecule affinity purification method is further tested varying: injection concentrations, volumes, wash/bind times, elution gradients, antibody/protein-contaminant combinations, and varying injection buffers. The benefits can be listed as i) reduced column cost, ii) increased column capacity, iii) increased life-time for the column due to more durable materials, iv) resin and capture molecule stable to harsh regeneration steps to provide for a more pure product, and v) purified antibody retains its bivalently active state. This new small molecule affinity purification method moves to eliminate the need for immobilized protein based purification techniques that incorporate Protein A or Protein G affinity columns.

Fast LC-MS Proteomic Separations Using Ammonium Formate Modifier

Barry E. Boyes[1,2], Stephanie Schuster[1], Joe DeStefano[1], Darryl Johnson[2] and Ron Orlando[2]

[1]Advanced Materials Technologies, Inc.
3521 Silverside Road
Wilmington, DE 19810
BBoyes@advanced-materials-tech.com

[2]Complex Carbohydrate Research Center
University of Georgia
315 Riverbend Road
Athens, GA 30602

Novel superficially porous silica particles composed of a solid silica core and a porous silica layer have demonstrated high efficiency and high speed separations in HPLC. Columns packed with 2.7-micron diameter Fused-Core particles exhibit reduced plate heights reported as low as 1.5, which is comparable to sub-2-micron particle columns, but with less than one-half the column operating back pressure. The advantages of these columns have led to a rapid growth in the popularity of Fused-Core packing materials for a variety of pharmaceutical and small molecule separations. The utility of these materials has been extended by the recent introduction of wider pore size materials for biological molecule separations. The Halo Peptide ES-C18 packing material (1.7-micron core, with a 0.5-micron shell of 160 Å pore size silica) shows favorable mass transfer properties, yielding highly efficient columns for peptide and tryptic fragment separations, demonstrated by fast proteomic LC/MS analysis. The Halo Peptide ES-C18 column is prepared using fused-core particles that are surface modified with an extremely stable steric-protected C18 bonded phase, appropriate for the low pH conditions preferred for peptide separations. TFA is a most effective acid modifier for separations, while formic acid exhibits significant band broadening when used with the Halo Peptide ES-C18 columns; this is universally observed for high performance column packing materials of use for peptide separations. Ammonium formate is an effective modifier, showing reduced band broadening of bases, including peptides. We document the compatibility of this additive for LC/MS applications, including peptide separations and proteomic analyses.