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Separation, detection, and identification of peptides by ion-pair reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry at high and low pH

Hansjörg Toll [a], Herbert Oberacher [a,1], Remco Swart [b], Christian G. Huber [a,*]

[a] *Department of Chemistry, Instrumental Analysis and Bioanalysis, Saarland University, 66123 Saarbrücken, Germany*
[b] *LCPackings, 1046 AA Amsterdam, The Netherlands*

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Abstract

Bioactive peptides and tryptic digests of various proteins were separated under acidic and alkaline conditions by ion-pair-reversed-phase high-performance liquid chromatography (RP-HPIPC) in 200 μm I.D. monolithic, poly(styrene-divinylbenzene)-based capillary columns using gradients of acetonitrile in 0.050% aqueous trifluoroacetic acid, pH 2.1, or 1.0% triethylamine-acetic acid, pH 10.6. Chromatographic performances with mobile phases of low and high-pH were practically equivalent and facilitated the separation of more than 50 tryptic peptides of bovine serum albumin within 15–20 min with peak widths at half height between 4 and 10 s. Neither a significant change in retentivity nor efficiency of the monolithic column was observed during 17-day operation at pH 10.6 and 50 °C. Upon separation by RP-HPIPC at high-pH, peptide detectabilities in full-scan negative-ion electrospray ionization mass spectrometry (negESI-MS) were about two to three times lower as compared to RP-HPIPC at low-pH with posESI-MS detection. Tandem mass spectra obtained by fragmentation of deprotonated peptide ions in negative ion mode yielded interpretable sequence information only in a few cases of relatively short peptides. However, in order to obtain sequence information for peptides separated with alkaline mobile phases, tandem mass spectrometry (MS/MS) could be performed in positive ion mode. The chromatographic selectivities were significantly different in separations performed with acidic and alkaline eluents, which facilitated the fractionation of a complex peptide mixture obtained by the tryptic digestion of 10 proteins utilizing off-line, two-dimensional RP-HPIPC at high pH × RP-HPIPC at low pH and subsequent on-line identification by posESI-MS/MS.

Keywords: Peptides; Monoliths; Positive mode; Negative mode; Liquid chromatography; Electrospray ionization mass spectrometry

1. Introduction

With the advent of high-throughput and highly sensitive protein identification technology based on mass spectrometry (MS) the efficient separation of complex mixtures of proteins and peptides has become more important than ever [1,2]. Csaba Horváth was not only one of the founders of modern high-performance liquid chromatography (HPLC) but also a pioneer in the development of high-resolution chromatographic techniques for peptide separation and analysis. His involvement in peptide separations began in the 1970's with the reversed-phase HPLC of bioactive peptides using octadecyl-silica columns and gradients of acetonitrile in phosphate buffer or perchloric acid [3]. In the years to follow, his investigations into reversed-phase HPLC of peptides included, among many others, high-speed analysis on micropellicular sorbents [4,5], displacement chromatography [6,7], separations at temperatures above 100 °C [8], the comparison of mobile phase additives [9], the separation of conformers [10], the use of monoliths for micro-HPLC and capillary electrochromatography [11,12], and applications to proteomic analysis [13–15].

* Corresponding author at: Instrumental Analysis and Bioanalysis, Saarland University, P.O. Box 151150, 66041 Saarbrücken, Germany. Tel.: +49 681 302 2433; fax: +49 681 302 2963.
E-mail address: christian.huber@mx.uni-saarland.de (C.G. Huber).
[1] Present address: Institute of Legal Medicine, Innsbruck Medical University, 6020 Innsbruck, Austria.

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The applied chromatographic conditions exert significant influence on the performance of peptide separations in a number of different ways, as has been clearly shown by Csaba Horváth and many other researchers. In order to tune retentivity and selectivity on reversed-phase stationary phases, various additives including inorganic or organic acids and bases have been incorporated into the chromatographic eluent [9,16–21]. In ion-pair reversed-phase HPLC (RP-HPIPC), which is performed in the presence of amphiphilic additives, peptide retention is effectuated by a combination both of solvophobic and electrostatic interactions [22]. Since peptides are generally amphoteric, RP-HPIPC can be in principle carried out at acidic pH using negatively charged amphiphiles such as acetate, trifluoroacetate, or perchlorate, or at basic pH with positively charged amphiphiles such as trietylammonium-, tetrabutylammonium-, or decyltrimethylammonium ion. The large majority of RP-HPIPC separation is carried out today at acidic pH in the presence of trifluoroacetic acid (TFA) or formic acid as additives [23], although a number of reports have been published on the separation of peptides at basic pH [17,18,24–26].

Recently, microcapillary RP-HPLC using alkaline mobile phases has been interfaced to electrospray ionization tandem mass spectrometry (ESI-MS/MS) for the analysis of complex mixtures of histocompatibility leukozyte antigen associated peptides [27]. A significant difference in chromatographic selectivity was demonstrated for peptide separations at alkaline pH as compared to acidic pH. Moreover, it was shown that separations at alkaline pH can be successfully interfaced to ESI-MS in the positive ion detection mode. Nevertheless, stability of the silica-based separation column under alkaline conditions was rather limited, necessitating its replacement already after one week of continual use. We have recently shown that monolithic stationary phases based on poly(styrene-divinylbenzene) (PS-DVB) are suitable for robust and high-resolution separations of peptides at elevated temperatures using TFA, heptafluorobutyric acid, and formic acid as mobile phase additives [28,29]. The nature of the additive has been shown to have a significant influence on the performance of the analytical system and the use of TFA as mobile phase additive represented the best compromise between optimal chromatographic and mass spectrometric conditions.

Since PS-DVB-based monolithic capillary columns can be anticipated to be stable at elevated pH, we investigated in this report their robustness and applicability to high-resolution peptide separations under basic conditions. ESI-MS(/MS) detection was performed both in positive and negative ion detection mode in order to shed light on the differences with respect to analyte detectabilities and the possibility to deduce sequence information about the separated peptides from the MS/MS spectra. Finally, separations under alkaline and acidic conditions were combined with the aim to establish a scheme for two-dimensional separation of complex peptide mixtures.

2. Experimental

2.1. Chemicals and samples

Acetonitrile (HPLC gradient-grade) was obtained from Riedel-de Haën (Seelze, Germany). Acetic acid, hydrochloric acid, imidazole, piperidine, ammonia, triethylamine, butyldimethylamine (all analytical reagent grade), and dithiothreitol were purchased from Aldrich (Milwaukee, WI, USA). Trifluoroacetic acid (TFA, for protein sequence analysis), iodoacetic acid, urea and ammonium carbonate were purchased from Fluka (Buchs, Switzerland). For preparation of all solutions, deionized water (18.2 MΩ cm) from a Purelab Ultra Genetic system (Elga, Griesheim, Germany) was used. Adjustment of the eluent pH was always performed on the neat aqueous solution (eluent A) by titrating a given concentration of amine with acid. Subsequently, the same concentration of additives was dissolved in acetonitrile, which served as the gradient former (eluent B).

Catalase (from bovine liver), cytochrome *c* (from horse heart), myoglobin (from horse heart), α-lactalbumin (from bovine milk), transferrin (human), carbonic anhydrase (from bovine erythrocytes), β-lactoglobulin A (from bovine milk), lysozyme (from chicken egg), serum albumin (bovine), ribonuclease A (bovine), oxytocin, and the mixture of nine bioactive peptides (P2693) were obtained from Sigma (St. Louis, MO, USA). Trypsin (sequencing grade modified) was purchased from Promega (Madison, WI, USA).

2.2. Preparation of tryptic digests

Proteins were denatured and reduced for 4 h at 37 °C in the presence of 8.0 mol/L urea, 0.50 mol/L ammonium hydrogencarbonate and 0.010 mol/L dithiothreitol followed by alkylation with iodoacetic acid (0.040 mol/L) for 20 min at 37 °C. The reduced and alkylated proteins were dialyzed against water in dialysis sacks (Sigma). Subsequently, trypsin was added to the protein mixture at an enzyme-to-substrate ratio of 1:50 and digestion was allowed to proceed for 12 h at 37 °C before stopping digestion by the addition of trifluoroacetic acid (1%, v/v). The final concentrations in the tryptic digests based on intact protein were as follows: 67.6 pmol/μL in the bovine serum albumin digest and 16.0 pmol/μL cytochrome *c*, 7.5 pmol/μL bovine serum albumin, 11.0 pmol/μL α-lactalbumin, 6.9 pmol/μL carbonic anhydrase, 3.5 pmol/μL catalase, 14.0 pmol/μL lysozyme, 11.0 pmol/μL myoglobin, 15.0 pmol/μL ribonuclease A, 2.5 pmol/μL transferrin, 14.0 pmol β-lactoglobulin A in the digest of 10 proteins.

2.3. High-performance liquid chromatography-electrospray ionization mass spectrometry

Monolithic capillary columns (60 mm × 0.20 mm I.D., 60 mm × 0.10 mm I.D. and 50 mm × 0.53 mm I.D.) were prepared according to the published protocol [30].

A 50 mm × 0.20 mm I.D. and 50 mm × 0.10 mm I.D. monolithic capillary columns are available from LC-Packings/Dionex (Monoliths, Amsterdam, The Netherlands). The Ultimate capillary HPLC system with a Famos autosampler (LC-Packings/Dionex) was used for HPLC–ESI-MS(/MS) experiments. Pneumatically assisted ESI-MS was performed with a quadrupole ion trap mass spectrometer (esquire HCT, Bruker Daltonics, Bremen, Germany) equipped with a pneumatically assisted electrospray ion source. The metal needle in the electrospray source was replaced by a 90 μm O.D./20 μm I.D. fused silica capillary, which was grounded and connected to the capillary column by means of a stainless steel microtight union (Fritz Gyger Swiss, Gwatt-Thun, Switzerland). Fully automated peptide identifications and database searches were performed using the MASCOT software package (Version 1.8, Matrix Science, London, UK). Because comprehensive detection of most of the peptides eluting from the column was very important to us in order to get an impression of the chromatographic performance of the analytical system peptides were considered as positively identified when the correlation between measured and simulated MS/MS spectrum yielded Mowse scores of ≥15. The acceptability of this relatively low Mowse score is also supported by the fact that the peptides expected in the protein mixture were well known and that false negatives were therefore very unlikely.

3. Results and discussion

3.1. RP-HPIPC of peptides under basic conditions

A number of different additives, including tetraethylammonium chloride or phosphate [17,24], tetrabutylammonium hydrogensulfate [18], dodecylamine [24], or decyltrimethylammonium bromide [25] proved useful for the separation of peptides under alkaline conditions. Interestingly however, all separation systems that have been realized so far in combination with ESI-MS detection were based on reversed-phase HPLC utilizing solutions of ammonia at pH 9.7–10.5 [26,27,31,32]. The starting point in our search for a positively charged amphiphile for RP-HPIPC–ESI-MS of peptides was triethylammonium ion, because it has been shown to facilitate very efficient separations of oligonucleotides and double stranded DNA on PS-DVB monoliths and to be compatible with sensitive ESI-MS detection [30].

Injection and elution of a mixture of nine bioactive peptides with a gradient of acetonitrile in 1.0% (v/v, corresponding to 72 mmol/L) aqueous triethylamine, pH 11.9, resulted in a very poor chromatogram with broad and unresolved peaks showing little retention (Fig. 1a). Upon titration of a 0.50% (36 mmol/L) triethylamine solution with acetic acid to pH

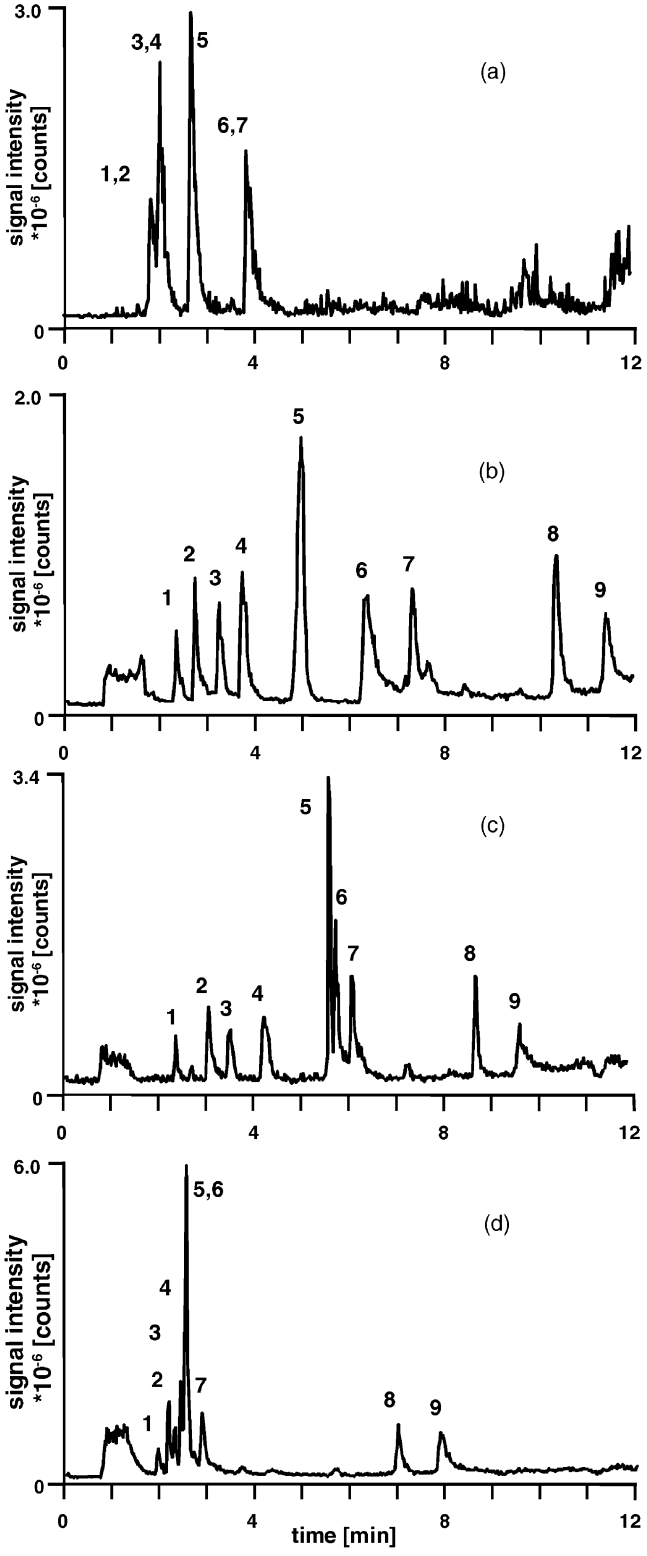

Fig. 1. RP-HPIPC–ESI-MS analysis nine bioactive peptides at alkaline pH. Column, PS-DVB monolith, 60 mm × 0.20 mm I.D.; mobile phase, 15-min linear gradient of 0–50% acetonitrile in (a) 72 mM aqueous triethylamine, pH 11.9, or in aqueous triethylamine-acetic acid, pH 10.6, of the following concentrations (b) 36/25, (c) 72/50, (d) 216/150 mmol/L; flow rate, 2.0 μL/min; temperature, 50 °C; detection, negative-mode ESI-MS, 3.5 kV spray voltage, *m/z* 500–1500 scan range; sample, (1) bradykinin fragment 1–5, (2) [Arg^8]-vasopressin, (3) methionine enkephalin, (4) leucine enkephalin, (5) oxytocin, (6) bradykinin, (7) luteinizing hormone releasing hormone (LHRH), (8) bombesin, (9) substance P, 1.25 ng each.

10.6, the chromatogram depicted in Fig. 1b was obtained, in which all nine peptides were completely separated to baseline. Because the eluent needed to be volatile for compatibility with ESI-MS, acetic acid was utilized instead of the more common phosphoric acid to titrate the eluent to the desired pH. With a gradient of 0–50% acetonitrile in 15 min, the separation window for the nine peptides was significantly broader in the basic eluent as compared to an acidic eluent containing 0.10% trifluoroacetic acid or heptafluorobutyric acid (compare Fig. 3 in ref. [29]). Nevertheless, the peaks were also broader and considerably tailing under the basic conditions, so that the total peak capacity of the two separation systems was quite similar.

In an attempt to improve peak shapes, the concentration of triethylamine was raised form 0.50 to 1.0% (72 mmol/L), resulting in the chromatogram shown in Fig. 1c. Both peak widths and peak asymmetries were significantly reduced. Compared to the separation with 0.50% triethylamine-acetic acid, the retention of the early eluting peptides remained practically unchanged, while that of the later eluting compounds was smaller. The average peak width at half height calculated for all nine peptides decreased from 8.2 s in Fig. 1b to 5.6 s in Fig. 1c. A further increase in the triethylamine concentration up to 3.0% (216 mmol/L) had relatively little influence on the average peak width (4.5 s), whereas separation selectivity was severely impaired as seven of the nine peaks eluted only partly resolved close to the void volume (Fig. 1d). The reduction in retention with increasing concentration of triethylamine in the eluent can be rationalized by the concomitant increase in acetic acid concentration needed to keep the pH constant. This results both in competitive adsorption of positively and negatively charged species at the hydrophobic surface as well as the weakening of electrostatic interactions due to the increase in the ionic strength of the mobile phase. In consequence, we decided to perform further optimization with an eluent containing 0.75–1.0% triethylamine.

3.2. Influence of type of amine and counterion on negESI-MS of peptides under basic conditions

The next step of optimization involved the investigation of different bases as potential amphiphiles. Fig. 2 illustrates the signal-to-noise ratios of the singly charged ions of four different peptides measured by direct infusion in 1.0% solutions of three different amines and ammonia. The average signal-to-noise ratios of 23 and 22, respectively, for the four peptides with 1.0% (72 mmol/L) triethylamine and 1.0% (72 mmol/L) butyldimethylamine were practically equivalent. The decrease in signal-to-noise ratios with 1.0% (101 mmol/L) piperidine is not very dramatic and to the most part a consequence of the higher ionic strength in the electrosprayed solution. For comparison, we also show in the diagram the signal-to-noise ratios with 1.0% ammonia, although it was not considered as a mobile phase additive because of its low tendency to adsorb to the stationary phase and act as an amphiphile. Since the difference between the

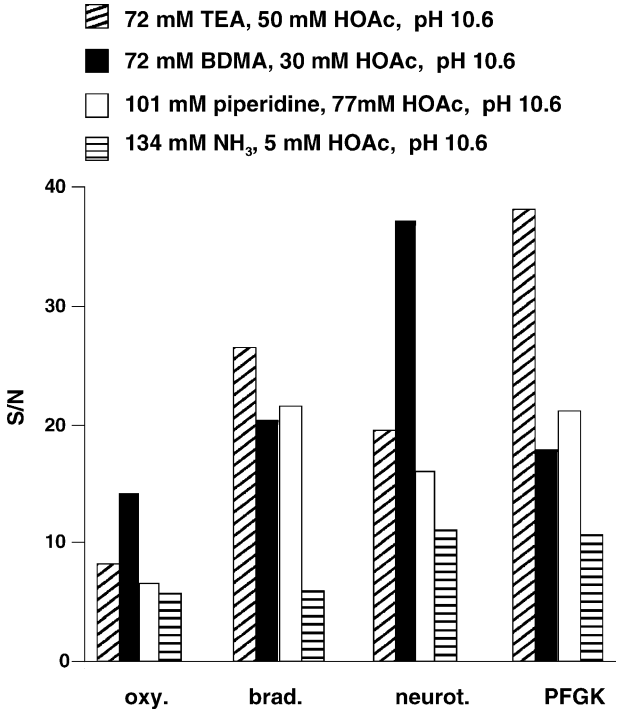

Fig. 2. Signal-to-noise ratios in the mass spectra obtained by direct infusion of peptides in solutions of different amines at pH 10.6. Direct infusion at 3 μL/min of 0.10 ng/μL oxytocin, bradykinin, neurotensin and PFGK in a water–acetonitrile (80:20) solution of 1.0% amine (TEA, triethylamine; BDMA, butyldimethylamine), titrated to pH 10.6 with acetic acid; detection, negative-mode ESI-MS.

amines was rather moderate, we continued optimization with triethylamine and butyldimethylamine.

In RP-HPIPC–ESI-MS of oligonucleotides we have seen that the counterion to the amphiphile exerted a considerable effect on detectability of the analytes by ESI-MS [33]. In due consequence, we examined the effect of different counterions on ESI-MS of peptides in the presence of triethylammonium ion as amphiphile. The mass spectra of 0.10 ng/μL oxytocin, which was electrosprayed from a solution of 1.0% triethylamine in water–20% acetonitrile, adjusted to pH 10.6 with formic-, acetic-, trifluoroacetic-, heptafluorobutyric-, and hydrochloric-acid, are shown in Fig. 3. The highest absolute signal as well as the largest signal-to-noise ratio was observed with triethylamine-acetic acid (Fig. 3b). While the mass spectrum with triethylamine-formic acid was quite similar (Fig. 3a), extensive adduction was detected with trifluoroacetic- and heptafluoroisobutyric-acid (Fig. 3c and d). The intensities of the mono-, bis- and tris-adducts with heptafluorobutyric acid relative to the non-adducted species were 70, 80, and 20%, respectively. This observation is in concordance with our previous findings in positive-mode RP-HPIPC–ESI-MS of peptides, in which heptafluorobutyric acid showed significant cluster-formation in the *m/z* range of 500–1000 [29]. In the spectrum recorded with chloride as counter ion, a hydrogen chloride adduct of 10% relative intensity was present. From a mass spectrometric point of

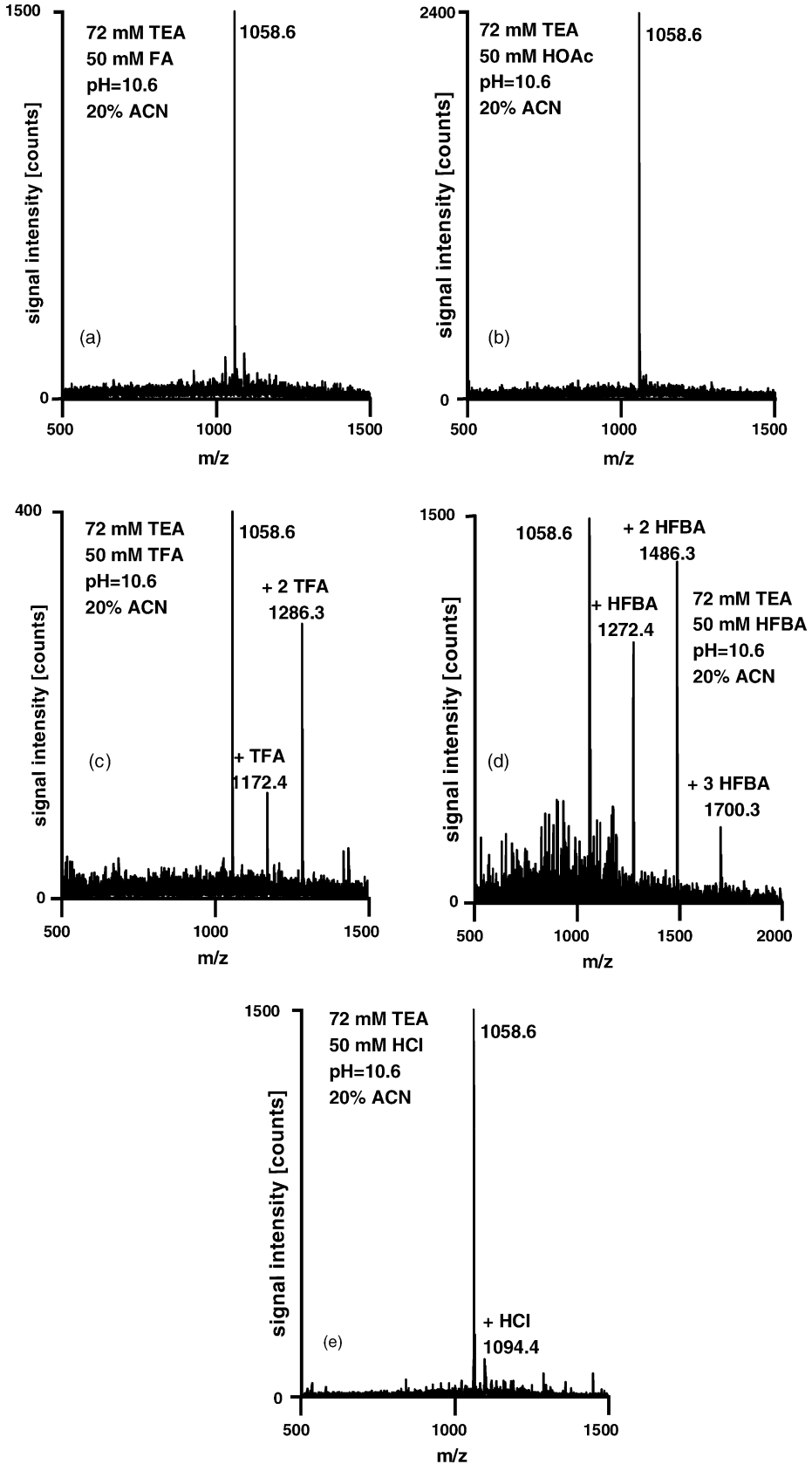

Fig. 3. Negative-mode mass spectra of bradykinin upon direct infusion in a 1.0% triethylamine (TEA) solution in water-acetonitrile (80:20) adjusted to pH 10.6 with (a) formic- (FA), (b) acetic- (HOAc), (c) trifluoroacetic (TFA)-, (d) heptafluorobutyric- (HFBA), (e) hydrochloric-acid (HCl). Direct infusion at 3 μL/min.

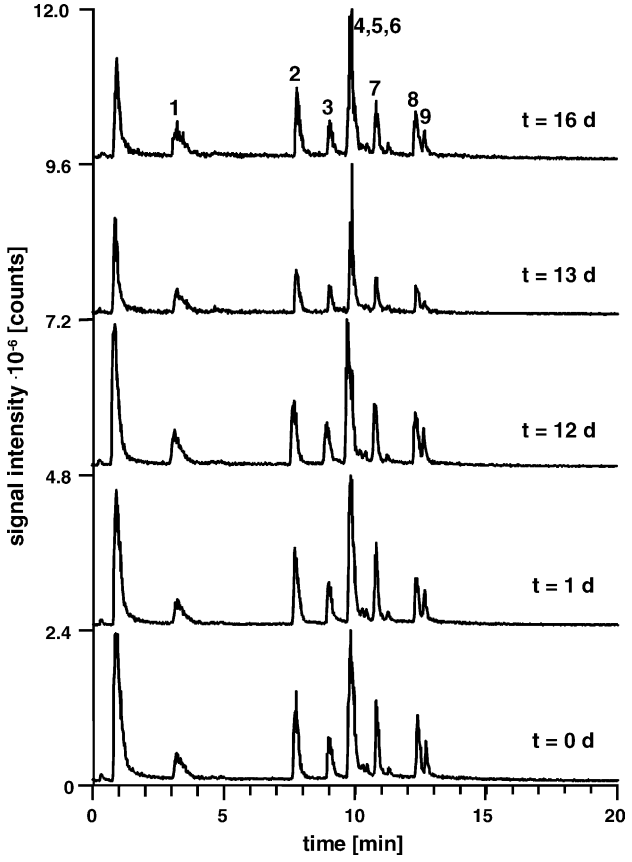

Fig. 4. Stability of monolithic separation columns operated at alkaline conditions. Mobile phase, 15-min linear gradient of 0–35% acetonitrile in 72 mmol/L triethylamine–50 mmol/L acetic acid, pH 10.6; temperature, 50 °C; detection, negative-mode ESI-MS; other conditions and peak identification as in Fig. 1.

view, we concluded that triethylamine-acetic acid represents a practicable combination in terms of ESI-MS detectability of peptides separated at alkaline pH.

3.3. Column stability at alkaline pH

In RP-HPIPC with silica-based stationary phases at alkaline pH, column stability represents a major concern [34]. Aqueous solutions of amines are particularly known to rapidly dissolve silica leading to a serious decrease in and final loss of column performance during extended use of the column with mobile phases having a pH above 8.0, especially at elevated column temperatures. With stationary phases based on organic polymers such as PS-DVB monoliths on the other hand, pH stability is not an issue. However, high-pH mobile phases may attack the fused silica tubing containing the monolith and destroy the chemical bonds that immobilize the porous monolith onto the wall of the capillary.

Nevertheless, we did not see any signs of column degradation or mobilization of the column bed during the extensive use of the monolithic columns at elevated temperature and pH, as illustrated in Fig. 4 through repeated separations of the nine-peptide mixture at pH 10.6 and 50 °C. Neither a decrease in retention nor in column efficiency could be observed over a 17-day period of permanent operation under alkaline conditions. This column stability study also revealed the high day-to-day reproducibility of peptide separations at alkaline pH on PS-DVB monolithic columns. The relative standard deviations in the retention times of the peptides [Arg^8]-vasopressin (2), methionine enkephalin (3), and luteinizing hormone releasing hormone (7) determined from 17 measurements were between 0.46 and 0.73%, while the variation in peak widths at half height for the same peaks was 3.5–7.7%. This data specify RP-HPIPC at high pH with monolithic PS-DVB columns as a very robust separation system for peptides.

3.4. Comparison of separation selectivity and analyte detectability under acidic and alkaline conditions

The retention of peptides in RP-HPIPC is strongly affected by eluent pH because of the interplay of solvophobic and electrostatic interaction. The latter heavily depends on the net charge of the peptides, which is determined by peptide sequence and solution pH. Hence, we can expect that a change in the pH of the chromatographic eluent exerts a significant impact on the chromatographic selectivity, which might eventually result in complementary selectivities in separations of peptide mixtures at acidic and alkaline pH. To elucidate the effect of eluent pH and amphiphile on selectivity, a tryptic digest of bovine serum albumin was chromatographed at pH 9.5 with triethylammonium ion and at pH 2.1 with trifluoroacetate as amphiphiles. For comparability of the retentivity of both separation systems, a gradient of 0–40% acetonitrile was applied in both experiments.

As can be deduced from the chromatograms shown in Fig. 5, retention times were larger and the separation window under an identical acetonitrile gradient was narrower with 0.050% (6.5 mmol/L) trifluoroacetic acid as additive as opposed to 0.75% (54 mmol/L) triethylamine-acetic acid. On the other hand, the average peak widths at half height of the eluting peptides in extracted ion chromatograms were 5.9 s under alkaline versus 4.8 s under acidic conditions. Altogether, there was only a small and most probably insignificant difference between the peak capacities in a given time window with the two separation systems (172 versus 156 for triethylamine-acetic acid and trifluoroacetic acid, respectively). The total number of tryptic peptides of bovine serum albumin without any missed cleavages, detected on the basis of their intact molecular masses under alkaline and acidic conditions, was 47 and 48, respectively, covering a practically identical portion of the total sequence of serum albumin (81.5 and 82.9%, respectively). Due to the scan range setting to *m/z* 500–1500, the peptide DTHK was detected only in posESI-MS ($[M+H]^+$, *m/z* 500.2) and not in negESI-MS ($[M-H]^-$, *m/z* 498.2). Including peptides containing missed cleavage sites, the total number of detected peptides increased significantly, but again, there was no significant difference between RP-HPIPC at low- and high-pH, respectively.

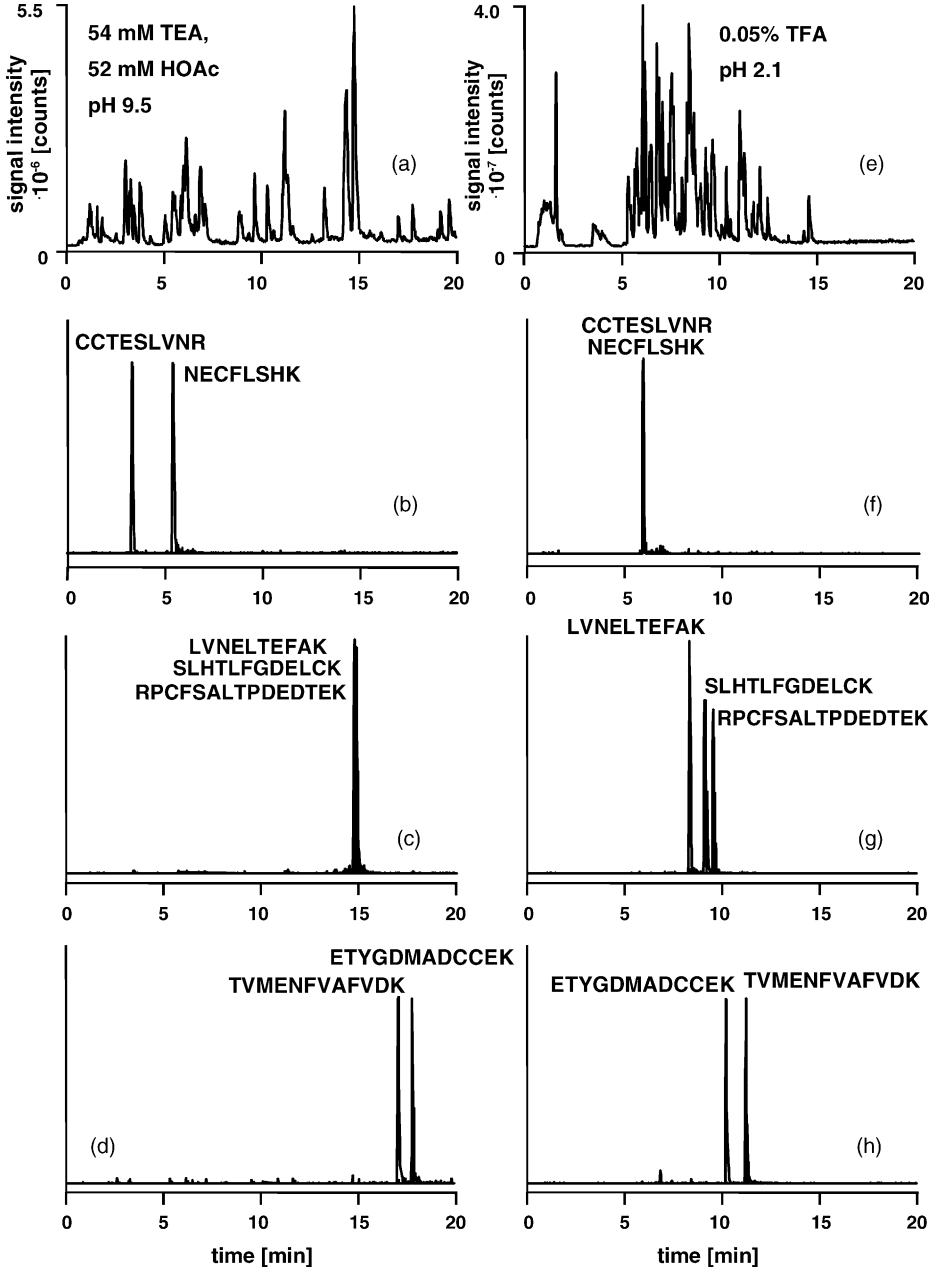

Fig. 5. RP-HPIPC-MS analysis of a tryptic digest of bovine serum albumin at alkaline (a–d) and acidic (e–h) conditions. Mobile phase, 20-min linear gradient of 0–40% acetonitrile in (a–d) 54 mM triethylamine–52 mM acetic acid, pH 9.5, or (e–h) 0.05% trifluoroacetic acid, pH 2.1; sample, 2 pmol tryptic digest of bovine serum albumin; (a) and (e), reconstructed total ion current chromatograms, (b–d) and (f–h), extracted ion chromatograms of selected peptides, detection, negative-mode ESI-MS (a–d), positive-mode ESI-MS (e–h), other conditions as in Fig. 1.

Despite the similar performances of RP-HPIPC at low- and high-pH it is interesting to have a closer look at the retention times and elution orders of individual peptides in both separation systems. The two peptides CCTESLVNR and NECFLSHK, for example, were fully separated with a resolution of 9.9 under basic conditions (Fig. 5b), whereas they completely coeluted at acidic pH (Fig. 5f). A strong contribution of solvophobic interaction to retention can be deduced from the fact that the longer peptide elutes first and that both peptides contain the same number of acidic residues, giving rise to similar electrostatic interactions.

Another example for the difference in selectivity are three peptides of different length (decapeptide, dodecapeptide, and pentadecapeptide) coeluting with triethylamine-acetic acid (Fig. 5c) and completely separated with trifluoroacetic acid (Fig. 5g). Finally, we could find peptides of identical length (ETYGDMADCCEK and TVMENFVAFVDK) that were well separated in both chromatographic systems, however, with reversed elution order (Fig. 5d and h). Comparing the sequences or the two peptides, one will find that ETYGDMADCCEK contains two glutamates and two aspartates, whereas TVMENFVAFVDK contains only one each. As expected, the

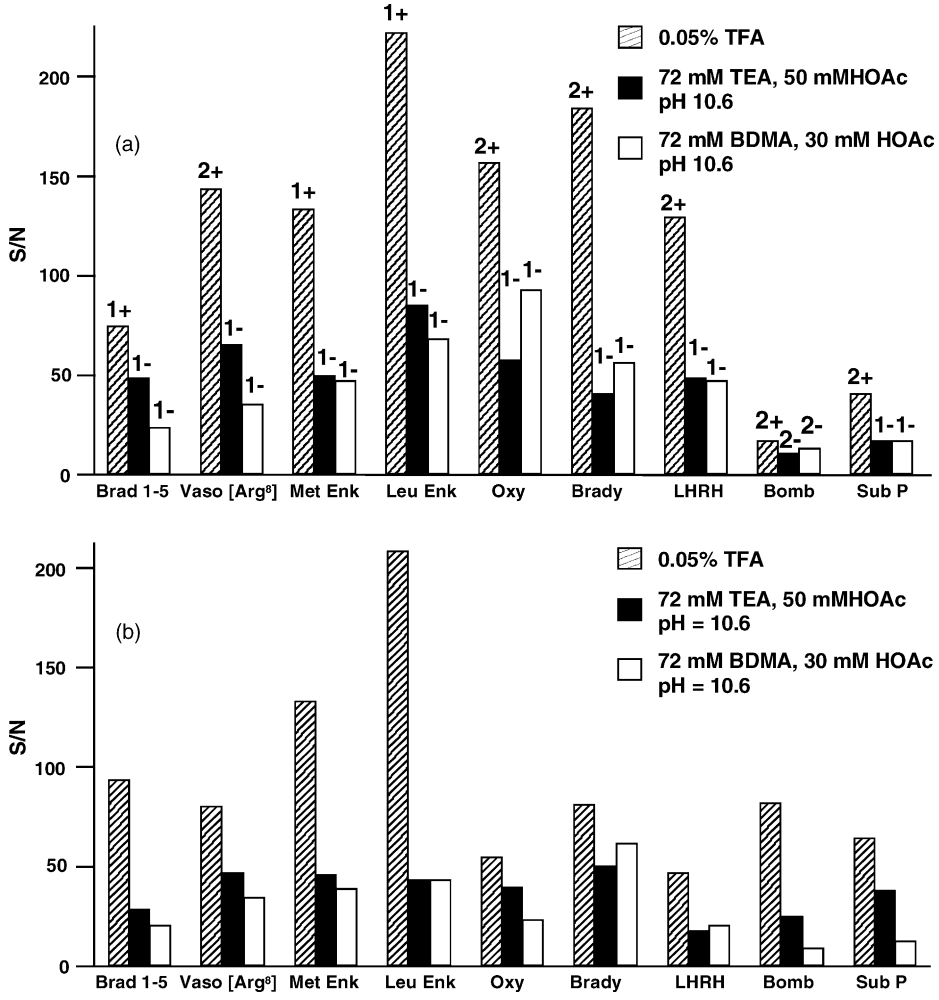

Fig. 6. Signal-to-noise ratios in the extracted ion chromatograms (a) and mass spectra (b) of peptides analyzed at basic and acidic conditions. Mobile phase, 15-min linear gradient of 0–50% acetonitrile in 0.05% trifluoroacetic acid, pH 2.1, 72 mmol/L triethylamine-50 mmol/L acetic acid, pH 10.6, or 72 mmol/L butyldimethylamine-30 mmol/L acetic acid, pH 10.6; detection, positive-mode ESI-MS with trifluoroacetic acid, negative-mode ESI-MS with triethylamine-acetic acid and butyldimethylamine-acetic acid, other conditions as in Fig. 1. Numbers over the bars indicate the charge of the most abundant peptide ion.

peptide with the higher number of acidic residues elutes later under the basic conditions (Fig. 5d).

The differences in the chromatographic and mass spectrometric detectabilities with the various eluents were evaluated by measuring the signal-to-noise ratios for the nine bioactive peptides both in extracted ion chromatograms and extracted full-scan mass spectra, analogously to the comparison recently performed on acidic additives [29]. Fig. 6a clearly reveals that the signal-to-noise ratios in the extracted ion chromatograms were generally highest with trifluoroacetic acid as mobile phase additive with posESI-MS (average signal-to-noise ratio of 122), while triethylamine-acetic acid and butyldimethylamine-acetic acid gave 2.6–2.7 times lower average ratios in negESI-MS (average ratios of 47 and 45, respectively). The picture was quite similar in the mass spectra extracted at the chromatographic peak maxima (Fig. 6b), yielding average signal-to-noise ratios of 86, 34, and 27 for trifluoroacetic acid, triethylamine-acetic acid, and butyldimethylamine-acetic acid, respectively.

Looking at the sequences of the detected peptides it is interesting to note that the detectabilities do not seem to be related to amino acid sequence or charge state of the peptide. In positive ion mode, the distribution of singly and doubly charged ions was almost balanced, whereas predominantly singly deprotonated peptides were observed in the negative ion mode. Surprisingly, the only doubly deprotonated peptide bombesin has an amidated carboxyterminus and does neither contain aspartate nor glutamate. The two peptides showing the largest difference in signal-to-noise ratios between acidic and alkaline conditions, specifically methionine enkephalin (YGGFM) and leucine enkephalin (YGGFL), do not contain any basic nor acidic amino acid but have an aminoterminal tyrosine, which could be deprotonated at pH values higher than 10. The observed general decrease in detectabilities of peptides by a factor of about 2–3 upon changing from acidic to basic conditions is mainly due to the higher ionic strength in the alkaline eluents. However, a reduction in ionic strength of the alkaline eluents is achievable only at the cost

of a decrease in chromatographic performance (see Fig. 1). Hence, we considered an eluent containing 0.75–1.5% (54–72 mmol/L) triethylamine as the best compromise between optimal chromatographic and mass spectrometric performance.

3.5. Peptide identification by MS/MS in positive and negative ion mode

One of the most successful experimental approaches to high-throughput peptide identification is fractionation by RP-HPIPC at acidic pH and subsequent peptide fragment fingerprinting by MS/MS in positive ion mode [35]. Recently, it was reported that peptides can be separated by high-pH RP-HPIPC and subsequently detected by posESI-MS/MS, taking advantage of the broader separation window of the former and higher detection sensitivity of the latter technique [27]. Other investigations revealed that peptides can be successfully sequenced by ESI-MS/MS using collision-induced fragmentation and mass analysis in the negative ion mode [36]. Nevertheless, compared to fragmentation and mass analysis in the positive ion mode it was frequently found that ion series obtained from deprotonated peptide ions frequently show gaps or even truncation, which makes the deduction of useful sequence information especially for longer peptides challenging, if not impossible [37]. As far as we know, experiments to get peptide sequence information in the negative ion mode were always carried out by direct infusion of the peptides in alkaline solution, which facilitates relatively long periods of measurement and the accumulation of many mass spectra. During HPLC–ESI-MS/MS analysis on the other hand, the recording of high-quality mass spectra by spectrum accumulation becomes far more challenging, as the transient peaks of only a few seconds eluting from the columns only allow the recording of a few scans.

For a direct comparison of the performance of peptide identifications using various combinations of separation and ion detection modes, we prepared tryptic peptides from a mixture of 10 proteins, separated them by RP-HPIPC at low- and high-pH and detected the eluting peptides by MS/MS in positive and negative ion mode. Exhaustive trypsin-digestion of the 10 proteins yields 229 peptides and even more peptides could be expected to be present as a result of missed cleavages. Fig. 7a illustrates the base peak chromatogram of the peptide mixture separated at pH 2.1 and detected in the positive ion mode. Detection and identification were accomplished by data-dependent MS/MS, in which one full scan mass spectrum was recorded for precursor ion selection followed by three MS/MS scans on the three most abundant precursor ions. Fully automated database searches using the MASCOT software package yielded 115 distinct peptide identifications, which covered 46.5% of the total sequence of the 10 proteins in the mixture. A typical MS/MS spectrum of the peptide KVPQVSTPTLVEVSR form bovine serum albumin together with MASCOT identification data and the fragment ion annotations is depicted in Fig. 7b.

The separation performed at high pH and monitored by negESI-MS clearly gave more chromatographic peaks (Fig. 7c) as compared to those obtained at low pH. Again, precursors were automatically selected from full scan mass spectra in negative ion mode and subjected to fragmentation and mass analysis. Due to the lack of software routines that allow database searching and identification of peptides in negative ion mode, MS/MS spectra were extracted from the total ion current chromatogram and screened manually for fragment ions of the α- and β-type, which are the equivalent to b- and y-type fragment ions common in positive ion MS/MS spectra of peptides. As can be seen from the MS/MS spectrum of KVPQVSTPTLVEVSR illustrated in Fig. 7d, however, only two abundant signals were present and none of the signals could be annotated to α/β fragment ions, so that it was impossible to obtain useful sequence information under these conditions.

Another spectrum extracted from the base peak chromatogram around 2 min clearly showed a number of α-type fragment ions, which could be readily assigned to the peptide SLGKVGTR from bovine serum albumin (Fig. 7e). Nonetheless, careful inspection of a high number of MS/MS spectra revealed that interpretable sequence information was rather the exception than the rule and mostly restricted to rather short peptides. Moreover, a change in fragmentation energy had no significant impact on the quality of mass spectra. Hence, we cannot consider RP-HPIPC at high pH in combination with negESI-MS/MS as a viable alternative to RP-HPIPC at low pH with posESI-MS/MS detection for high-throughput peptide identification based on peptide fragment fingerprinting.

Finally, peptide separation at high pH was hyphenated to detection using ESI-MS/MS in the positive ion mode, as recently suggested [27]. Not unexpectedly, the chromatogram depicted in Fig. 7f shows a high similarity with that in Fig. 7c in terms of peak number and retention times, whereas the pattern of peak intensities was significantly altered due to differences in ionization efficiencies in positive- and negative-ion mode. A comparison of the posESI-MS/MS mass spectra of the peptide KVPQVSTPTLVEVSRA electrosprayed form acidic (Fig. 7b) and alkaline eluents (Fig. 7g) revealed some differences in the annotated fragment ions, which, however, both enabled the unequivocal identification of the peptide in both MS/MS spectra. The total number of peptide identifications in this mode was 37 and hence, significantly smaller than that found in RP-HPIPC at low pH posESI-MS/MS, most probably due to a decrease in ionization efficiency in posESI of peptides electrosprayed from alkaline solutions. The results obtained here are in contrast to those observed by Tomlinson and Chicz [27], who reported a significant increase in the detectabilities of peptides separated at high pH and detected in posESI-MS compared to separations at low pH. Nevertheless, we believe that this has to be attributed to a rather high ionic strength in the eluent utilized for elution at low pH.

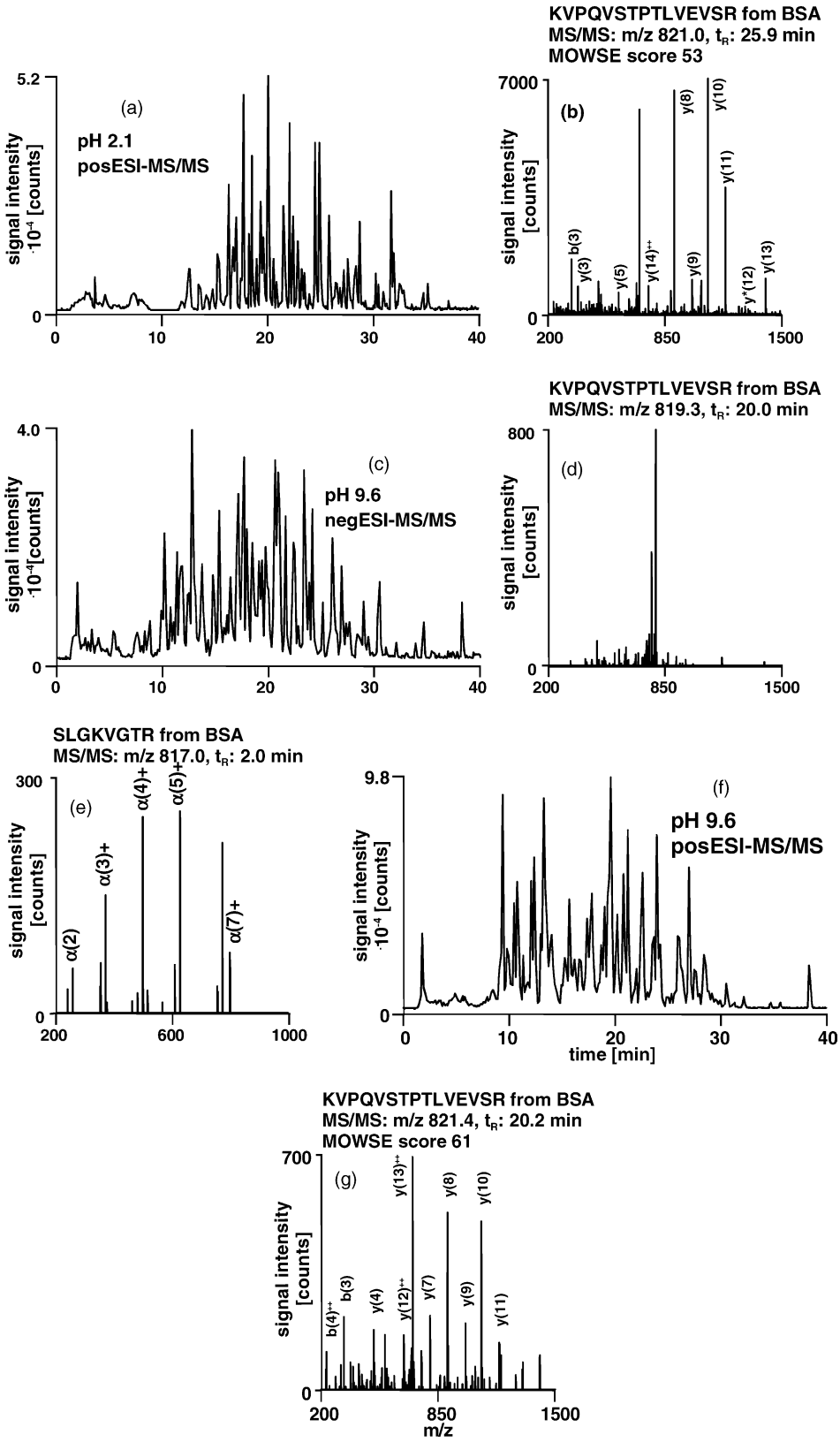

Fig. 7. Shotgun identification of proteins by separation of peptides under acidic and alkaline conditions and ESI-MS/MS detection in positive and negative ion mode. Mobile phase, 40-min linear gradient of 0–40% acetonitrile in (a) 0.05% trifluoroacetic acid, pH 2.1 and (c and f) 54 mmol/L triethylamine–52 mmol/L acetic acid, pH 9.6; MS/MS detection, data dependent MS/MS of the three most intense precursor ions with 1.5 V collision energy; detection, positive-mode ESI-MS/MS (a, b, f, g), negative-mode ESI-MS/MS (c–e); sample, 1:25 diluted tryptic digest of 10 proteins.

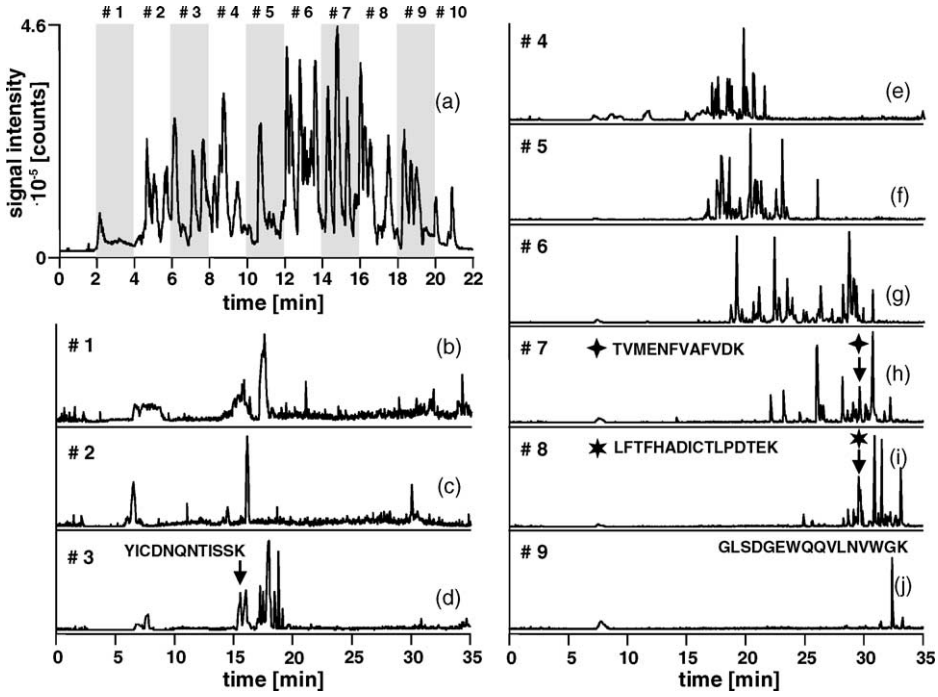

Fig. 8. Two-dimensional separation and identification of tryptic peptides from a mixture of 10 proteins by RP-HPIPC at high pH × RP-HPIPC–posESI-MS at low pH. First dimension: 50 mm × 0.53 mm I.D. PS-DVB monolith; linear gradient 0–30% acetonitrile in 72 mmol/L triethylamine–65 mmol/L acetic acid, pH 10.0 in 20 min; flow rate, 18 μL/min; temperature, 50 °C; sample, 0.50 μL of undiluted tryptic digest of 10 proteins; detection, negative-mode ESI-MS. Second dimension: 50 mm × 0.10 mm PS-DVB monolith; linear gradient, 0–35% acetonitrile in 6.5 mmol/L TFA, pH 2.1 in 20 min; flow rate, 500 nl/min; temperature, 50 °C; detection, positive-mode ESI-MS; sample, 10 fractions from first dimension, 1.0 μL injected.

3.6. Two-dimensional separations using monolithic columns

The experiments described in Section 3.4 have clearly demonstrated that peptide separations at acidic and alkaline pH have a strongly complementary nature. In due consequence, we examined the potential of a combination of RP-HPIPC at high pH with RP-HPIPC–posESI-MS at low pH for the comprehensive analysis of complex peptide mixtures. The first dimension of separation comprised a 50 mm × 0.53 mm I.D. monolithic capillary column that was loaded with tryptic peptides from a mixture containing between 1.2 pmol (transferrin) and 8.1 pmol (cytochrome *c*) of 10 different proteins. The peptides were eluted with a gradient of 0–30% acetonitrile in triethylamine-acetic acid and collected in 2-min intervals into 10 fractions of approximately 36–40 μL. Because UV detection at 215 nm was not sensitive in the eluent containing triethylamine and acetic acid, we show in Fig. 8a a representative base peak chromatogram, in which the peptides in the column effluent were detected by negative-mode ESI-MS. In order to remove acetonitrile from the fractions, which would otherwise serve as a strong starting eluent in the second dimension, they were evaporated to half the volume under a gentle stream of nitrogen and reconstituted with 20 μL water to give a final volume of 40 μL. Subsequently, 1-μL aliquots of the fractions were re-injected onto a 50 mm × 0.10 mm I.D. monolithic column and eluted in the second dimension under acidic conditions with a gradient of 0–35% acetonitrile in trifluoroacetic acid.

It can be recognized from the base peak chromatograms shown in Fig. 8b–j that peptides isolated in one fraction of RP-HPIPC at high-pH were properly separated in the second dimension of RP-HPIPC at low-pH. The earliest eluting peptide in all runs of the second dimension was YICDNQDTISSK from bovine serum albumin at 15.5 min in fraction 3 (Fig. 8d), and the latest eluting peptide was GLSDGEWQQVLNVWGK from myoglobin at 32.7 min in fraction 9 (Fig. 8j), indicating a total effective separation window in the second dimension of approximately 17 min. The relatively late elution of the first peptide is because of the large gradient delay time with the 0.10 mm I.D. monolithic column. The mechanism of ion-pair reversed-phase separation under the two conditions is not fully orthogonal because strongly hydrophobic peptides generally tend to retain more strongly on reversed-phase stationary phases. Nonetheless, an influence of peptide hydrophobicity on retention is also observable to a considerable extent in peptide separations by ion-exchange HPLC, which represents at the time the most commonly utilized mode for combination with RP-HPIPC in multidimensional peptide separation schemes. A major advantage with our off-line two-dimensional separation scheme rests within the total absence of salts that are necessary to elute the peptides from ion-exchange columns and which have to be removed by extensive washing protocols before transfer of the peptides to the second dimension.

Table 1
Number of peptides and their average molecular masses in the fractions obtained by high-pH RP-HPIPC

	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8	Fraction 9
Number of identified peptides	2	1	4	13	23	29	11	12	2
Average M_r	1374.1	1751.6	1697.7	1378.3	1381.3	1476.0	1782.9	1688.1	1814.9

As an example for the benefits of two-dimensional separation we look at the identification of two peptides contained in fractions numbers 7 and 8 of RP-HPIPC at high-pH, namely the peptides TVMENFVAFVDK and LFTFHADICTLPDTEK coming from bovine serum albumin (Fig. 8h and i). They eluted at exactly the same position in the chromatogram of the reversed phase separation at low-pH (29.6 min) and there was a good chance that only one of them would be selected for fragmentation if they were not present in two different fractions. Nevertheless, both peptides were automatically selected in the two fractions as precursors for fragmentation. High-quality MS/MS spectra were recorded in positive ion mode, which enabled their confident identification by automated database search yielding Mowse scores of 83 and 50, respectively.

Table 1 summarizes the number of peptides that were identified by low-pH-RP-HPIPC–posESI-MS/MS in the 10 fractions of high-pH RPIPC. The lack of an influence of peptide molecular mass on retention in the first dimension can be inferred from the average molecular masses found in the different fractions, which did not show any correlation with the fraction number (Table 1). As expected from the first-dimension separation (Fig. 8a), most of the peptides were found in fractions numbers 3–8, which accounted for 92 of total 97 peptides identified. None of the peptides was detected in more than two adjacent fractions, indicating that all peptides eluted as well defined bands in the first dimension of separation. This is in contrast to ion-exchange fractionations, in which some peptides were found to be distributed over several fractions [38] or even elute in isolated fractions. Several unique peptides were detected for all 10 proteins present in the mixture. The sequence coverages for the individual proteins ranged between 23% for catalase and 53% for bovine serum albumin, the average sequence coverage being 40%.

4. Conclusions

High-resolution peptide separations can be carried out at elevated temperature and both at acidic and alkaline pH due to the high chemical and mechanical stability of PS-DVB-based monolithic capillary columns. Using volatile acids and/or bases as mobile phase additives, detection can be accomplished in negative or positive ion detection mode. In terms of column efficiency and peak capacity, both separation modes perform equivalent, whereas on-line detection utilizing positive-ion ESI-MS is two to three time more sensitive than negative-ion ESI-MS. Studies into the feasibility of peptide fragment fingerprinting by RP-HPIPC–negESI-MS/MS revealed that the obtained MS/MS spectra did not generally contain interpretable sequence information, especially for longer peptides.

The previous demonstration of the applicability of chromatographic separation at high pH in combination with positive ion ESI-MS/MS is confirmed in our experiments. However, we observe a significant reduction in the number of successful peptide identifications when this mode of peptide separation and detection was utilized. Although the selectivities of separations performed at low- and high pH are not fully orthogonal, the two separation modes show a considerable degree of complementarity making them highly suitable for implementation in an off-line, two-dimensional separation scheme that is applicable to complex peptide mixture analysis. Compared to ion-exchange chromatography, which is frequently applied as a first dimension in multidimensional peptide separation schemes, high-pH-RP-HPIPC holds the advantages of higher column efficiency, the absence of any salt that has to be removed carefully before mass spectrometric investigation, the possibility of concentrating the fractions before transfer to the second dimension effortlessly by evaporation of the hydro-organic eluent, and minimal carryover between the fractions because of well defined and relatively sharp chromatographic peak profiles.

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