Journal of Chromatography A, 1218 (2011) 1822–1827

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Retention mechanism divergence of a mixed mode stationary phase for high performance liquid chromatography

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ARTICLE INFO

Article history:
Received 10 November 2010
Received in revised form 20 January 2011
Accepted 25 January 2011
Available online 4 March 2011

Keywords:
2D-HPLC
Mixed mode stationary phase
β-Lactoglobulin digest
Mobile phase pH
Scherzo SM-C18

ABSTRACT

Mixed mode stationary phases utilize secondary retention mechanisms to add a dimensionality to the surface of high performance liquid chromatography (HPLC) adsorbents. This approach was used by several authors to improve the separation performance of single dimension separations. We explored the magnitude of these secondary interactions by performing an off-line two-dimensional (2D)-HPLC separation with a Scherzo SM-C18 column of a β-lactoglobulin tryptic digest with a mobile phase pH of 7 in the first dimension and 2 in the second. Mechanism divergence was determined using the peak capacity and a geometric approach to factor analysis, to measure the correlation. This separation was repeated with a C18 stationary phase as a control. It was found that the C18 column had a correlation coefficient of 0.784, smaller than the mixed mode column, 0.884. This indicated that the retention mechanisms of the C18 column were more divergent under these two pH environments than the mixed mode column. However, the SM-C18 still provided alternative selectivity of the peptides to that of the C18 and could be considered as a good alternative for further 2D-HPLC separations.

1. Introduction

For a successful two-dimensional high performance liquid chromatography (2D-HPLC) separation, two things are required: (1) a high peak capacity in both dimensions, capable of separating the sample mix; and (2) a sufficiently large divergence between the retention mechanisms so that the maximum amount of 2D separation space could be utilized [1]. Peptides have a sample dimensionality that makes them ideal for studying separations by 2D-HPLC [2]. These compounds have a charge that is governed by the number of amino acids, so they can be separated on the basis of their charge, i.e. by a strong cation exchange mechanism, and by their hydrophobicity, with a C18 stationary phase [3].

Mixed mode columns have a surface modification that allows for multiple retention processes to occur simultaneously. These are typically used in one dimension separations where the secondary selectivity provides enough retention to separate similar compounds that would normally co-elute [4–6].

The aim of this paper was to investigate the mechanism divergence of the Scherzo SM-C18 column, which is packed with C18 bonded particles, the surface of which reportedly works via both anion and cation exchange mechanisms but can also provide separations by normal and reversed phase modes. The extent of the anionic and cationic interactions was gauged by performing the separations of peptides at different mobile phase pH. The retention mechanism is affected by the mobile phase composition. For example Gilar et al. [7] performed a 2D-HPLC separation of a peptide digest with a C18 bridged-ethyl hybrid stationary phase, using different mobile phase pH in each dimension. They could achieve a certain degree of orthogonality. By comparing the 2D-HPLC profile obtained against that of a separation made on a standard C18 stationary phase, under the same separation conditions, the correlation coefficients [8] were used to gauge the influence of the secondary retention mechanisms. The results are similar to those Mnatsakanyan et al. [9] whereby the optimization of the second dimension of a 2D-HPLC separation was completed by evaluating the differences in separation performance with those provided by a constant C18 stationary phase in the first dimension. It is expected that if the cation and the anion exchange modes of the single column are significantly different, there will be less correlation between these dimensions than for the C18 phase.

2. Theory

2.1. Peak capacity

The peak capacity of a one dimension gradient is defined by how many peaks of a given width can fit side-by-side within a retention

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doi:10.1016/j.chroma.2011.01.078

window, according to the following equation [10]:

$$n_c = 1 + \frac{\sqrt{N}}{4}\frac{S\Delta\varphi}{1 + S\Delta\varphi t_0/t_G} \quad (1)$$

where the peak capacity, n_c, in the gradient elution mode is influenced by the gradient time, t_G, the range of the mobile phase composition, φ during the gradient, the slope, S, and the hold up time, t_0.

The number of theoretical plates of a column has a large influence on n_c. Modern HPLC column technology provides columns packed with core-shell particles that are highly efficient and are capable of producing a large number of theoretical plates [11]. However, even with such columns, the peak capacity is insufficient to adequately separate samples of complex mixtures in a reasonable amount of time.

The peak capacity of 2D-HPLC is much greater than that of traditional HPLC because, theoretically, the 2D-HPLC peak capacity is the product of the two dimensions peak capacities (i.e. ${}^2n_c \approx {}^1n_{c,1} \times {}^1n_{c,2}$). However, the maximum potential peak capacity can only be achieved when the two dimensions are orthogonal, that is, when the first dimension separates the mixture via a different mechanism than the one acting in the second dimension. For an extensive analysis of 2D-HPLC peak capacity, see References [12,13].

2.2. Geometric approach to factor analysis

A geometric approach to factor analysis (GAFA) yields information that is visually simple to interpret and for which calculations can be easily automated with minimal programming knowledge [14]. GAFA is commonly used to examine variations within data sets, in the context of 2D-HPLC. Liu et al. [8] used a geometric approach to factor analysis to assess orthogonality and to estimate peak capacity. Correlation matrices can be constructed from the scaled retention times of solutes in each dimension. This permits a practical visualization of the peak capacity.

The correlation matrix (C) is calculated according to Eq. (2):

$$C = \left(\frac{1}{N-1}\right) M'^T M' \quad (2)$$

where N is the number of scaled retention times, M' is a matrix of scaled retention times and M'^T is the transposed matrix of M' and yields a square correlation matrix in the form of Eq. (3):

$$C = \begin{vmatrix} 1 & C_{12} \\ C_{21} & 1 \end{vmatrix} \quad (3)$$

where $C_{12} = C_{21}$ is a measure of the correlation between two sets of retention time data and of the orthogonality of a two-dimensional system. Complete correlation exists in a chromatographic system when $C_{21} = 1$. When $C_{21} = 0$ the chromatographic system is totally orthogonal.

The practical peak capacity of a 2D-HPLC separation is approximated by using the peak spreading angles, β, in which the region of correlation is calculated and then subtracted from the product of the theoretical peak capacity in each dimension [8]. The creation of a geometric plot using these calculations demonstrates the unavailability of part of the two-dimensional retention space due to the correlation. The practical peak capacity is given by the following Eq. (4):

$$N_p = N_T - (A + C) \quad (4)$$

where N_p is the practical two-dimensional peak capacity, N_T the theoretical two-dimensional peak capacity and A and C are the unavailable separation areas due to the correlation.

The key metrics that GAFA yields include the degree of correlation between each dimension, the peak spreading angle (i.e. the measure of difference between the two separation vectors), the theoretical and practical peak capacities and the degree of utilization of the separation space. Total orthogonality, thus maximal coverage of the separation space, coincides with a spreading angle of 90°, and total correlation with a spreading angle of 0°. Further information regarding GAFA can be found in Reference [8].

3. Experimental

3.1. Chemicals and reagents

HPLC grade acetonitrile (ACN) and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The first dimension mobile phase pH was adjusted with a phosphate buffer (25 mM, pH 7), made from potassium dihydrogen phosphate and dipotassium hydrogen phosphate. The pH of the second dimension was adjusted with trifluoroacetic acid (TFA, 0.1% v/v, pH 2.2). These ionogenic salts were from Sigma, St. Louis, MO, USA.

3.2. Protein enzymatic digestion

The procedure used to prepare the samples of protein digests was modified from an in-solution digestion protocol obtained from the Proteomics Center at Stony Brook (New York State University, NY, USA). The digestion of β-lactoglobulin was carried out with trypsin (1 mg/mL in 1 mM HCl). Neither reducing nor alkylating steps were used in this procedure. β-Lactoglobulin were solubilised in a 40 mM Tris–HCl buffer solution (pH 8.5). The amount of trypsin added was calculated in order to have a final ratio of 1:50 (w/w) between the enzyme and the substrate. The digestion was performed at 37 °C for 24 h and then stopped with a 10 mM HCl solution. A reference solution, containing Tris–HCl buffer and the same amount of trypsin as the previous digestion solution, was kept at the same temperature and for the same time to evaluate the extent of a possible autodigest processes.

3.3. Instrumentation

The first dimension separation was performed on an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a mobile-phase degasser, an austo sampler, a binary pump and a column compartment. 100 μL of the sample was injected into the first dimension with a Rehodyne 7725i manual injector (IDEX Health & Science, Oak Harbor, WA, USA). The mobile phase gradient of the first dimension went from 5% to 40% ACN over 10 min at a flow rate of 0.5 ml/min and was held for 1 min.

Fractions were collected every 15 s (125 μL) for re-injection using a Gilson model 203 fraction collector (Middleton, WI, USA) and stored in the laboratory freezer at −11 °C until injected onto the second dimension.

The second dimension separation was performed on a Hewlett-Packard 1090 liquid chromatograph (now Agilent, Palo Alto, CA, USA). This instrument included a multi-solvent delivery system, an auto-sampler with a 250 μL sample loop, a diode-array UV-detector (set to record at 210 nm), and a data station. Compressed nitrogen (National Welders, Charlotte, NC, USA) was connected to the instrument to allow the continuous operations of the pump and the auto-sampler. The mobile phase gradient of the second dimension went from 5% to 40% ACN over 5 min at a flow rate of 1 ml/min and was held for 1 min before returning to the initial concentration for a re-equilibration step that lasted 4 min. The second dimension injection volume was 100 μL.

For the two 2D-HPLC separations, the Scherzo SM-C18 column (generously donated by Imtakt, Philadelphia, PA, USA) (150 × 4.6 mm, 3 μm particle diameter, S/N IF09MBF) and a Luna C18(2) column (Phenomenex, Torrance, CA, USA) (150 × 4.6 mm,

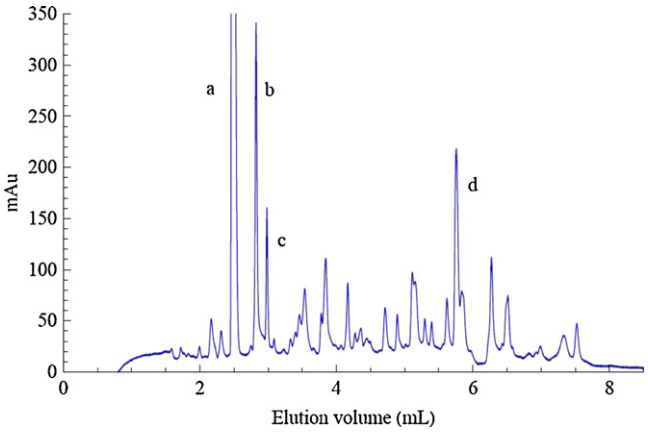

Fig. 1. Separation of β-lactoglobulin tryptic digest: mixed mode stationary phase, mobile phase pH 7. Peaks assigned a through d are matched to those in Fig. 2 based on relative peak heights and were confirmed in Fig. 3 by examining retention times.

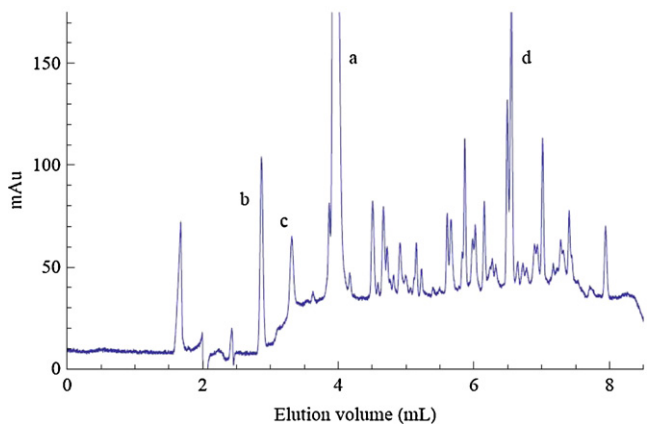

Fig. 2. Separation of β-lactoglobulin tryptic digest: mixed mode stationary phase, mobile phase pH 2. See Fig. 1 for definitions of peaks a through d.

3 μm particle diameter, S/N 380692-5) were used as the stationary phase in both separation dimensions. All measurements were carried out at a constant temperature of 24 °C, fixed by the laboratory air conditioner.

One-dimensional separations were conducted according to the corresponding methods with injection volumes of 5 μL for all 2D-HPLC dimensions.

3.4. Data analysis

The retention times for 2D-HPLC peaks, geometric approach to factor analysis calculations and graphics were constructed with Wolfram Mathematica 7 (distributed by Hearn Scientific Software, Melbourne, VIC, Australia) using algorithms built in-house. The algorithms employed to determine retention times were able to distinguish between strongly overlapping and shouldering peaks. A detailed description of operations of the peak picking algorithm are presented in Reference [15]. The thresholds used were held constant throughout the analysis, the minimum signal height was restricted to 20 and the signal height of the first derivative was 0.2. System peaks, that is solvent peaks are formed from the cut, and other gradient related artefacts, were manually removed prior to further analysis at the analysts discretion.

The sample peak capacity was measured according to Eq. (5), where t_g is the gradient time and W is the average width of the peaks measured at 4σ [10,13].

$$n_c \approx \frac{t_g}{W_{4\sigma}} \tag{5}$$

4. Results and discussion

Mixed mode columns contain ligands that allow for multiple retention mechanisms to occur simultaneously. In the case of the Scherzo SM-C18 column used here, the stationary phase behaves following both anion and cation exchange mechanisms, thus it performs differently depending on the mobile phase environment and its pH. Figs. 1 and 2 illustrate this observation with the separation of a tryptic digest of β-lactoglobulin with mobile phase pH of 7 and 2, respectively. Both separations were performed with an ACN/water gradient that began at 5 and finished at 40% ACN. The gradients increased the ACN concentration at a rate of 7% per mL mobile phase while the mobile phase flow rate is 0.5 mL/min for the pH 7 separation and 1 ml/min for the pH 2 separation.

From visual examination of these separations it is apparent that the retention characteristics of the peptides in the two separations were entirely different. Several peaks were matched in Figs. 1 and 2, based on their relative intensities (the peak assignments were also confirmed by 2D-HPLC analysis, see Fig. 3) and their relative retention characteristics can be compared. The elution orders of peak a and both peaks b and c are changed. The distance between peaks b and c is larger under acidic conditions, with both these peaks eluting earlier at pH 2, though the change in elution volume was greater for peak b. Conversely peak d has a greater elution volume in an acidic mobile phase. Overall retention is stronger with an acidic mobile phase. The peak capacity of these separations was 47 at pH 7 and 125 at pH 2. The results are summarised in Table 1. There are several explanations as to why the peak capacities are so different with these different mobile phases. For instance the decreased peak capacity in the separation at pH 7 is possibly due to the band broadening of the now polar solutes interacting with the exposed silanol groups on the surface. At low pH, the solutes are protonated, which decreases their polarity and weakens these unwanted interactions. Also, TFA is a chaotropic agent that somewhat disrupt the geometry of the peptide and, thus physically changes the shape of the solute

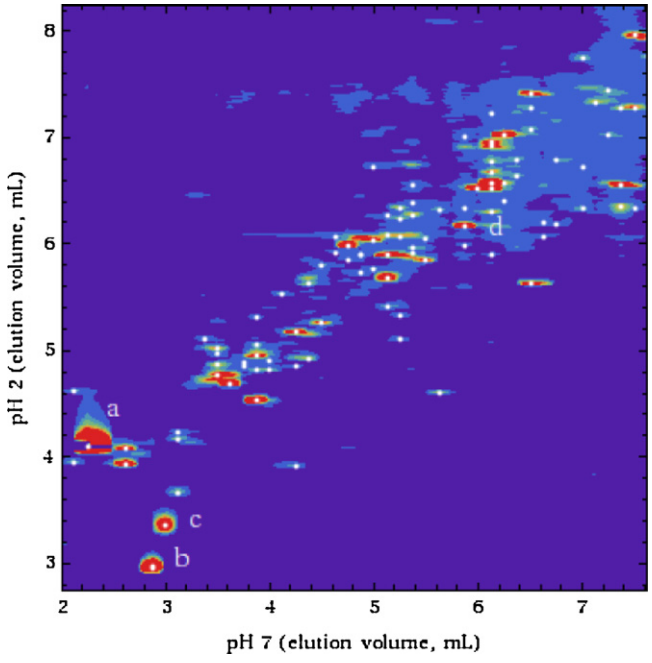

Fig. 3. 2D-HPLC separation of β-lactoglobulin tryptic digest: mixed mode stationary phase. Mobile phase: first dimension pH 7, second dimension pH 2. White points indicate peaks that have been identified by the peak picking algorithm. Peaks labelled a through d are the same as Figs. 1 and 2.

Table 1
Results of data analysis calculations of the 2D-HPLC separations with the Scherzo SM-C18 and Luna C18(2) columns.

Stationary phase	Mixed mode		C18	
pH	7	2	7	2
Sample n_c[a]	47	125	52	90
2D peaks	104		85	
Theoretical 2D n_c	5875		4680	
Practical 2D n_c[b]	2670		2498	
Correlation coefficient	0.884		0.784	
Spreading angle	27.8°		38.4°	

[a] Measured according to Eq. (5).
[b] According to the geometric approach to factor analysis, see Eq. (4).

under low pH environment. Gilar et al. [16] recently published on the influence of the concentration of TFA on the retention strength of peptides in HPLC.

Although it was not possible to map all the peaks in this sample with the changes mentioned above and the visual differences in both Figs. 1 and 2, it can be assumed that the retention mechanisms are different, as expected, when the peptides are eluted with mobile phases of different pH. The extent of this effect could be further investigated by performing a 2D-HPLC separation with mobile phases at different pH in each dimension, see Fig. 3. The locations of peaks a and b clearly illustrate the differences in the retention mechanisms of these two modes, as both compounds are retained longer when elution is made at pH 7. The peaks in Fig. 3 are largely spread around the diagonal, with a spreading angle of 27.8° and a correlation coefficient of 0.884. This is not an ideal result for a 2D-HPLC separation. However, when considering that the only difference between the two dimensions is the eluent pH, any significant divergence in elution order can be considered as providing a successful separation.

The 2D-HPLC separation on the mixed mode column produced 104 detectable peaks (represented by the white points in Fig. 3). This does not mean that there are only 104 components in the sample, but just that with the given set thresholds, the algorithm was able to detect 104 components. It is certain that there are strongly co-eluting peaks and that small peaks have escaped detection. For this purpose, however, it is not important that all components be accounted for, it is sufficient that enough peaks can be detected to provide an accurate determination of the correlation between retention, that will be discussed later.

To determine how much of the separation differences that were observed are due to the mixed retention mode, not to the changes made in the sample, the same separation was completed on a standard C18 stationary phase . All the other factors were held constant such as the column physical parameters, the particle diameter, the mobile phase and the gradient rate. However, the columns were obtained from different manufacturers, thus the underlying silica composition was different, resulting in slightly different retention characteristics.

Figs. 4 and 5 are one-dimensional chromatograms in each of the two separation dimensions. Unlike in Figs. 1 and 2, it is difficult to visually match the peaks and to determine if there are any significant alterations in the retention patterns. However, the two chromatograms do appear to be different. The peptides are less strongly retained on the C18 column than on the mixed mode column in a pH 7 mobile phase where the last eluted peak requires approximately 1 mL more of the mobile phase to be eluted (i.e. retention time difference of approximately 2 min). Retention was slightly longer on the C18 column with a mobile phase at pH 2. The peak capacity of the pH 7 and pH 2 separations were 52 and 90, respectively. The end-capping procedure is the likely reason why the peak capacity at these two pH values are much closer than those of the mixed mode column. If the exposed silanols are protected, less unfavorable interactions may take place at high pH, resulting in an efficiency similar to the one observed at low pH. The exact chemistry of the Scherzo SM-C18 is unknown to us and we cannot verify that the end-capping step is the only contributor to the different column efficiencies at different pH. It is quite likely that the C18 chain is also functionalized, thus impacting separation performance.

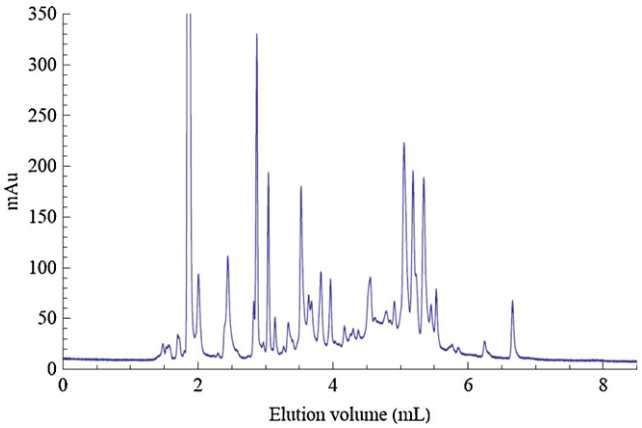

Fig. 4. Separation of β-lactoglobulin tryptic digest: C18 stationary phase, mobile phase pH 7.

The 2D-HPLC separation on the C18 stationary phase is illustrated in Fig. 6. Due to the large volume injected onto the second dimension and to the buffer solution being weakly retained, the unretained, peaks with an elution volume less than 2 mL are 'hidden' in the solvent peak (which had been cropped also from Fig. 6), thus the large peak occurring after passing about 2 mL of eluent in either dimensions is not apparent. This separation provided 85 detectable peaks.

A geometrical approach to factor analysis was used to quantify and compare the 2D separation performance of these separations by measuring the correlation between the two separation dimensions. Correlation coefficients between these dimensions close to 1 were expected as there is little difference between them, the only difference being the pH of the mobile phase. The correlation coefficient of the mixed mode phase is 0.884, that of the C18 stationary phase is 0.784, see Table 1. This agrees well with what can be visually approximated in Figs. 3 and 6. Surprisingly a larger fraction of the separation space is occupied with the standard C18 stationary phase (angle of 38.4°) than with the mixed mode column (27.8°). In both cases, the number of detected peaks is approximately equal to 3.5% of the practical peak capacity.

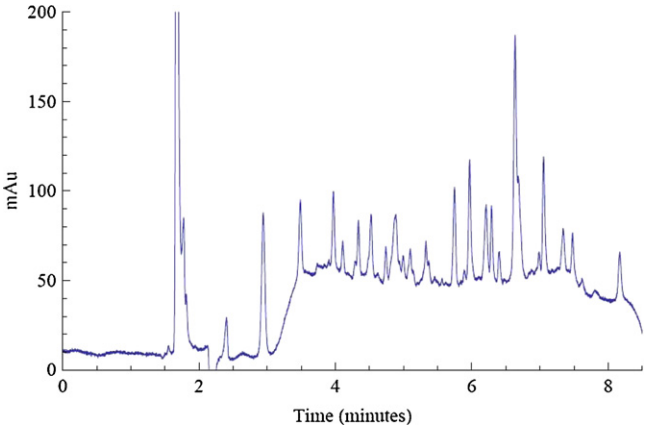

Fig. 5. Separation of β-lactoglobulin tryptic digest: C18 stationary phase, mobile phase pH 2.

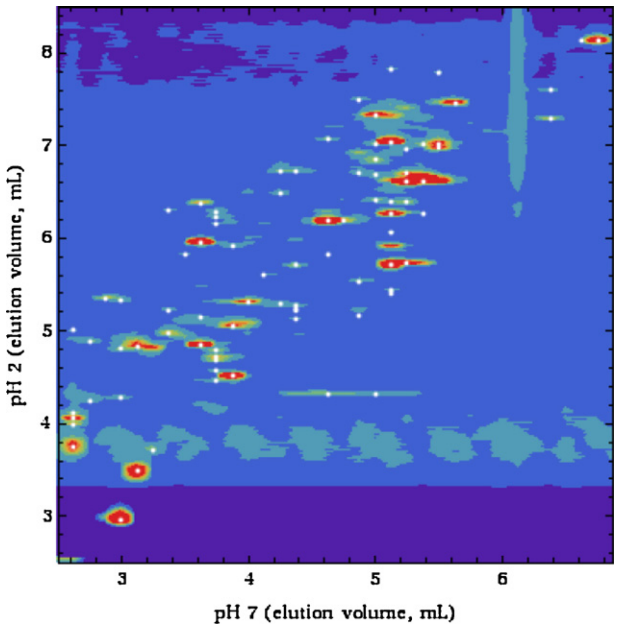

Fig. 6. 2D-HPLC separation of β-lactoglobulin tryptic digest: C18 stationary phase. Mobile phase: first dimension pH 7, second dimension pH 2. White points indicate peaks that have been identified by the peak picking algorithm.

On the C18 surface the only system changes brought by the pH variation is the polarity of the solute, due to protonation and de-protonation of the amino acid groups, thus its preference to partition in the stationary or in the mobile phase. The C18 stationary phase itself (especially when shielded by an end capping group) should not alter the retention process. Alterations to the solute molecule, i.e. its protonation in the acidic environment and de-protonation in the neutral environment, above the peptides isoelectric constant, p*I*, occurs in the same way on the mixed mode column, and does not factor into the differences of the retention mechanism.

When solute molecules are in their ionic or polar form, they prefer to remain in the mobile phase rather than to partition into the stationary phase [17]. In the case of retention on a C18 surface at low pH, when the amino acids are protonated, the peptides are less charged and will preferentially partition into the stationary phase. However, at pH 7 the peptides are charged and the retention mechanism changes as the solutes are attracted to the partial charges of the polar mobile phase, giving the results observed in Fig. 6. The p*I* of peptides varies from 3 to 12 [7], thus 2D-HPLC dimension divergence should be more pronounced at higher pHs. However, for column protection, we chose to run the first dimension at pH 7. It is suspected that a mixed mode column operating in both anion and cation exchange modes is possible, due to end-capping agents containing charged functional groups. This might decrease the effects observed on the C18 phase because, at low pH the charged molecules will still be attracted into the bulk stationary phase where the primary retention mechanism, i.e. hydrophobic interactions, then dominates the retention process.

The examination of the 2D-HPLC profiles of these separations shows that the retention mechanisms of these stationary phases are different. For example, the final peak of the separation made on the mixed mode column provides several peaks in the second dimension. However, the final peak of the separation made on the C18 column produces only one peak in the second dimension, the other peaks having migrated to earlier fractions. The two stationary phases seem to behave similarly at neutral pH, as reflected by the similar sample peak capacities of 47 and 52. However, the mixed mode column performs much better with an acidic pH, providing an n_c of 125, in contrast with the 90 obtained on the C18. The theoretical peak capacity alone suggests that the mixed mode column is more effective at separating peptides than the C18 column, providing a value of 5875 peaks opposed to 4680 peaks. This improved performance can be attributed to the additions to the C18 chain, be that the end capping agent or embedded functionality, that provide the different mechanisms for different modes of chromatography. The average peak width is narrower at acidic pH on the mixed mode column suggesting that the mass transfer is faster than with the C18 chain under these conditions. The peak width is smaller for the C18 stationary phase at pH 7, but not to as large an extent.

The geometrical approach to factor analysis suggests that the two modes of the Scherzo SM-C18 are not significantly diverse. However, these results do suggest that the secondary interactions are strong enough for the mixed mode column to be an alternative to the C18 dimension in a 2D-HPLC separation, and these differences in selectivity will enhance the overall 2D-HPLC performance.

5. Conclusion

The comparison of a mixed mode stationary phase and a C18 stationary phase for the 2D-HPLC separation of a tryptic digest of β-lactoglobulin provides interesting results. First, the sample peak capacities of both columns at high pH were similar (47 and 52), but they were significantly different at low pH (125 and 90). The greater efficiency of the mixed mode column under acidic conditions gave it a larger theoretical peak capacity than the C18. This also suggests that the retention processes are different under different pH. However, this possibility was not confirmed by a multidimensional separation whereby each dimension was operated at a different pH. The geometrical approach to factor analysis calculations confirmed that the retention mechanisms on the C18 stationary phase under different pH conditions were more divergent than those for the mixed mode column with correlation coefficients of 0.784 and 0.884, respectively.

There are indeed differences between the elution profiles of the separations made with the mixed mode column and the standard C18. However, the full extent of these differences cannot be assessed without the prior identification of each peak with a mass spectrometer, or possibly, using standards. As the differences between the separations obtained in the anion and the cation exchange modes are less on the mixed-mode than on the conventional C18 column, the primary retention process must be hydrophobic in nature, however, other factors such as solute hydrophobicity or basic amino acid content will influence the retention processes. For a complex sample matrix the use of a mixed mode column might still be favorable since the secondary interactions provide different selectivities to assist with the separation, as well as stronger retentions and a higher peak capacity when operated under an acidic environment.

Finally, these separations illustrate the power of adjusting the mobile phase pH to improve the selectivity of peptide separations. This was evidenced particularly by the separations achieved on the C18 phase, Fig. 6, whereby the *only* difference between the two separation dimensions was the pH and this resulted in a 2D-HPLC separation with a correlation coefficient of 0.784.

Acknowledgements

This work was supported in part by Grant DE-FG05-88-ER-13869 of the US Department of Energy and by the cooperative agreement between the University of Tennessee and Oak Ridge National Laboratory. We acknowledge the generous donation of the Scherzo SM-C18 column by the manufacturers.

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