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Mixed-mode chromatography for fractionation of peptides, phosphopeptides, and sialylated glycopeptides

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Abstract

A mixed-mode chromatographic (MMC) sorbent was prepared by functionalizing the silica sorbent with a pentafluorophenyl (PFP) ligand. The resulting stationary phase provided a reversed-phase (RP) retention mode along with a relatively mild strong cation-exchange (SCX) retention interaction. While the mechanism of interaction is not entirely clear, it is believed that the silanols in the vicinity of the perfluorinated ligand act as strongly acidic sites. The 2.1 mm × 150 mm column packed with such sorbent was applied to the separation of peptides. Linear RP gradients in combination with salt steps were used for pseudo two-dimensional (2D) separation and fractionation of tryptic peptides. An alternative approach of using linear cation-exchange gradients combined with RP step gradients was also investigated. Besides the attractive forces, the ionic repulsion contributed to the retention mechanism. The analytes with strong negatively charged sites (phosphorylated peptides, sialylated glycopeptides) eluted in significantly different patterns than generic tryptic peptides. This retention mechanism was employed for the isolation of phosphopeptides or sialylated glycopeptides from non-functionalized peptide mixtures. The mixed-mode column was utilized in conjunction with a phosphopeptide enrichment solid phase extraction (SPE) device packed with metal oxide affinity chromatography (MOAC) sorbent. The combination of MOAC and mixed-mode chromatography (MMC) provided for an enhanced extraction selectivity of phosphopeptides and sialylated glycopeptides peptides from complex samples, such as yeast and human serum tryptic digests.

Keywords: Mixed-mode; Two-dimensional; Cation-exchange; Reversed-phase; Phosphopeptides; Sialylated glycopeptides

1. Introduction

Mixed-mode chromatography (MMC) is a promising tool for the separation and analysis of variety of compounds. The driving force behind the development of mixed-mode stationary phases is to achieve an alternative separation selectivity compared to conventional chromatographic modes. Because two or more separation principles are utilized in conjunction, the method development may be more complicated and less understood.

The impact of mixed-mode liquid chromatography was recognized earlier during the development of reversed-phase (RP) stationary phases [1]. Incompletely capped silanol groups exhibited an ion-exchange activity, causing peak tailing and retention shift for basic compounds [2,3]. Although generally unwanted, the silanol interaction has, in selected cases, a positive impact on the peak resolution [4].

More recently, Zhu et al. [5,6] have utilized ion-exchange columns for the mixed-mode separation of peptides. When using a significant amount of organic modifier as a mobile phase additive, both strong cation-exchange (SCX) and hydrophilic interaction chromatography (HILIC) modes were simultaneously contributing to the peptide retention and overall separation selectivity. This method was applied to the separation of cyclic and helical peptides and offered a complementary selectivity to RPLC [7,8].

Mixed-mode solid phase extraction (SPE) devices were commercialized at the end of the twentieth century [9,10] and are used successfully for selective extractions of pharmaceutical compounds from complex matrixes. The combination of retention modes improved the extraction selectivity [11,12] and allowed for the reduction of the so-called matrix effect on electrospray ionization used in LC–mass spectrometry analysis [13].

Several new mixed-mode chromatographic (MMC) sorbents have been commercialized by SIELC Inc. under the Primesep trade name. The column choices include combinations of RP with cation, anion, and zwitter ion functionalities. The alterna-

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tive selectivity of these columns has been successfully utilized in two-dimensional (2D) chromatography applications [14].

MMC separation can in principle be realized by combining two different sorbents in a single column [15] or connecting separately packed columns in a series, as described by Strege et al. [16]. A similar approach with a biphasic column has recently been utilized for the separation of a complex peptide mixture for proteomic analysis [17,18]. However, the definition of mixed-mode is blurred here, since the dual-phase SCX-RP sorbent packed columns were utilized for 2D LC separations. Essentially, the authors used salt step gradients of various elution strengths to sequentially displace the peptides from the SCX sorbents packed in the first portion of a column. The peptides were eluted from the second (RP) section of the column prior to application of another salt step gradient. In this case, the classification of the experiment as 2D LC seems to be more fitting [19].

Nogueira et al. described a novel stationary phase combining RP and weak anion-exchange (WAX) interactions [20,21]. The column was utilized for purification of peptides. The resolution was enhanced compared to RP columns as long as the synthetic impurities and the purified compound had different net charges. The combination of attractive and repulsive forces permitted resolution of the target peptide from impurities of closely related structures.

Bell et al. [22,23] have recently described a pentafluorophenyl (PFP) RP sorbent offering a significant ion-exchange interaction with basic solutes at neutral and moderately acidic mobile phase pH. The ion-exchange properties of the sorbent were attributed to ionized surface silanols. No such interaction was detected for sorbents with C_{18} ligands immobilized on the same base silica with similar surface coverage [23].

During the preparation of this manuscript a report was published describing MMC, more specifically the combination of HILIC and ion-exchange modes for the separation of peptides and phosphopeptides, among other analytes [24]. Both attractive and electrostatic forces were utilized to selectively improve or reduce the retention of analytes. The selective isolation of phosphopeptides was proposed.

In this paper we describe an SCX-RP mixed-mode separation of peptides carried out on a silica-based PFP MMC column. Various modes of operation were evaluated, including pseudo 2D LC elution of the peptides. Selective isolation of classes of peptides with negative charge, such as phosphopeptides and sialylated glycopeptides was achieved in the MMC separation mode. A combination of metal oxide affinity chromatography (MOAC) SPE and MMC was utilized for a highly selective extraction of phosphopeptides and sialylated glycopeptides from complex biologic samples.

2. Experimental

2.1. Materials and reagents

Formic acid (FA), and ammonium formate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium hydroxide and HPLC grade acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA). A Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare deionized water (18 MΩ cm) for HPLC mobile phases. RapiGest™ SF, an acid labile surfactant, was obtained from Waters (Milford, MA, USA). A synthetic MassPREP™ phosphopeptide mixture standards, the MassPREP™ nine peptide standard, and protein tryptic digestion MassPREP™ standards of enolase, ADH, phosphorylase b, hemoglobin, BSA, were from Waters (Milford, MA, USA). The MOAC SPE 96-well micro-elution plate (also under the trade name of MassPREP™) was used to extract phosphorylated and sialylated peptides from protein tryptic digests. Other chemicals and reagents were purchased from Sigma, unless specified otherwise.

2.2. HPLC instrumentation, columns, and conditions

Chromatographic experiments were carried out using a 2796 Alliance Bio HPLC system equipped with a 2996 photodiode array detector, and a single quadrupole mass detector (ZQ™, Waters). All experiments were performed using 2.1 mm × 150 mm column packed in house with 5 μm PFP silica-based sorbent. The sorbent was prepared in house by modifying silica in two steps with pentafluorophenylpropyltrichlorosilane and trimethylchlorosilane, respectively. The starting silica sorbent had a surface area of 341 m^2/g, the PFP ligand surface concentration was 3.33 μmole/m^2. The mobile phases used for general peptide separations were (A) water, (B) acetonitrile, and (C) 100 mM ammonium formate, pH 3.25. The mobile phases used for the separation of phosphopeptides and sialylated glycopeptides were (A) 0.1% FA, pH 2.6, (B) acetonitrile with 0.08% FA, and (C) 100 mM ammonium formate, pH 3.25. The ammonium formate buffer was prepared as follows: 1.58 g of ammonium formate salt was dissolved in 245 g of water, adjusted with concentrated FA (approximately 2.8 mL) to pH 3.25, and the final volume was adjusted to 250 mL.

Nano-LC analysis of tryptic peptides was performed with a Waters nanoACQUITY™ UPLC system equipped with a Waters NanoEase™ Atlantis™ C_{18}, 75 μm × 15 cm column. The separation temperature was 35 °C. The aqueous mobile phase (mobile phase A) contained 0.1% FA and the organic mobile phase (mobile phase B) contained 0.1% FA in acetonitrile. Peptides were eluted from the column with a gradient of 0–50% mobile phase B over 30 min at 300 nL/min flow rate, followed by a 5 min rinse with 80% of mobile phase B. The column was immediately re-equilibrated at initial conditions (0% B for 20 min).

2.3. Tryptic digestions of human serum, yeast, and bovine alpha-casein

Approximately 60 μL of human serum (~80 mg/mL protein concentration, based on biochemical assay), was denatured with 0.1% RapiGest detergent, and reduced and alkylated with DTT/iodoacetamide [25]. Overnight digestion was performed using Promega trypsin (50:1, w:w, proteins:trypsin) at 37 °C. The digested sample was acidified with TFA (0.5%, v/v) to

hydrolyze the RapiGest SF surfactant [25] and centrifuged prior to MOAC extraction of sialylated glycopeptides.

Similarly, 2.4 mg of soluble yeast proteins (Sigma) were denatured, reduced, and alkylated followed with tryptic digestion. The phosphopeptides from the yeast protein digests were enriched using the MOAC SPE procedure specified in the next chapter.

A single protein, bovine alpha-casein was prepared as 2 mg/mL in 50 mM ammonium bicarbonate (500 μL, pH 7.8). Trypsin was added to the alpha-casein solution in a 50:1 ratio (w:w). The sample was incubated overnight at 37 °C. Alpha-casein protein contains multiple phosphorylated sites that are well known [26,27]. Therefore, the alpha-casein is a suitable sample for phosphopeptide separation and enrichment method validation.

2.4. SPE of phosphopeptides and sialylated glycopeptides

The alpha-casein tryptic digest spiked with 4 synthetic phosphopeptide standards was purified using MOAC SPE. Either a 96-well micro-extraction SPE plate or 1cc SPE cartridges were used to process the samples. The 96-well plate was operated using a vacuum manifold, each well packed with 2.5 mg of MOAC sorbent. The well was conditioned with 200 μL of water and equilibrated with 200 μL of 0.5% TFA in 80% acetonitrile in water. The sample was solubilized in the same solution and loaded into a well. The well was washed with 200 μL of 0.5% TFA in 80% acetonitrile and washed again with 200 μL of water. Elution was carried out with 100 μL of 2% triethylamine solution in water; the pH was approximately 11. The eluent was neutralized with FA and dried down.

Enrichment of phosphopeptides from yeast was performed using a 1cc SPE cartridge packed in house with 50 mg of MOAC sorbent (the same as in the MassPREP phosphopeptide enrichment kit). The cartridge was washed twice with 1 mL of deionized water, and equilibrated twice with 1 mL of 0.5% TFA in 80% acetonitrile in water. The sample (∼2.4 mg of protein digest) was dissolved in 0.5 mL of 0.5% TFA, 80% acetonitrile containing 50 mg/mL of enhancer (supplied in the MassPREP phosphopeptide enrichment kit) and slowly loaded onto a cartridge. The cartridge was washed with 0.5 mL of 0.5% TFA in 80% acetonitrile twice, followed with 0.5 mL of deionized water. Phosphopeptides were eluted with 1 mL of 2% triethylamine solution in water, pH ∼11. The eluent was neutralized with FA and lyophilized.

Extraction of the sialylated glycopeptides from human serum was similar to that of the yeast phosphopeptide extraction. The total sample loaded was 2.4 mg per well.

3. Results and discussion

3.1. MMC on PFP column

PFP-modified silica sorbents have been described in the literature as an alternative RP stationary phase for the separation of small molecules and biopolymers. There is a distinguishable difference in selectivity of phenyl, PFP and alkyl silica stationary phases, although the precise rationale is not known [28,29]. It is expected that PFP has a different selectivity compared to alkyl-based RP sorbents. Therefore, we have included a PFP column in the earlier published study, whose goal was to identify sorbents with orthogonal selectivity useful for the 2D LC separation of peptides [30]. Indeed, PFP offered moderately dissimilar selectivity compared to a C_{18} sorbent. During this study we identified a surprising retention pattern of tryptic peptides on the PFP column. Specifically, a 10–20 mM background concentration of salts in the RP gradient was necessary to successfully elute the peptides from the silica PFP sorbent. We speculated that the PFP sorbent provides some cation-exchange interaction with peptides; the cation-exchange sites appeared to be active even at pH 2.6.

This observation was further clarified by the following experiment. A 2.1 mm × 150 mm column packed with 5 μm silica PFP sorbent was utilized for the separation of tryptic digests of selected proteins. The acetonitrile content was kept constant (50%) throughout the analysis with the goal to eliminate the RP interaction of peptides with the stationary phase. A gradient of 1–40 mM ammonium formate buffer, pH 3.25 in 39 min (1 mM/min) was used to elute the adsorbed peptides. Fig. 1 shows the separation of the phosphorylase B digest; the eluting peptides were detected with a single-quadrupole mass detector. An additional four samples, enolase, hemoglobin, bovine serum albumin (BSA), and alkaline dehydrogenase (ADH) protein digests were analyzed in a similar fashion. The retention times of the peptides were recorded.

Fig. 2 compares the retention times of 196 reliably identified peptides with the retention data obtained earlier [30,31] on a PolySULFOETHYL SCX column (PolyLC, Columbia, MD, USA). The correlation coefficient $R^2 = 0.659$ suggests that the retention relies on similar principles. For comparison, the correlation between peptide retention times on PolySULFOETHYL columns packed with 200 and 300 Å SCX sorbents was $R^2 = 0.876$. The PFP ligand is known to be a strong Lewis acid [28]. However, we do not believe the Lewis acidity is responsible for the cation-exchange properties of the silica PFP sorbent. In fact, from three commercially available PFP columns (Fluorophase PFP, Thermo Scientific, Waltham, MA, USA; Pursuit PFP, Varian, Palo Alto, CA, USA; and Curosil PFP, Phenomenex, Torrance, CA, USA) only the Fluorophase PFP offered a minor SCX-like activity. During the writing of this manuscript we have found two reports describing an ion-

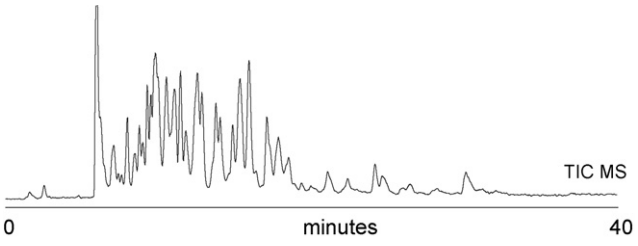

Fig. 1. Separation of phosphorylase B tryptic digest on the mixed-mode silica PFP column. The acetonitrile content in the mobile phase was 50%. Peptides were eluted with 1–40 mM ammonium formate gradient, pH 3.25, in 39 min.

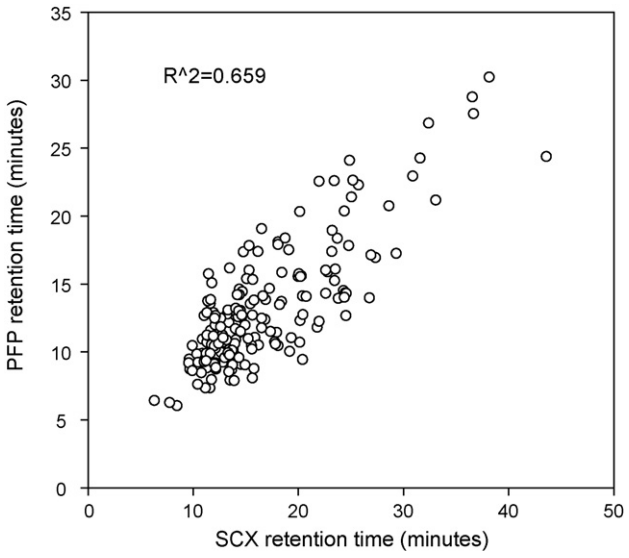

Fig. 2. Peptide retention correlation between SCX-RP mixed-mode silica PFP column and polySULFOETHYL aspartamide SCX column. The PFP column was operated as described in the caption of Fig. 1. Peptides were eluted from the 50 mm × 4.6 mm polySULFOETHYL SCX column using a 1.5 mM NaCl gradient; the mobile phase contained 5% acetonitrile. For details see reference [31].

exchange secondary interaction for the silica-based Discovery HS F5 column (Sigma–Aldrich, St. Louis, MO, USA) [22,23].

The titration of in house prepared silica PFP sorbent revealed only 0.05 meq/g ion-exchange capacity. This is consistent with our experimental data where a relatively low concentration of buffer was needed for a peptide to elute from the column. Therefore, we will refer to the in house prepared PFP sorbent from this point on as MMC PFP. As the most electronegative element, the fluorine is known to have a pronounced impact on the acidity (pK_a) of the nearby functional groups [32]. This is the case even for non-covalent structures via inter- and intra-molecular interactions, such as hydrogen bonds [33]. While it is possible to speculate about the increased silanol acidity in the proximity of the PFP ligand [32,34], this hypothesis does not completely explain why the SCX retention mode was observed for the silica MMC PFP sorbent used in the presented work, but not for the other PFP columns tested. Arguably, the type of silica, the specific PFP ligand, and the bonding chemistry may play an important role. However, the details about the nature of sorbents and the type of endcapping reaction are typically not provided by the manufacturers. The lack of such data precludes a pertinent discussion.

One of the reviewers suggested that the uncapped silanols are unlikely to exist on the silica surface. Instead, rearrangement of the SiO_2 network may create pairs of peroxide linkages nearby non-bridging oxygen hole center, a defect that provides for a stable cation-exchange site [35]. The magnitude of the polarization may depend on the proximity of PFP group. The same rearrangement also produces positively charged centers. However, no anion-exchange properties of PFP sorbent were observed in our experiments.

Despite the limited understanding of the nature of the SCX interaction, both Figs. 1 and 2 support the same conclusion. The silica MMC PFP sorbent utilized here offers two retention mechanisms: RP as well as cation-exchange interactions. This conclusion is supported by earlier published reports observing mixed-mode interaction for Discovery HS F5 column [22,23].

3.2. 2D LC separation of peptides using MMC

The mixed-mode retention mechanism and its impact on the separation of peptides were further investigated using a pseudo 2D elution approach. Fig. 3 shows the analysis of a rather complex peptide sample consisting of an equimolar mixture of five

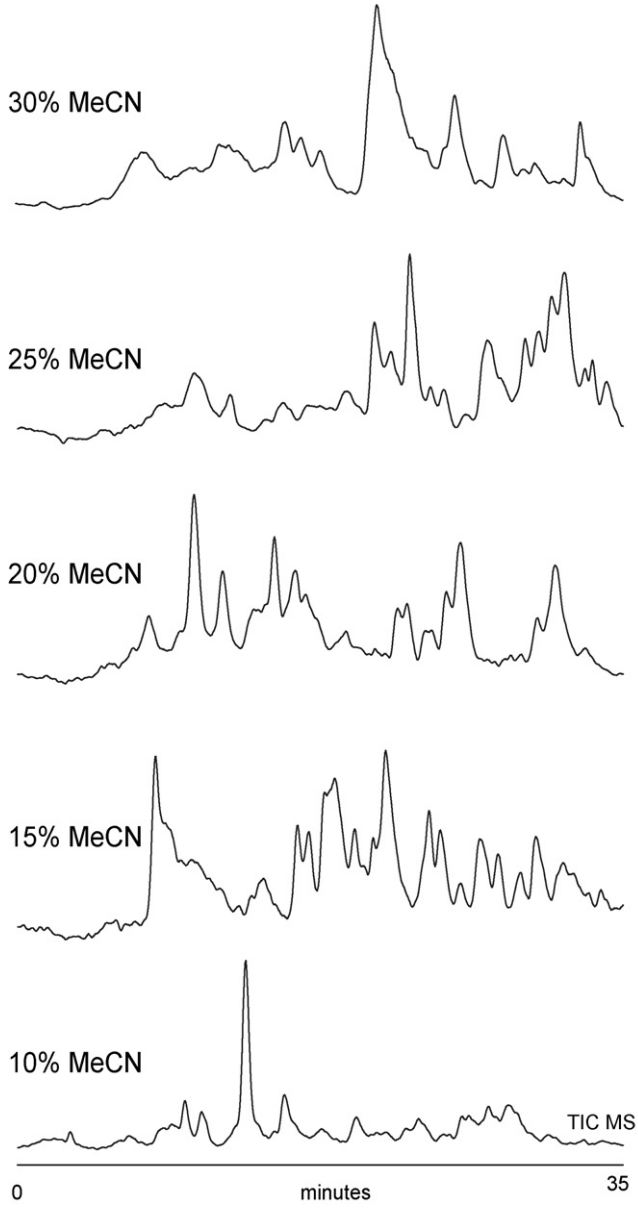

Fig. 3. Pseudo 2D separation of tryptic peptides using the mixed-mode silica PFP column. The sample was loaded and sequentially eluted with a series of ion-exchange gradients (1–25 mM in 25 min followed by 25–50 mM between 25 and 30 min, ammonium formate, pH 3.25). The gradient was executed with a progressively greater content of acetonitrile in the mobile phase background. Both cation-exchange and RP separation interaction contributed to the peptide retention.

protein digests plus nine additional peptides (MassPREP standards). After the initial sample injection, the peptides were eluted with a series of SCX gradients, with stepwise changes in the acetonitrile background concentration. The SCX gradient was as follows: 1–25 mM in 25 min followed by 25–50 mM between 25 and 30 min, ammonium formate, pH 3.25. Subsequent SCX gradients had a greater percentage of acetonitrile than the previous ones, which promoted an elution of more hydrophobic peptides.

Fig. 3 illustrates the separation of a complex peptide mixture in a pseudo 2D approach; both SCX and RP retention mechanisms of MMC PFP sorbent were utilized. The uniform SCX gradients were sequentially performed with 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, and 60% acetonitrile content in the mobile phase. Only the selected chromatograms are shown in Fig. 3. After each SCX gradient, the column was re-equilibrated with 1 mM ammonium formate buffer. An acetonitrile step gradient was executed at the beginning of the subsequent linear ion-exchange gradient. In this scenario the majority of peptides were exposed to multiple SCX gradient cycles, before their elution. As a consequence, a certain degree of peak broadening can be observed in Fig. 3.

An alternative elution scheme was evaluated, using a repetitive linear RP gradient with a fixed concentration of ammonium formate in the mobile phase background. Fig. 4 illustrates the scenario of five consecutive RP gradients where the ammonium formate buffer background was raised in steps from 1, 5, 10, 20, to 40 mM; pH 3.25. The gradient table for this pseudo 2D LC experiment is presented in Table 1.

Both of the described pseudo 2D LC approaches (Figs. 3 and 4) are potentially suitable for the fractionation of complex peptide samples. Peptides eluted as distinct peaks with little or no overlap in between fractions. Fig. 4 illustrates this fact on extracted ion chromatograms of selected peaks. As expected, the peptides with lower charge eluted prior to the more charged ones. This is consistent with the SCX retention order (peptide net charge is assumed to be equal to the sum of the number of arginines, lysines, and histidines in the sequence, plus one charge contributed by the peptide NH_2-terminus). Peptide retention times in Fig. 4 are provided as a supplementary table.

Table 1
Gradient conditions for mixed-mode 2D LC experiment

Minutes	A%	B%	C%	Gradient shape[a]
0.00	99.0	0.0	1.0	
25.00	49.0	50.0	1.0	6
25.50	99.0	0.0	1.0	6
30.00	95.0	0.0	5.0	11
80.00	45.0	50.0	5.0	6
80.50	95.0	0.0	5.0	6
85.00	90.0	0.0	10.0	11
135.00	40.0	50.0	10.0	6
135.50	90.0	0.0	10.0	6
140.00	80.0	0.0	20.0	11
190.00	30.0	50.0	20.0	6
190.50	80.0	0.0	20.0	6
195.00	60.0	0.0	40.0	11
233.00	10.0	50.0	40.0	6
243.00	0.0	60.0	40.0	6

Mobile phase, A: 0.1% FA in water; B: 0.08% FA in acetonitrile; C: 100 mM ammonium formate, pH 3.25. Five consecutive gradients of 0–50% acetonitrile were performed with the 1, 5, 10, 20, and 40 mM ammonium formate background. For chromatogram see Fig. 4.

[a] The value 6 indicates a linear gradient, e.g. 0–50% linear gradient of acetonitrile from 0 to 25 min. Value 11 is a step gradient executed at the end of the interval, e.g. the composition of C changed stepwise from 1% to 5% at 30 min.

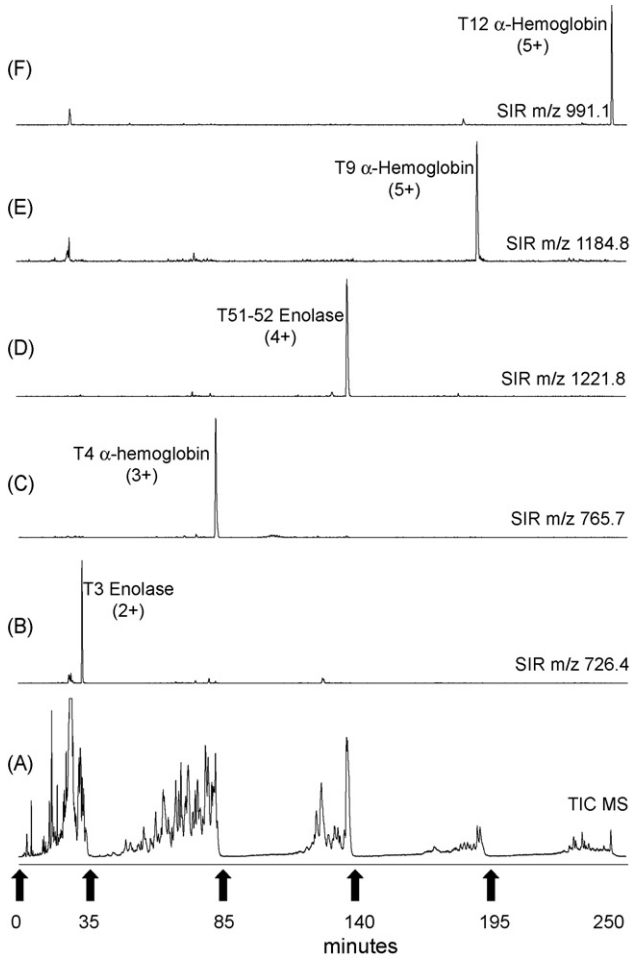

Fig. 4. Pseudo 2D separation of the five protein digestion standards using the mixed-mode silica PFP column. The sample was loaded and sequentially eluted with a series of RP gradients. Arrows indicate the beginning of each RP gradient, which coincides with the salt steps. Gradient conditions are listed in Table 1. RP gradients were executed with a progressively greater concentration of the ammonium formate buffer in the mobile phase (1, 5, 10, 20, and 40 mM ammonium formate background). Selected ion traces were extracted for one peptide in each gradient segment.

Although the peptide separations shown in Figs. 3 and 4 are intriguing, they may not be practical for proteomic analysis. First, a desirable salt background necessary for peptide elution causes ion-suppression in MS. Second, the cation-exchange binding capacity of the sorbent may not be sufficient for large sample loads. Third, some peak broadening is induced by repetitive RP gradients. However, the majority of these problems can be solved in an off-line fractionation mode. The collected fractions can be analyzed in nano-LC mode, provided that the

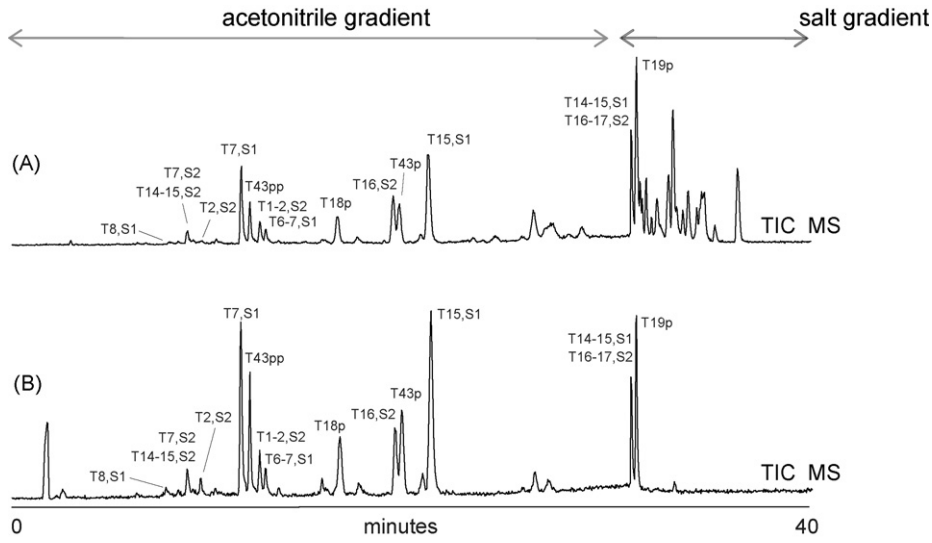

Fig. 5. Separation of phosphopeptides from tryptic peptides on a 2.1 mm × 150 mm MMC PFP column. (A) Bovine alpha-casein tryptic digest spiked with four additional synthetic phosphopeptides (see Table 2). (B) The mixed-mode LC separation of the same sample, except that the sample was processed using the MOAC SPE device to extract the phosphopeptides prior to LC. Phosphopeptides are labeled in the figure. For sequences and further information see Table 2. Gradient conditions were as follows: RP gradient from 0% to 50% acetonitrile in 30 min; cation-exchange gradient 0–25 mM of ammonium formate, pH 3.25, from 30 to 45 min. The acetonitrile content was kept constant (50%) during the ion-exchange gradient. The flow rate was 0.2 mL/min, and the column temperature was 30 °C.

separation selectivity of mixed-mode is dissimilar compared to a C_{18} sorbent [30]. This will be discussed in Section 3.5.

3.3. MMC separation and fractionation of phosphopeptides

MMC typically utilizes the attractive forces and combines the retention of both retention modes [5,6]. For example, mixed-mode SCX-HILIC separation of peptides relies primarily on SCX with a secondary retention and selectivity contribution provided by HILIC interaction [7,8]. However, the repulsive forces such as ion-exclusion phenomena can also influence the retention behavior and selectivity, as recently reported by Nogueira et al. [20] and Alpert [24].

We have utilized the RP-SCX mixed-mode silica PFP sorbent for the separation of phosphorylated peptides, which carry a negatively charged phosphate group(s) at the mobile phase pH used for the separation. The phosphopeptides are expected to be weakly retained or not retained by SCX interaction due to the charge-to-charge repulsion [36–38]. However, they should be retained by the RP mechanism.

Fig. 5A shows the separation of the bovine alpha-casein tryptic digest spiked with four synthetic phosphopeptides. The primary amino acid sequence of the detected phosphopeptides is shown in Table 2. The analytes were eluted from the MMC PFP column using two consecutive gradient segments. First, a 30 min gradient of 0–50% acetonitrile was used to elute the analytes not

Table 2
Sequences of phosphorylated peptides used in the study

Peptide assignment[a]	Source	Sequence	Peptide mass	No. of phosphorylations	Peptide net charge[b]
T8, S1:59–79	α-Casein	QMEAEpSIpSpSpSEEIVPNpSVEQK	2719.91	5	−3
T7, S2:46–70	α-Casein	NANEEEYSIGpSpSpSEEpSAEVATEEVK	2617.90	4	−2
T14–15, S2:126–137	α-Casein	EQLpSTpSEENSKK	1538.59	2	+1
T2, S2:2–21	α-Casein	NTMEHVpSpSpSEESIIpSQETYK	2617.90	4	−1
T7, S1:43–58	α-Casein	DIGpSEpSTEDQAMEDIK	1926.68	2	0
T6, S1:37–42	α-Casein	VNELpSK	768.34	1	+1
T43pp	Synthetic	VNQIGTLpSEpSIK	1447.63	2	0
T1–2, S2:1–21	α-Casein	KNTMEHVpSpSpSEESIIpSQETYK	2745.99	4	0
T6–7, S1:37–58	α-Casein	VNELpSKDIGpSEpSTEDQAMEDIK	2677.02	3	0
T18p	Synthetic	NVPLpYK	812.38	1	1+
T16, S2:138–149	α-Casein	TVDMEpSTEVFTK	1465.60	1	1+
T43p	Synthetic	VNQIGpTLSESIK	1367.67	1	1+
T15, S1:106–119	α-Casein	VPQLEIVPNpSAEER	1659.79	1	1+
T14–15, S1:104–119	α-Casein	YKVPQLEIVPNpSAEER	1950.95	1	2+
T16–17, S2:138–150	α-Casein	TVDMEpSTEVFTKK	1593.70	1	2+
T19p	Synthetic	HLADLpSK	862.38	1	2+

Peptides are listed in the order of elution in Fig. 5.

[a] Tryptic peptides, S1 and S2 denote the α-casein sub-unit, with trypsin cleavage site. Synthetic peptides sequence is derived from the yeast enolase tryptic peptides.

[b] K, R, and H and amino terminus are assumed to be 1+ charged, phosphate groups are assumed to carry 1− charge at pH 3.25.

retained by cation-exchange interaction. The remaining peptides were eluted in a second segment with 0–25 mM linear gradient of ammonium formate (1.67 mM/min, pH 3.25) The acetonitrile concentration was maintained constant at 50%.

Fig. 5B shows a similar experiment for a sample containing only the phosphopeptides. Phosphopeptides were extracted from the sample as described in Section 2.4 using the phosphopeptide enrichment kit. The comparison of chromatograms in Fig. 5 reveals that the majority of phosphopeptides elute during a RP gradient, well resolved from non-phosphorylated tryptic peptides. The non-phosphorylated tryptic peptides were generally retained by the SCX mechanism and eluted later with the salt gradient (Fig. 5A).

Fig. 5 and Table 2 illustrate that the retention order of phosphopeptides loosely correlates with the number of phosphate moieties (the net peptide charge). The multiply phosphorylated peptides elute before monophosphorylated ones. One expects that multiply phosphorylated peptides with zero or net negative charge (Table 2) are not retained by the SCX mechanism. Nevertheless, they are still retained by the RP mechanism provided by the mixed-mode sorbent. Several singly phosphorylated peptides eluted during the SCX gradient segment. This is because their overall positive charge (2+) was comparable to tryptic peptides and encompasses SCX interaction.

Chromatograms shown in Fig. 5 suggest that the unique selectivity of RP-SCX MMC columns is suitable for the separation of phosphopeptides from non-phosphorylated ones. We propose that RP-SCX MMC can be used separately or in conjunction with other available phosphopeptide enrichment methods (for highly complex samples). The rationale for the multi-step sample preparation prior to nano-LC–MS/MS phosphopeptide sequencing is twofold. (i) No phosphopeptide enrichment method is absolutely selective. Non-phosphorylated peptides are known to be co-extracted with both immobilized metal affinity chromatography (IMAC) and MOAC SPE devices [27,39,40]. The MMC has the potential to further enhance the selectivity of the enrichment process. (ii) MMC can be used for peptide fractionation. Reducing the sample complexity improves the prospects for a successful LC–MS/MS peptide identification.

Soluble yeast proteins were digested with trypsin and enriched with MOAC SPE as described in Section 2.4. The extract was injected, separated and fractionated on the mixed-mode column; LC conditions were the same as in Fig. 5. The fractionation interval was 3 min; fractions were analyzed in nano-LC chromatography.

The LC–MS/MS peptide sequencing results are summarized in Table 3. We detected 501 peptides, from which 268 were phosphorylated with some redundancy between fractions. About 151 phosphoproteins were identified based on 198 unique phosphopeptides with a Mascot score greater than 25 (some proteins were identified by two or more phosphopeptides). The first two fractions did not contain peptides and were discarded. Fractions 12 through 14 contained significant numbers of non-phosphorylated peptides eluted from the MMC PFP column at the beginning of ion-exchange gradient. This observation is consistent with the data presented in Fig. 5A. For more information see supplementary data.

Table 3
Phosphopeptides identified in yeast using MOAC SPE enrichment followed by RP-SCX mixed-mode fractionation[a]

MMC PFP fraction	Phosphopeptides	Non-phosphopeptides
RP gradient segment		
3	12	13
4	29	9
5	43	4
6	33	1
7	31	1
8	24	2
9	19	0
10	11	1
11	16	1
SCX gradient segment		
12	52	55
13	17	107
14	4	39
Sum	268	501

[a] For RP-SCX LC conditions see Fig. 5 captions.

3.4. Utility of MMC for separation and fractionation of sialylated glycopeptides

We have attempted to extract and fractionate phosphopeptides from human serum tryptic digest using the method as described in the previous section. However, an overwhelming background of non-phosphorylated peptides was detected. The characteristic signature MS fragment ions *m/z* 292 and 657 observed at elevated MS collision chamber energy suggested that these are sialylated glycopeptides. It appears that the glycopeptides containing sialic acid residues ($pK_a \sim 2.6$) are effectively extracted by the MOAC SPE and further resolved with the silica PFP mixed-mode column. Although MOAC SPE was designed for the extraction of phosphopeptides, it appears to be highly effective for the enrichment of sialylated glycans and glycopeptides. This observation was recently corroborated by Larsen et al. [41].

The retention of sialylated peptides on a mixed-mode sorbent is assumed to be governed by similar principles as for phosphopeptides. This is due to the strong acidity of sialic or phosphoric acids. The combination of the repulsive forces of the negatively charged sialic acid moiety with a RP interaction is believed to contribute to the overall retention on the MMC PFP column.

Human serum digest was enriched by MOAC SPE (see Section 2.4) and fractionated on the MMC PFP column using the same LC conditions as in Fig. 5. The fraction collection interval was 3 min. The collected peptide fractions were enzymatically deglycosylated with PNGase F, which liberated the N-linked glycans while simultaneously converting the asparagine to aspartic acid (N → D, deamidation). The deamidation causes a peptide mass shift by +1 Da, which can be detected by a mass spectrometer.

Peptide fractions were analyzed by nano-LC–MS/MS with the goal to identify the primary sequence of the original sialylated glycopeptides, as well as the corresponding glycosylation site. The knowledge of the glycosylation motif NXS/T and the

Table 4
Sialylated glycopeptides enriched from human serum with MOAC SPE followed by RP-SCX mixed-mode fractionation[a]

MMC PFP fraction	Glycopeptides	Non-glycopeptides
RP gradient segment		
3	14	14
4	8	4
5	19	14
6	14	7
7	16	18
8	18	9
9	16	4
10	15	6
11	14	19
SCX gradient segment		
12	4	10
13	14	39
14	7	33
Sum	159	177

[a] For RP-SCX LC conditions see Fig. 5 captions.

expected (N → D) conversion simplified the *N*-glycosilated site assignment, and strengthen the validity of the peptide identification.

Table 4 lists the number of identified sialylated glycopeptides from human serum in fractions collected from the MMC separation; a total of 159 glycosylated and 177 non-glycosylated peptides were identified. Due to a redundancy (fraction overlap), overall 41 sialylated glycoproteins were identified by 63 unique peptides using a Mascot score cut-off at 25. A large number of non-glycosylated peptides were identified in fractions 13 and 14, being eluted with the salt gradient. This pattern is consistent with the RP-SCX mixed-mode retention selectivity as observed previously (Fig. 5A and Table 3, see also supplementary data).

3.5. Orthogonality of MMC PFP and C_{18} separations

Examples of nano-LC–MS analyzes of selected MMC PFP fractions are shown in Fig. 7. The glycopeptides in fraction 6 (see Fig. 6) as well as phosphopeptides in fraction 7 (the chromatogram of phosphopeptide MMC PFP isolation is not shown) were analyzed using a nano-LC column packed with C_{18} sorbent. A good resolution of peptides is achieved, which suggest that the separation selectivity of mixed-mode PFP and C_{18} columns is sufficiently different. Arguably, the separation in Fig. 7A may be in part achieved due to the fact that the peptides were deglycosylated prior to the nano-LC analysis. In other words, the peptides isolated with the PFP column are not identical to the ones analyzed in nano-LC. Nevertheless, this is not the case for the phosphopeptide analysis. The yeast phosphopeptide fractions isolated by the MMC PFP column were directly analyzed in the

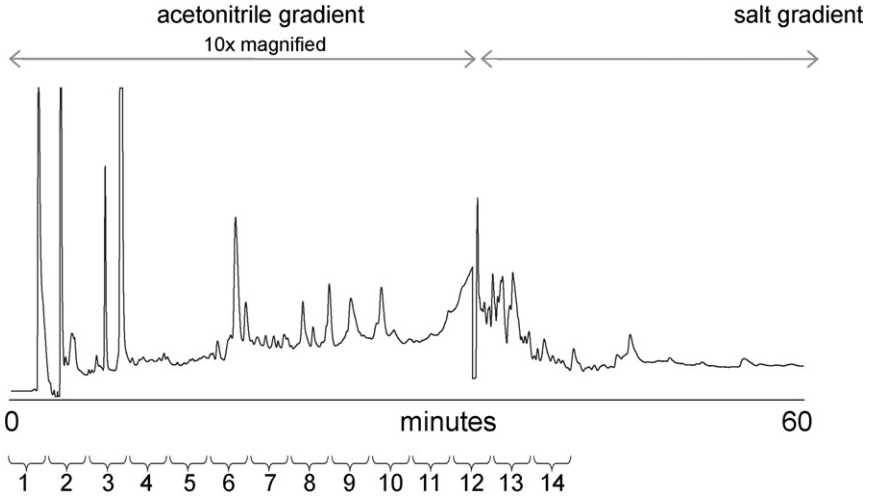

Fig. 6. Separation and fractionation of sialylated glycopeptides from human serum on the MMC PFP column. Gradient conditions are the same as in Fig. 5.

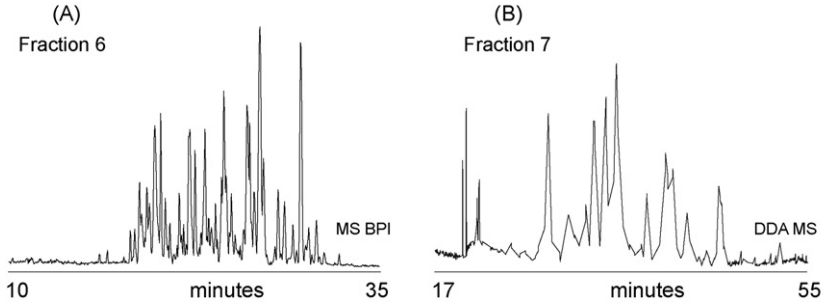

Fig. 7. Nano-LC–MS analysis of selected MMC PFP fractions. (A) peptides from fraction 3 shown in Fig. 6 and (B) fraction 6 of phosphopeptides (MMC PFP chromatogram is not shown).

nano-LC dimension. Fig. 7B shows the phosphopeptide resolution in the second (C_{18}) separation dimension. The overall peak shape is affected by the low sampling rate of the data-dependent analysis (DDA) MS/MS experiment; however, the dissimilar selectivity between MMC PFP and C_{18} columns is evident. All MMC PFP phosphopeptide fractions were sufficiently resolved in the nano-LC dimension; the achieved selectivity was comparable to the scenario shown in Fig. 7B.

These observations suggest that the selectivity of the mixed-mode PFP column and conventional RP sorbents is sufficiently dissimilar. The combination of MMC and RP chromatographic columns is a suitable option for 2D-LC [14].

4. Conclusions

The silica-based PFP column was found to provide for MMC interactions with peptides. Both cation-exchange and RP mechanisms appear to contribute to the retention. The pseudo 2D separation of peptides was demonstrated using either a linear RP gradient in conjunction with an ion-exchange step gradient or vice versa. In addition, the mixed-mode column was utilized for the separation of peptides with strong negative moieties, such as phosphopeptides and sialylated glycopeptides. Surprisingly, the peptides with net negative charges, presumably repulsed from SCX sorption sites, were still retained by in RP mode and could be selectively isolated from remaining tryptic peptides with typical 2+ charge or greater. The method for the selective purification and fractionation of phosphopeptides and sialylated glycopeptides was applied to analyze complex samples. The combination of MOAC SPE enrichment and MMC provided for improved selectivity for extraction of targeted peptides. We believe that RP-SCX MMC is applicable also for the enrichment and isolation of sulfated peptides and other types of molecules differing in charge.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2008.01.061.

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