ZIC®-HILIC for Multidimensional Protein Identification Technology (MudPIT)

ZIC®-HILIC is a hydrophilic zwitterionic stationary phase that is an extremely powerful tool for the two–dimensional separation of peptides and proteins (MudPIT). The stationary phase provides separations that are orthogonal with respect to reverse phase and leads to a higher peak capacity than SCX stationary phases which are commonly used for MudPIT separations. In addition, it reduces the clustering of peaks and allows for a better distribution so more comprehensive data can be obtained from complex peptide mixtures.

Introduction

Analysis of digested cell lysates require selective and highly efficient separation prior to detection as the samples are complex (i.e. contain numerous peptides and proteins). A broad range of separation techniques including SDS-PAGE, isoelectric focusing, size exclusion chromatography and reverse phase chromatography have been evaluated. In recent years, Multidimensional Protein Identification Technology (MudPIT) has proven to be a viable alternative as it provides superb resolving power. MudPIT uses a two-dimensional liquid chromatography (2D-LC) approach and combines orthogonal techniques to separate the compounds of interest. As an example, a strong cation exchange column (SCX) can be used for the first dimension and a reverse phase (RP) column for the second dimension to maximize the resolving power.

Hydrophilic interaction liquid chromatography (HILIC) also is orthogonal to RP chromatography, and provides an elution order similar to normal phase chromatography. A significant advantage of HILIC relative to normal phase is that water miscible solvents can be used.

ZIC®-HILIC is a hydrophilic stationary phase with a permanent zwitterionic functional group which is particularly capable of solvating polar and charged compounds via weak electrostatic interactions (as opposed to the strong electrostatic interactions obtained with silica or amino HILIC phases) and being much more selective than a neutral diol phase. In a recent study, PJ Boersma and coworkers[1] have shown that ZIC®-HILIC can provide a very powerful approach to the separation of peptides and proteins and is very useful as the first stage of a MudPIT separation.

Experimental

Protein Samples: A model standard peptide mixture consisting of protein digests from bovine serum albumin, and •- and •-casein was employed. In addition, proteins were obtained from the nuclei of *Murine erythrolukemia* (MEL) cells and digested.

One Dimensional LC-Separation Conditions: One-dimensional ZIC®-HILIC separations were performed using a 150 x 1.0 mm, 3.5 µm, 200 Å column (SeQuant, Umeå, Sweden) with a flow rate of 40 µL/min with a buffered Formic Acid (FA)/Acetonitrile gradient.

Two Dimensional LC-Separation, First Dimension: HILIC Separations: The first separation was performed using a ZIC®-HILIC trapping column (SeQuant, Umeå, Sweden), 200 µm x 5 mm, 3.5 µm, 200 Å at a flow of 2 µL/min and the analytical separation was performed using a 200 µm x 160 mm, 3.5 µm, 200 Å ZIC®-HILIC column at a flow of 1.5 µL/min. *SCX Separations:* The first separation was performed using a polysulfoethyl A trapping column (PolyLC, Columbia, MD), 200 µm x 32 mm, 5µm, 200 Å and the analytical separation was performed using a polysulfoethyl A column, 200 µm x 32 mm, 5µm, 200 Å.

Trapping was done at 2 µL/min for 10 min and the analytical separation was done at 1.5 µL/min. One min fractions were collected in a titer plate with a Probot Microfraction Collector (LC Packings, Amsterdam) and 40 µL of 5% FA was added to each fraction to make the sample compatible with RP and reduce evaporation of the sample.

Two Dimensional LC-Separation, Second Dimension: The second separation was performed using a Aqua trapping column (Phenomenex, Torrance, CA) 50 µm x 10 mm, 5 µm, 120 Å trapping column and a Reprosil (Dr. Maisch GmbH, Ammerbuch, Germany) 50 µm x 254 mm, 3 µm, 120 Å column. Trapping was performed at a flow rate of 5µL/min and the analytical separation was performed at a flow rate of 100 µL/min using a gradient of 0-45% B in 45 min, 45-100% in 1 min, 100% B in 4 min. with a flow of 4 nL/min.
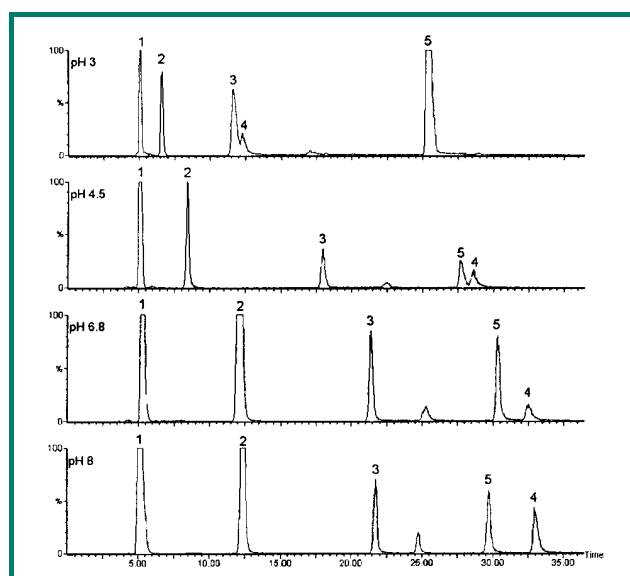

Figure 1: Ion Chromatograms of peptides from standard mixture 1) GPFPIIV, 2) LTV-DLTK, 3) ADLAK, 4) FQpSEEQQQTEDELQDG, 5) LKECCDKPLLEK. Separation with ZIC®-HILIC column, see Experimental for mobile phase.

Detection was performed with a Q-TOF Micromass (Micromass UK, Ltd, Manchester, UK) in positive ion mode) or a Orbitrap MS system (Thermo, San Jose, CA). Protein identification was performed using the Marcot software platform (Matrix Science, London, UK). Spectra were searched against the UniProt-Swiss-Prot 50.4 database.

Results

1D: ZIC®-HILIC Separations: Figure 1 presents the ion chromatograms of peptide mixtures at various pH's using ZIC®-HILIC. The charge on the stationary phase does not change between pH 3 and 8, so the nature of the separation depends on the charge on the peptides.

2D: ZIC®-HILIC-RP Separations: 2D experiments were performed with 4 buffers to determine the orthogonality at various pH's. A dot plot is used to demonstrate the efficiency of 2D separations, with the two chromatographic conditions as the axes. If the two separations have a "perfect" reverse elution pattern, a downward diagonal would be expected, and any deviation from a downward trendline is an indication that the ZIC®-HILIC is the reverse of RP. At all four pH's ZIC®-HILIC performed well with RP to separate the peptides. The dot plots at pH 3 and 8 have negative coefficients (Figures 2 and 3).
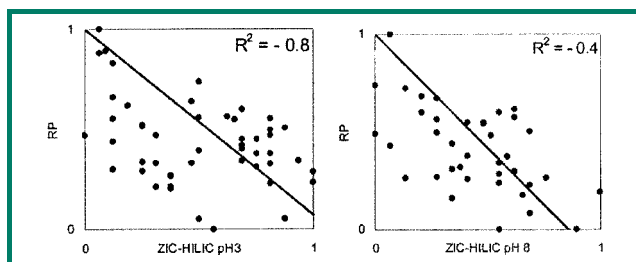

Figure 2: Normalized Peptide Retention Time Plots comparing ZIC®-HILIC vs. RP at pH 3 (left) and pH 8 (right).
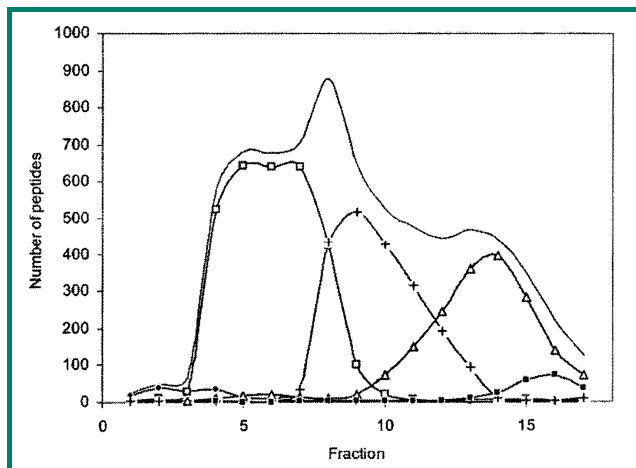

Figure 3: Distribution of peptides from nuclear extract digest, ZIC®-HILIC fractionation at pH3. The number of peptides/fraction are as follows (-) total, net charge ( ) +1, □ (+2), (+) +3, (Δ) +4, ■ (>+4).

At pH 3, the ZIC®-HILIC column functions similar to that of a SCX column, but also provides the highest orthogonality to RP. The authors note that with the SCX column, all of the +1 peptides were separated first, then the +2 peptides and then all the +3 peptides., but when ZIC®-HILIC was employed, the separation of like charged peptides was

less distinct and there is a positive effect on the distribution of similar peptides over the entire chromatogram. When SCX was used, the +2 and +3 peptides eluted over about 1/3 rd of the chromatogram, with ZIC®-HILIC, they were more evenly distributed, and the possibility of separating them in more fractions is preferred.

Separation of a Nuclear Fraction of a Cellular Lysate

The analysis of the nuclear fraction of a cellular lysate involves the detection of a large number of proteins which are present in a range of concentrations that may range over ten orders of magnitude. Boersma and co-workers employed 2D-ZIC®-HILIC-RP for the separation. When the first dimension (ZIC®-HILIC) was run at pH 3, 1040 proteins with 4973 unique peptides were identified, while at pH 6.3, 1284 proteins with 6625 peptides were identified (when only RP was used only 367 proteins could be confidently identified). The distribution of peptides (at pH 3) is shown in Figure 3, it can be seen that there is a broad distribution range with respect to peptide charge.

Conclusions

The characteristics of ZIC®-HILIC provides significant benefits for MudPIT separations compared to the use of SCX. It provides a higher peak capacity than SCX and significantly reduces the clustering of +2 and +3 peptides. The reduction of clustering improves the chromatographic resolution and the peak capacity. The nature of the separation is pH dependent; at pH 3, the orthogonality with RP is maximized and at higher pH the separation power is highest.

References

1: This application note is condensed from the scientific paper "*Evaluation and Optimization of ZIC-HILIC-RP as an Alternative MudPIT Strategy*" by P. J. Boersema, N. Divecha, A. J. R. Heck, and S. Mohammed, *J. Proteome Research*, 8 (2007) 937-946. Figure 1-3 are reprinted with permission from American Chemical Society, © (2007).

About ZIC®-HILIC Chromatography

The ZIC®-HILIC stationary phase is based on the covalently bonded zwitterionic sulfobetaine group indicated in Figure 4. It is available with a silica support in 3.5, 5 and 10 μm particle sizes in various column dimensions on glass lined stainless steel for capillary separations, as well as PEEK and stainless steel for analytical and preparative separations, In addition, it is available with a polymeric support in 5 μm particles (ZIC®-*p*HILIC).
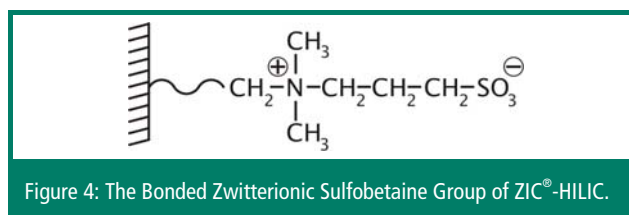

Figure 4: The Bonded Zwitterionic Sulfobetaine Group of ZIC®-HILIC.

About SeQuant

SeQuant is a Swedish company that develops a broad range of innovative products for separation and purification of complex samples via chromatography. The company was founded in 1987 by research workers from Umeå University. Products include silica based and polymer based ZIC®-HILIC columns for HPLC, Membrane Suppressors and Suppressor Regeneration Systems for Ion Chromatography, Proteomic Calibration Kits and Post Column Reaction Delay and Mixing Coils. These products are supported by distributors world-wide.