Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography A, 1191 (2008) 162–170
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

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

Martin Gilar*, Ying-Qing Yu, Joomi Ahn, Jennifer Fournier, John C. Gebler

Waters Corporation, 34 Maple Street, Milford, MA 01757, USA

Available online 31 January 2008

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-

* Corresponding author. Tel.: +1 508 482 2000; fax: +1 508 482 3625.
E-mail address: Martin_Gilar@waters.com (M. Gilar).

0021-9673/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2008.01.061

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