



The benefits of mixed-mode chromatography columns for separation of peptides and protein digests

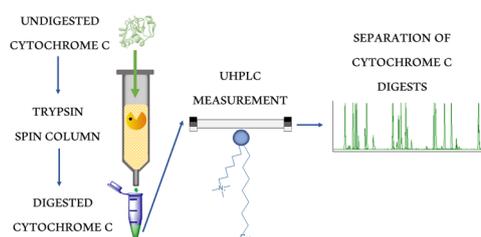
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Abstract

In this work, the evaluation and comparison of mixed-mode chromatography and reversed-phase chromatography for separation of peptides and protein digests have been performed. The effects of pH of aqueous part of mobile phase as well as the effects of organic modifier on retention, resolution, and peak shape were investigated on several columns including three mixed-mode columns possessing reversed-phase/anion-exchange mechanism, two reversed-phase octadecyl columns, and one column with mixed-mode reversed-phase/anion-exchange character only in defined pH range. The set of peptides varying in their polarity, length, amino acid sequence, and charge state, namely dipeptides, *N*-blocked dipeptides, and oligopeptides, was selected to describe the chromatographic behavior under different conditions properly. These measurements showed the potential of mixed-mode chromatography columns for analysis of differently charged peptides in a single run. The applicability of the tested conditions has been verified by the analysis of cytochrome C digested fragments. Two types of samples were analyzed and compared, i.e., commercial cytochrome C digested standard and cytochrome C digested via trypsin spin columns. The obtained results point to the necessity of using mass spectrometry detection because of large number of unknown peaks in cytochrome C digested standard, probably originating from chymotryptic and miscleavage activities.

Graphical abstract



Keywords High-pressure liquid chromatography · Cytochrome C · Resolution · Mass spectrometry detection

Introduction

Mixed-mode chromatography is a promising tool for the analysis and separation of a wide range of compounds [1, 2]. The reason is utilization of at least two different types of interactions between the analyte and the stationary phase

simultaneously, which can significantly affect the retention and separation [3]. Therefore, it is possible to analyze a variety of compounds differing in their physico-chemical properties (such as polarity, charge state) in a single chromatographic run [4]. This can be very advantageous for analysis of peptides/protein digests, since these analytes are usually multiply charged and differ largely in polarity [5, 6].

Mixed-mode chromatography is not an entirely new concept. For example, “hydrophobic interactions” were observed in ion-exchange chromatography and affinity chromatography, and electrostatic interactions may occur in size exclusion chromatography [7]. Before mixed-mode

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chromatography has been reckoned as a novel individual chromatographic approach, secondary interactions (such as originating from dissociated silanols [8]) in traditional chromatography modes were often considered undesirable—they were identified as the main causes of peak tailing, and therefore there was an effort to eliminate or at least minimize them [9–11]. Mixed-mode chromatography differs from other single chromatographic modes by providing two or more significant different types of interactions, and thus all interactions contribute to the retention [3]. Generally, mixed-mode chromatography can be achieved by several approaches: (i) serial connection of two columns with different stationary phase/retention modes [12], (ii) mixing of two types of packing materials in one column [13], (iii) chemical bonding of a functional group in a ligand chain or support [14]. Covalent modification of a carrier or ligand with different types of functional groups within a single stationary phase is the dominant approach to obtain mixed-mode stationary phases nowadays.

In this work, mixed-mode stationary phases combining reversed-phase and anion-exchange retention mechanisms were used. Therefore, both “hydrophobic interaction” between the analyte and C18 ligand, and electrostatic interaction between the analyte and positively charged moiety of the stationary phase contribute to the overall retention [15]. Whether it will be electrostatic attraction or repulsion is determined mainly by the pK_a of the functional groups of the analytes and by the pH of the aqueous part of the mobile phase [16]. Hence, it is clear that mixed-mode chromatography provides an increased number of tunable mobile phase parameters, which makes the method development more flexible, but also complex [17, 18].

Specifically, three columns containing octadecyl ligand and positively charged modifier, i.e., pyridyl group for column XSelect CSH C18, quaternary alkylamine for column Atlantis PREMIER BEH C18 AX, and positively charged moiety (details not available from the manufacturer) for column Luna Omega PS C18 were used and compared in this work. Column XSelect CSH C18 is marketed as a reversed-phase column; however, the presence of pyridyl groups brings a positive charge to the stationary phase at mobile phase $pH < 6$ [19]. The other two mixed-mode columns should provide permanent positive charge, but our previous work reveals the similarity of the column Luna Omega PS C18 with column XSelect CSH C18 in terms of the pH range in which the electrostatic interaction with a positively charged moiety is applied [5]. The schematic structures and basic properties of stationary phases evaluated in this work are summarized in Table 1.

Mixed-mode stationary phases have been mainly used for separation of biologically active molecules, including peptides and proteins [5, 20–23]. For more complex protein molecules, digestion into smaller fragments/peptides is a

necessary step preceding their analysis [1, 24]. Digestion of proteins is usually executed by trypsin, which cleaves proteins at their C-terminal arginine or lysine residues (unless proline follows) [25]. Trypsin digestion can be performed by several ways, e.g., in solution, via trypsin spin columns or on-line (immobilized trypsin reactor coupled with liquid chromatography or capillary electrophoresis systems) [26–28]. While digestion in solution requires the presence of a buffer (Tris–HCl), strict pH, and temperature control ($pH = 7.8$, temperature $37.0\text{ }^\circ\text{C}$) [29], and is very time consuming, digestion via spin column may be a suitable alternative, which is easy and fast [30].

As mentioned above, tested mixed-mode stationary phases combine the advantages of two separation modes (reversed phase and anion exchange) and are able to separate complex mixtures of analytes based on their polarity and charge simultaneously. For this reason, the set of various peptides differing in their properties (polarity, charge, length, amino acid sequence) was chosen for investigating the effects of chromatographic parameters on the retention, separation, and peak shape. The single mode C18 column was used for comparison. Based on the obtained results, optimal conditions for analysis of digested cytochrome C were selected.

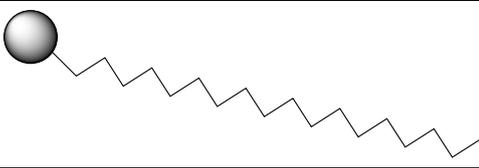
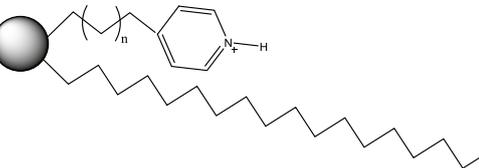
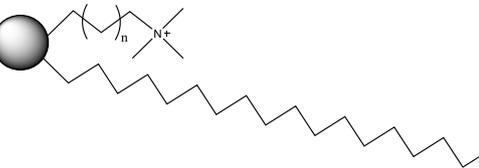
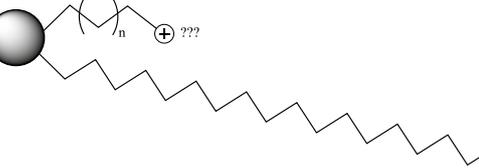
Results and discussion

HPLC measurements

First of all, preliminary measurements of a set of 22 various peptides were performed on an HPLC system. The effects of pH of aqueous part of mobile phase on retention and resolution of individual groups of peptides (dipeptides, *N*-blocked dipeptides, and oligopeptides) were investigated on three different columns—XBridge C18 (reversed phase), XSelect CSH C18 (mixed-mode character at $pH < 6$), and Atlantis PREMIER BEH C18 AX (mixed mode). The goal of this study was to investigate the retention behavior of various peptides in wide pH range to describe the mixed-mode stationary phases properly and to compare mixed-mode chromatography and reversed-phase chromatography in terms of retentivity and selectivity for peptides.

Figure 1 clearly shows the importance of using gradient elution for the analysis of mixture of various peptides—while dipeptides need highly aqueous mobile phase to be retained (95 vol% of aqueous part), blocked dipeptides exhibit sufficient retention even in mobile phase composed of 60–40% by volume of acetonitrile, depending on the used column and pH of aqueous part of mobile phase. Blocked dipeptides contain benzoyl protecting group at *N*-terminus, and thus they are much more hydrophobic than their non-blocked counterparts. The “hydrophobic interaction”

Table 1 Schematic structures of stationary phases and their basic properties

Column name	Stationary phase structure	Interaction properties
XBridge C18 (HPLC)		reversed-phase column
PREMIER BEH C18 (UHPLC)		reversed-phase column with anion-exchange character at pH < 6 p <i>K</i> _a of pyridyl group ~ 5-6
Atlantis PREMIER BEH C18 AX		mixed-mode column (reversed-phase + anion-exchange mechanism) permanently positively charged quaternary alkylamine interactions with negatively charged dissociated silanols prevails at pH ≥ 6.8
Luna Omega PS C18		mixed-mode column (reversed-phase + anion-exchange mechanism) positively charged moiety (no details available) similar interaction behavior as column XSelect CSH C18

between the benzoyl group and C18 ligand (present in each tested stationary phase) results in higher retention in comparison with the non-blocked dipeptides. pK_a values of carboxy groups of *N*-blocked dipeptides vary within the range 3.5–3.9, i.e., as the dipeptides are more charged with increasing pH, they are becoming more polar, and thus their log *D* values decrease (Table S1 in Supporting material). This is the reason of decreasing retention of *Z*-Phe-Trp-OH on reversed-phase XBridge C18 column (Fig. 1A) with increasing pH (pH=2.1 and pH=3.0 exhibits comparable retention). For column XSelect CSH C18 (Fig. 1B), the situation is very similar, even though the stationary phase surface should be positively charged at pH < 6. In fact, pyridyl groups on CSH particles are only partially positively charged at pH=4.7, and thus higher dipeptide polarity prevails over

the electrostatic attraction (as was for peptides already shown in [5]) and the retention decreases with increasing pH.

This is not the case of mixed-mode column Atlantis PREMIER BEH C18 AX (Fig. 1C), where the highest retention was observed at mobile phase with aqueous part of pH=4.7, where the strong electrostatic interaction between negatively charged *N*-blocked dipeptide and positively charged stationary phase surface is applied. At mobile phase with aqueous part of pH=6.8, the electrostatic repulsion with dissociated free silanols prevails and the decrease in retention was observed. But the retention is still higher in comparison with stationary phases without positively charged moiety. The higher retention of *N*-blocked dipeptides at mobile phase with aqueous part of pH=2.1 (where no electrostatic

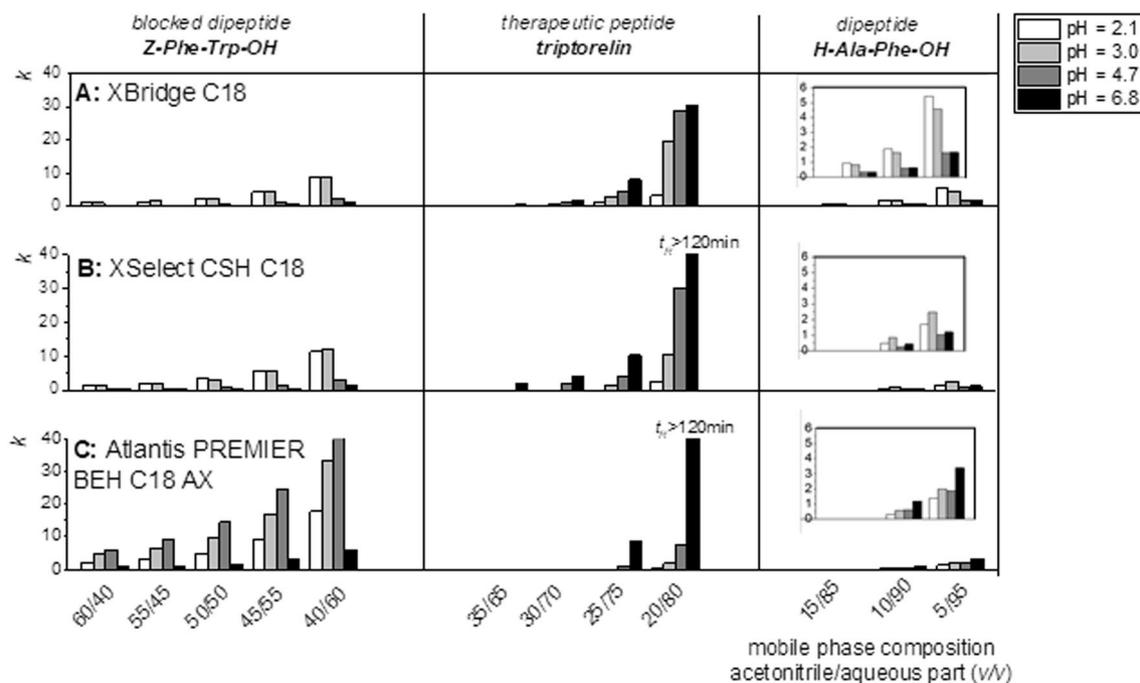


Fig. 1 Retention factors of selected representatives of peptides (Z-Phe-Trp-OH as blocked dipeptide, triptorelin as therapeutic oligopeptide, and H-Ala-Phe-OH as dipeptide) in dependence on the content of acetonitrile in mobile phase (5–60 volumetric percent-

ages), the pH of the aqueous part of mobile phase (pH=2.1, pH=3.0, pH=4.7, and pH=6.8) and used stationary phase (A: XBridge C18, B: XSelect CSH C18, C: Atlantis PREMIER BEH C18 AX)

interactions take a role) on mixed-mode column in comparison with other columns can be caused by the difference in pore size (XBridge C18 pore size 130×10^{-10} m; XSelect CSH C18 pore size 130×10^{-10} m; Atlantis PREMIER BEH C18 AX pore size 95×10^{-10} m). The retention trends for other tested *N*-blocked dipeptides were the same (Fig. S1 in Supporting material). pK_a values of carboxy groups of non-blocked dipeptides vary within the range 3.5–3.8 and pK_a values of amino groups are in the range 8.0–8.5 (Table S1 in Supporting material). Log D values are much lower in comparison with *N*-blocked dipeptides (negative, because of missing benzoyl group) and their trend depending on pH is exactly opposite, i.e., with increasing pH, log D value increases and the values are almost the same for mobile phases with aqueous part of pH=4.7 and pH=6.8 (Table S1 in Supporting material). It may be explained by the similar charge distribution, at mobile phase with aqueous part of pH=4.7, all amino groups are positively charged and only a part of the carboxy groups is negatively charged. Similarly, at mobile phase with aqueous part of pH=6.8, all carboxy groups are negatively charged and only a part of the amino groups is positively charged. This is the reason why the retention of dipeptides on XBridge C18 column is not decreasing in a whole pH range, but only up to pH=4.7 (Fig. 1A). As in the case of *N*-blocked dipeptides, retention behavior on XSelect CSH C18 column is very similar to

the classical reversed-phase column (Fig. 1B). At mobile phase with aqueous part of pH=2.1 electrostatic repulsion between positively charged pyridyl groups on the stationary phase surface and positively charged amino groups further reduces the already very low retention. No positive charge on the stationary phase surface is available above pH=4.7, and thus only electrostatic repulsion between the negatively charged carboxy groups and negatively charged free silanols can apply. This results in very low retention in all tested pH values. Mixed-mode column Atlantis PREMIER BEH C18 AX exhibits increasing retention with increasing pH of the aqueous part of mobile phase. No retention of dipeptides was observed at mobile phase with aqueous part of pH=2.1, because of the repulsion of positively charged amino group and positively charged stationary phase surface. With increasing pH of aqueous part of mobile phase, larger part of carboxy groups (capable of electrostatic attraction with the positively charged stationary phase surface) is dissociated and thus the retention is increasing. Moreover, free silanol groups can interact with the charged amino groups, and thereby the retention increases. The retention trends for other tested non-blocked dipeptides were similar (Fig. S2 in Supporting material).

Since oligopeptides/therapeutic peptides contain more functional groups enabling multiple charging, the description of retention behavior is more complex and differs for

individual peptides. All three tested columns exhibit no retention of oligopeptides at mobile phase with aqueous part of pH=2.1 (the highest polarity of oligopeptides, log *D* values are summarized in Table S1 in Supporting material). Columns XBridge C18 and XSelect CSH C18 provide the same trends—the increasing retention with increasing mobile phase pH for triptorelin (Fig. 1), leuprolide, and goserelin. Individual trends in retention of other oligopeptides are shown in Fig. S3 in Supporting material. Mixed-mode column Atlantis PREMIER BEH C18 AX exhibits the same trend for all tested oligopeptides—increasing retention with increasing mobile phase pH (Fig. S3 in Supporting material).

Based on the obtained results, we can assume appropriate conditions for separation of mixture of all the tested peptides, i.e., dipeptides, blocked dipeptides, and oligopeptides. It was revealed that oligopeptides exhibit no retention at mobile phase aqueous part of pH=2.1. In addition, pH=2.1 shows very low selectivity for *N*-blocked dipeptides in comparison with pH=6.8 (Fig. S4 in Supporting material). Therefore, mobile phase with aqueous part of pH=2.1 was not used for analysis of the peptides' mixture. For non-blocked dipeptides, it seems to be advantageous to use rather higher pH of aqueous part of mobile phase (pH=6.8) in combination with a mixed-mode column (if pH=2.1 was excluded). Moreover, these conditions are also suitable for analysis of blocked dipeptides.

Figure 2 shows the comparison of the best obtained results for separation of mixture of peptides. Columns XSelect CSH C18 and Atlantis PREMIER BEH C18 AX at mobile phase aqueous part of pH=6.8 exhibited the highest selectivity and suitable resolution of most of the tested peptides in comparison with column XBridge C18 and other tested pH values of the aqueous part of the mobile phase. However, it is clearly visible that mixed-mode column Atlantis PREMIER BEH C18 AX provides higher retention times, thus inferior peak shapes (which can be obviously subjected to further optimization).

Further investigation of these two columns (XSelect CSH C18 and Atlantis PREMIER BEH C18 AX) revealed that for analysis of cytochrome C digested fragments, column Atlantis PREMIER BEH C18 AX provides better selectivity (no co-elution was observed—Fig. S5 in Supporting material).

UHPLC measurements

The obtained results from HPLC measurements revealed great potential of mixed-mode chromatography for analysis of peptides/cytochrome C digests. Therefore, we were interested in the further evaluation of two different columns marketed as mixed-mode—Luna Omega PS C18 (similar to XSelect CSH C18 column, losing positive charge at pH > 6) and Atlantis PREMIER BEH C18 AX (permanently positively charged). Just to confirm the benefits of mixed-mode

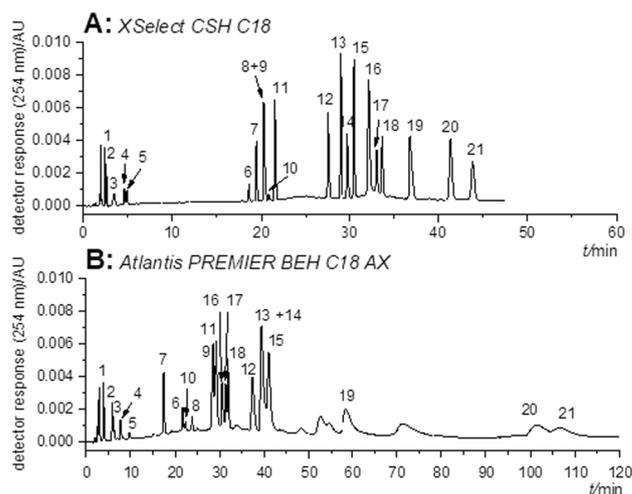
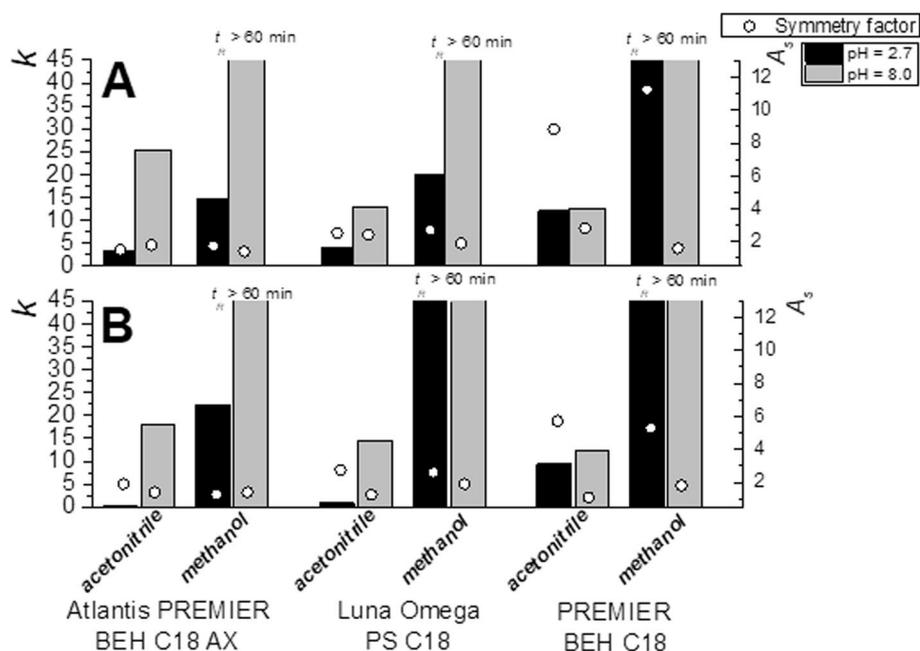


Fig. 2 Comparison of separation of mixture of peptides. **A** column XSelect CSH C18; **B** Atlantis PREMIER BEH C18 AX. Gradient elution— t_0 : acetonitrile/10 mM ammonium acetate, pH=6.8, 5/95 (v/v); $t_{30\text{min}}$: acetonitrile/10 mM ammonium acetate, pH=6.8, 30/70 (v/v). Detection wavelength 254 nm. Analytes: 1: H-Ala-Tyr-OH; 2: H-Tyr-Ala-OH; 3: H-Val-Tyr-OH; 4: H-Ala-Phe-OH; 5: H-Phe-Ala-OH; 6: [Met³]-enkephalin; 7: [Lys⁸]-vasopressin; 8: Z-Tyr-Ala-OH; 9: [Leu⁵]-enkephalin; 10: angiotensin II; 11: Z-Ala-Tyr-OH; 12: Z-Ala-Phe-OH; 13: Z-Ala-Trp-OH; 14: Z-Phe-Ala-OH; 15: Z-Trp-Ala-OH; 16: goserelin; 17: leuprolid; 18: triptorelin; 19: Z-Phe-Leu; 20: Z-Trp-Phe-OH; 21: Z-Phe-Trp-OH

chromatography for our purpose, reversed-phase column PREMIER BEH C18 was also tested. Since UHPLC methods exhibit higher efficiency, the following experiments were carried out at UHPLC instrumentation, which additionally enables us the analysis of some other peptides, which are not UV detectable, and thus different detection, in our case mass spectrometry detection, is needed. To show the potential of mixed-mode stationary phases for separation of peptides, the effects of pH of aqueous part of mobile phase and moreover the effect of type of organic solvent (methanol vs. acetonitrile) on retention, separation, and peak shape were investigated. Two different pHs of aqueous part of mobile phase were tested: acidic pH (0.1% solution of formic acid: 26.5 mM, pH=2.7, which is usually used for peptide analysis [31, 32]), and basic pH (26.5 mM ammonium formate, pH=8.0, which would be appropriate for possible future on-line protein digestion by trypsin).

Figure 3 confirms the differences between mixed-mode chromatography and reversed-phase chromatography, as well as the differences between different mixed-mode columns (also previously described in [5]). For all tested peptides (the two examples are shown in Figs. 3A, B), mixed-mode columns at mobile phase aqueous part of pH=2.7 exhibited significantly lower retention in comparison with reversed-phase column (applies for both methanol and acetonitrile). The reason is electrostatic repulsion between predominantly

Fig. 3 The effects of pH and organic solvent on retention (k) and peak symmetry (A_s) for all tested columns. **A:** Val-Tyr-Val, mobile phase composition: organic solvent/aqueous part 5/95 (v/v); **B:** bradykinin, mobile phase composition: organic solvent/aqueous part 15/85 (v/v)



positively charged peptide and mixed-mode stationary phase surface. On the other hand, at mobile phase with aqueous part of pH = 8.0, exactly opposite trend was observed, i.e., electrostatic attraction between predominantly negatively charged peptide and positively charged mixed-mode stationary phase result in higher retention on mixed-mode columns than on reversed-phase column; however, only a small difference between Luna Omega PS C18 and reversed-phase column PREMIER BEH C18 was observed (because Luna Omega loses at pH > 6 positive charge). In addition, electrostatic repulsion between the negatively charged analytes and dissociated silanol groups on the stationary phase surface also contribute to the retention. The overall retention depends on the number of free residual silanol groups, and the retention is, therefore, the result of the combination of following interaction mechanisms—“hydrophobic” interaction, electrostatic repulsion, and electrostatic attraction.

Not surprisingly, mobile phases with methanol provide significantly higher retention in comparison with acetonitrile. Comparing the symmetry factors, no meaningful differences between methanol and acetonitrile were observed (Fig. 3). Regarding the effect of mobile phase aqueous part pH on the retention and peak symmetry, it was observed that higher pH (pH = 8.0) provides higher retention in comparison with lower pH (pH = 2.7). This effect is very significant for mixed-mode columns while retention on reversed-phase column is not very affected by pH of aqueous part of mobile phase, which indicates the essential role of stationary phase charge. In addition, it was observed that higher pH mostly provides better peak shape (lower symmetry factor), which was the most significant for reversed-phase column PREMIER BEH C18 (Fig. 3).

Another goal was to find suitable conditions for separation of mixture of 12 peptides in UHPLC system. The large number of gradient types for each column in combination with each aqueous part pH and each organic modifier has been tested. Table 2 clearly shows that achieving the baseline separation of all peptides of interest was the most problematic on reversed-phase column at pH = 8.0 and on column Luna Omega PS C18. Under these conditions, it was not possible to resolve both pairs leuprolide–carbetocin and angiotensin II–leucine enkephalin—the change in gradient conditions leads to the loss of the resolution of one or the other pair. But we were able to baseline separate all the peptides on mixed-mode column Atlantis PREMIER BEH

Table 2 The success of the gradient separation of the mixture of 12 peptides and cytochrome C digests. Summary of the shortest obtained analysis times. The corresponding gradients are given in Table S2 in Supporting material

	Mixture of 12 peptides		Cytochrome c	
	pH = 2.7	pH = 8.0	pH = 2.7	pH = 8.0
Mixed-mode column Atlantis PREMIER BEH C18 AX				
Acetonitrile	✓ 7.5 min	✓ 13 min	✓ 11 min	✓ 20 min
Methanol	✓ 5.5 min	✓ 18 min	✓ 30 min	✓ 36 min
Mixed-mode column Luna Omega PS C18				
Acetonitrile	✓ 7.5 min	✗ 14 min	✓ 12 min	✓ 20 min
Methanol	✗ 10.5 min	✓ 13 min	✓ 30 min	✗ 50 min
Reversed-phase column PREMIER BEH C18				
Acetonitrile	✓ 8 min	✗ 13 min	✗ 25 min	✓ 25 min
Methanol	✓ 10.5 min	✗ 12 min	✓ 35 min	✓ 33 min

✓ baseline separated, ✗ not baseline separated

C18 AX, even within 5.5 min while using mobile phase with methanol (the fastest obtained analysis of peptide mixture is depicted in Fig. S6 in Supporting material). Generally, in mobile phases with aqueous part of pH = 8.0, very low signals from UV detection for Gly-Glu and Lys-Lys-Lys were observed (because of the significant peak deterioration); thus, usage of mass spectrometry detection is very advantageous.

Cytochrome C was selected as a model protein due to the availability of standard of its digests, which enables the comparison of digested standard and cytochrome C digested via spin columns. The list of cytochrome C digests declared in standard and corresponding m/z values is shown in Table S3 in Supporting material. From the HPLC measurements, it turned out that the conditions suitable for the separation of peptides may not be suitable for the separation of cytochrome C digests at the same time. Thus, analysis of cytochrome C digests (from digested standard and obtained by digestion via spin column) was performed in previously tested UHPLC conditions, i.e., three columns, acetonitrile vs. methanol, aqueous part of pH = 2.7 vs. pH = 8.0, and in large number of gradients (differing from the gradients used for analysis of 12 peptides).

Table 2 summarizes obtained results, i.e., whether it was possible to baseline separate all cytochrome C digests and the shortest analysis time. The fastest analysis of cytochrome C digested fragments was obtained using mixed-mode column Atlantis PREMIER BEH C18 AX (mobile phase with acetonitrile and aqueous part of pH = 2.7), where all 13 fragments declared in standard certificate were baseline separated within 11 min (Fig. 4A). Figure 4B shows the

comparison with different mobile phase (but the same stationary phase), i.e., mobile phase with methanol in combination with aqueous part of pH = 8.0. These conditions may be very advantageous for on-line protein digestion. The comparison of Figs. 4A, B shows that in both conditions, we can achieve baseline separation of all 13 fragments, but with different elution order (different mobile phase pH).

Fragment T19C originates from chymotryptic activity (Table S3 in Supporting material, the amino acid sequence does not end with lysine or arginine), which may occur during trypsin digestion as a result of impure trypsin or trypsin autolysis [33]. This fragment was not found in the sample of cytochrome C digested via spin column, which corresponds to the statement of the manufacturer that trypsin spin columns are highly purified and immobilization prevents the trypsin autolysis [34]. Fragments T9-10, T12-13, and T13-14 are the consequence of miscleavage activity, their presence is declared in cytochrome C digested standard and they were observed also in sample digested via spin column.

The missing chymotryptic fragment is not the only difference between cytochrome C digested standard and cytochrome C digested via spin column. The comparison of analysis of these two samples (digested standard vs. digested via spin column) revealed increased number of peaks for cytochrome C digested standard, which indicates number of impurities (Fig. 5). Impurities in digested protein sample are often a consequence of chymotryptic or miscleavage activity, which is common while using in-solution digestion, especially for extended time of digestion [35]. On the other hand, lower chymotryptic and miscleavage activity can be achieved by digestion via spin columns. Obtained data point

Fig. 4 Separation of 13 cytochrome C digested fragments on column Atlantis PREMIER BEH C18 AX. **A** mobile phase A—26.5 mM formic acid in acetonitrile, mobile phase B—26.5 mM formic acid in water, pH = 2.7, gradient elution: 0 min—0% A; 8 min—25% A; 10.5 min—30% A; 12 min—40% A. **B** mobile phase A—26.5 mM ammonium formate in methanol, mobile phase B—26.5 mM ammonium formate in water, pH = 8.0, gradient elution: 0 min—0% A; 40 min—75% A

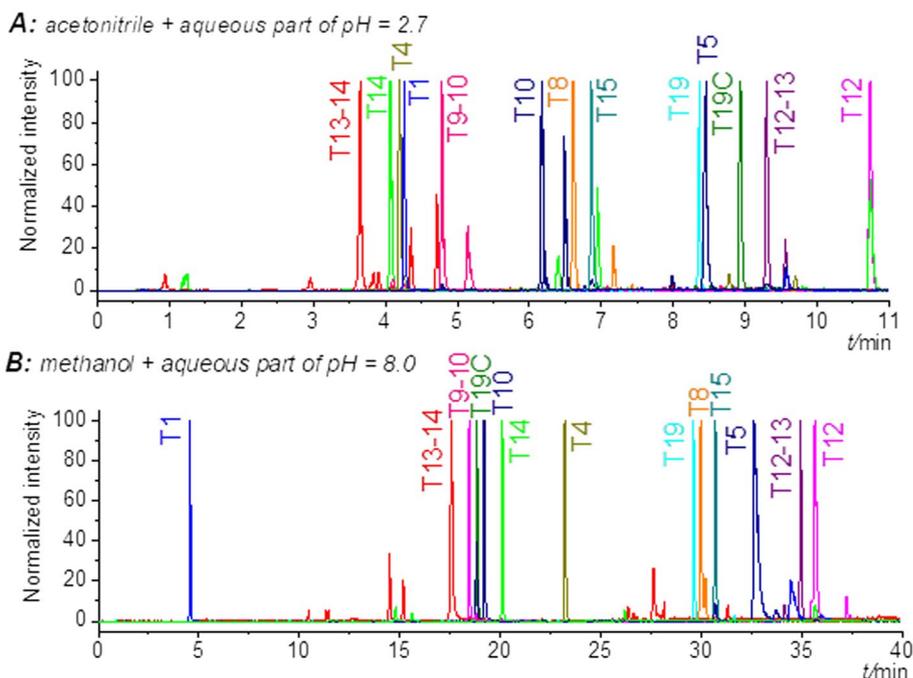
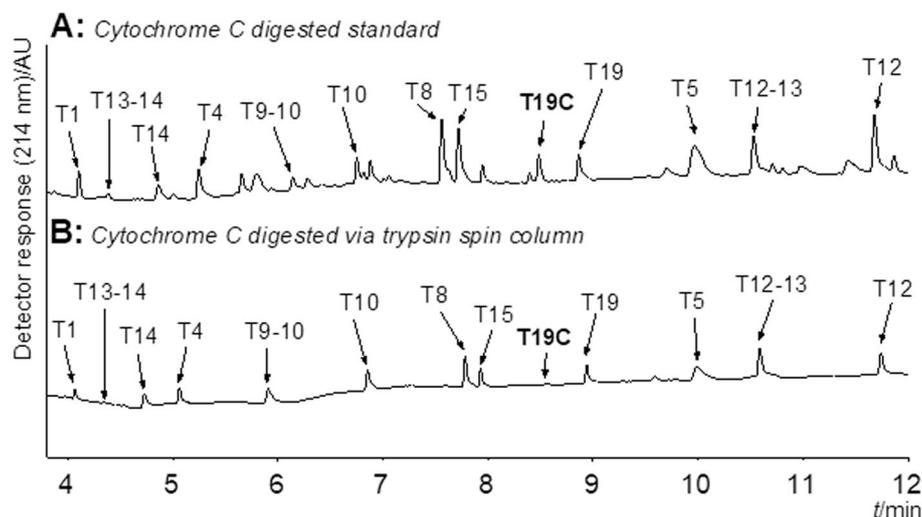


Fig. 5 Comparison of the signals from UV detection (214 nm) for **A** cytochrome C digested standard and **B** cytochrome C digested via trypsin spin column. Column: Luna Omega PS C18, mobile phase A—0.1% formic acid in acetonitrile, mobile phase B—0.1% formic acid in water, gradient elution: 0 min—0% A; 8 min—25% A; 10.5—30% A; 12 min—40% A. Table S3 in Supporting material contains information for identification of individual fragments



to the problematic nature of the analysis of protein digestion samples without mass spectrometry detection. Besides the chymotryptic peak and the number of impurities, no significant differences between the spin digestion and digested standard were observed, i.e., comparable retention of 12 fragments was achieved.

Conclusion

The goal of our study was to show the great potential of mixed-mode chromatography for analysis of peptides and protein digests. Several columns, including mixed-mode and reversed-phase columns, were used in HPLC and UHPLC systems. The effect of pH of aqueous part of mobile phase and the effect of the type of organic modifier on the retention, selectivity, resolution, and peak shape were investigated.

Analysis of dipeptides with easily defined charge state contributed to the description of mixed-mode retention behavior. It was shown that mixed-mode column Atlantis PREMIER BEH C18 AX exhibits the highest retention of negatively charged dipeptides at mobile phase aqueous part of pH = 4.7. At higher pH, the electrostatic repulsion with negatively charged residual silanols prevails. It was confirmed that column XSelect CSH C18 (marketed as reversed-phase) possesses significant mixed-mode character only in mobile phase of aqueous part of pH = 2.1 and pH = 3.0. Using HPLC, the baseline separation of various 21 peptides has been achieved on column XSelect CSH C18. In terms of the peak shape, PREMIER BEH C18 column in acidic pH (2.7) was found as the most inappropriate (symmetry factors higher than 8 for mobile phase with acetonitrile, for methanol even higher).

The analysis of cytochrome C digests on UHPLC points to the necessity of mass spectrometry detection due to

the number of miscleaved/chymotryptic fragments in cytochrome C digested standard. It was shown that Atlantis PREMIER BEH C18 AX column is the most universal from the tested columns. The both acetonitrile and methanol with acidic (pH = 2.7) and basic (pH = 8.0) aqueous parts of mobile phase are suitable for baseline separation of cytochrome C digests. Mixed-mode column Luna Omega PS C18 showed comparable results except for mobile phase composed of methanol and ammonium formate buffer, pH = 8.0.

Experimental

Chemicals and materials

Acetonitrile (LC–MS grade) and methanol (LC–MS grade) were supplied by VWR International (Radnor, USA), water for LC was purchased from Honeywell (Charlotte, USA)—used for UHPLC measurements. Acetonitrile (Chromasolv® gradient grade, for HPLC, ≥ 99.9%), methanol (Chromasolv® gradient grade, for HPLC, ≥ 99.9%), ammonium acetate (purity ≥ 98%), ammonium formate (purity ≥ 97%), formic acid (purity ≥ 95%), acetic acid (purity ≥ 99%), ammonium hydroxide solution (28.0–30.0% NH₃), and trifluoroacetic acid (purity 99%) were purchased from Sigma-Aldrich (St. Louis, USA). Deionized water was purified with Rowapur and Ultrapur system from Watrex (Prague, Czech Republic). All dipeptides were purchased from Bachem (Bubendorf, Switzerland), except Z-Phe-Leu and H-Val-Tyr, which were supplied by Sigma-Aldrich (St. Louis, USA). Goserelin acetate salt, leuprolide acetate salt, bradykinin acetate salt, and carbetocin were purchased from Bachem (Bubendorf, Switzerland). Lys-Lys-Lys, Val-Tyr-Val, angiotensin I, angiotensin II, [Lys⁸]-vasopressin, [Met⁵]-enkephalin acetate salt hydrate, and [Leu⁵]-enkephalin acetate salt hydrate were

supplied by Sigma-Aldrich (St. Louis, USA). Cytochrome C digestion standard was supplied by Waters (Milford, USA). Cytochrome C from bovine heart (purity > 95%), trypsin spin columns, and protein extraction reagent type 4 were purchased from Sigma-Aldrich (St. Louis, USA). The list of all tested analytes, their structures, log *D* and p*K*_a values is presented in Table S1 in Supporting material. Marvin software (product of ChemAxon company) was used for calculation of log *D* values in corresponding pH of aqueous part of mobile phase and for calculation of p*K*_a values of peptides.

Instrumentation and chromatographic conditions—HPLC measurements

All HPLC measurements were performed using the Waters Alliance system (Waters, Milford, USA) consisting of 2690 D Separation Module, 2487 Dual λ Absorbance Detector, 717 Plus autosampler, and Waters Alliance Series column heater. Empower 2 software was used for system control and data acquisition. Following columns were used: XSelect CSH C18, XBridge C18 and Atlantis PREMIER BEH C18 AX. All tested columns, particle size 5 μm , 150 \times 4.6 mm, were obtained from Waters (Milford, USA).

Stock solutions of most of the analytes were prepared by dissolving the sample in methanol at concentration 1 mg cm⁻³. More polar peptides (H-Val-Tyr-OH, H-Ala-Tyr-OH, H-Tyr-Ala-OH) and angiotensin II were dissolved in mixture of methanol and water (50/50 (v/v)) at concentration 1 mg cm⁻³. Several peptides (Z-Ala-Phe-OH, Z-Ala-Tyr-OH, Z-Phe-Ala-OH, Z-Phe-Leu, and Z-Tyr-Ala-OH) needed to be dissolved at higher concentration 5 mg cm⁻³, because of very low UV response. The first system peak was used as a dead time marker. All measurements were performed in triplicate.

Mobile phases composed of acetonitrile and aqueous part in volume ratios from 5/95 to 60/40 (v/v) with 5 volume percentage steps were used. For analysis of mixtures of peptides and cytochrome C digests, gradient elution was used. The following aqueous parts of mobile phases were used: 365 mM formic acid, pH = 2.1; 10 mM ammonium formate buffer, pH = 3.0; 10 mM ammonium acetate buffer, pH = 4.7 and pH = 6.8. For calculation of buffer components concentrations, and corresponding pH values, PeakMaster software was used [36]. Basic chromatographic conditions were set as follows: mobile phase flow rate 1 cm³ min⁻¹, injection volume 5 mm³, column temperature 25.0 °C, sample temperature 20.0 °C, detection wavelengths 254 nm and 280 nm. For analysis of cytochrome C digests, injection volume was 15 mm³ and detection wavelength was 214 nm.

Instrumentation and chromatographic conditions—UHPLC measurements

Waters Acquity UPLC H-Class system (Waters, Milford, USA) was used for UHPLC measurements. The system was equipped with a quaternary solvent manager, an autosampler, a column thermostat, a photodiode array detector and a QDa mass detector. The Empower 3 software was used for system control, data acquisition, and results processing. Following columns were tested: Atlantis PREMIER BEH C18 AX; PREMIER BEH C18 (Waters, Milford, USA), both columns dimensions were 100 \times 2.1 mm; particle size 1.7 μm ; column Luna Omega PS C18 (Phenomenex, Torrance, USA) with dimensions 100 \times 2.1 mm; particle size 1.6 μm .

Stock solutions of the peptides were prepared by dissolving the sample in deionized water at concentration 1 mg cm⁻³. The first system peak was used as a dead time marker. All measurements were performed in triplicate.

Mobile phases were composed of acetonitrile or methanol and aqueous part in volume ratios from 0/100 to 60/40 (v/v) with 5 volume percentages steps. For analysis of mixture of peptides and cytochrome C digests, gradient elution was used. As aqueous part of mobile phase (mobile phase (B)), 0.1% formic acid, pH = 2.7 was used (corresponds to 26.5 mM solution of formic acid). Similarly, aqueous part of pH = 8.0 was prepared as 26.5 mM ammonium formate with addition of ammonium hydroxide to reach the pH = 8.0. In both cases, the same amount of formic acid/ammonium formate + ammonium hydroxide as was added to the mobile phase B was added also to the mobile phase A (to keep the ionic strength constant during the gradient). Mobile phase A contains organic solvent—pure methanol (with formic acid or ammonium formate + ammonium hydroxide), pure acetonitrile (with formic acid) or mixture acetonitrile/water, 80/20 (v/v) (ammonium formate + ammonium hydroxide), because of a low solubility of ammonium formate in pure acetonitrile. Basic chromatographic conditions were set as follows: mobile phase flow rate 0.3 cm³ min⁻¹, injection volume 1 mm³, column temperature 37.0 °C, sample temperature 10.0 °C, detection wavelengths 214 nm and 220 nm + QDa detection (positive mode, cone voltage 15 V, probe temperature 600 °C).

Cytochrome C trypsin digests

Two samples of cytochrome C digests were analyzed: (i) standard of digested cytochrome C, (ii) cytochrome C digested via spin column. Standard of digested cytochrome C was dissolved in 200 mm³ of 0.055% trifluoroacetic acid. Spin digestion was performed exactly according to the manual enclosed to the trypsin spin columns (from Sigma-Aldrich). Digestion via spin column includes following steps: protein denaturation (using mixture of urea, thiourea,

and detergent protein extraction reagent type 4), spin column washing and equilibration (using 100 mM ammonium bicarbonate reaction buffer is a part of the spin column package), and digestion itself (100 µg of protein is applied, 15 min take the digestion). The products of digestion are washed from the spin column by deionized water and the sample is ready for LC analysis.

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Data availability The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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