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Operational variables in high-performance liquid chromatography–electrospray ionization mass spectrometry of peptides and proteins using poly(styrene–divinylbenzene) monoliths

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Abstract

Capillary reversed-phase high-performance liquid chromatography (RP-HPLC) utilizing monolithic poly(styrene–divinylbenzene) columns was optimized for the coupling to electrospray ionization mass spectrometry (ESI-MS) by the application of various temperatures and mobile phase additives during peptide and protein analysis. Peak widths at half height improved significantly upon increasing the temperature and ranged from 2.0 to 5.4 s for peptide and protein separations at 70 °C. Selectivity of peptide elution was significantly modulated by temperature, whereas the effect on proteins was only minor. A comparison of 0.10% formic acid (FA), 0.050% trifluoroacetic acid (TFA), and 0.050% heptafluorobutyric acid (HFBA) as mobile phase additives revealed that highest chromatographic efficiency but poorest mass spectrometric detectabilities were achieved with HFBA. Clusters of HFBA, water, and acetonitrile were observed in the mass spectra at *m/z* values >500. Although the signal-to-noise ratios for the individual peptides diverged considerably both in the selected ion chromatograms and extracted mass spectra, the average mass spectrometric detectabilities varied only by a factor of less than 1.7 measured with the different additives. Limits of detection for peptides with 500 nl sample volumes injected onto a 60 mm × 0.20 mm monolithic column were in the 0.2–13 fmol range. In the analysis of hydrophobic membrane proteins, HFBA enabled highest separation selectivity at the cost of lower mass spectral quality. The use of 0.050% TFA as mobile phase additive turned out to be the best compromise between chromatographic and mass spectrometric performance in the analysis of peptides and proteins by RP-HPLC–ESI-MS using monolithic separation columns.
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Keywords: Monolithic columns; Proteins; Peptides; Poly(styrene–divinylbenzene)

1. Introduction

The concept of comprehensive proteome analysis by utilizing combined high-resolution separation methods and mass spectrometry (MS) has revolutionized modern biochemical, biological, and biomedical research [1]. Mass spectrometric analysis of proteins has benefited tremendously form several major technical advances during the past two decades, including the introduction of electrospray ionization (ESI) [2] and matrix-assisted laser desorption/ionization (MALDI) [3], the development of fragmentation-based sequencing methods for peptides [4,5], as well as the availability of high-performance mass analyzers [6,7] such as quadrupole ion trap, Fourier-transform ion cyclotron, or delayed extraction reflectron time-of-flight mass analyzers. Successful separation and purification of the analytes before mass spectrometric investigation, on the other hand, remains the bottleneck of proteome analysis in the face of extreme sample complexity, ranging from a few thousand proteins in cell organelles [8] to a few hundred thousand proteins in blood serum [9]. In due consequence, major advances in separation technology are mandatory.

Two-dimensional gel electrophoresis (2D-GE) [10] has been the standard method for high-resolution protein separations over the past 25 years, mainly because of its unique

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ability to fractionate several thousand proteins in a single run. Gel-electrophoretic methods have been complemented by high-performance liquid chromatography (HPLC) [11], which offers the additional advantages of full automation, quantitative capability, compatibility with very small, very large, and strongly hydrophobic proteins, and the possibility for direct interfacing to MS, however, at the cost of lower separation performance. The latter limitation was overcome in part by multidimensional HPLC upon combination of two, ideally orthogonal, chromatographic separation modes [12,13].

The most popular chromatographic mode for high-resolution separation of peptides or proteins is reversed-phase (RP) chromatography, involving gradients of acetonitrile in aqueous solutions of organic acids in conjunction with hydrophobic stationary phases. Nevertheless, acidic additives dissociate forming carboxylate ions, which adsorb to the stationary phase due to their amphiphilic properties with a tendency increasing with their hydrophobicity. The result is the formation of a surface potential which is characteristic for ion-pair reversed-phase (IP-RP) chromatography [14,15]. Consequently, both solvophobic interaction between hydrophobic sections of the analytes and the hydrophobic surface as well as electrostatic interaction between charged analyte functionalities and amphiphilic ions adsorbed onto the hydrophobic surface contribute to chromatographic retention.

Despite considerable advances, column technology still holds the most promising potential for significant improvements in chromatographic separation performance and miniaturization. Macroporous butyl- or octadecyl-silica particles having 10–30 nm pores represent the most common stationary phases for RP separations, but slow mass transfer entails analysis times of several hours for complex protein or peptide mixtures [16]. Polymeric stationary phases based on poly(styrene–divinylbenzene) (PS–DVB) have been shown to represent an alternative to silica-based stationary phases for rapid and efficient peptide and protein separations. In order to enhance mass transfer, these stationary phases have been applied in the configurations of totally non-porous particles [17,18], particles with a bimodal pore size distribution [19], or as monolithic column beds [20,21]. The polymers are chemically stable even at elevated column temperatures, which is desirable for rapid and high-resolution separations. The rapid mass transfer in micropellicular, monolithic column beds has enabled highly efficient peptide and protein separations at 50–70 °C with peak capacities of 80–130 within 10 min [22].

The operational variables for chromatographic peptide and protein separations have been studied in detail with silica-based reversed-phase stationary phases, for which the suppression of silanophilic interactions with basic analytes such as peptides and proteins by means of suitable mobile phase additives plays an important role [23,24]. However, such electrostatic secondary interactions do not need to be considered with polymeric, PS–DVB-based stationary phases, leaving more freedom in the choice of separation conditions. If the chromatographic separation is to be hyphenated to ESI-MS [25,26], the mobile phase composition needs to be optimized with respect to sensitive analyte detection, which is more than ever important for proteomic applications [27]. In order to fully exploit the potential of PS–DVB stationary phases of monolithic column configuration for high-resolution peptide and protein separations, we studied here the effects of column temperature and mobile phase composition both on chromatographic and mass spectrometric performance in RP-HPLC–ESI-MS systems.

Table 1
Standard proteins and peptides used in this study

Number[a]	Protein or peptide	Origin or sequence[b]	Molecular mass
Proteins			
1	Trypsin inhibitor	Soybean	20094.0
2	Ribonuclease A	Bovine pancreas	13682.3
3	Cytochrome *c*	Horse heart	12360.2
4	Lysozyme	Chicken egg	14305.2
5	Transferrin	Human	79593.6
6	β-Lactoglobulin B	Bovine milk	18276.3
7	β-Lactoglobulin A	Bovine milk	18362.4
8	Catalase	Bovine liver	57585.6
9	Ovalbumin	Chicken egg	43000–45000
Peptides			
1	Bradykinin fragment 1–5	RPPGF	572.3
2	[Arg^8]Vasopressin	**C**YFQN**C**PRG-amide	1083.5
3	Methionine enkephalin	YGGFM	573.3
4	Leucine enkephalin	YGGFL	555.3
5	Oxytocin	**C**YIQN**C**PLG-amide	1006.5
6	Bradykinin	RPPGFSPFR	1059.6
7	Luteinizing hormone releasing hormone (LHRH)	pEHWSYGLRPG-amide	1181.6
8	Bombesin	pEQRLGNQWAVGHLM-amide	1617.9
9	Substance P	RPKPQQFFGLM	1347.8

[a] Numbers used to identify the peaks in the chromatograms.
[b] Cysteine residues in bold letters indicate disulfide bridges; pE denotes pyroglutamic acid.

2. Experimental

2.1. Chemicals and samples

Acetonitrile (HPLC gradient-grade) was obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA, for protein sequence analysis), heptafluorobutyric acid (HFBA, analytical reagent grade) and formic acid (FA, analytical reagent grade) were purchased from Fluka (Buchs, Switzerland). All standard proteins (Table 1) and the peptide standard (P2693, Table 1) were obtained from Sigma (St. Louis, MO, USA). The sample of Lhcb proteins isolated from spinach was kindly donated by L. Zolla and A.M. Timperio (University of Viterbo, Italy). Trypsin (sequencing grade modified) was obtained from Promega (Madison, WI, USA). Water was purified by a NANOpure-Infinity water deionization system from Barnstead (Dubuque, IA, USA). Concentrations of the mobile phase additives are given as % (v/v) throughout the text.

2.2. High-performance liquid chromatography and interfacing with electrospray ionization mass spectrometry

The Ultimate fully integrated capillary HPLC system (LC Packings, Amsterdam, The Netherlands) with a column oven

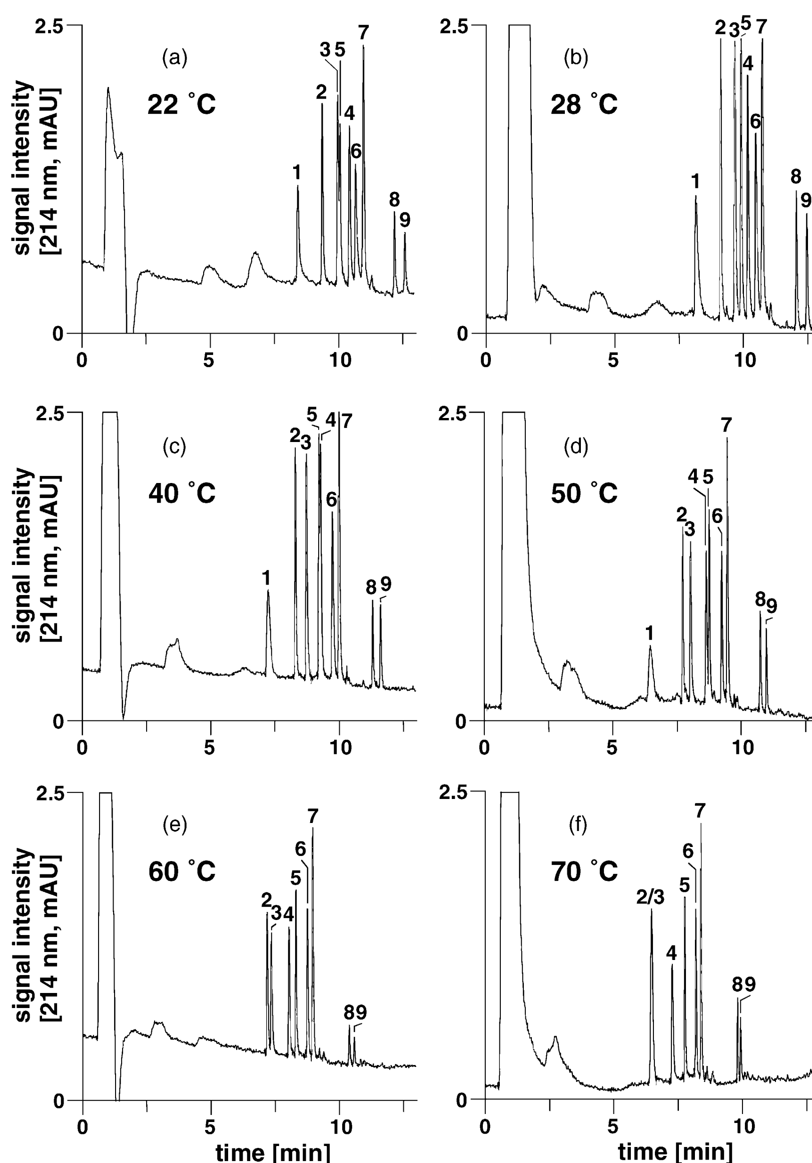

Fig. 1. Separation of nine peptides by IP-RP-HPLC on monolithic capillary columns at different temperatures. Column: PS–DVB monolith, 60 mm × 0.20 mm i.d.; mobile phase: (A) 0.050% trifluoroacetic acid in water, (B) 0.050% trifluoroacetic acid, 50% acetonitrile in water; linear gradient: 0–60% B in 10 min; flow rate: 1.8–2.0 μl/min; temperature: (a) 22 °C, (b), 28 °C, (c) 40 °C, (d) 50 °C, (e) 60 °C, (f) 70 °C; detection: UV, 214 nm; sample: mixture of nine peptides, 309–874 fmol each, peak identification in Table 1.

(model CTO-2A, Shimadzu, Kyoto, Japan) was used for all chromatographic experiments with UV detection. The HPLC system was controlled by a personal computer with the Ultichrom software version 1.2 (LC Packings). The detection cell was a 3 nl ULT-UZ-N10 cell from LC Packings. The system used for HPLC–ESI-MS experiments consisted of a low-pressure gradient mixing micropump (model Rheos 2000, Flux Instruments, Basel, Switzerland) controlled by a personal computer, a vacuum degasser (Knauer, Berlin, Germany), a column thermostat made from 3.3 mm o.d. copper tubing which was heated by means of a circulating water bath (model K 20 KP, Lauda, Lauda-Königshofen, Germany), and a microinjector (model C4-1004, Valco, Houston, TX, USA) with a 500 nl internal sample loop. ESI-MS was performed on a quadrupole ion trap mass spectrometer (esquire HCT, Bruker Daltonics, Bremen, Germany, used for the experiments illustrated in Figs. 5 and 6 or an LCQ classic from Thermo Finnigan, San Jose, CA, USA, used for the experiments illustrated in Figs. 7 and 8) equipped with a triaxial electrospray ion source.

Monolithic capillary columns (60 mm × 0.20 mm i.d.) were prepared according to the published protocol [28] and are commercially available for peptide and protein separations from LC Packings (Monoliths, Amsterdam, The Netherlands). The capillary column was directly connected to the spray capillary (fused silica, 90 μm o.d., 20 μm i.d., Polymicro Technologies, Phoenix, AZ, USA) by means of a microtight union (Upchurch Scientific, Oak Harbor, WA, USA). For analysis with pneumatically assisted ESI, an electrospray voltage of 1–3 kV and a nitrogen sheath gas flow were employed. Mass calibration and tuning were performed according to the manufacturer's recommendations. Fine-tuning for ESI-MS of peptides and proteins [29] was accomplished by infusion of a 0.9 pmol/μl solution of bradykinin, a 0.4 pmol/μl solution of cytochrome *c*, or a 6.9 pmol/μl solution of carbonic anhydrase in 0.050% aqueous TFA solution containing 20% (v/v) acetonitrile at a flow rate of 3.0 μl/min.

3. Results and discussion

3.1. Effect of column temperature on peptide and protein separation by IP-RP-HPLC

Column temperature has been known as a major determinant of separation efficiency and selectivity in peptide and protein separations for a long time [30]. Nevertheless, silica-based columns are preferentially operated at temperatures around ambient because of chemical instability of the bonded stationary phase during extended use at elevated temperatures. Stationary phases based on PS–DVB copolymers, on the contrary, are very stable up to temperatures of 90 °C and more. Hence, in order to optimize separation speed and selectivity, one may take advantage of a considerably broad range of operable column temperatures with PS–DVB-based monolithic columns.

Fig. 1 illustrates the separation of a mixture of nine synthetic, bioactive peptides at column temperatures between 22 and 70 °C with a gradient of acetonitrile in 0.050% aqueous TFA. At 22 °C, separation of the nine peptides was achieved within 13 min, and merely methionine enkephalin and oxytocin were partially separated (Fig. 1a). Peptide retention decreased continually upon increasing the temperature to 70 °C (Fig. 1b–f). However, the relative decreases for the individual peptides were different, which facilitated a separation to baseline of all nine peptides both at 28 and 50 °C. At 40 °C, oxytocin and leucine enkephalin were only partially separated (Fig. 1c) and at 50 °C, they exchanged elution order (Fig. 1d) to become separated to baseline again at 60 °C (Fig. 1e). Bradykinin fragment 1–5 was only poorly retained at 50 °C and eluted in the injection peak at 60 and 70 °C. [Arg[8]]Vasopressin and methionine enkephalin coeluted at 70 °C (Fig. 1f). Temperatures higher than 70 °C were not practicable because of beginning evaporation of acetonitrile.

Under constant gradient conditions, the average peak width at half height was 3.6 s at 28 °C and reached a minimum of 2.8 s at 50–70 °C with a concomitant slight decrease

Table 2
Peak widths at half height of peptides and proteins as a function of temperature

Peptide[a]	$b_{1/2}$ (s)			Protein[a]	$b_{1/2}$ (s)		
	22 °C	50 °C	70 °C		23 °C	50 °C	70 °C
1	–	–	–	1	3.1	5.0	3.5
2	3.2	2.8	n.d.[b]	2	8.2	3.1	3.0
3	3.8	3.3	n.d.	3	3.5	3.3	3.6
4	3.8	3.1	4.1	4	n.d.	3.2	3.1
5	3.4	2.5	2.0	5	4.7	5.0	5.1
6	4.3	3.5	3.1	6	n.d.	3.1	3.1
7	3.5	2.6	2.5	7	n.d.	3.0	2.8
8	3.1	2.4	2.5	8	5.4	4.1	4.0
9	3.4	2.5	2.6	9	6.6	5.4	5.4
Average	3.6	2.8	2.8	Average	5.3	3.9	3.7

Peak identification in Table 1.
[a] Chromatographic conditions as in Figs. 1 and 2.
[b] Not determined because of coeluting peaks.

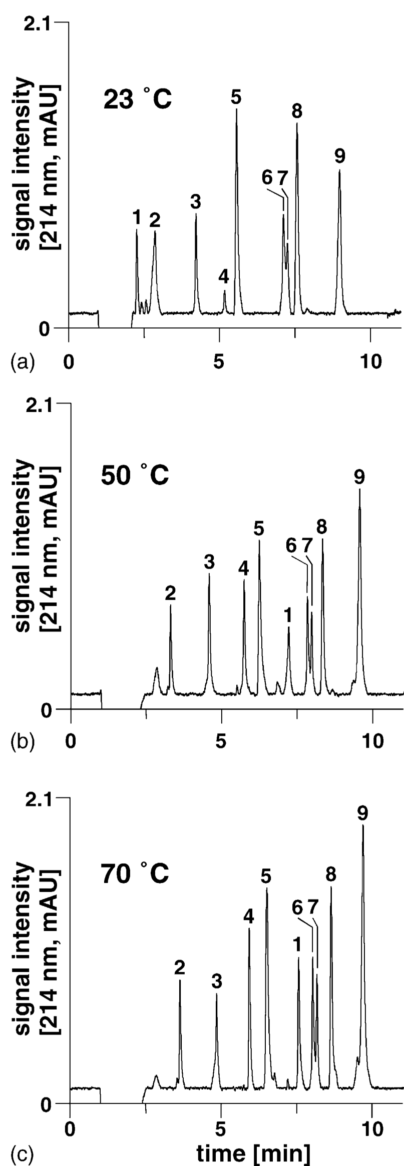

Fig. 2. Separation of nine proteins by RP-HPLC on monolithic capillary columns at different temperatures. Column: PS–DVB monolith, 60 mm × 0.20 mm i.d.; mobile phase: (A) 0.050% trifluoroacetic acid in water, (B) 0.050% trifluoroacetic acid, 80% acetonitrile in water; linear gradient: 20–70% B in 10 min; flow rate: 1.7–2.3 μl/min; temperature: (a) 23 °C, (b) 50 °C, (c) 70 °C; detection: UV, 214 nm; sample: mixture of nine proteins, 10–60 fmol of each protein, peak identification in Table 1.

in the available separation window. The results indicate that the optimal temperature for the separation of the nine standard peptides was between 50 and 60 °C (Table 2). Nevertheless, differences in separation performance at various column temperatures were minor and hence, variation in column temperature constitutes a simple means to fine-tune selectivity of peptide elution on PS–DVB-based monoliths.

Nine proteins were chosen to study the effect of temperature on protein separations in monolithic columns. All proteins, except the pair β-lactoglobulin A/B, were completely separated at all temperatures (Fig. 2) with peak widths at half height ranging from 3.1 to 6.6 s (Table 2). The average peak width at half height was minimal at 70 °C. The best separation of β-lactoglobulin A and B was achieved at 60 and 70 °C with a resolution of 1.7. A baseline separation of β-lactoglobulin A and B, which is a good indicator for the performance and selectivity of reversed-phase columns, could be achieved at 80 °C with a shallower acetonitrile gradient (see Fig. 2 in [31]).

The early elution of trypsin inhibitor before ribonuclease A at 23 °C (Fig. 2a), 28, and 40 °C was remarkable, considering its comparatively large molecular size. At 50 °C (Fig. 2b), however, the trypsin inhibitor peak suddenly shifted between transferrin and β-lactoglobulin B, indicating a transition of two different conformations of the protein at a temperature between 40.0 and 50.0 °C. For the rest of the proteins, elution order remained constant and resolution values varied only to a minor extent in the investigated temperature range, which indicates that column temperature has only a minor effect on separation selectivity with proteins. Columns proved to be very stable at temperatures up to 70 °C, as no untoward effect on separation efficiency or column back-pressure was detectable even after extended use for several months.

3.2. *Influence of mobile phase additive and additive concentration on the chromatographic separation of peptides*

The surface concentration of the amphiphilic ion and the ionic strength in the eluent play a major role in determining the interaction of peptides and proteins with the stationary phase in RP-HPLC. At high surface concentrations of amphiphiles, most of the hydrophobic surface is covered and electrostatic interactions between the generated surface potential and charged groups of the peptides dominate retention. Moreover, an increase in the hydrophobicity of the pairing ion usually entails an increase in retention times of peptides, which is desirable especially for small and hydrophilic peptides that are poorly retained on RP columns.

Adsorption isotherms for TFA and HFBA on octadecylsilica have shown that their surface concentration may differ by more than one order of magnitude at similar concentrations in the mobile phase [32]. Previous studies with monolithic PS–DVB columns revealed that di-, tri- and some tetrapeptides were not retained with 0.050% TFA as mobile phase additive [23,33]. Consequently, we attempted to increase the retentivity of the RP-HPLC system by using HFBA as alternative mobile phase additive. Peptide separations performed with eluents containing 0.010–0.10% HFBA were compared with separations using TFA under otherwise identical conditions at the optimum column temperature of 55 °C (Fig. 3). At this point, it is appropriate to emphasize that due to the difference in molecular mass of the acids and density of the neat acids, the same concentrations of additive in volume percent correspond to different molar concentrations. Hence, 0.010% (v/v) concentrations of TFA and HFBA convert into molar concentrations of 13 and 7.7 mmol/l, respectively.

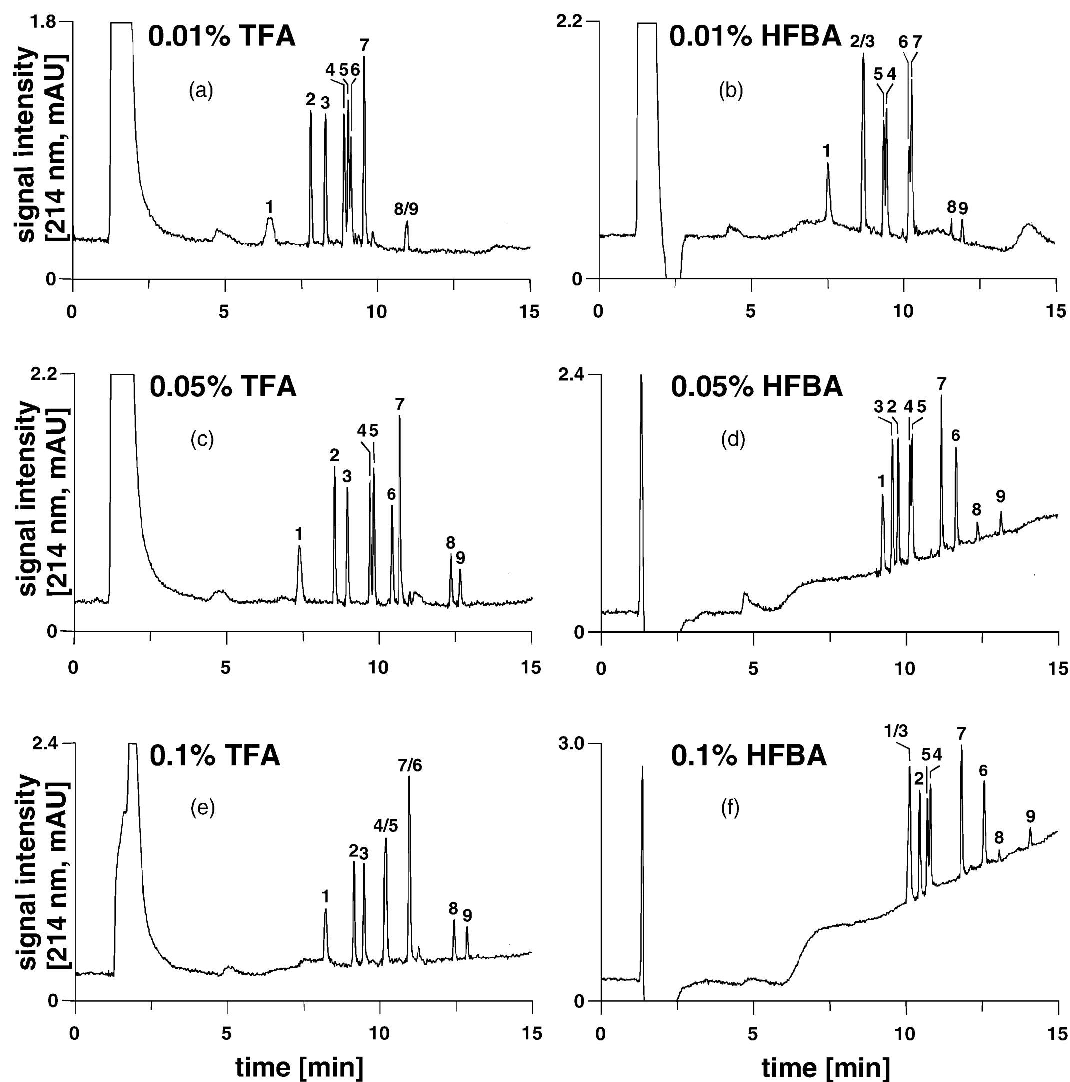

Fig. 3. Separation of nine peptides by IP-RP-HPLC on monolithic capillary columns with different mobile phase additives and mobile phase additive concentrations. Column: PS–DVB monolith, 60 mm × 0.20 mm i.d.; mobile phase: (A) 0.010–0.10% perfluorinated carboxylic acid in water, (B) 50% acetonitrile, 0.010–0.10% perfluorinated carboxylic acid in water; linear gradient: 0–50% B in 15 min; flow rate: 1.8–2.0 μl/min; temperature: 55 °C; detection: UV, 214 nm; sample: mixture of nine peptides, 309–874 fmol each, peak identification in Table 1.

Bradykinin fragment 1–5 was only poorly retained and eluted as a broad peak in a 0.010% TFA eluent (Fig. 3a) compared to 0.010% HFBA, where the peptide eluted as a sharp peak at 7.5 min (Fig. 3b). This is a consequence of a significant increase in surface potential with adsorbed heptafluorobutyrate ions, resulting in enhanced interaction of the hydrophilic peptide containing one basic arginine residue. As can be seen in Fig. 3, the use of HFBA instead of TFA resulted not only in an increase in chromatographic retention, but also in a change in selectivity. At 0.050% TFA and HFBA, respectively, [Arg^8]vasopressin and methionine enkephalin exchanged elution position because [Arg^8]vasopressin contains both a basic arginine and the aminoterminus, whereas methionine enkephalin has no additional basic amino acid residue for electrostatic interaction. Upon a further increase in the HFBA concentration from 0.050 to 0.10%, the small and hydrophilic bradykinin fragment 1–5 coeluted with methionine enkephalin (Fig. 3f). For bradykinin (containing two basic amino acids) and luteinizing hormone releasing hormone (LHRH, containing one basic amino acid), this effect was even more pronounced. Bradykinin showed less retention than LHRH with 0.010% HFBA, indicating predominant solvophobic adsorption for both peptides having similar hydrophobicity at this concentration of pairing ion. In an eluent containing 0.050 or 0.10% HFBA, however, bradykinin eluted after LHRH as a consequence of the more pronounced shift in retention due to electrostatic interaction of the triply charged peptide.

The separation of the peptide mixture using formic acid as additive is illustrated in Fig. 4. Since the formate anion is very polar, its surface concentration can be anticipated to be rather low, resulting in low retentivity for small and/or hydrophilic

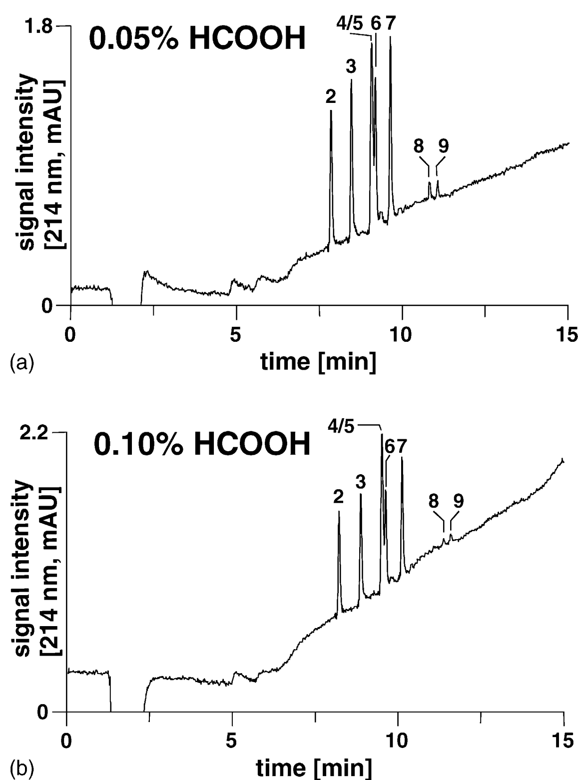

Fig. 4. RP-HPLC analysis of nine peptides on monolithic capillary columns with different formic acid concentrations. Column: PS–DVB monolith, 60 mm × 0.20 mm i.d.; mobile phase: (A) 0.050–0.10% FA in water, (B) 50% acetonitrile, 0.050–0.10% FA in water; linear gradient: 0–50% B in 15 min; flow rate: 1.8–2.0 μl/min; temperature: 55 °C; detection: UV, 214 nm; sample: mixture of nine peptides, 309–874 fmol each, peak identification in Table 1.

peptides. In fact, bradykinin fragment 1–5 could not be retained on the monolithic column at FA concentrations of 0.050 and 0.10% (Fig. 4a and b). Moreover, the retention window was significantly compressed compared to TFA and HBFA. None of the investigated FA concentrations facilitated a complete separation of the peptide mixture. Bradykinin 1–5 eluted as a very broad peak during the isocratic part of the gradient. Bradykinin was only partially separated from the two coeluting peptides leucine enkephalin and oxytocin. Furthermore, the separation selectivity remained unchanged upon an increase in the concentration from 0.050 to 0.10%, leading to the conclusion that solvophobic interaction is mainly responsible for chromatographic retention with FA as additive. A comparison of the chromatographic performances with the three acidic additives measured at 55 °C revealed a decrease in the average peak widths at half height in the order FA (3.6 s at 0.10% concentration) > TFA (3.3 s at 0.050%) > HFBA (3.1 s at 0.050%).

3.3. Influence of mobile phase additive on the mass spectrometric detection of peptides

Since ESI-MS is performed directly from a liquid phase, it lends itself to the on-line interfacing with liquid chromatographic separation [34]. Nevertheless, solution conditions need to be optimized carefully in order to obtain good mass spectrometric detectability while, however, retaining maximum chromatographic performance. Low flows of solutions having low surface tension and low electrical conductivity have turned out to enable the most sensitive detection of biopolymers by ESI-MS [27,35]. This is of special importance in proteomic analyses, because the amounts of accessible sample are usually very low. TFA has turned out to be the most practical additive for RP-HPLC of peptides and proteins with photometric detection, mainly because of its good transmittance down to the low UV range. In combination with mass spectrometric detection, however, weaker acids such as formic acid or acetic acid have been shown to offer lower detection limits [27], primarily because strong acids tend to suppress ion formation in ESI-MS [36,37].

Table 3
Estimated detection limits of bioactive peptides in RP-HPLC–ESI-MS with different eluent additives

Peptide	Detection limit (fmol)[a]		
	0.05% HFBA	0.05% TFA	0.1% FA
Bradykinin fragment 1–5	4	4	–
[Arg^8]Vasopressin	6	2	13
Methionine enkephalin	4	3	3
Leucine enkephalin	3	2	2
Oxytocin	6	1	4
Bradykinin	0.5	0.4	0.9
LHRH	0.3	0.3	0.2
Bombesin	1	0.4	0.4
Substance P	0.8	0.8	0.3

[a] Values represent the average of three determinations.

In order to characterize the influence of the mobile phase additives on detection performance of RP-HPLC–ESI-MS with monolithic capillary columns both from a chromatographic and mass spectrometric point of view, we evaluated the signal-to-noise ratios in the chromatograms and mass spectra. FA was added to the eluent at a concentration of 0.10% in order to ensure acceptable chromatographic performance, while TFA and HFBA were present in concentrations of 0.050%. Fig. 5a–c illustrate the total ion current chromatograms resulting from the separation of nine bioactive peptides (25 pg each, corresponding to 15–43 fmol) with the different mobile phase additives. Selected ion chromatograms were extracted in the *m*/*z* range of the most abundant charge state of the peptides to characterize the chromatographic detectabilities of the peptides, which is essential for their quantitative determination. Full-scan mass spectra were extracted at the maxima of the chromatographic peaks, which are important for peptide identification based on molecular mass. Moreover, a full scan spectrum (Fig. 5d–i) usually forms the basis for selection of precursor ions for subsequent fragmentation and peptide identification by tandem MS.

The signal-to-noise ratios in the selected ion chromatograms ranged from 5 (vasopressin with FA) to 418

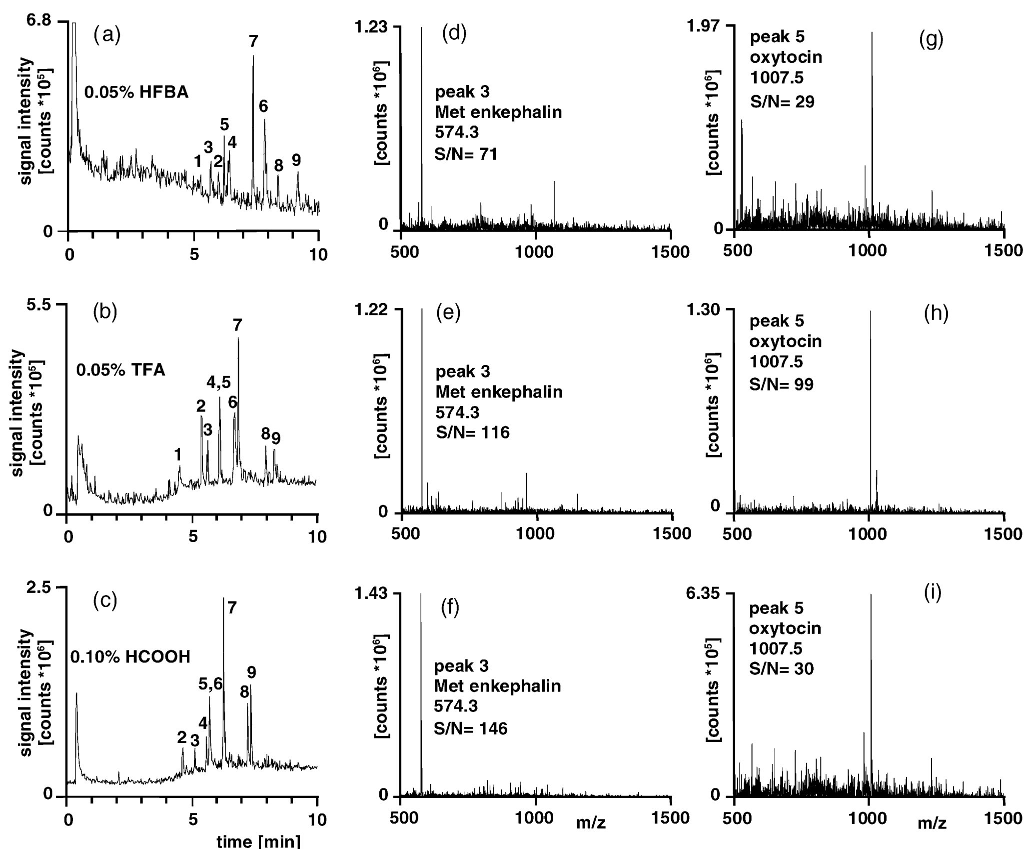

Fig. 5. IP-RP-HPLC–ESI-MS analysis of nine peptides with different solvent additives. Column: PS–DVB monolith, 60 mm × 0.20 mm i.d.; mobile phase: (A) 0.050% HFBA (a, d and g), 0.050% TFA (b, e and h), and 0.10% FA (c, f and i) in water, (B) 50% acetonitrile in (A); linear gradient: 0–50% B in 15 min; flow rate: 2.0 μl/min; temperature: 50 °C; detection: MS, scan, 500–1500 u; electrospray voltage: 3.5 kV; nebulizer gas: nitrogen, 1.4 bar; sample: mixture of nine peptides, 25 pg (15–43 fmol) each, peak identification in Table 1.

(luteinizing hormone releasing hormone with FA, Fig. 6a) while values of 7 (Vasopressin with HFBA) to 310 (luteinizing hormone releasing hormone with FA, Fig. 6b) were observed in the extracted mass spectra. With 0.050% HFBA as mobile phase additive, bradykinin fragment 1–5 could not be detected in the total ion current chromatogram (Fig. 5a) although a clear peak with a signal-to-noise ratio of 32 was seen in a selected ion chromatogram extracted at m/z 573. The extracted mass spectrum showed an abundant mass signal at m/z 573.3 with a signal-to-noise ratio of 90. All other peptides were detected in the mass spectra with signal-to-noise ratios ranging from 7 (vasopressin) to 162 (luteinizing hormone releasing hormone). Using 0.050% TFA as additive, all peptide peaks were clearly distinguishable in the total ion current chromatogram (Fig. 5b). Compared to HFBA, signal-to-noise ratios were mostly higher with TFA both in the selected ion chromatograms and extracted mass spectra (Figs. 5 and 6). This is also reflected in average signal-to-noise ratios of 97 versus 66 in the selected ion chromatogram and 140 versus 87 in the extracted mass spectra with TFA and HFBA, respectively, as mobile phase additives.

The largest difference in detection performance with the different additives was observed in the extracted mass spectrum of substance P, which showed signal-to-noise ratios of 201 and 73 with 0.10% FA and 0.050% HFBA, respectively. It is interesting to note that FA typically yielded the highest signal-to-noise ratios for peptide peaks eluting as a single compound (e.g. Met-enkephalin, Leu-enkephalin, luteinizing hormone releasing hormone, Fig. 6a and b) whereas the ratio dropped significantly for coeluting peptides (e.g. oxytocin and bradykinin, Fig. 6a and b). This is a consequence of signal suppression during the simultaneous electrospray ionization of several peptides, which demonstrates that efficient separation prior to mass spectrometric detection significantly enhances the detectabilities of peptides. The average signal-to-noise ratio for formic acid of 123 in the extracted mass spectra was significantly smaller than that found with TFA (140). Ratios of 108 and 97 for the selected ion chromatograms were, on the other hand, quite similar for both additives.

Finally, the signal-to-noise ratios in the selected ion chromatograms and the amounts of injected peptide were utilized to calculate estimates for the limits of detection at a signal-to-noise ratio of 3:1 (Table 3). The estimated limits of detection ranged from a few hundred attomoles to a few femtomoles and were practically identical with TFA and FA, whereas lowest detectable amounts were slightly higher with HFBA.

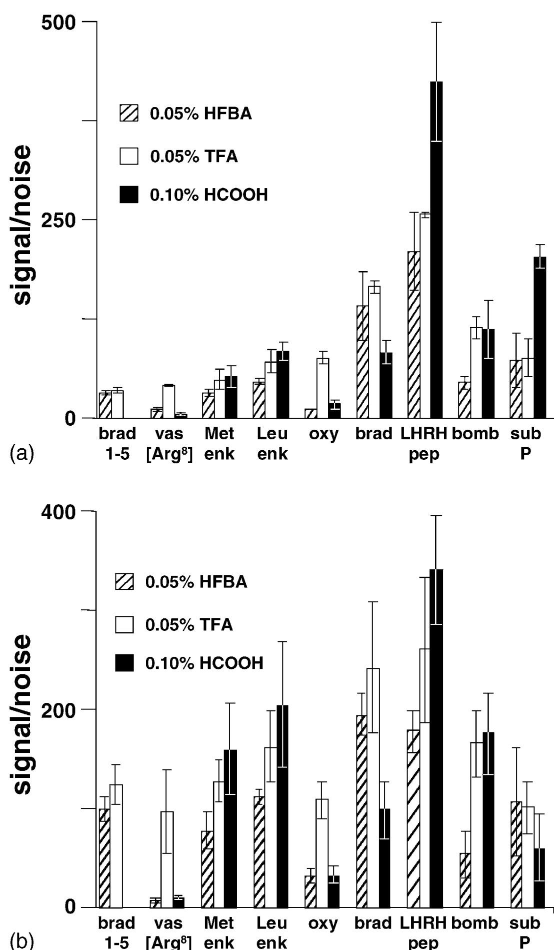

Fig. 6. Signal-to-noise ratios of peptide mass signals in the mass spectrum extracted from the chromatographic peak maximum. Signal-to-noise ratios were determined in (a) from the selected ion chromatogram extracted in a *m/z* window spanning the isotopic cluster of the most abundant charge state of the peptides with a mass window of whereas they were determined in (b) from a single mass spectrum extracted at the chromatographic peak maximum. Conditions as in Fig. 5.

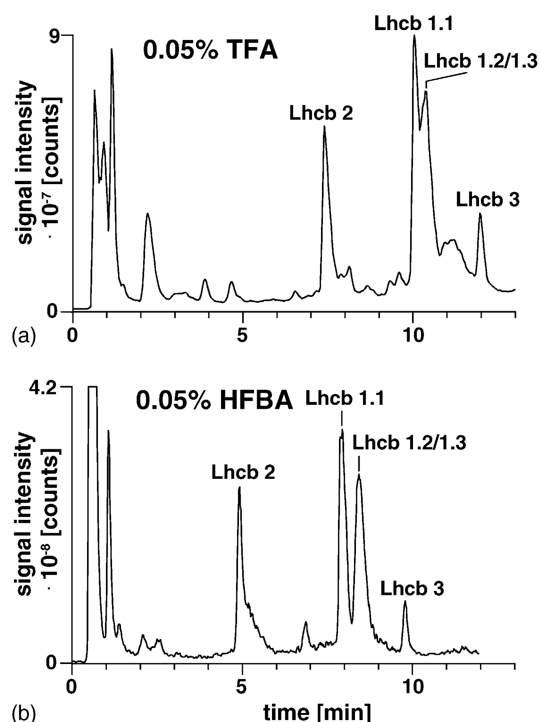

Fig. 7. IP-RP-HPLC–ESI-MS analysis of membrane proteins of the photosystem II from spinach. Column: PS–DVB monolith, 60 mm × 0.20 mm i.d.; mobile phase: (A) 0.050% TFA or HFBA in water, (B) 0.050% TFA or HFBA in acetonitrile; linear gradient: 36–48% B in 20 min (a), 42–54% B in 20 min (b); flow rate: 2.0 μl/min; temperature: 60 °C; detection: MS, scan: 600–2000 u; electrospray voltage: 1.6 kV; sheath gas: 100 units; sample: major antenna proteins of photosystem II from spinach.

3.4. Influence of mobile phase additive on the mass spectrometric detection of proteins

Antenna proteins of the photosystem II from spinach were used to study the effect of mobile phase additive on chromatographic and mass spectrometric performance for hydrophobic membrane proteins [38]. The Lhcb 1.1 protein was only partially resolved from the coeluting Lhcb 1.2 and Lhcb 1.3 proteins with an eluent containing 0.050% TFA (Fig. 7a). A change to HFBA enabled a baseline resolution of Lhcb 1.1, whereas Lhcb 1.2 and Lhcb 1.3 still coeluted (Fig. 7a). The proteins were separated with HFBA with significantly higher selectivity. Moreover, they eluted at higher concentrations of acetonitrile in a mobile phase containing HFBA compared to eluents prepared with TFA as additive (36–48% versus 42–54% acetonitrile). This increase in retention results from a more efficient denaturation of the strongly hydrophobic membrane proteins by heptafluorobutyric acid in combination with a stronger electrostatic interaction with the stationary phase.

The quality of protein mass spectra obtained with TFA and HFBA is compared in Fig. 8. Even though the absolute signal intensities were more than three times higher with eluents containing HFBA, a significant increase in chemical noise level was obvious in the spectra recorded from HFBA eluents. This increase is most probably due to cluster formation of the organic acid with solvent molecules. In mass spectra extracted from the chromatograms at positions that were free of eluting proteins, some of the mass signals could be annotated to clusters of HFBA with water and acetonitrile. Because of the higher molecular mass of HFBA, these clusters fall within the mass range chosen for the observation of protein signals, while the molecular masses of clusters with TFA or FA are below the investigated mass range of *m/z* 600–2000. The signal-to-noise ratios for the most abundant charge states in the protein mass spectra were 33 (Lhcb 1.1) to 13 (Lhcb 3) with TFA and 19 (Lhcb 1.1) to 5.8 (Lhcb 3) with HFBA. The intact molecular masses could be calculated from the mass spectra for all antenna proteins, which were in excellent agreement with the molecular masses expected from their DNA sequences [39].

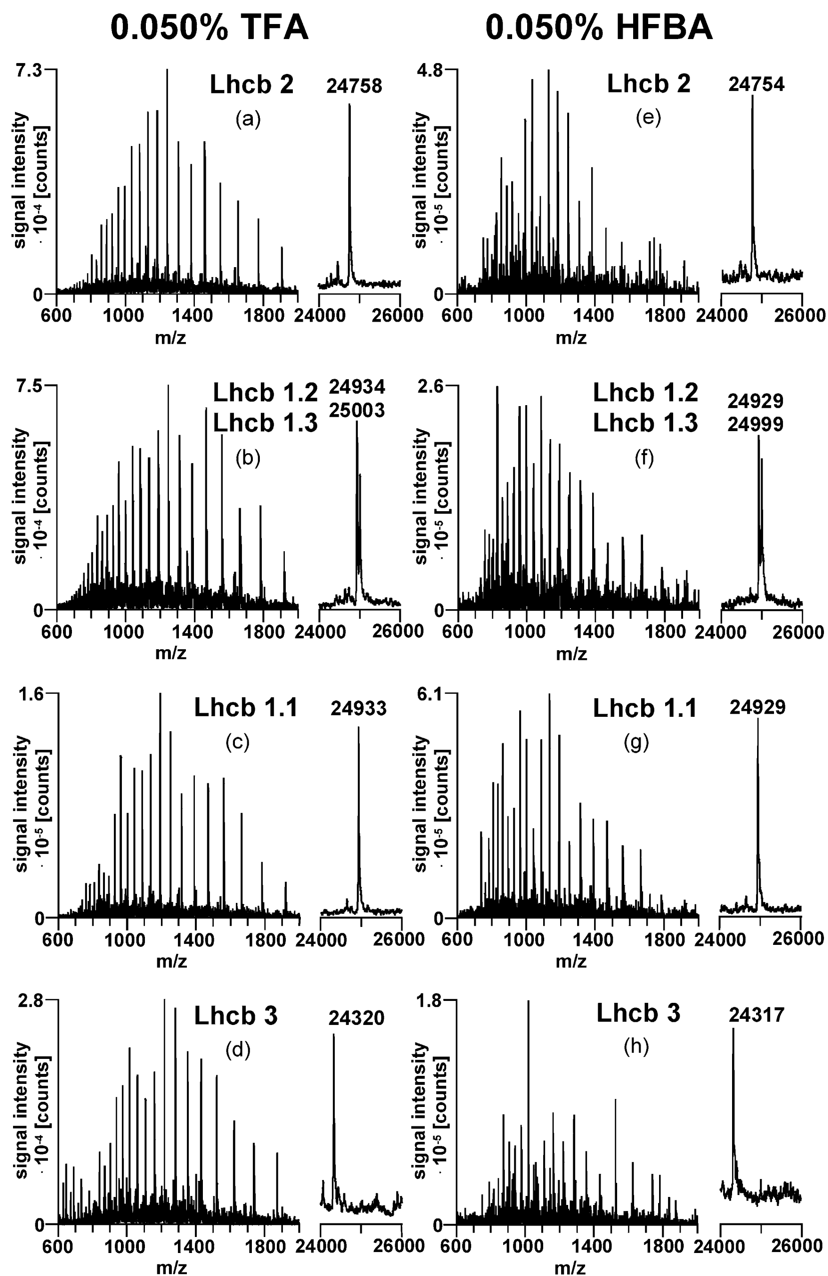

Fig. 8. Mass spectra of major antenna proteins of the photosystem II from spinach. Conditions as in Fig. 7.

4. Conclusions

Variation of column temperature and type of pairing ion are suitable means for fine-tuning retentivity and selectivity of PS–DVB-based monolithic columns for peptide and protein separations. The influence of temperature and mobile phase additive on selectivity is far more pronounced with peptides compared to proteins. From a chromatographic point of view, HFBA turned out to be the most favorable mobile phase additive, which enables the most efficient separations, the retention even of small, hydrophobic peptides, as well as significant changes in elution order as function of additive concentration. While the chromatographic efficiencies with 0.050% TFA and 0.050% HFBA were similar, separations performed with FA as additive not only resulted in an increase in chromatographic peak width but also in a decrease in chromatographic selectivity.

From a mass spectrometric point of view, 0.10% FA and 0.050% TFA were found to be essentially equivalent in terms of detectability and spectral quality for peptides. TFA should be preferred as additive in RP-HPLC–ESI-MS systems due to its significantly better separation efficiency and selectivity. Nevertheless, the variations in detectabilities between the different additives were generally rather small, contrasting the

results of previous investigations [27]. Cluster formation in a mass range relevant for peptide and protein detection considerably impairs the quality of mass spectra recorded with HFBA as mobile phase additive. This drawback of HFBA is only tolerable when separation efficiency and selectivity are of great importance, or when very small and hydrophilic peptides have to be retained on monolithic columns.

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