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Evaluation and Optimization of ZIC-HILIC-RP as an Alternative MudPIT Strategy

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Abstract: In proteomics, a digested cell lysate is often too complex for direct comprehensive mass spectrometric analysis. To reduce complexity, several peptide separation techniques have been introduced including very successful two-dimensional liquid chromatography (2D-LC) approaches. Here, we assess the potential of zwitterionic Hydrophilic Interaction Liquid Chromatography (ZIC-HILIC) as a first dimension for the analysis of complex peptide mixtures. We show that ZIC-HILIC separation is dramatically dependent on buffer pH in the range from 3 to 8, due to deprotonation of acidic amino acids. ZIC-HILIC exhibits a mixed-mode effect consisting of electrostatic and polar interactions. We developed a 2D-LC system that hyphenates ZIC-HILIC off-line with reversed-phase (RP). The two dimensions are fairly orthogonal, and the system performs very well in the analysis of minute amounts of complex peptide mixtures. Applying this method to the analysis of 10 μg of a cellular nuclear lysate, we were able to confidently identify over 1000 proteins. Compared to strong cation exchange chromatography (SCX), ZIC-HILIC shows better chromatographic resolution and absence of clustering of prevalent +2 and +3 charged peptides. At pH 3, ZIC-HILIC separation allows best orthogonality with RP and resembles conventional SCX separation. A significant enrichment of N-acetylated peptides in the first fractions is observed at these conditions. ZIC-HILIC separation at high pH (6.8 and 8), however, enables better chromatography, resulting in more comprehensive data acquisition. With this extended flexibility, we conclude that ZIC-HILIC is a very good alternative for the more conventional SCX in multidimensional peptide separation strategies.

Keywords: two-dimensional liquid chromatography mass spectrometry • peptide separation • zwitterionic hydrophilic interaction chromatography • strong cation exchange chromatography • N-acetylation • enrichment of post-translationally modified peptides • MEL cells

Introduction

Two of the main challenges facing comprehensive proteomics analyses are the sheer complexity of the proteome and the huge dynamic range in protein expression.[1] To accommodate these challenges, a number of protein and peptide separation strategies are implemented to reduce the complexity for the final step of mass spectrometric detection and protein identification. Depending on what level the first dimension is performed, most separation techniques can be placed in one of two categories: protein level or peptide level. On the protein level, techniques such as SDS-PAGE, isoelectric focusing (IEF), size exclusion chromatography (SEC), ion exchange chromatography (IEC), and reversed-phase (RP) chromatography have been implemented.[2] On the peptide level, digestion is performed immediately after cell lysis, and peptides are then subjected to a number of, most usually, chromatographic separations. Recently, also IEF and capillary electrophoresis (CE) approaches have been shown to be suitable for peptide-level first-dimension separation.[3−6]

The end point of most proteomics experiments is a peptide mixture separated on RP at nanoliter flow rates on a liquid chromatography (LC) system coupled with a mass spectrometer (nanoLC−MS).[2] The use of peptides rather than proteins allows for an easier automation of the process, minimizes nonspecific adsorption onto separation devices, and allows a higher chromatographic resolving power.[7] Although RP chromatography has one of the highest separation powers available, it alone cannot sufficiently reduce the complexity of most peptide mixtures for comprehensive analysis by MS. A number of laboratories have attempted to improve single-dimension separation by using longer columns (over 50 cm) and increasing gradients (up to 10 h).[8] However, such systems typically operate at ultrahigh pressures, that is, in excess of 1000 bar and, thus, cannot be implemented using routine HPLC instrumentation.

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The use of chromatographic techniques in tandem to allow a higher separation power and improve comprehensiveness of an analysis has proven to be successful. The most prominent strategy to use multiple chromatographic separations is often referred to as multidimensional protein identification technology (MudPIT).[9] Originally called direct analysis of large protein complexes (DALPC),[10] the typical approach is to create a column containing two phases (strong cation exchange (SCX) and RP) to increase resolving power. The peptides bind initially on SCX material and are eluted stepwise onto the RP part, where each fraction is further separated and analyzed by mass spectrometry.[9] Recently, MudPIT has been implemented on a ultrahigh-pressure scale allowing improvements in separation.[11] Compared to an off-line system, online hyphenation displays advantages such as minimal loss of sample, no vial contamination, and no sample dilution.[12,13] Despite the convenience brought by automation by online 2D-LC, it has been found to be rigid and a compromise on separation efficiency. Off-line fractionation removes some of these limitations; that is, loading an appropriate level of material for the second dimension is no longer an issue as it is for online approaches, where overloading of the second dimension can easily occur. Peptide separations have also been shown to be generally superior for off-line coupling, since a conventional gradient can be implemented.[14] It is also possible to perform selective, in-depth analysis of certain SCX fractions allowing to focus on a subgroup of peptides such as those that are phosphorylated.[15] The pH of the SCX elution buffer can also be modified from the necessary low pH for electrospray, which normally causes the majority of tryptic peptides to elute in a narrow window.[16] Finally, an additional advantage of off-line coupling is the use of different column materials that are not directly compatible with each other in terms of required solvents.[12,14]

Over time, a number of 2D-LC configurations have been developed, including the off-line[17] and online[18] coupling of SEC in the first dimension and RP in the second. Although SEC is compatible with RP and separation may be orthogonal, this combination is not very widespread due to the low separation power of SEC.[18] The combination of RP at pH 10 in the first dimension and RP at pH 2.6 in the second is an interesting setup for its compatibility with MS and surprisingly high orthogonality.[19] However, because the separation is correlative, this is still not the optimal 2D-LC system. It has been suggested that hydrophilic interaction liquid chromatography (HILIC) is a good alternative candidate as first dimension of a hyphenated system, showing an orthogonal separation to RP, with a separation power similar to RP and compatibility with MS.[20,21] HILIC is characterized by the use of a hydrophilic stationary phase and a hydrophobic organic mobile phase, conditions that have been in use since 1975.[22] The order of elution of peptides is reversed to RP, but rather than choosing the historical name normal phase (NP), the descriptive acronym HILIC was used to differ NP from HILIC, since NP is performed with nonaqueous, non-water-miscible solvent buffers, whereas HILIC is performed with water-miscible solvents and elution is achieved by a water gradient.[23−26] Although discussion still exists about the exact separation mechanism, it is generally accepted that an aqueous layer is formed around the hydrophilic functional groups of the HILIC material. The separation of peptides can be explained by a partitioning mechanism between the aqueous layer and the hydrophobic buffer,[23] by hydrogen bonding with the HILIC material,[27] or a mechanism somewhere in between, with both partitioning and hydrogen bonding.[28]

At present, a number of different materials are commercially available for HILIC, including underivatized silica that contains functional groups such as siloxanes, silanols, and a small quantity of metals[24,25,29,30] and derivatized silica, such as polysulfoethyl A,[23,26] the weak anion exchanger polycat A,[31] TSKgel amide 80,[27,32] and ZIC-HILIC.[33] They are all capable of generating an aqueous layer around their functional groups. The charge that most functional groups carry contributes to the hydrophilicity of the stationary phase.[23] For a more comprehensive and detailed overview of different HILIC materials, see Hemström and Irgum.[24]

In this paper, we report on the evaluation and optimization of off-line hyphenation of ZIC-HILIC with RP for the separation of minute amounts of (complex) peptide mixtures for subsequent MS analysis. The optimized system is then used to characterize a cellular nuclear lysate.

Materials and Methods

Chemicals and Reagents. Formic acid, acetic acid, and sodium chloride were purchased from Merck (Darmstadt, Germany). Acetonitrile was purchased from Biosolve (Valkenswaard, Netherlands). Dithiothreitol and ammonium bicarbonate were purchased from Fluka (Buchs, Switzerland). Trypsin and endoprotease LysC were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Water used in these experiments was obtained from a Milli-Q purification system (Millipore, Bedford, MA). All other chemicals were from Sigma (St. Louis, MO).

Preparation of Protein Standard Mixture. For the optimization and evaluation of ZIC-HILIC, we prepared a model standard peptide mixture consisting of combined protein digests from bovine serum albumin (BSA) and α- and β-casein. Each protein was digested separately. A total of 31 μL of 45 mM DTT was added to 125 μL of 4 μg/μL dissolved protein, and the mixture was incubated at 50 °C for 30 min. A total of 31 μL of 110 mM iodoacetamide was added, and the mixture was kept at room temperature for 30 min. The mixture was diluted 5 times with 50 mM ammonium bicarbonate, and 10 μg of trypsin was added for overnight digestion at 37 °C. Digests were subsequently mixed 1:1:1, and the mixture was dried *in vacuo* ('Speedyvac', Thermo, CA) and reconstituted in buffer A for subsequent LC separation.

Cell Culture, Subcellular Fractionation, and Sample Preparation. Murine erythroleukemia (MEL) cells were maintained in routine culture using 10% fetal calf serum (FCS) in Dulbecco's modified Eagle medium (DMEM). Nuclei isolation was performed as published previously.[34,35] Briefly, cells were fractionated by using a detergent-free hypotonic buffer. Separation using a sucrose cushion yields a mixed cytosol/membrane fraction and intact nuclei. A pellet of 1200 μg of nuclei was dissolved in 300 μL of 8 M urea and 400 mM ammonium bicarbonate by two 5 s sonication bursts on ice. A total of 40 μg of sample was reduced in 2.5 μL of 45 mM DTT for 30 min at 50 °C. A total of 2.5 μL of 100 mM iodoacetamide was added, and the mixture was kept at room temperature for 30 min. LysC (0.5 μg) was added, and the mixture was incubated at 37 °C for 4 h. The mixture was diluted 7 times, 1 μg of trypsin was added, and the mixture was incubated at 37 °C overnight. Sample was desalted using C18 ZipTip (Millipore, Bellerica, MA), filled with extra Aqua C18 beads. The eluent was dried *in vacuo* and reconstituted in buffer A of which one-fourth (~10 μg) was used for subsequent LC separation.

LC Buffers. pH 3 buffer A was 80% acetonitrile (ACN) and 0.05% formic acid (FA), and buffer B was 40% ACN and 0.05% FA. pH 4.5, 6.8, and 8 buffer A was 80% ACN/20% water and 20 mM ammonium acetate (overall), and buffer B was 40% ACN/60% water and 20 mM ammonium acetate (overall). SCX buffer A was 30% ACN and 0.05% FA; buffer B was 30% ACN, 0.05% FA, and 0.5 M NaCl. Buffer A for nanoLC-LTQ-Orbitrap analysis was 0.5% acetic acid; buffer B was 80% ACN and 0.5% acetic acid.

LC−MS. One-dimensional ZIC-HILIC separation was performed using an Agilent 1100 series LC system, with a ZIC-HILIC column (SeQuant, Umeå, Sweden) 1.0 mm × 150 mm, 3.5 μm, 200 Å. The flow was passively split from 800 to 40 μL/min before the column. Gradient elution was performed for all pH conditions similarly: 0−100% B in 43 min. Column output was coupled with a Q-TOF Micromass spectrometer (Micromass UK Ltd., Manchester, U.K.) via a passive split reducing the flow to 250 nL/min. Nanospray was achieved using a distally coated fused silica emitter (New Objective, Cambridge, MA) (360 μm o.d./20 μm i.d./10 μm tip i.d.) biased to 2.5 kV. The mass spectrometer was operated in the positive ion mode with a resolution of 4500−5500 full-width half-maximum (fwhm) using a source temperature of 80 °C and a counter current nitrogen flow rate of 150 L/h. Data dependent analysis was employed (three most abundant ions in each cycle): 1 s MS (m/z 350−1500) and max 2 s MS/MS (m/z 50−2000, continuum mode), 30 s dynamic exclusion. A charge state recognition algorithm was employed to determine optimal collision energy for low-energy CID MS/MS of peptide ions. External mass calibration using NaI resulted in mass errors of less than 50 ppm, typically 5−15 ppm in the m/z range 50−2000.

2D-LC−MS, First Dimension. First-dimensional ZIC-HILIC separation was performed on a Famos/Ultimate LC instrument (LC Packings, Naarden, Netherlands), using a vented column setup.[36] The trapping column was ZIC-HILIC, 200 μm × 5 mm, 3.5 μm, 200 Å; analytical column ZIC-HILIC, 200 μm × 160 mm, 3.5 μm, 200 Å. For SCX separation, the trapping column was polysulfoethyl A (PolyLC, Columbia, MD) 200 μm × 32 mm, 5 μm, 200 Å; the analytical column was polysulfoethyl A 200 μm × 105 mm, 5 μm, 200 Å. All columns were packed in-house. Trapping was performed at 2 μL/min for 10 min, analytical separation at 1.5 μL/min, passively split from 250 μL/min. Gradient elutions were chosen such that peptides elute in a similar time frame, generally 0−80% B in 30 min, 80−100% B in 2 min, 100% for 5 min. One-minute fractions were collected in a titer plate using a Probot Microfraction collector (LC Packings), adding 40 μL of 5% FA per fraction. No additional sample modification was performed before second-dimensional LC−MS.

2D-LC−MS, Second Dimension. A volume of 8 μL of collected fractions was used for subsequent nanoLC-LTQ-Orbitrap-MS (Thermo, San Jose, CA). An Agilent 1100 series LC system was equipped with an Aqua (Phenomenex, Torrance, CA), 50 μm × 10 mm, 5 μm, 120 Å trapping column and a Reprosil (Dr. Maisch GmbH, Ammerbuch, Germany), 50 μm × 254 mm, 3 μm, 120 Å analytical column. Trapping was performed at 5 μL/min for 10 min; elution was achieved with a gradient of 0−45% B in 45 min, 45−100% B in 1 min, 100% B for 4 min, with a flow rate of 0.4 mL/min passively split to 100 nL/min. Nanospray was achieved using a distally coated fused silica emitter (New Objective, Cambridge, MA) (360 μm o.d./20 μm i.d./10 μm tip i.d.) biased to 1.8 kV. The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS. Survey full scan MS spectra (from m/z 350−1500) were acquired in the FT-Orbitrap with resolution R = 60 000 at m/z 400 (after accumulation to a target value of 500 000 in the linear ion trap). The two most intense ions were fragmented in the linear ion trap using collisionally induced dissociation at a target value of 10 000.

Table 1. Charge State of Peptide Residues at the Different pH Values Used in This Paper

	pH 3	pH 4.5	pH 6.8	pH 8
K	+	+	+	+
R	+	+	+	+
H	+	+	0	0
D	0	0/-	-	-
E	0	0/-	-	-
N-term	+	+	+	+
N-term acetylated	0	0	0	0
pS, pT, pY	-	-	-	-

Protein Identification. Data analysis was carried out using the Mascot (version 2.1.0) software platform (Matrix Science, London, U.K.). Q-TOF Micromass spectra from the protein standard mixture were searched against the UniProt-Swiss-Prot 50.4 database with taxonomy: other mammalia, trypsin with maximal 2 missed cleavages, carbamidomethyl (C) as fixed modification and oxidation (M), N-acetylation (N-terminus), and phosphorylation (S, T, Y) as variable modifications. Peptide tolerance was set to 50 ppm with 1+, 2+, and 3+ peptide charges and MS/MS tolerance of 0.9 Da.

LTQ Orbitrap spectra from protein standard mix and nuclear lysate were searched against, respectively, UniProt-Swiss-Prot 50.4 database with taxonomy: other mammalia and IPI-Mouse 3.19 database. Further settings: trypsin with 2 missed cleavages, carbamidomethyl (C) as fixed modification, oxidation (M), N-acetylation (N-terminus), and phosphorylation (S, T, Y) as variable modifications. Peptide tolerance was set to 5 ppm for 2+ and 3+ charged peptides, and MS/MS tolerance was 0.9 Da. A minimum peptide score was set to 20 and expect value ≤0.05. If a peptide was found in more than one fraction, the retention time was determined to 0.5 min precision, calculated from peptide intensities. Minimum Mascot protein score of 60 was used for confident identification.

Results

1D ZIC-HILIC-MS. For this research, ZIC-HILIC was chosen over alternative HILIC materials due to its zwitterionic functional group. It has been suggested that such a material creates weaker ionic interactions when compared to charged HILIC materials such as polysulfoethyl A and silica, allowing the use of buffers with lower ionic strengths.[37] The nature of ZIC-HILIC has an additional benefit in that between pH 3 and 8 the charge of the chromatographic material will not change.[24] However, changing pH within this range does have an effect on the charge state of peptide residues. Table 1 lists the charge of certain amino acids at the pH levels used in this study. Below pH 4, only basic residues and N-termini (when not acetylated) are charged, while above pH 4, acidic residues are deprotonated and become negatively charged. Increasing pH over 6 will cause hisitidines to be deprotonated and lose their charge. To assess the effect of pH and peptide charge on peptide separation, the two extreme pH conditions the silica-based ZIC-HILIC could handle (pH 3 and 8) and two intermediate pH levels (pH 4.5 and 6.8) were selected.

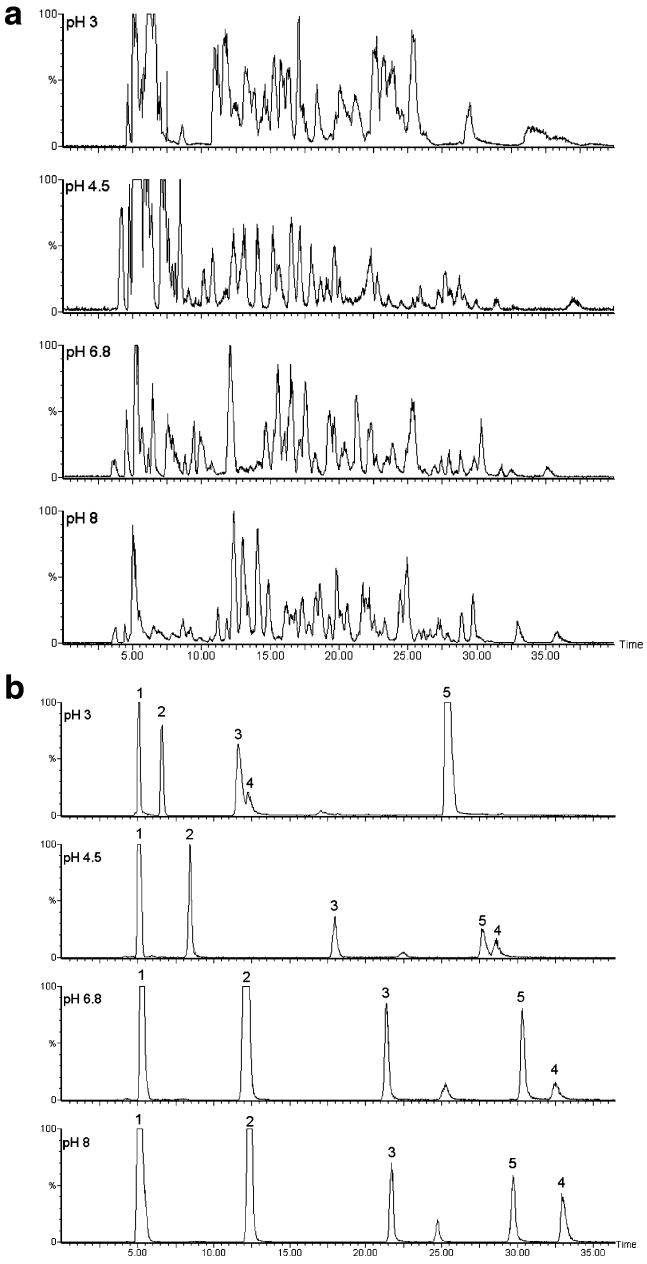

Figure 1. LC−MS chromatograms of a standard peptide mixture using a ZIC-HILIC, 1.0 mm × 150 mm, 3.5 μm, 200 Å column online connected to a Q-TOF Micromass mass spectrometer. Buffer pH was as indicated. (a) Base peak chromatograms; (b) extracted ion chromatograms of peptides (1) GPFPIIV, (2) LVTDLTK, (3) ADLAK, (4) FQpSEEQQQTEDELQDK, and (5) LKECCDKPLLEK.

A peptide mixture consisting of BSA and α- and β-casein in a 1:1:1 ratio was applied onto a ZIC-HILIC column, 1.0 mm × 150 mm, separated under the four different pH conditions and analyzed by MS. Peptide elution was performed with a 80−40% ACN gradient in approximately 40 min. LC−MS chromatograms are depicted in Figure 1a, and additional information on retention times of individual peptides is provided in the Supporting Information Table 1. Examination of the retention times of the peptides shows a trend of longer retention with increasing pH. This is probably due to the fact that the interaction with ZIC-HILIC material is increased upon deprotonation of acidic residues. To further investigate trends relating to peptide net charge changes, retention times of 'model peptides' from each subgroup were tracked with buffer pH change, and their extracted ion chromatograms are shown in Figure 1b. The phosphopeptide FQpSEEQQQTEDELQDK (peptide 4) carries three charges at pH 3: the N-terminus, a lysine, and the phospho-group. When the buffer reaches above pH 4, the aspartic acid and glutamic acid residues are deprotonated, adding six extra charges to the peptide. The interaction with the chromatographic material is thus improved, consequently increasing the retention time dramatically. On the other hand, the peptide GPFPIIV (peptide 1) does not contain any acidic groups, and therefore, its retention is not affected by the pH of the buffer. The basic peptide LKECCDKPLLEK (peptide 5) carries four positive charges at pH 3, but the addition of three extra charges when increasing the pH up to 8 seems not to affect the retention of this peptide dramatically.

The chromatographic performance of ZIC-HILIC is visibly improved upon changing the buffer. Through the use of ammonium acetate buffers at higher pH, peaks are generally sharper and more peaks are baseline-separated as can be seen in Figure 1a. This improved separation can be partly explained by the nature of the tryptic peptide pool used for evaluation. At pH 3, acidic residues are neutral, so regular tryptic peptides generally carry only two or three positive charges. At higher pH levels, the acidic residues are charged, so the variation in net charge of peptides increases; consequently, coelution is less likely to happen, improving chromatographic performance of ZIC-HILIC.

In all four conditions, the peak intensity diminished over the run, which is probably due to the decreasing ACN gradient. The magnitude of this effect is most likely related to the suboptimal performance of the electrospray conditions applied in the test bed system.[25] In other words, lower signals for late-eluting peptides are not due to the ZIC-HILIC column or separation but due to the ionization process as could be confirmed by UV detection (data not shown).

Figure 2 compares peptide retention times at different pH values. Little difference is observed between pH 6.8 and 8, which is not surprising since no pK_a or pK_b is passed. A large difference in retention times was observed between pH 3 and pH 8, confirming the effect of changing the net charge state of peptides. Peptides with a longer retention time at pH 8 usually have at least two acidic residues, while peptides with a higher retention time at pH 3 generally contain more than two basic residues or lack acidic residues.

2D ZIC-HILIC-RP LC−MS. Generally, the separation power of 2D-LC is superior over 1D-LC, since the peak capacity of the system is increased by the addition of an extra column. A common way to gauge the theoretical peak capacity of a 2D-LC system is by multiplying the peak capacity of both columns.[38] It can also be visualized as a dotplot with the two chromatographic dimensions making up the axes. In an ideal situation, dots will cover the whole dotplot area. However, this will not occur since the separation of peptides is not carried out in an 'orderly fashion'.[39] Furthermore, the peak capacity in 2D-LC is dependent on dimensionality of the sample and column and the orthogonality of the two columns.[20,39]

The high organic content of the buffers used in ZIC-HILIC separations does not allow a direct hyphenation with RP. To overcome this problem, we developed an off-line 2D-LC system, based on the vented column design,[36] in which peptides are initially separated over a homemade 200 μm i.d. ZIC-HILIC column operating at a flow rate of 1−2 μL/min. Instead of directly collecting the peptides, the eluent was diluted via a

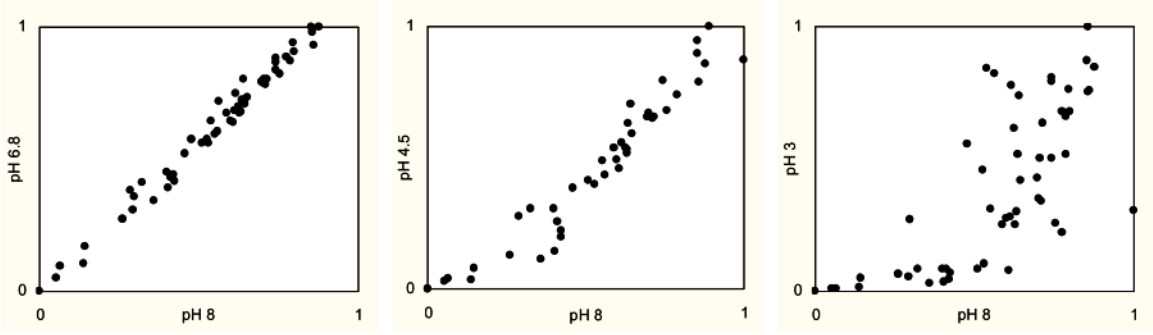

Figure 2. Normalized peptide retention times of all peptides detected in Figure 1 at different pH conditions. All peptides eluted between 5 and 40 min.

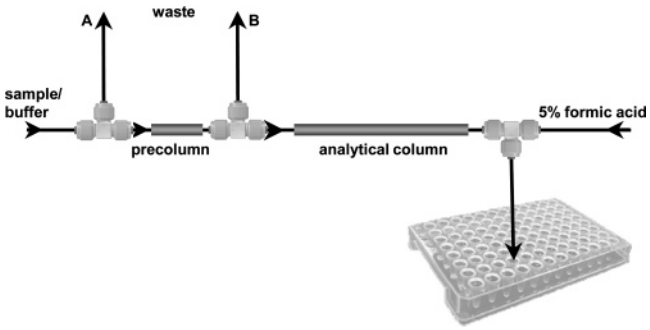

Figure 3. Schematic presentation of the first-dimensional ZIC-HILIC separation/fractionation system. During sample loading, waste line A is closed, and the flow is 2 μL/min. During analysis, waste line B is closed, and the flow is split by the first T-piece from 250 to 1.5 μL/min. The eluent is mixed with 5% formic acid, and 1-min fractions are collected in a titer plate which can be directly used for subsequent nanoRP-LC−MS analysis.

T-piece with a make-up solution of 5% formic acid operating at 80 μL/min (Figure 3). Thus, 1 min fractions would now be sufficiently large and aqueous to reduce evaporation and become compatible with nanoRP chromatography. Additionally, the acidified fractions were directly placed in a 96 well plate, reducing sample handling. The second dimension was also based on a vented column system, where peptides are trapped first on a RP precolumn and then analyzed at nano flow rates. It is expected that certain hydrophilic peptides, such as those that are glycosylated and might have been retained on ZIC-HILIC, are not retained on a RP column.[40]

As shown above, changing buffer pH influences the retention times of peptides. The impact of this variation on the orthogonality of ZIC-HILIC with RP was investigated by applying the peptide mixture to the 2D-LC system. The results, presented as dotplots (Figure 4), provide an impression of the system's orthogonality at different ZIC-HILIC pH conditions. A system that merely has a reverse elution pattern compared to RP would show a downward diagonal from left to right. The apparent deviation from such a downward trend line indicates that ZIC-HILIC is not simply the reverse of RP. In fact, at pH 3 and 8, the correlation coefficient (R^2) is negative, demonstrating that the trend line was forced to be a diagonal and is far from the best fit. In conclusion, ZIC-HILIC seems to perform well as a first dimension at all four pH conditions, displaying the highest orthogonality at pH 3.

As implied by the results from the one-dimensional ZIC-HILIC experiments, ZIC-HILIC at pH 3 shows an elution profile that resembles that of an SCX system. To allow detailed comparison of ZIC-HILIC with SCX, the same peptide mix was analyzed using the off-line fractionation system in which the ZIC-HILIC column was replaced by a polysulfoethyl A column. Under the modified conditions using SCX, a distinctive separation of the charged peptide subgroups is observed, with nearly no overlap. Peptides with a net charge of 1+, including three phosphopeptides, elute first, followed by peptides with a net charge of 2+, 3+, and finally those that contain more than 3+ charges. When performing ZIC-HILIC at pH 3, peptides with a net charge of 1+ also elute first, followed by peptides with a net charge of 2+, 3+, and more than 3+. Figure 5 highlights the resemblance of ZIC-HILIC at pH 3 with SCX. However, with ZIC-HILIC, separation with respect to peptide charge is less distinct and more overlap between differently charged peptides occurs. The three phosphopeptides with a net charge of 1+ are coeluting with 2+ peptides. A hydrophilic effect is also visible: within a group of similarly charged peptides, a reverse correlation is apparent. This results in a positive effect on the distribution of similar peptides over the chromatogram. When using SCX conditions, peptides with a net charge of 2+ and 3+ elute during about one-third of the gradient time, whereas with ZIC-HILIC, those peptides elute during almost the whole gradient. Since those peptides are most prevalent, the possibility of separating them in more fractions, as made possible by ZIC-HILIC, is preferred.

Analysis of a Nuclear Fraction of a Cellular Lysate. One of the challenges of proteomics is to identify proteins in a very large dynamic range that can be more than 10 orders of magnitude.[41] Nuclear fractions represent one of the most difficult proteomes to analyze. One way to cope with this complexity is to use extensive separation methods. We examined the practicality of the optimized 2D-ZIC-HILIC-RP system to investigate the nuclear proteome. Nuclei were isolated from Murine erythroleukemia cells, and proteins were digested in-solution with trypsin, yielding a sample that is complex in the number of proteins and the dynamic range at which they are expressed, with histones being highly abundant. Separation of 10 μg of nuclear proteins was performed in the first dimension using ZIC-HILIC at pH 3 (showing best orthogonality with RP) and pH 6.8 (showing best separation), while the second dimension consisted of a 60 min gradient analysis on a nanoRP-LC−MS. The use of ZIC-HILIC at pH 3 as a first dimension allowed the identification of 1040 proteins with 4973 unique peptides and 1284 proteins with 6625 peptides at pH 6.8 (MS/MS spectra and information on protein and peptide scores are available at https://bioinformatics.chem.uu.nl/supplementary/boersema_jpr). Applying the same sample solely on the second RP dimension, only 367 proteins could be confidently identified

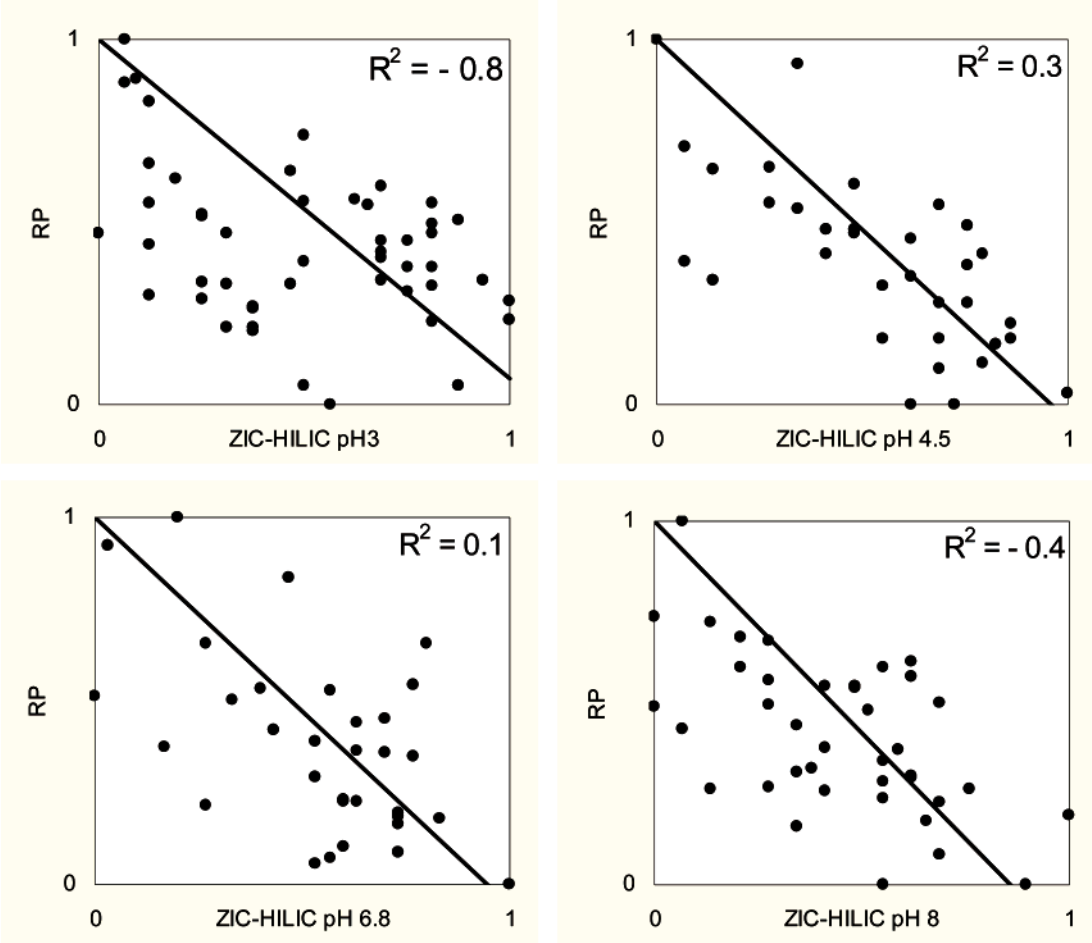

Figure 4. Normalized peptide retention time plots, comparing ZIC-HILIC versus RP at different pH conditions. Peptides elute between 10 and 50 min from ZIC-HILIC and between 5 and 40 min from RP. The downward diagonal is the trend line, and R^2 is the correlation coefficient.

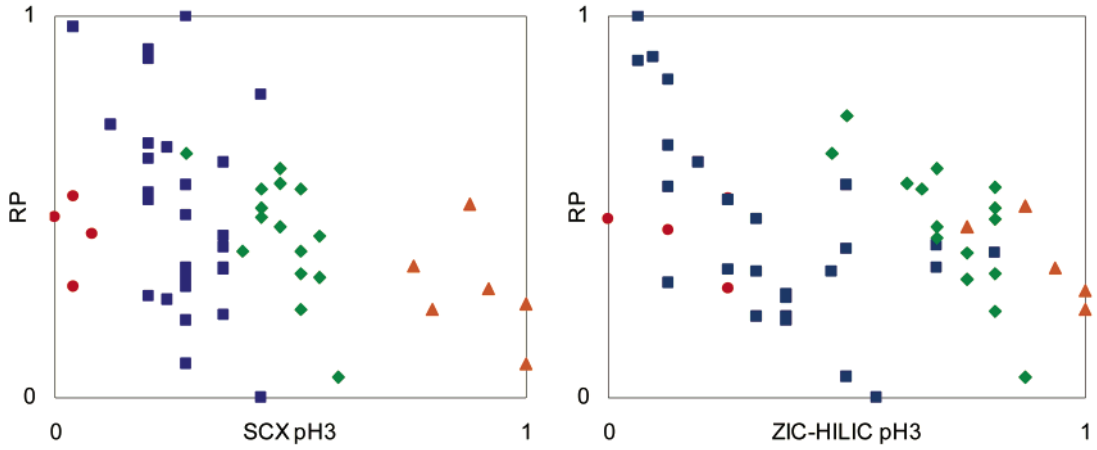

Figure 5. Normalized retention time plots for SCX−RP and ZIC-HILIC-RP. The net charge of the peptides are indicated with (●) 1+, (■) 2+, (♦) 3+, and (▲) >3+. (Left panel) SCX pH 3 versus RP; (right panel) ZIC-HILIC pH 3 versus RP.

with 1230 unique peptides. In a comparison between the analyses at the two different pHs, an overlap of 772 proteins (50%) was observed (Figure 6). In total, 268 proteins were exclusively identified at pH 3, and 512 proteins were exclusively identified at pH 6.8. This might be explained by either undersampling, separation power, or both. It has been shown many times that the overlap between two subsequent 'MudPIT' type experiments is poor, often below 50%. Such a low level of reproducibility is related to the speed of sequencing by the mass spectrometer and the overwhelmingly complex proteolytic analyte.[42] For instance, the reproducibility and level of random sampling was investigated for MudPIT LCQ-MS analysis of proteins of a yeast cell lysate.[42] In total, 24% of proteins were found in just one analysis, and only 35.4% of proteins

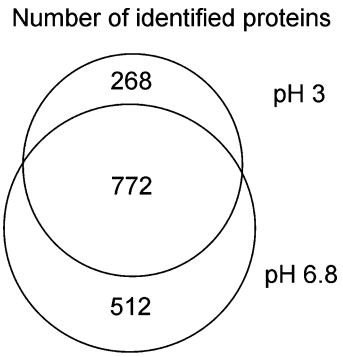

Figure 6. Venn diagram showing the number of proteins identified under either or both pH ZIC-HILIC separation conditions.

were identified in all of the analyses. Although this situation of low overlap between subsequent MudPIT experiments is constantly improving with new and faster mass spectrometers with better dynamic range as Shen et al.[43] (among others) have shown, improvements are still required. However, the increase in identifications observed by increasing the pH of the 2D-LC system cannot be solely explained by undersampling. The identification of nearly an extra 1700 peptides is significant and most likely an effect of the change of conditions. As shown in Figure 1, the peptide standard mixture revealed a better separation with the higher pH conditions. Such an improvement in chromatography will help the analysis of complex samples by reducing the emphasis on the mass spectrometer, therefore, allowing more peptides to be sequenced and identified. The observations suggest that ZIC-HILIC at pH 6.8 is better than pH 3 for a more comprehensive analysis. As discussed previously, RP has one of the highest separation powers. A 2D system combining RP at different pH levels would also result in comprehensive results. However, in such a system,[19] a pH level as high as 10 needs to be used; under such conditions, there is a risk peptides such as those that are phosphorylated may start to degrade.

Apart from the identification of highly abundant proteins such as histones, tubulin, actin, and an almost complete set of ribosomal proteins and proteins involved in the citric acid cycle, also lower abundant proteins were identified. Those lower-expressed proteins include transcription factors and members of the SWI−SNF complex and the phosphatidyl inositol signaling pathway. The observation of these proteins indicate that the ZIC-HILIC-RP method presented here allows a reasonable dynamic range coverage. Functional annotation and categorization of more than 70% of the identified proteins was carried out with the online tool DAVID Bioinformatic Resources 2006 (Supporting Information Figure 1; http://david.abcc.ncifcrf.gov/).[44] As expected, most proteins identified are involved in DNA or protein binding, including histones and ribosomal proteins, which are the most abundant proteins in the nucleus. On the other hand, also a number of proteins with a more specialized or rare functionality are identified.

The complexity of biological samples in proteomics is further increased by the existence of post-translational modifications (PTMs) of proteins. Identification of these typically lower abundant modifications can be critical for the elucidation of cellular processes. Recently, the ability of SCX to isolate peptides with a specific net charge has been exploited for the enrichment of phosphorylated peptides, based on the fact that the net charge of a phosphorylated peptide is lower than its analogous nonphosphorylated peptide.[15] The nuclear fraction analysis provides a far larger dataset for elution trend analysis than that provided by the peptide standard mixture. Analysis of the effect of peptide net charge on retention time once again demonstrates the similarity of ZIC-HILIC at pH 3 to SCX (Figure 7). Although ZIC-HILIC separation resembles SCX separation,

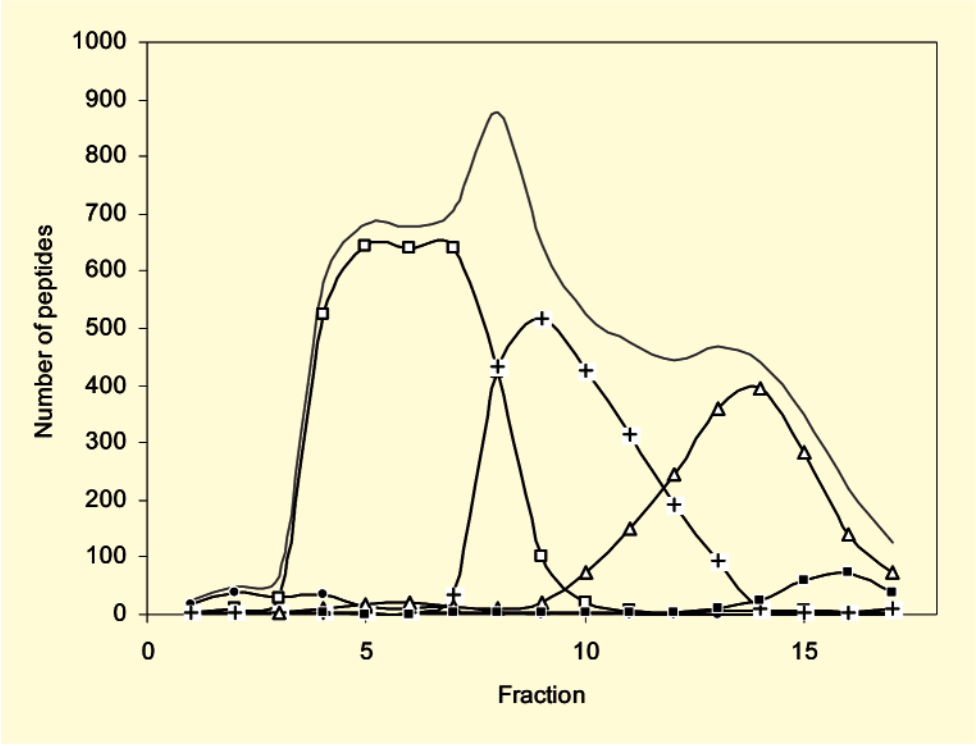

Figure 7. Distribution of peptides after ZIC-HILIC fractionation at pH 3 of MEL-cells nuclear extract digest. The number of peptides per fraction are plotted as indicated: (−) total, net charge (-●-) 1+ , (-□-) 2+, (-+-) 3+, (-△-) 4+, and (-■-) >4+.

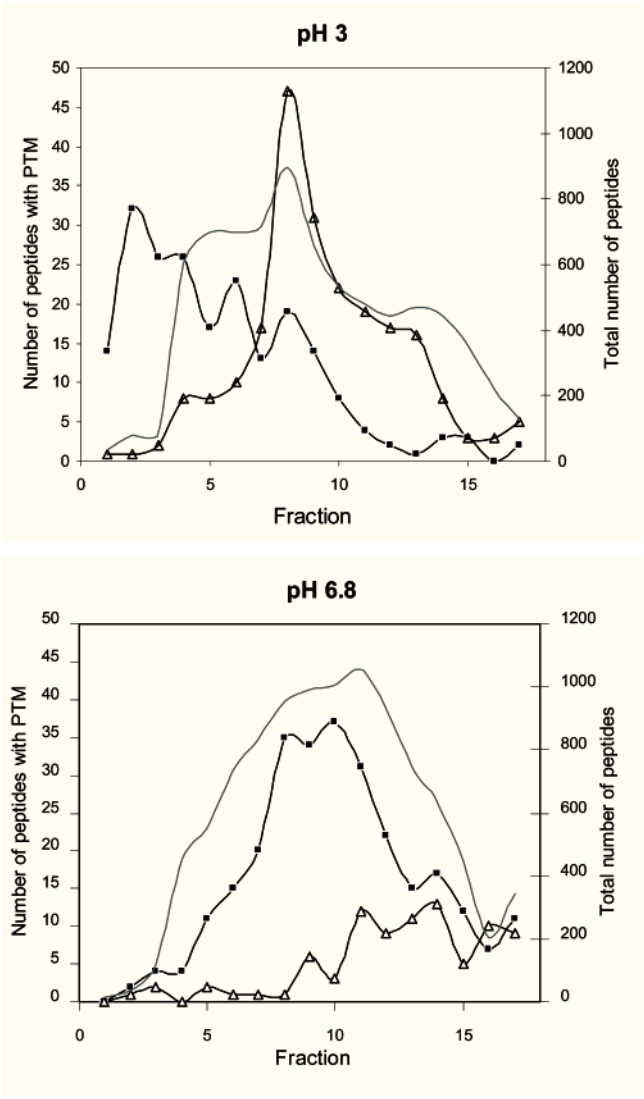

Figure 8. Distribution of phosphorylated and N-acetylated peptides over ZIC-HILIC fractions. First dimension: ZIC-HILIC, 200 μm × 160 mm, 3.5 μm, 200 Å. Flow rate 1.5 μL/min after splitting, 1 min fractions. Number of peptides: (−) total, (-■-) N-acetylated, and (-△-) phosphorylated.

no obvious enrichment of phosphorylated peptides was observed at either of the pH conditions (Figure 8), although it must be stated that phosphopeptides in our study represent only a few percent of the total peptide amount. The functional group of SCX material only contains a negative charge, repelling the similarly charged phospho-group. ZIC-HILIC, however, also contains a positive group that can attract the phospho-group. Moreover, the phospho-moiety increases the hydrophilicity of the peptide, enhancing the retention instead of decreasing it. Total phosphopeptide statistics need to be improved before firm conclusions can be made.

Another PTM that influences the charge of a peptide is N-acetylation. The positive charge of the N-terminus of a peptide is removed by the addition of an acetyl group, resulting in a reduction of both the hydrophilicity and the number of charges on the peptide. At pH 3, N-acetylated peptides tend to cluster in the first fractions of the ZIC-HILIC separation, indicating that enrichment can be performed. Sixty percent of the peptides in the first two fractions are N-acetylated peptides. At pH 6.8, such a fractional enrichment for N-acetylated peptides disappears. It is possible that deprotonated acidic residues could compensate for the loss of one charge by N-acetylation at this pH. The use of ZIC-HILIC appears more suitable for analysis of such N-terminal peptides than the use of SCX, since in ZIC-HILIC, N-acetylated peptides are more exclusively separated, while with SCX similar elution profiles for both phosphorylated and N-acetylated peptides are achieved.

The formation of N-terminal pyroglutamic acid is a peptide modification that is analogous to N-acetylation in that it also removes the N-terminal charge. As was expected, these modified peptides also show up in the first fractions of the ZIC-HILIC separation (Supporting Information Figure 2).

C-terminal peptides lack a basic residue and thus carry the same charge as an N-acetylated peptide. However, it seems that C-terminal peptides are not enriched in the very first fractions with, admittedly, a number of identified C-terminal peptides that is too low for reliable statistics (Supporting Information Figure 2). One can hypothesize that the loss of a positive charge in the case of N-terminal acetylation is different from the lack of one positive charge of a tryptic C-terminal peptide and that the charge distribution on a C-terminal peptide makes it more polar than an N-acetylated peptide.

Discussion

Our results obtained for the protein standard mixture and the cellular nuclear lysate reveal that ZIC-HILIC separation shows a mixed-mode effect of both polar and electrostatic interactions. An obvious indicator that electrostatic interactions were playing a role was the need to remove all salts before analysis; otherwise, little retention was observed. A similar mixed-mode effect was reported before, with the use of the SCX material polysulfoethyl A at high (>50%) ACN conditions, where elution was achieved using a sodium perchlorate gradient.[45] Separation with this material was only based on hydrophilicity and positive charges, but was believed to rival RP for the separation of peptides. Furthermore, a setup that combines both a weak anion exchanger and weak cation exchanger was used for separation at high ACN (>50%) conditions. Such a system required a combined aqueous and pH gradient for elution.[46] Here, we report a system that shows a mixed-mode HILIC/ion exchange separation with an aqueous gradient which is sufficient for elution with a superior separation to SCX using a salt gradient.

The use of SCX in 2D-LC systems has been shown to be useful for the separation of complex peptide mixtures.[9,11,14] However, the separation power of SCX is not optimal, and unwanted clustering of the most prevalent peptides (net charge +2 and +3) is observed.[19] The peak capacity of HILIC is shown to be higher than that of SCX,[20] and we demonstrated here that the +2 and +3 charged peptides elute over a wider time window due to the mixed-mode separation of ZIC-HILIC at pH 3. With ZIC-HILIC at pH 6.8 or 8, this effect is nearly abolished, whereby chromatographic resolution has improved. All this increases the separation power of the 2D-LC setup and decreases the eventual coelution into the mass spectrometer, allowing more comprehensive data acquisition.

The separation on a ZIC-HILIC column shows dependency on the pH of the buffer, caused by changes in peptide composition rather than chromatographic material. The effect of acidic residues underlying the improvement in separation, has been previously noted.[47] A sudden decrease in retention was observed when the pH of the buffer dropped below the

pK_a of the acid, an effect that was also observed with different HILIC materials, including ZIC-HILIC. We also observed that when using ZIC-HILIC the basic residues appear to have a larger effect on retention time than that observed for acidic residues, possibly due to the fact that the sulfonic group on ZIC-HILIC is a distal charged moiety, giving the material a low negative excess charge.[24] It is possible that reversing the positioning of the acidic and basic components on the silica particles can further enhance the acidic-influenced separation of tryptic peptides at pH 8.

Conclusions

Although the zwitterionic charge of ZIC-HILIC is pH-independent, the ability to separate peptides is influenced by buffer pH. The primary reason for pH influence is the different peptide net charge distributions at different pH levels. The change in peptide net charge not only changes the interaction of the peptide with the chromatographic surface but also has the knock-on effect of producing a different separation. At higher pH conditions (pH 6.8 and 8), separation power is highest; at pH 3, orthogonality with RP is best. The off-line hyphenation of ZIC-HILIC with RP showed successful in the analysis of the nuclear proteome. Although the most comprehensive analysis is achieved at pH 6.8 in the first dimension, separation at pH 3 can be used for the enrichment of certain PTMs. N-acetylated peptides clustered in the fist few fractions of ZIC-HILIC. We can hypothesize that enrichment for other PTMs can be achieved by ZIC-HILIC. Peptides that are formylated, carboxylated, hydroxylated (all N-terminal charge removed), palmitoylated, myrisotylated (N-terminal charge removed and hydrophobic group attached), and glycosylated (hydrophobic group attached) will all show up in the first fractions of a ZIC-HILIC run at pH 3.

Although ZIC-HILIC, unlike SCX, cannot be coupled directly with RP, it shows better peak capacity, and no unwanted clustering of most prevalent peptides occurrs. Consequently, ZIC-HILIC-RP should allow more comprehensive data acquisition from complex peptide mixtures.

Therefore, we conclude that the presented ZIC-HILIC-RP LC−MS setup is a useful alternative for 2D-LC in proteomics, with separation that, to some extent, can be tailored to the research question. Most comprehensive results can be obtained by using ZIC-HILIC at pH 6.8, but pH 3 can be used when the interest is PTMs.

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Supporting Information Available: A table listing peptides identified in one-dimensional ZIC-HILIC-MS including retention times at different pH conditions, a pie chart displaying the functional annotation of proteins identified in the MEL cells nuclear lysate, and a figure displaying the distribution of C-terminal and N-terminal pyroglutamic peptides over ZIC-HILIC fractions at pH 3. This material is available free of charge via the Internet at http://pubs.acs.org. MS/MS spectra are available as a Scaffold-file at https://bioinformatics.chem.uu.nl/supplementary/boersema_jpr. This file includes protein and peptide scoring and information on PTMs. The Scaffold viewer is also available for download.

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