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Review

New trends in reversed-phase liquid chromatographic separations of therapeutic peptides and proteins: Theory and applications

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ABSTRACT

In the pharmaceutical field, there is considerable interest in the use of peptides and proteins for therapeutic purposes. There are various ways to characterize such complex samples, but during the last few years, a significant number of technological developments have been brought to the field of RPLC and RPLC–MS.

Thus, the present review focuses first on the basics of RPLC for peptides and proteins, including the inherent problems, some possible solutions and some directions for developing a new RPLC method that is dedicated to biomolecules. Then the latest advances in RPLC, such as wide-pore core–shell particles, fully porous sub-2 μm particles, organic monoliths, porous layer open tubular columns and elevated temperature, are described and critically discussed in terms of both kinetic efficiency and selectivity. Numerous applications with real samples are presented that confirm the relevance of these different strategies. Finally, one of the key advantages of RPLC for peptides and proteins over other historical approaches is its inherent compatibility with MS using both MALDI and ESI sources.

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1. Introduction

Most traditional pharmaceuticals are chemically synthesized low-molecular-weight compounds, and only a few of them were originally isolated from biological sources. In addition to these "chemical" substances, there are a number of substances that are produced from biological sources (i.e., biological systems or biological molecules). These products of pharmaceutical biotechnology are known as "biopharmaceuticals" and include recombinant peptides, proteins or glycoproteins (e.g., cytokines, monoclonal antibodies (mAbs)) [1].

The pharmaceutical potential of numerous proteins (e.g., interferons, interleukins, and growth factors) that are naturally produced in the body, originally demonstrated more than 40 years ago, presents obvious advantages, including high efficacy, high specificity, wide therapeutic range, limited side effects, and exceptional chemical and biological diversity. However, their widespread medical application has historically been impractical because of the very small quantities that are naturally produced. This statement is no longer valid because of the development of recombinant DNA technology (i.e., cloning of human genetic material and development of in vitro biological production) during the 80s that marked the beginning of a new era of the pharmaceutical sciences [2]. Besides this point, the promise and hype of biotechnology often exceed its ability to deliver the final product [2]. Indeed, there are a number of additional shortcomings, including low bioavailability, low stability in plasma, poor transfer across biological membranes, high conformational flexibility, structural complexity and the high cost of biopharmaceuticals that have yet to be resolved. Depending on the source of information, between 20 and 40% of the new drugs that enter the market today are biopharmaceuticals, and there are several hundred potential biopharmaceuticals that have been evaluated in clinical trials for the treatment of genetic diseases, cancer and infectious diseases [2–4].

Because the patents of the oldest approved biopharmaceuticals (e.g., recombinant human growth hormone, insulin, erythropoietin, and interferon) have expired, these drugs can be copied and marketed by other biotech companies. As a result, the first generic biologics entered the market a few years ago. However, because two cell lines that have been developed independently cannot be considered as identical, the term "biosimilar" is employed for any generic biopharmaceutical in recognition of the fact that the two products are similar but not identical [2].

Because the development of biopharmaceuticals and biosimilars is quite complex, regulatory bodies such as the FDA (Food and Drug Administration) and EMA (European Medicines Agency) require a demonstration of the drug substance characterization (e.g., verifying primary structure and appropriate post-translational modifications, secondary and tertiary structure), lot-to-lot and batch-to-batch comparisons, stability studies, impurity profiling, glycoprofiling, determination of related proteins and excipients such as polysorbates or determination of protein aggregates [5].

For this purpose, a single analytical technique is generally not sufficient, and a variety of methods (orthogonal methods) are required to fully describe such a complex sample, as has been summarized elsewhere [6–9]. The most relevant approaches include (i) spectrophotometry, (ii) electrophoresis, (iii) chromatography, and (iv) mass spectrometry. Spectrophotometric methods include UV–vis spectrophotometry, fluorescence spectrophotometry and circular dichroism (CD), which are commonly employed for the determination of protein concentration and for the characterization of secondary/tertiary structure. Electrophoresis is one of the key techniques for protein analysis, and different modes can be employed in the slab gel or capillary format. In SDS-PAGE, the proteins are separated according to their electrophoretic mobility (a function of the length of a polypeptide chain and its charge). Isoelectric focusing (IEF) is able to distinguish charge differences (isoelectric point) among proteins through the use of a pH gradient. Finally, capillary zone electrophoresis (CZE) has several well-established attractive features for the characterization of such complex samples because of its high resolving power and elevated throughput. Chromatography is another interesting alternative for the characterization of proteins [10]. The three most common types of HPLC are size exclusion chromatography (SEC), which separates proteins based on their molecular size, ion exchange chromatography (IEX), able to separate proteins based on the charge of the protein, and reversed-phase chromatography (RPLC), where separation occurs on the basis of hydrophobicity and which presents a high resolving power, compared to SEC and IEX. There are several other basic modes of HPLC that are currently used for peptide and protein analysis and purification, such as normal phase chromatography (NPLC), hydrophobic interaction chromatography (HIC), hydrophilic interaction chromatography (HILIC), and affinity chromatographic techniques.

Finally, mass spectrometry (MS) is a method that has increasingly been used to characterize proteins because of its significant sensitivity and selectivity and its ability to be used in conjunction with electrophoretic and chromatographic methods. With all of these different approaches, it is possible to determine the primary sequence, post-translational modifications, aggregation, batch purity, charge heterogeneity, PEGylations, degradation products, isoforms, and size variants of proteins [6].

In conclusion, there are a number of well-established strategies for protein characterization, including UV–vis, CD, IEF, SDS-PAGE, SEC, IEX, which will not be covered in this review. Because there have recently been a considerable number of technological developments in the field of RPLC and RPLC–MS, the present contribution focuses on RPLC of peptides and proteins, including the description of the latest advances in RPLC and RPLC–MS and their applications to real samples.

2. Conventional RPLC

2.1. Inherent problems with RPLC

Because of its versatility, flexibility and robustness, RPLC is one of the dominant approaches used for the analysis of peptides and proteins [11]. High-molecular-mass compounds such as intact proteins may have numerous different conformations, post-translational modifications, or multiple isoforms that can cause broadened peak shapes and shifted retention times in the chromatograms. Another reason for broadened peaks is the very low values of the molecular diffusion coefficients (D_m) of these compounds due to their large size. Even by means of multiple dimension techniques combined with mass spectrometry (MS), it is impossible to fully resolve all of the variants that are present in the different complex protein-based products. Another serious problem in

biopharmaceutical analysis is the lack of high-purity reliable reference materials [12]. Having no proper standards, it is impossible to perform absolute quantitation and method validation according to the existing guidelines. Currently, there is no specific procedure for the analytical validation of complex biomedicals. Q2(R1) was originally developed for low-molecular-weight products, so it cannot be applied easily for biotechnology-based pharmaceuticals, while Q6B does not describe true validation guidelines [13,14]. Recently EMA releases a guideline on bioanalytical method validation which is planned to come into effect in February 2012 [4,5].

In the case of RPLC, solute binding to the stationary phase is mediated predominantly through hydrophobic interactions between the nonpolar amino acid residues of peptides or proteins and the immobilized n-alkyl ligands. The solute mixture is initially applied to the sorbent in the presence of aqueous buffers, and the solutes are eluted by the addition of organic solvent to the mobile phase. Generally, the gradient elution mode is preferred, whereby the amount of organic solvent is increased over a period of time. The solutes are, therefore, eluted in order of increasing molecular hydrophobicity [15,16]. The retention of proteins is strongly dependent on small changes in the solvent strength; a very small change (<1%) in the organic modifier content could lead to a significant shift in protein retention. For this reason, isocratic conditions are impractical, and gradient elutions are mandatory in practice.

It is necessary to mention that RPLC can sometimes cause an irreversible denaturation of protein samples, reducing the potential recovery of material in a biologically active form. The three-dimensional structure of proteins can also be sensitive to the often harsh conditions that are employed in RPLC. As a consequence, RPLC is less commonly employed for the isolation of proteins when it is important to recover the protein in a biologically active form [17].

In spite of several problems related to RPLC, this technique has several advantages compared to other chromatographic techniques. It was shown very early (in 1977) that RPLC is one of the most promising analytical technique on the field [18]. The efficiency of RPLC is superior to that obtained on IEX or SEC columns [18]. Moreover, the separation time can be drastically shortened compared to IEX or SEC, and one of the main advantages is the straightforward coupling to mass spectrometric detection (MS).

2.2. Basic requirements of conventional RPLC stationary phases

The chromatographic packing materials that are generally used in conventional RP separations are based on microparticulate (2–5 μm) porous silica, which allows the use of high linear flow rates, resulting in favorable mass transfer properties and rapid analysis times [19,20]. The silica is generally chemically modified with a derivatized silane that bears an n-alkyl hydrophobic ligand. The most common ligand is n-octadecyl (C18), but n-butyl (C4) and n-octyl (C8) are commonly used for the analysis of more hydrophobic proteins. Additionally, phenyl and cyanopropyl ligands can provide alternative selectivity [21]. The process of chemical immobilization of the silica surface results in approximately half of the surface silanol groups being modified. Therefore, the sorbents are generally subjected to further silanization with a small reactive silane to produce an end-capped packing material. The type of n-alkyl ligand significantly influences the retention of peptides and proteins and can therefore be used to manipulate the retention, recovery and, to a lesser extent, selectivity for peptides and proteins. Although the detailed molecular basis of the effect of ligand structure is not fully understood, a number of factors, including the relative hydrophobicity of the ligand, surface coverage, ligand density, carbon load, flexibility, and the degree of exposure of the surface silanols, play a significant role in the retention process. In addition, the choice of ligand can also influence the recovery and conformational integrity of the protein samples. Generally, higher protein recoveries are obtained with shorter and less hydrophobic n-butyl ligands. However, proteins have also been recovered in high yield from n-octadecyl silica in certain applications [22–24].

Silica-based packings are also susceptible to dissolution at pH values greater than 7–8. Therefore, alternative stationary phases that possess greater stability at alkaline pH values, such as cross-linked polystyrene-divinylbenzene [25,26], porous zirconia [27,28], or hybrid silica-based stationary phases should be considered for basic conditions.

The pore size of the RPLC stationary phase is also an important factor that must be considered. For the analysis of peptides and small proteins (i.e., smaller than 14 kDa), a pore size between 100 and 200 Å is generally acceptable. However, porous materials with pore sizes of more than 200 Å are mandatory for the separation of larger proteins or monoclonal antibody fragments, as the solute molecular diameter must be approximately one-tenth the size of the pore diameter to avoid the restricted diffusion of the solute and to allow the total surface area of the sorbent material to be accessible. In this context, the development of materials with 6000–8000 Å pores that contain a network of smaller pores of 500–1000 Å has allowed very rapid peptide and protein separations [29,30].

2.3. Method development in RPLC separations of proteins

The RPLC separations of proteins can easily be tuned by changing the gradient slope, operating temperature, additives, pH, or organic modifier [15,16]. The optimization of protein separations in RPLC has generally been achieved via the manipulation of the mobile phase with a given column; however, the use of different stationary phases, preferably with complementary selectivities, has also been successful.

The major difference in method development of small analytes and large molecules such as proteins is related to the number of interactions (e.g., hydrophobic and ionic) that occur between the mobile and the stationary phase, the latter being strongly influenced by the conformations of proteins. Unfortunately, the changes in protein conformation that occur as the chromatographic conditions are varied are very complex and unpredictable. Therefore, method development that utilizes automated computer-assisted methods for predicting the retention properties on the basis of protein structure cannot be employed. However, computer simulations of the retention behavior of peptides and proteins that is based on experimental chromatographic runs can still be a useful tool. There are a few instances found in the literature of applications of DryLab software for the computer-assisted method development of RPLC protein separations. Predictions in gradient elution mode were very valuable for protein separations, as demonstrated by Dolan and Ghrist [31–33]. Molnar et al. found during their study of ribosomes that the predictions of DryLab were highly reliable, even for 54 different ribosomal proteins of *Thermus* ricians *aquaticus* from the 30*S* and the 50*S* subunits. Proteins from the bacterium *Thermus aquaticus* that maintain their biological activity at 80 °C are held together by strong ionic and hydrophobic forces between themselves and ribosomal ribonucleic acid (rRNA) and were precisely studied with DryLab [34]. In contrast, computer-assisted method development is commonly used for peptide mapping [35].

2.3.1. Classical approach, improving the selectivity

For the separation of the diverse components of a sample that contains peptides or proteins of an unknown composition, an initial scouting gradient is typically run to determine the intervals of the variables to be optimized [11,17]. In many cases, the nature of the components is unknown. However, there are some models that describe the retention behaviors of peptides and proteins on the basis of their amino acid sequence. The most commonly adapted concepts are based on the solvophobic theory [36,37] and

the linear solvent strength theory [38]. These concepts allow for the development of fast and robust separation methods [39].

The best approach to improve selectivity and thus resolution for peptides and proteins is to change the chemical nature or concentration of the organic modifier (e.g., acetonitrile, methanol, or isopropanol) and to select a suitable ion-pairing reagent [40].

A good starting point is the solvent selectivity triangle approach. Here, solvents are classified according to their relative dipole moment, basicity, or acidity in a triangle. Blends of three different solvents, plus water to provide an appropriate retention (k) range, are selected to differ as much as possible in their polar interactions. This selection permits the solvent combinations to mimic the selectivity that is possible for any given solvent and confines the boundaries of the triangle [41,42]. Four-solvent mobile phase optimization using three organic solvents and water provides more possibilities for tuning the selectivity. If different organic solvents are used, the different eluotropic strengths [43,44] must be considered to elute the sample within an appropriate k range.

The retention of peptides and proteins can be influenced by adding ion-pairing reagents to the mobile phase [37,45–47]. The ion-pairing reagents interact with the ionized groups of the proteins. Anionic counterions (e.g., hexanesulfonic acid, orthophosphoric acid, and trifluoroacetic acid) interact with the basic residues (i.e., arginine, lysine, and histidine) of a protein and with the protonated *N*-terminus. Cationic counterions (e.g., triethylammonium and tetrabutylammonium) interact with ionized acidic residues (i.e., glutamic and aspartic or cysteic acid) and ionized free C-terminal carboxylic groups. The actual effect on retention depends strongly on the hydrophobicity and concentration of the ion-pair reagent and the number of oppositely charged groups on the protein. An additional factor to consider when employing ion-pairing reagents is their compatibility with MS and their reactivity towards the stationary phase.

Once the initial conditions of the mobile and stationary phase are fixed, further optimization should concentrate on less relevant parameters, such as the mobile phase temperature and gradient profile that could help improve the resolution.

Indeed, the separation temperature can be a significant parameter in method development because it influences both the conformation of the peptides and proteins and decreases the resistance to the mass transfer. Generally, elevated temperatures lead to improved peak symmetry and peak widths for proteins. Thus, the main advantage of temperature is the improvement in kinetic efficiency; therefore, higher resolution can be expected at elevated temperatures.

To optimize the gradient profile, the best approach is to perform two linear gradient conditions that differ by a factor of 3 in their gradient run times t_G, all other chromatographic parameters being held unchanged. This helps to evaluate the influence of t_G on the overall resolution, and these two experiments can also be employed to predict the RPLC retention times of each protein as a function of the gradient program using optimization software [48–50].

2.3.2. Another approach, improving the kinetic efficiency

In real-life applications, proteins with very similar molecular weights and nearly identical structures (conformations) often must be separated. A typical example is the separation of oxidized, deamidated or reduced forms of a given intact protein. The difference in molecular structure is relatively low; therefore, similar retention behaviors of the different forms are expected. In many cases, the selectivity cannot be improved. As a result, the kinetic efficiency must be considered. In this approach, the stationary phase and temperature are the two most relevant parameters in method development. By utilizing the latest technologies of stationary phases, such as core–shell-type materials, sub-2 μm porous particles or wide-pore monolithic columns; the separation power can be considerably increased (see later). Another possibility involves changing the stationary phase chemistry to exhibit a weaker interaction between the protein and the alkyl ligands. Generally, when a C18 stationary phase is changed to C4, a considerable improvement in the kinetic efficiency is observed [5]. The use of a mobile phase temperature between 60 and 90 °C typically further improves the performance (see later). Finally, it is also possible to adjust the column length to achieve the required kinetic efficiency in terms of plate count or peak capacity [51].

A recent systematic study showed the effect of column length on peak capacity in intact protein separations [52]. In agreement with the theory, the peak capacity increased with the square root of the column length.

2.4. Applications of conventional RPLC in protein analysis

Generally the term "conventional HPLC" refers to separations achieved at pressures lower than 400 bar and by using columns packed with 2.5–5 μm porous particles. Several applications of conventional reversed-phase separations can be found in the literature, which demonstrates the dominance of this separation mode in the field of peptide and protein analysis. Bonfatti et al. presented a method for the separation and quantification of the most common genetic variants of bovine milk proteins. All of the most common casein and protein genetic variants were separated in less than 40 min [53]. Umrethia et al. compared different techniques for the evaluation of bovine serum albumin concentrations in pharmaceutical polymeric formulations and found that RPLC was the most suitable method [54]. A conventional HPLC method that preserved the heterodimer of human follicle-stimulating hormone (hFSH) preparations (pituitary, urinary, and two commercial recombinant hFSH preparations) was established for the qualitative and quantitative analysis of hFSH. This method was capable of detecting different degrees of heterogeneity in these preparations [55]. Specific HPLC conditions were reported by the same group for the analysis of recombinant and native human luteinizing hormone and human chorionic gonadotropin preparations [56]. A rapid and easy-to-use method was developed for the quantitative analysis of interferon-beta-2b in pharmaceuticals [57]. Another method was developed and applied for the determination of recombinant human interferon omega in the fermentation broth of the yeast *Pichia pastoris* [58]. Insulin and insulin-related peptides (oxidized and deamidated forms) were separated and determined by various RPLC methods [59–61]. A neutral pH RPLC method has been described that allows for the rapid separation of several human growth hormone (hGH) variants [62]. Using an optimized shallow gradient method, oxidized recombinant human interleukin11 (rhIL-11) was effectively separated from native rhIL-11 [63]. A method for determining the isomerization of asparagine (Asp) residues in proteins was described and applied for the quantitation of the isomerization of Asp^{151} in recombinant human alpha-A-crystallin [64]. Additionally, a perfusion® RPLC–electrospray mass spectrometry (ESI-MS) method was employed for the characterization of soybean cultivars through the analysis of intact soybean proteins [65]. Perfusion® chromatography uses media (Poros® stationary phases) that consists of rigid cross-linked poly(styrene-divinylbenzene) flow-through particles with pore structure optimized for very rapid mass transport.

3. Improvements to conventional RPLC

Higher separation efficiencies and throughput have always been of great interest in high-performance liquid chromatography (HPLC). They have become increasingly important in recent years

Table 1
Recent, commercially available very efficient medium- and widepore RPLC columns for protein separations.

Column name	Surface chemistry	Particle size/macropore size	Pore size/mesopore size	Max temperature	pH range	Max pressure (bar)
Monoliths						
Thermo Scientific Proswift RP-1S	Phenyl	1 μm	Information not available	70 °C	1–14	200
Thermo Scientific Proswift RP-2H	Phenyl	2.2 μm	Information not available	70 °C	1–14	200
Thermo Scientific RP-3U	Phenyl	5.1 μm	Information not available	70 °C	1–14	200
Thermo Scientific RP-10R	Phenyl	Information not available	Information not available	80 °C	1–10	300
Merck Chromolith 2nd generation	C18	1.2 μm	150 Å	60 °C	1–8	200
Porous sub-2 μm						
Waters Acquity BEH	C18, C4	1.7 μm	300 Å	80 °C	1–12	1000
Agilent ZORBAX 300SB RRHD	C18, C8	1.8 μm	300 Å	80 °C	1–8	1200
Thermo Hypersil GOLD	C18, C8, CN, aQ, PFP, Phenyl	1.9 μm	175 Å	60 °C	2–9	1000
Core–shell type						
Halo Peptide ES	C18	2.7 μm (0.5 μm thickness)	160 Å	100 °C	1–9	600
Supelco Ascentis Peptide ES	C18	2.7 μm (0.5 μm thickness)	160 Å	100 °C	1–9	600
Perkin-Elmer Brownlee SPP Peptide ES	C18	2.7 μm (0.5 μm thickness)	160 Å	90 °C	1–8	600
Phenomenex Aeris Widepore	C18, C8, C4	3.6 μm (0.2 μm thickness)	Information not available	90 °C (C18, C8), 60 °C (C4)	1.5–9	600
Phenomenex Aeris Peptide	C18	3.6 μm (0.5 μm thickness) 1.7 μm (0.2 μm thickness)	Information not available	90 °C	1.5–9	600 1000
Agilent Zorbax poroshell SB300	C18, C8, C3	5 μm (0.25 μm thickness)	300 Å	90 °C	1–8	600
Agilent Zorbax poroshell 300Extend	C18	5 μm (0.25 μm thickness)	300 Å	60 °C	2–11	600

and have mainly been driven by the challenges of more complex samples and increasing number of samples. The pharmaceutical industry is interested in using rapid and efficient procedures for qualitative and quantitative analyses to cope with the large number of samples and to reduce the time required for the delivery of results.

High kinetic efficiency is needed to reduce the analysis time and guarantee the quality of HPLC separations. In real-life separations with limited selectivity, the only way to improve the separation is to increase the kinetic efficiency. A general approach to improve the separation power is to enhance the column efficiency. The recently available wide- and medium-pore stationary phases for protein separations have been summarized in Table 1 and the potential of these columns is discussed below.

3.1. Kinetic performance

3.1.1. Mobile phase temperature

In LC, the selectivity and retention are easily controlled by the mobile phase composition and pH. Thus, temperature does not play a key role and typically remains near ambient. However, it has been demonstrated that temperature can drastically improve chromatographic separations, and various positive effects have been reported [66–68].

First, the mobile phase viscosity is strongly reduced at increased temperatures. For example, a mixture of 50:50 MEOH:H_2O (v/v) possesses a viscosity of 1.47 cp at 30 °C but only 0.46 cp at 100 °C, leading to a considerable increase in the diffusion coefficients [69]. As the B and C terms of the Van Deemter equation both depend on D_m to various extents, a decrease in the kinetic performance is generally observed at flow rates less than the optimal values, and an enhancement of the kinetic performance occurs at flow rates above the u_{opt} [70]. These effects manifest in a shift of the optimal flow rate to higher velocities. This increase with D_m is particularly beneficial for peptides and proteins compared to small molecules because these large molecules possess rather low D_m values, and the experiments are always conducted in the C-term region of the Van Deemter curve (i.e., extremely low u_{opt} with large molecules) where the kinetic performance is improved at elevated temperature [71,72].

Second, the surface tension of the mobile phase (which is a function of polarity) is decreases with increased temperature. With small molecules, it has previously been demonstrated that similar retention factors are obtained when the amount of organic modifier is reduced, on average, by 10% each 30 °C temperature increase [67,73]. This significantly reduces the organic solvent consumption and explains why LC at very elevated temperatures can be considered a greener strategy. However, this can be a potential problem with hydrophilic compounds, as their retention would be too low [74], but this would also help elute the most hydrophobic compounds. In the case of large biomolecules, the effect of temperature on retention is not so simple, and we previously observed that little or even no change in retention occurred with therapeutic peptides and insulin [71]. In some cases – with therapeutic proteins, especially with pegylated ones – the retention increases when the temperature is increased. Depending on the possible conformational changes – caused by the temperature – the retention behavior of large biomolecules could be very different. Moreover, this is certainly related to the significant number of charges on such large biomolecules as well. Indeed, it has previously been demonstrated that the simple Van't Hoff equation could not be applied to small basic drugs, as the models for $\log k$ vs. $1/T$ were sometimes sigmoidal because of the simultaneous change in pK_a values with temperature [66,75]. Therefore, it is possible to observe a decrease, increase or no change in retention as a function of temperature, depending on the investigated compounds and the temperature range. In the case of peptides or proteins, which contain many charged amino acids, it is much more difficult to predict the evolution of retention with temperature. Therefore there is a need to measure and model retention behaviour in the analysis of peptides and proteins, preferably with the help of modelling software packages. According to our experience, changes in retention between 30 and 90 °C are usually observed, but this would certainly deserve some additional theoretical studies.

Third, an improvement of secondary ionic interaction kinetics between negatively charged residual silanols and positively charged analytes is commonly observed at elevated temperatures, significantly reducing the observed tailing. This improvement is related both to the increase of D_m of the charged molecules and also to the reduction in the basicity and acidity of the positively charged analytes and the negatively charged residual silanols, respectively. Indeed, it has been demonstrated that the pK_a of a base decreases, on average, by 1 unit for every 30 °C temperature increase, while that of an acid increases by 1 unit for every 30 °C increase [75]. Such tailing reduction has been demonstrated

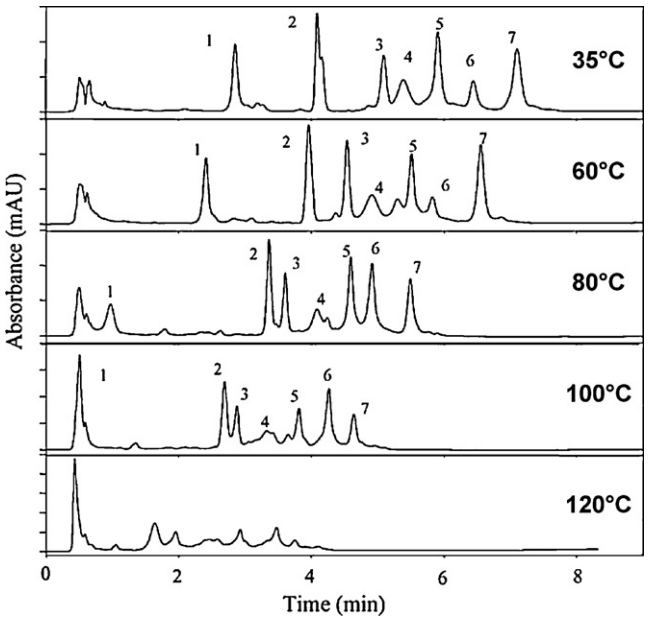

Fig. 1. The effect of mobile phase temperature on model protein separations. Proteins: (1) ribonuclease A; (2) insulin; (3) lysozyme; (4) apo-transferrin; (5) lactalbumin; (6) chymotrypsin; (7) concanavalin A. All the chromatograms are on the same absorbance scale.
Adapted from Ref. [80] with permission.

in the past with small charged compounds [73] and with large biomolecules [76].

Of course, there are also a few limitations when using high-temperature LC, but some solutions exist for tackling these different problems. First, conventional HPLC instruments require slight modifications to be compatible with temperatures above 60 °C. Particularly, it is necessary to add a preheater device before the column inlet to avoid a thermal mismatch, which could generate peak distortions and broadening. The new generation of instruments include passive (length of tubing included in a piece of conductive metal) or active preheaters (heated coat surrounding a small length of tubing) in combination with ovens that withstand temperatures up to 90–100 °C [77]. Second, the column should also be able to resist elevated temperatures, but it is well known that silica can be dissolved at high temperature. There are a variety of alternative materials (e.g., carbon, zirconia, titania, or polymeric) that are compatible with temperatures up to 150–200 °C. However, the behaviors of these materials are not fully understood, and only few applications with intact proteins using these alternative materials have been published until recently [66,78]. To solve this problem, some of the latest generations of silica-based wide-pore core–shell and hybrid UHPLC columns can be employed, as they exhibit good performance with large biomolecules (as shown in Sections 3.1.2 and 3.1.4) and are fully compatible with temperatures as high as 80–100 °C [73,79]. Finally, the most critical factor when using elevated temperatures is the thermal stability of the sample. For proteins, it has been demonstrated that intramolecular disulfide bonds can be broken and that the amide backbone of the proteins can be hydrolyzed (in the temperature range above 100 °C), as illustrated in Fig. 1 [80]. It has also been shown with small-molecular-weight compounds [81] and proteins [80] that the degradation rate depends on the temperature and residence time. Thus, to limit the degradation of intact proteins, the temperature should be increased to a reasonable range (60–100 °C) and the analysis time should be reduced as much as possible, which is now possible with the core–shell and UHPLC approaches [76].

3.1.2. Core–shell particles

Columns that are packed with totally porous particles have constraints in separation speed because of limitations in the stationary phase mass transfer that result from the relatively long diffusion times required for macromolecules to traverse the porous structure for interacting with the stationary phase [82]. The concept of shell particles was first applied by Horvath and co-workers in the late 60s, leading to the start of HPLC [83,84]. They were initially intended for the analysis of macromolecules such as peptides and proteins. Later, Kirkland showed that 30–40 μm diameter superficially porous packing provided much faster separations compared with the large, fully porous particles that were used earlier in liquid chromatography [85]. The rationale behind this concept was to improve the column efficiency by shortening the diffusion path that the analyte molecules must travel and to improve their mass transfer kinetics.

Several brands of superficially porous particles were developed and became popular in the 70s. However, major improvements in the manufacturing of high-quality, fully porous particles took place at the same time, particularly by making them smaller and more homogeneous. This inhibited the success of the shell particles, and they eventually disappeared. Recently, the need for improved analytical throughput forced particle manufacturers to find a better compromise between the demands for higher column efficiency and the need for columns to be operated using conventional LC instruments with moderate column back pressures [86]. This led to the development of a new generation of columns that were packed with shell particles [86]. Today, core–shell packing materials are commercially available in various diameters (5 μm, 3.6 μm, 2.7 μm, 2.6 μm and 1.7 μm) and with different shell thicknesses (0.5 μm, 0.35 μm, 0.25 μm, 0.23 μm and 0.15 μm) [87–90]. The thickness of the porous layer plays a major role in governing the porosity of the particles [91].

Indeed, the kinetic efficiencies (such as achievable plate numbers or peak capacity) of columns that are packed with these shell particles increase as the porous shell thickness decreases. However, the optimum shell thickness in reality is likely to be a compromise between efficiency, sample loading capacity and analyte retention.

The second generation of wide-pore core–shell particles (particle diameter d_p = 5 μm, average pore size of 300 Å and 0.25 μm shell thickness) showed excellent efficiency in macromolecule separations [87]. This material, called Poroshell, was launched in the year 2000 and was dedicated to protein separations. Fig. 2 shows a schematic profile of the Poroshell particles and a very efficient separation of model peptides and proteins. It appears that the structure of the third generation of shell-particles (d_p < 3 μm) is very close to the optimum column efficiency and loadability. In addition, media with pore sizes of 300 Å, which are required for macromolecule separations, are also commercially available. A 160 Å packing was introduced in 2010 by Advanced Material Technology (AMT) and Supelco under the brand names of Halo Peptide ES-C18 and Ascentis Express Peptide ES-C18, respectively [89,92]. An average pore size of 160 Å allows the unrestricted access of molecules up to approximately 15 000 Da, depending on the molecular conformation [93]. Kirkland et al. compared the efficiency of the 160 Å Halo Peptide ES-C18 column to the original 90 Å Halo-C18 column for mixtures of peptides and small proteins [92]. Small proteins (i.e., ribonuclease, insulin, cytochrome C and lysozyme) exhibited broadened peaks with the 90 Å Halo-C18 column, indicating restricted diffusion, but they had narrow peaks with the 160 Å Halo Peptide ES-C18 column. Gritti and Guiochon also investigated the potential of the 160 Å Halo Peptide ES packing [89]. Their results also demonstrated that the Halo Peptide ES column, which was designed to resolve mixtures of large molecules, provided markedly better kinetic performance than the first generation of Halo particles. The sample diffusivity in the porous shells was indeed increased. The results

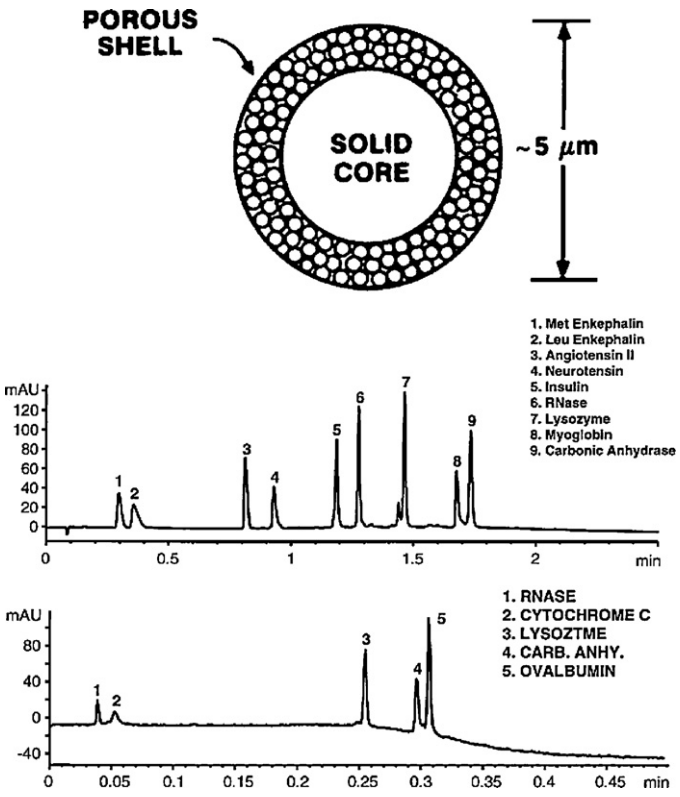

Fig. 2. Schematic figure of a Poroshell superficially porous particle and chromatograms of some very fast peptide and protein separation by using Poroshell column.

Adapted from Ref. [87] with permission.

that were obtained from these two studies also demonstrated that the trans-particle mass transfer resistance term is not the limiting kinetic factor that controls the solid–liquid mass transfer resistance in the Halo particles. Actually, the mass transfer resistance of large molecules is mostly accounted for by a slow external film mass transfer (mass transport across the boundary mobile phase layer of the particles) [89]. It appears that the improvement in the column efficiency of large molecules is related to the easier access of these molecules to the internal volume. The improvement in column performance is also due to the eddy dispersion term of the Halo Peptide ES 160 Å column being 25% smaller than that of the first generation of Halo 90 Å column [89].

More recently, a new 3.6 μm core–shell wide pore material (0.2 μm shell thickness) was launched under the name of Aeris Widepore, and seems to be very promising in protein separations [94].

3.1.3. Monoliths

3.1.3.1. Monolithic columns. As alternatives to particle based-stationary phase formats for the liquid chromatographic separations of proteins, organic polymer-based monoliths offer several advantages, including high permeability, a wide range of available chemistries and rapid mass transfer [95]. Polymer monolithic stationary phases have shown great potential for the RPLC separations of large biomolecules, including intact proteins [96–99], oligonucleotides [100,101], and peptides [102–104]. Theoretically, this material is well suited to perform large-molecule gradient separations, as the mass transfer is mainly driven by convection, rather than diffusion, due to the absence of mesopores [105]. The fact that the solvent is forced to pass through the macropores of the polymer due to pressure leads to faster convective mass transfer compared to the slow diffusion process into the stagnant pore liquid that is present in porous beads-packed columns. Due to their open channel structure, monoliths generally possess a high permeability, allowing the application of high flow rates at moderate back pressure. It was previously demonstrated that polymeric stationary phases led to superior performance over silica-based materials in the reversed-phase analysis of very large proteins (MW > 50 kDa) [106].

The first attempts to prepare monolithic stationary phases date back to the late 60s and mid-70s [107,108], but the interest in this novel approach was quite limited at that time. At the end of the 80s, Hjerten et al. published their landmark study concerning macroporous compressed gels that were made by the copolymerization of acrylic acid and *N*,*N*′-methylenebisacrylamide [109]. Soon after, Svec and Frechet presented a new class of monolithic materials [110,111]. Their process resulted in highly crosslinked and thus rigid macroporous polymers that were suitable for HPLC application. Since then, several organic monolithic polymer materials have been prepared by the thermal in situ polymerization of styrenes [112–115] and acrylates [116,117] as well as the photochemically initiated polymerization of UV-transparent monomers [118]. Additionally, monolithic reversed-phase materials have been designed by ring-opening metathesis polymerization [119].

Porous polymer monoliths were recently employed at temperatures that exceeded 200 °C for the separation of a range of simple solutes using pure water as the mobile phase [120]. These promising results suggested that polymer monoliths were suitable support formats for the analysis of proteins at high temperatures (≥80 °C), allowing (i) the use of viscous organic modifiers such as 2-propanol, ethanol or methanol, (ii) the use of extended column lengths and (iii) the use of elevated linear velocities for fast separations. The use of a 5 cm-long poly(styrene-co-divinylbenzene) monolithic 1 mm I.D. column for the separation of intact proteins was previously reported [121]. When a 1 min-long gradient span was applied, peak widths at half height of only 1 s were achieved. At longer gradient durations (120 min), a maximum peak capacity of 475 was observed [121]. Using a 5 cm-long capillary poly(styrene-co-divinylbenzene) monolith that was coupled to an LTQ Orbitrap XL mass spectrometer, a limit of detection in the low femtomol range was achieved after injecting a mixture of nine proteins with molecular weights ranging from 5.7 and 150 kDa [122]. It was shown that when using long (25 cm) monolithic columns with optimized morphologies, a peak capacity of 620 could be achieved for the separation of intact proteins by applying a 120-min long gradient separation [52]. Monolithic capillary supports (200 μm I.D.) were prepared by the polymerization of methylstyrene and the use of 1,2-bis(*p*-vinylphenyl)ethane (MS/BVPE) as a crosslinker in the presence of inert diluents. These polymeric reversed-phase materials showed excellent mechanical stability. The chromatographic potential of monolithic MS/BVPE as a stationary phase for liquid chromatography was investigated for the separation of proteins and peptides under reversed-phase conditions (Fig. 3) [123].

3.1.3.2. On-chip monoliths and monolith discs. Due to the complexities of proteins and the small quantities of available samples, it would be attractive to design microsystems that afford high sensitivity, throughput, and excellent reproducibility for efficient protein analyses [124–126]. Microfluidic systems that integrate all parts of the separation device into a single chip are particularly attractive. Chips that are fabricated from synthetic polymers have recently increased in popularity due to the simplicity of their processes. They also possess a significant cost advantage when compared to their counterparts that are made of glass, quartz, or silica [127–132]. There are no fittings, adapters, connectors, or any other dispersive elements that are prone to leaking and can deteriorate the chromatographic performance of classical capillary or nano-LC systems [131,132]. Excellent peptide recovery was

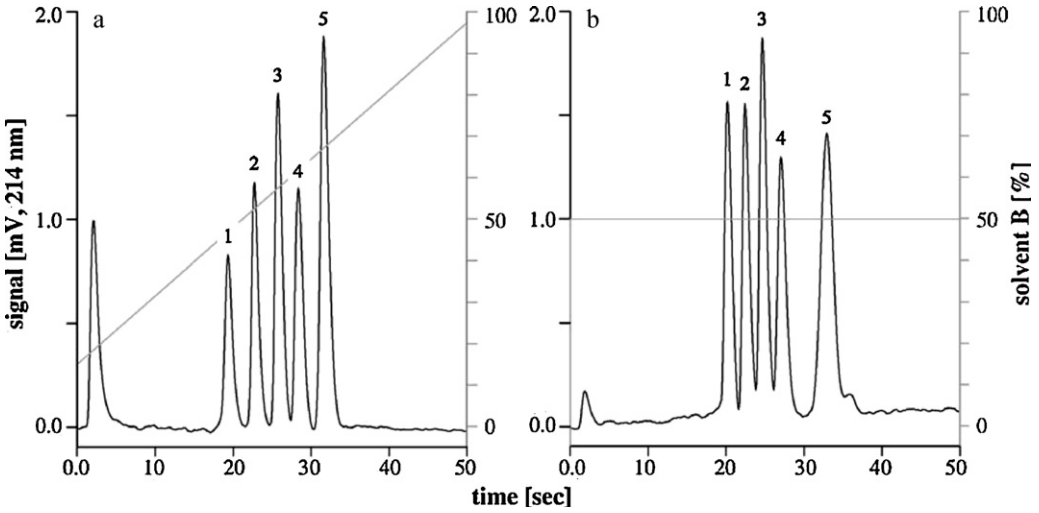

Fig. 3. Rapid protein separation on MS/BVPE monolith (80 mm × 0.2 mm) at high flow rate of 150 μl/min by applying (a) a steep linear gradient and (b) a single step gradient: (1) ribonuclease A; (2) cytochrome C; (3) α-lactalbumin; (4) β-lactoglobulin B; and (5) ovalbumin.
Adapted from Ref. [123] with permission.

observed due to the inertness of the polyimide material in contrast to fused silica microparticulate columns.

A recent study demonstrated that both methacrylate- and styrene-based monolithic stationary phases can be prepared with a polyimide HPLC chip [97]. Fig. 4 shows the scanning electron microscopic images of polyimide HPLC chips. This polymerization enables the preparation of monoliths with a wide variety of chemistries. Both types of monoliths enable efficient separations of proteins within a short period of time using a steep gradient of the mobile phase. Another option to further increase the speed and efficiency of the HPLC analyses in the chip would be to decrease the channel cross-section, leading to an increase in flow velocity at a specific flow rate [97].

Bio-Monolith HPLC columns (discs) were recently introduced, which provide high resolution and rapid separations of antibodies (IgG, IgM) and other macro-biomolecules. These monolith discs are 5.2 mm × 4.95 mm (100 μl column volume) with continuous channels, eliminating the diffusion mass transfer.

3.1.4. UHPLC (ultra-high-pressure liquid chromatography)

It was recognized very early (in the 1940s) that one potential approach for improving intact protein RP chromatography is the use of small particles [133]. It came to the practice in the late 90s that by using very fine particles (sub-2 μm and sub-1 μm), the performance (i.e., throughput, resolution, and sensitivity) is improved significantly but at the cost of pressure. The main advantage to this approach is that the analysis time could be reduced to a few minutes without a loss in resolution or sensitivity [134–136]. Conventional HPLC instruments have a maximum operating pressure limitation of 400 bar, leading to the common practice of using short columns packed with small particles to speed up the analysis [137,138]. Knox and Saleem discussed the compromise between separation speed and efficiency [139]. To overcome the pressure limitations of modern HPLC, the groups of Jorgenson [140,141] and Lee [142] constructed dedicated instrumentation and columns packed with nonporous material to allow analyses at very high pressures (up to 7200 bar). New nomenclatures have appeared to describe the higher back pressure requirement of the separation, including ultra-high-pressure liquid chromatography, ultra-high-performance liquid chromatography or very high-pressure liquid chromatography (UHPLC, UPLC, VHPLC or vHPLC). The first commercial system for ultra-high pressure separation was released in 2004. It was able to operate at pressures as high as 1000 bar (15 000 psi), and the system was known as ultra-performance liquid chromatography (UPLC™). Since then, several UHPLC systems

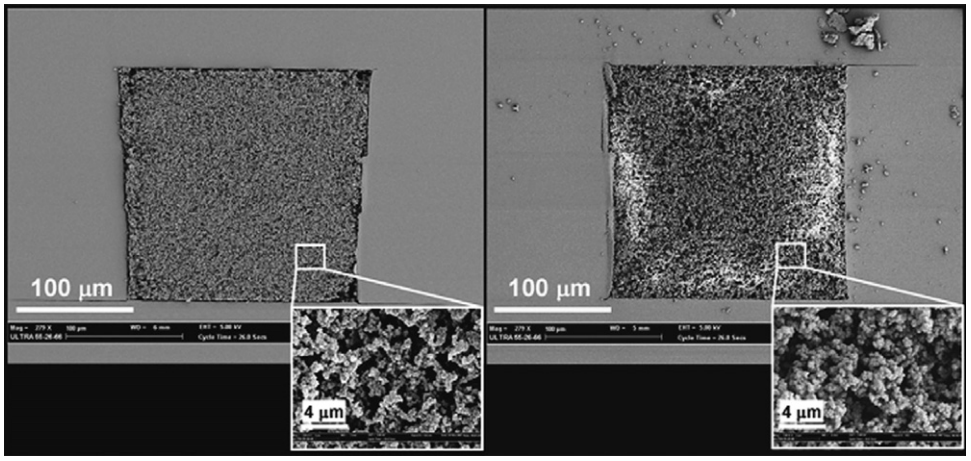

Fig. 4. Scanning electron microscopic images of the cross-section of the separation channel in a polyimide HPLC chip filled with poly(lauryl methacrylate-*co*-ethylene dimethacrylate) (left) and poly(styrene-*co*-divinylbenzene) monolith (right).
Adapted from Ref. [97] with permission.

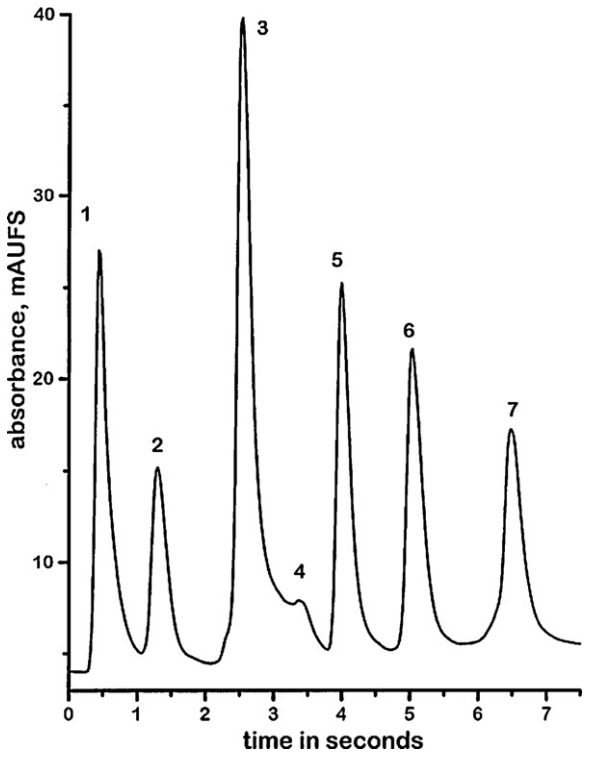

Fig. 5. Separation of a mixture of six proteins on a short non porous 1.5 μm (Micra NPS-RP) column. Analytes: (1) ribonuclease A; (2) cytochrome C; (3) lysozime; (4) unknown; (5) bovine serum albumin; (6) catalase; (7) albumin egg (ovalbumin). Adapted from Ref. [151] with permission.

have been commercialized that can withstand pressures up to 1200–1300 bar (18 000–19 500 psi).

A critical aspect of UHPLC is the effect of frictional heating, which causes significant temperature gradients within the columns at very high pressures ($\Delta P > 400$ bar). The radial temperature gradient, due to the heat dissipation at the column wall, can cause significant loss in column efficiency [143,144]. Gritti et al. concluded that both longitudinal and radial temperature gradients were more significant when the column length was decreased [145].

Nonporous and porous particles are the two major types of spherical packing materials that have been used for fast HPLC [140,142,146–149]. The major difference between these two types of particles is that the porous particles are resistant to mass transfer contributions from the stagnant mobile phase in the pores. Decreasing the particle size and increasing the diffusion coefficient can improve the mass transfer of solutes in the stagnant mobile phase. Very fine 1.5 μm nonporous silica particles, such as Micra C18, have been used in UHPLC systems [150]. Issaeva et al. demonstrated an extremely high-speed separation of proteins and peptides using the 1.5 μm Micra particles (Fig. 5) [151]. Barder et al. demonstrated that the column efficiency of nonporous silica particles (1.5 μm) was considerably higher than that of porous particles (3.5 μm), especially at high flow rates [148]. Nonporous particles can provide lower mass transfer resistance and higher efficiency than porous particles, but porous particles have greater surface area and can provide much higher sample loading capacities. Seifar et al. estimated a 50-fold higher sample capacity for porous particles vs. nonporous particles of the same size [152]. According to Wu et al., the loading capacity of 1.7 μm Acquity C18 porous particles is approximately 16.5 times larger than that of Micra C18 nonporous 1.5 μm particles [150]. Another issue is the very low retention of nonporous particles compared to totally porous particles.

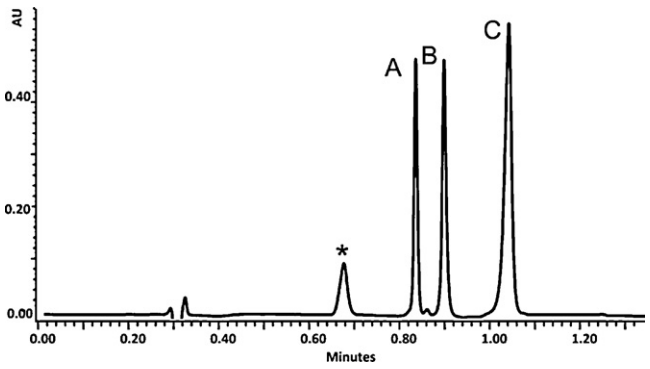

Fig. 6. Fast separation of insulin (A), α-lactalbumin (B), and ovalbumin (C) on Acquity C4 BEH 300 column (1.7 μm fully porous). Adapted from Ref. [76] with permission.

Columns that were packed with hybrid-type sub-2 μm wide-pore (300 Å) fully porous particles (Acquity BEH300) were used with great success in protein and peptide separations. Due to the intrinsic chemical stability of this hybrid particle technology (Ethylene Bridged Hybrid, BEH), a wide pH (pH 1–12) and temperature (up to 90 °C) range can be employed, enabling a versatile and robust separation technology for method development. These 1.7 μm hybrid particles are available in different pore sizes (130 Å, 200 Å and 300 Å), and several bonded phases for reversed-phase and hydrophilic interaction chromatography exist for both peptide and protein separations. Goetze et al. reported a very efficient glycan profile analysis using a 15 cm-long narrow-bore (2.1 mm I.D.) BEH300 C18 1.7 μm column [153]. The determination of methionine oxidation by peptide mapping of monoclonal antibodies was performed with a BEH300 C18 1.7 μm, 2.1 mm × 100 mm column [154]. In a theoretical study, a short narrow-bore BEH300 C4 column was used to investigate the plate counts of large molecules under reversed-phase gradient conditions [155]. The potential of UHPLC to enhance the separation of intact proteins was examined by Everley and Croley in a systematic study [156]. When UHPLC was applied to a mixture of 10 protein standards, the optimized method yielded improved chromatographic resolution, enhanced sensitivity, and a three-fold increase in throughput compared to conventional HPLC. A recent comparative study evaluated the potential of sub-2 μm fully porous particles and sub-3 μm shell particles in peptide and protein analyses [76]. To improve the separation of intact proteins, the hydrophobicity of the stationary phase-bonded alkyl chain (C4 and C18) and pore size were investigated. For the largest proteins, the C4 300 Å (Acquity BEH) column was the most effective because it combined good kinetic performance and appropriate pore size, yet it did not result in any improvement for the smaller proteins (<20 kDa) [76]. Fig. 6 illustrates an 1.2 min long separation of three protein standards on Acquity C4 BEH 300 column.

Silica-based wide-pore sub-2 μm particles in protein analysis are not yet widespread; however, a 175 Å stationary phase (1.9 μm) is commercially available. For example, olive pulp proteins have been separated with a Hypersil Gold 100 mm × 3 mm, 1.9 μm (175 Å) column under UHPLC conditions [157].

3.1.5. Porous-layer open-tubular (PLOT) columns

An alternative to packed and monolithic columns may be the so-called porous-layer open-tubular-type (PLOT) columns, which have been shown to provide very high column efficiency compared to conventional packed columns [158]. Open-tubular columns for liquid chromatographic applications were first investigated by Tsuda et al. in the late 70s [159]. Jorgenson and Guthrie first reported on open-tubular columns with an inner diameter of 15 μm

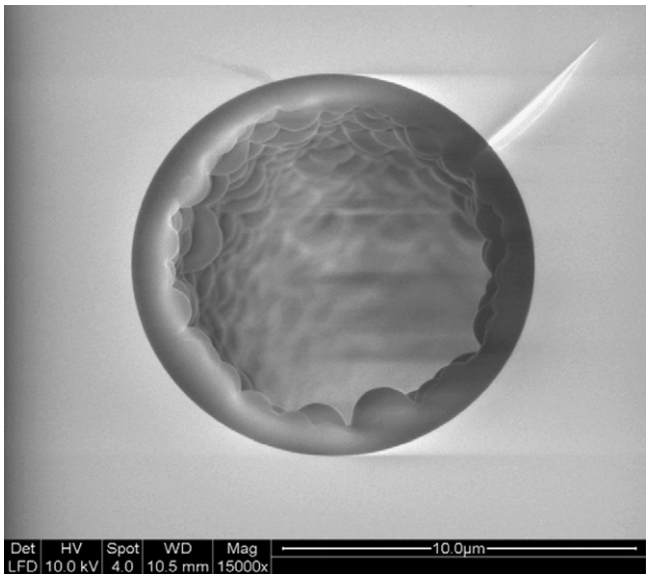

Fig. 7. SEM image of PLOT column used for separation of intact proteins. Adapted from Ref. [164] with permission.

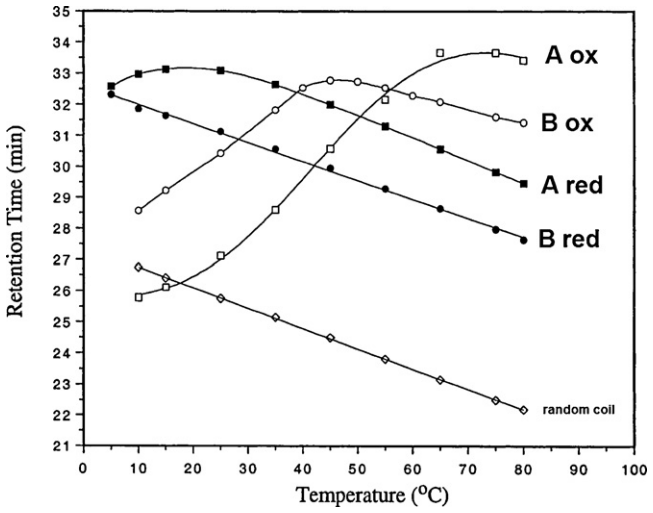

Fig. 8. Effect of temperature on RPLC of two oxidized and two reduced α-helical peptide analogues and a random coil peptide.
Adapted from Ref. [170] with permission.

[160]. The kinetic efficiency of PLOT columns is very promising, but the use of the narrow PLOT columns initially led to several technical problems, such as very high pressures, detector coupling difficulties and extra-column band broadening. PLOT columns have increased in popularity after they were successfully coupled to nanospray-MS [161]. Recently, 10 μm inner diameter PLOT polystyrene-divinylbenzene (PS-DVB) columns have been designed and used for high-resolution LC–MS separations of peptides [162,163].

Rogeberg et al. prepared and studied a 10 μm I.D. PS-DVB PLOT column for the separation of intact proteins [164]. The columns were prepared following the Karger group method [163]. Fig. 7 shows the SEM image of the PLOT column that was used for the separation of intact proteins. The authors found this column very promising because it provided narrow peaks, very low carryover and good repeatability. Hence, these columns have significant potential for the separation of more complex samples as encountered in top-down, bottom up and even middle down proteomics [164].

3.2. Thermodynamics, protein conformation, and retention

The conformation of proteins significantly depends on RPLC conditions. The hydrophobic interactions between proteins and the nonpolar RPLC stationary phase reflect the hydrophobicity and interactions between nonpolar residues, which are the major driving forces for protein folding and stability [165–167]. Because the effects of temperature, mobile phase organic modifier, mobile phase additives (pH, ionic strength) and applied pressure are crucial for protein conformation, these variables seriously affect the retention and peak shapes of proteins in RPLC; therefore, they are key factors in the optimization of selectivity.

3.2.1. Temperature

It was previously demonstrated that it is possible to manipulate polypeptide or protein separations by varying the temperature (5–80 °C) due to changes in the selectivity (and retention). Depending on the stability of the secondary structure, the molecules unfold to various extents at different temperatures and hence interact with the stationary phase with various strengths [168]. Due to the different conformation-dependent responses of peptides and proteins at elevated temperatures, the change in retention can be very different [169,170]. Therefore, temperature offers the ability to adjust the selectivity of the separation. It was previously shown that the response to a temperature increase in the case of a random coil configuration is not as marked as that of an α-helical structure (fully folded analogues) [169,171].

Both the hydrophobic nature of the column surface and the presence of organic solvent favor the rearrangement of the protein conformation to expose the normally internalized hydrophobic residues. If the protein is completely denatured into a random coil conformation, it will be eluted as a single sharp peak. However, under certain conditions, the native conformation and/or other intermediate conformations may be present during the analysis. Each of these will interact differently with the stationary phase, resulting in varying retention times and multiple peaks in the chromatogram [172–178].

A systematic study previously demonstrated the effect of temperature on the retention of different types of peptides, such as oxidized α-helical, reduced α-helical and random coil peptides [170]. At elevated temperature (80 °C), the disulfide-bridged peptides were eluted according to their hydrophobicity at the substitution site. The retention behavior of the random coil peptide showed a linear decrease in retention time with increasing temperature (on a polystyrene-divinylbenzene based RPLC stationary phase). As the temperature was decreased from 80 °C, the peptides become less retained and their elution order at 10 °C was opposite to that at 80 °C. In other words, the most hydrophobic peptide was eluted first, and the least hydrophobic peptide was eluted last (Fig. 8). The retention behavior of the reduced peptides was dramatically different from the disulfide-bridged peptides. Indeed, at 10 °C, the disulfide-bridged peptides were eluted substantially earlier than their reduced analogues (Fig. 8). This retention behavior suggested that the oxidized peptides were folding during chromatography, burying the hydrophobic residues. The disulfide bridge between two helical strands of a coiled coil has a dramatic effect on the stability of the coiled coil.

Another study demonstrated the thermally induced interconversion of insulin by temperature-dependent changes in the retention parameters [179]. The authors reported irreversible conformational changes for insulin at temperatures between 65 °C and 85 °C. Irreversible temperature-induced conformational transitions may have been responsible for the observed peak splitting of proteins under the RPLC conditions [179].

Temperature gradients represent attractive alternatives to gradient analysis, as a small change in temperature usually induces significant changes in the retention, peak width and resolution of macromolecules [180].

3.2.2. Organic modifier

The desorption and elution of proteins from RPLC columns is typically accomplished with aqueous solvents containing an organic modifier and an ion-pairing reagent or buffer. The organic modifier solubilizes and desorbs the proteins from the hydrophobic surface, while the buffer maintains the eluent pH and interacts with the proteins. Acetonitrile is the most commonly used organic modifier. Isopropanol is often used for large or very hydrophobic proteins because of its superior elution strength, but it suffers from a high viscosity. To reduce the viscosity of isopropanol while retaining its hydrophobic characteristics, a mixture of acetonitrile and isopropanol can be employed. Adding only 1–3% isopropanol to acetonitrile has been shown to increase protein recovery in some cases [181]. Ethanol and methanol are also often used and are preferred in process purifications and to elute hydrophobic proteins [182,183].

The addition of an organic solvent, such as acetonitrile, methanol or isopropanol, to the aqueous mobile phase is known to reduce the surface tension of the mixture [184]. Some studies have shown that adding acetonitrile or other solvents at relatively high concentrations can act as denaturants for many globular proteins at higher temperatures, yet the thermodynamic stability of the same proteins can be increased over a defined temperature range by reducing concentrations of these solvents [185,186]. Changes in the thermodynamic parameters associated with protein–ligand interactions or protein unfolding/refolding processes in water–organic solvents are generally described in terms of the Gibbs–Helmholtz equation.

Boysen et al. showed that in water–acetonitrile, the cytochrome C ligand interaction is enthalpy- or entropy-driven, or both, depending on the temperature [186]. The heat capacity/temperature dependence in conjunction with the Gibbs free energy balance was interpreted as an unfolding process of the protein with two states, the folded and the unfolded, which was characterized by a set of compensation points. The hydration effects and the van der Waals interactions contributed to the hydrophobic interactions between cytochrome C and ligand. With the described thermometric HPLC procedure using water–acetonitrile solvents, the thermodynamic stability of the hydrophobic core of the cytochrome C species could be measured and ranked. In contrast, in water–methanol, the cytochrome C–ligand interaction was entirely enthalpic. The heat capacity values were close to zero and lacked pronounced temperature dependence. This could be seen as evidence that cytochrome C existed in only one conformational state that was not necessarily native and that the conformation was certainly stabilized by the presence of a nonpolar ligand [186].

Sawicka et al. have shown that alcohols in the mobile phase can induce helical conformations, although pressure effects due to the different viscosities of the organic solvents cannot be discounted [178]. Both methanol and isopropanol induce conformational changes that can lead to the formation of a significantly altered but highly ordered conformational state at the secondary structural level that would be expected to have intermediate retention on the chromatographic surface compared to the compact native protein or a more fully denatured form [187]. The appearance of multiple peaks of similar retention may be caused by the presence of different conformations with different degrees of helix induction. In contrast, acetonitrile is not as disruptive to the protein's internal structure and therefore is not as strong of a modifier for inducing conformational change [177]. It has been reported to provide some stabilization of β-sheet structure in polypeptides. Additionally, in the presence of 60% acetonitrile, the protein interferon alpha-2b has been shown to lose its alpha-helical structure while still retaining its anti-parallel beta-sheets [188–190].

3.2.3. Mobile phase additives

Proteins contain many ionizable groups in the side chains of their amino acids and their amino- and carboxyl-termini. These include basic groups in the side chains of lysine, arginine and histidine and acidic groups in the side chains or glutamate, aspartate and cysteine. The pH of the solution, the pK_a of the side chain and the side chain's environment influence the charge on each side chain. The isoelectric point is the pH at which a protein carries no net electrical charge. Below the isoelectric point, proteins carry a net positive charge; above the isoelectric point, they carry a net negative charge. The isoelectric point is significant in protein analysis and purification because it is the pH at which solubility is often minimal. Therefore, the pH of the mobile phase is a very important factor, and peak tailing, peak width, retention and selectivity can be adjusted by varying the mobile phase pH and additive concentration. Ion-pairing reagents or buffers as mobile phase additives set the eluent pH and interact with the analytes.

Trifluoroacetic acid (TFA) at a concentration of 0.05–0.1% is commonly used for the analysis of peptides and proteins, as it provides excellent ion pairing and solvating characteristics and therefore inhibits peak broadening and tailing [191]. TFA concentrations up to 0.5% can be useful in solubilizing large or hydrophobic proteins, while lower concentrations are occasionally used for tryptic digest separations. In some cases, phosphate buffer results in sharper peaks than TFA and can change the elution order or selectivity. Phosphate interacts with the basic side chains of proteins, increasing the rigidity of the protein. Heptafluorobutyric acid (HFBA) is effective in separating basic proteins, and triethylamine phosphate (TEAP) has been used for preparative separations [192–195]. One study found that the sample capacity was greater using TEAP than TFA [196]. Formic acid has been used for the chromatography of very hydrophobic polypeptides and proteins. Formic acid is also gaining popularity in LC–MS separations of proteins and peptides because TFA partially suppresses the ion signal in the electrospray source and because formic acid has proven to be effective in the LC–MS of peptides and proteins. Very recently, a new particle technology (Charged Surface Hybrid, CSH™) has developed that can provide very sharp peaks through the use of more MS-friendly (volatile) additives such as FA instead of TFA and which could potentially be useful for peptides and intact proteins analysis. This CSH technology is based on hybrid particles and adding a low level charge to particle surface before functionalizing the bonded phase.

Guo et al. compared the use of TFA, HFBA and phosphoric acid in the elution of peptides and found that each gave somewhat different selectivity [197].

A hydrophilic counterion such as phosphate will neutralize the highly hydrophilic positively charged groups found in peptides, thus decreasing the overall peptide hydrophilicity. In contrast, more hydrophobic anions, such as perfluorinated acids, will not only neutralize the positively charged groups, thereby decreasing the peptide hydrophilicity, but will also further increase the affinity of the peptides for the hydrophobic reversed-phase stationary phase [197]. This affinity increases with the hydrophobicity of the anion, i.e., TFA < pentafluoropropionate < heptafluorobutyrate. Such acidic reagents have generally been employed at concentrations between 0.05% and 0.1% (v/v) for the majority of peptide/protein separations [197–200]. Higher concentrations have generally been avoided in the past due to, among other things, silica-based stationary phase hydrolysis under highly acidic conditions [201,202]. However, the advancement in recent years of reversed-phase, silica-based packings with excellent chemical

stability [203–205] has enabled us to revisit the question of the most suitable types and concentrations of acidic ion-pairing reagents for the separation of peptide and protein mixtures.

Shibue et al. showed the effect of increasing counterion hydrophobicity on the elution behavior of peptides at a constant concentration of different ion-pairing reagents [206]. The retention times of all peptides were higher with increasingly hydrophobic counterions. The elution ranges of all of the peptides remained very similar despite the large increase in overall peptide retention time [206]. Moreover, the authors demonstrated the reversal of elution order of some peptides as the additive concentration was raised from 1 mM to 2 mM and 20 mM. Increasing the additive concentration clearly resulted in improved peak shapes and increased peptide retention times [206].

Using TFA (the most commonly used additive) causes significant ion suppression in electrospray ionization mass spectrometry detection (ESI-MS). A relatively new approach based on the use of intermediate or high pH mobile phases gives noteworthy improvement in signal to noise ratio. Chromatographically, the use of high pH results in substantially different separation selectivities, which may prove useful in complex separations. In the intermediate pH range, ammonium formate (pH ~ 7) while in the high pH range, ammonium hydroxide or trisodium phosphate (pH ~ 9–11) could be appropriate additives.

3.2.4. Effect of pressure

For large molecules such as proteins, it has been observed that pressure can have a rather strong influence on retention [207–211]. The change of retention can be attributed to the fact that the partial molar volume of the analyte is smaller when it is adsorbed in the stationary phase than when it is in the liquid mobile phase [207–211]. The conformational change (unfolding or spreading) of a protein molecule upon adsorption is a well-known phenomenon that leads to the exposure of its hydrophobic core [209,210]. At higher pressures, the adsorption of proteins onto the stationary phase is thus more relevant.

Kirkland et al. found that the retention factor for insulin changed drastically with mobile phase velocity [87]. They speculated that this phenomenon was due to a conformational change of the protein as a result of the flow. At higher flow rates, and consequently higher shear forces, the protein tended to unfold, exposing more of its hydrophobic moiety that interacts with the reversed-phase stationary phase for increased retention. Similar results were found for carbonic anhydrase and other proteins [212].

A recent study described the dependence of the retention of insulin on the pressure [213]. Fig. 9 illustrates that the retention factor of insulin increased with the average column pressure for two different types of columns (a fully porous and a core–shell column). For polar and ionized analytes, the pressure dependence of the retention factor was rather pronounced with ultra-high-performance separations where the column inlet pressure reached 1000–1200 bar. Such analytes lose their hydration layer when entering the hydrophobic stationary phase; thus, the change of partial molar volume becomes substantial [211].

Because the decrease of the molar volume upon adsorption onto a hydrophobic surface is more pronounced for proteins, the pressure or flow rate can be an effective tool for changing the selectivity of RPLC protein separations.

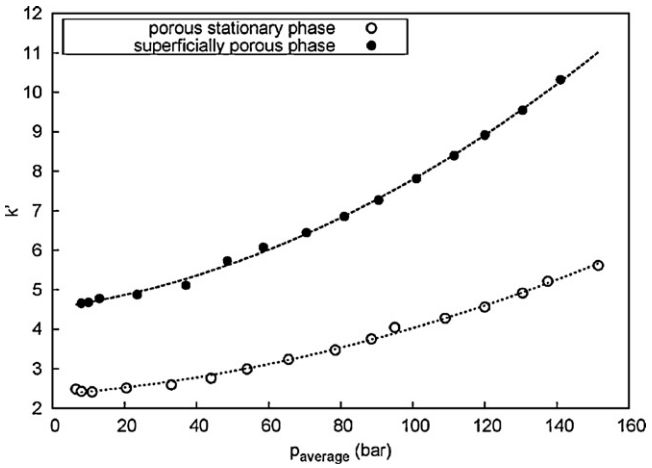

Fig. 9. Retention factor of human insulin against the average pressure drop. Adapted from Ref. [213] with permission.

3.3. Chromatographic system

The success of highly efficient separations depends on both column efficiency and on preserving the efficiency by minimizing instrument-induced extra-column band spreading. Every improvement in column technology requires considerable progress in instrument design and manufacturing [214]. Extra-column band spreading affects the measured performance of columns, especially for columns with an internal diameter smaller than the standard 4.6 mm [215]. Recently, several papers focused on the extra-column effect as a major factor that negatively impacted the apparent performance of columns that were packed with core–shell or sub-2 μm particles [214–216]. Further optimization of commercial UHPLC systems, such as using a smaller-volume needle seat capillary, narrower and shorter connector capillary tubes and a smaller volume detector cell, can provide a significant decrease in extra-column contribution. With these improvements the efficiency loss can be significantly reduced. According to a recent study [216], the latest LC systems can be classified into three groups: (1) optimized systems for fast separation with very low dispersion (extra-column peak variance (ECV) < 10 μl^2), (2) hybrid LC systems, which are recommended for both fast and conventional separations (ECV = 10–30 μl^2), and (3) conventional LC systems with an extra-column variance greater than 50 μl^2. These major differences in extra-column peak variance have a significant impact on the measured column performance and the achievable analysis time.

However, in real-life protein separations, the gradient elution mode is necessary. In this mode, the negative effects of extra-column band broadening are not as important as they are in the isocratic mode. In the gradient elution mode, the samples focus at the inlet of the column; therefore, only the contribution of the connecting tube after the column and the detector cell contribute to the peak broadening. This extra-column peak variance in gradient elution mode is thus negligible in most cases. Another feature to be accounted for is the low diffusivity of proteins. Because large analytes move more slowly in the mobile phase than the small analytes do, the contributions of the extra-column volumes to the total peak broadening are not as critical as they are for small analytes.

A possible issue in protein analysis is the adsorption of proteins onto the HPLC instrument (injector, tubes or detector cell). It is suggested to avoid the use of polyether ether ketone (PEEK) for connection tubing and the injection needle. PEEK is a hydrophobic material that can cause strong protein adsorption. Inert materials such as titanium, stainless steel or PEEK-Sil (fused silica inside, PEEK outside) are preferable, though fused silica and stainless steel do not completely eliminate protein adsorption [217]. Recently, Agilent Technologies introduced the 1260 Infinity Bio-inert HPLC system, which is dedicated to biomolecule analysis. This system is iron- and steel-free in solvent delivery, and the sample-contacting surface is completely metal-free, minimizing undesired surface interactions. Waters Corporation (Milford, USA) recently launched a new UHPLC system (ACQUITY UPLC® H-Class Bio System) that also features an

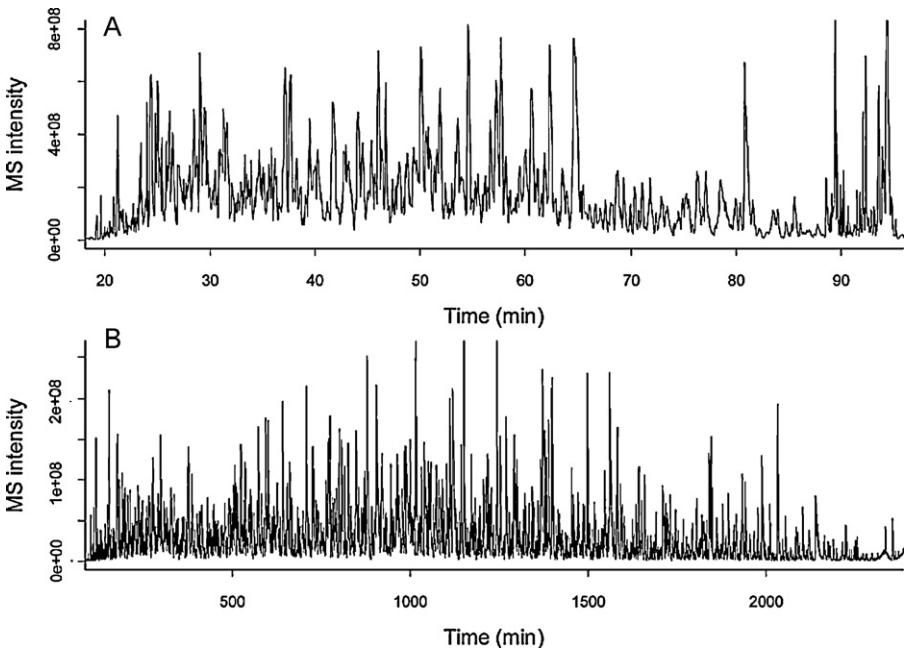

Fig. 10. Base peak chromatograms for the analysis of *E. coli* cell lysate using the 15 cm long C18 silica particle-packed column (A) and the 350 cm long monolithic silica C18 column (B). Tryptic peptides in 4 μg of *E. coli* cell lysate were loaded onto each column. Gradients of 70 and 2470 min were applied to the 15 cm long particle-packed column and the 350 cm long monolithic silica column, respectively.

Adapted from Ref. [229] with permission.

inert flow path. Other vendors offer bioinert LC systems, such as the Shimadzu LC-10Ai HPLC system or the Dionex Ultimate 3000 system. Jasco recommends some bioinert LC modules for their LC-2000 system.

3.4. Coupling RPLC to MS

Mass spectrometry (MS) has become increasingly popular for the characterization of peptides and proteins. The popularity of this technique is related to the introduction of two highly sensitive and "soft" ionization techniques that enable the transfer of intact proteins into the gas phase without fragmentation, electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) and to the continuous improvements of instrumentation that are capable of high mass and high sensitivity detection [218]. In addition, MS devices have become easier to use, more robust and better able to discriminate proteins with close m/z ratios because of the very high resolution that can be attained (e.g., maximal resolution of 50 000 for time of flight (TOF), 240 000 for Orbitrap [219] and up to several millions with Fourier transform ion cyclotron resonance (FTICR) [220]) in comparison with quadrupole-based instruments (resolution of 1000).

An obvious advantage of RPLC over the traditional chromatographic approaches for protein characterization (e.g., IEX, SEC, and affinity chromatography) is related to its inherent compatibility, in terms of mobile phases, with mass spectrometry. Indeed, the two types of ionization techniques, namely ESI and MALDI can be combined with RPLC, while the other chromatographic approaches are only compatible with MALDI, which is more tolerant to salts. MALDI is a solid-state technique in which a laser vaporizes a mixture of sample and matrix that has been spotted and dried onto a metal target plate. Thus, the combination with RPLC is exclusively off-line and consists of the collection of spots that originated from the RPLC column [221]. In contrast, ESI is a flow-based strategy where the liquid sample is transformed into an aerosol and ionized, to produce gas-phase ions. In this case, ESI can be coupled in-line with RPLC [222]. The two ionization techniques each have certain pros and cons for protein sample characterization. In the case of ESI, the analysis is convenient, as sample is injected into the column and analyzed directly as it is eluted. The limitation is that the time for performing MS experiments is limited by the peak width, which is particularly critical in UHPLC or with core–shell material [222–224]. Another important difference between the two techniques is that MALDI mostly produces ions with a charge of +1, while the ESI produces a range of charge states for each protein, making the ESI spectra more difficult to interpret. However, larger proteins are more easily accessible with ESI than with MALDI, and the resolving power is also significantly higher in ESI because of the lower m/z ratios of the multi-charged ions. Finally, it is also worth mentioning that although each technique has its advantages and limitations, it is clear that some proteins will only ionize with one technique and not with the other. For this reason, both ESI and MALDI are important and complementary to mass spectrometry of proteins.

Whatever the ionization technique, several approaches for identifying and characterizing proteins have been reported in the literature. The simplest one consists of directly analyzing the intact proteins. If ESI is selected, it is necessary to deconvolute the signal of multiply charged ions to determine the average molecular weight of the species [225]. In the case of MALDI, the molecular weight can be directly obtained. There are two additional approaches that are employed in the field of proteomics [226]. These approaches are known as (i) "bottom-up proteomics", in which a complex mixture of proteins is subjected to proteolytic cleavage and the peptides produced are analyzed by MS, (ii) "middle-down proteomics" in which long polypeptides in the mass range of 3–20 kDa is subjected to analyze by MS and (iii) "top-down proteomics", in which intact proteins are subjected to gas-phase fragmentation for MS analysis. These three proteomic approaches have been widely described and reviewed [227,228] and will not be discussed in the present contribution. It is, however, possible to find in the literature some impressive peptide mass fingerprinting by RPLC, as presented in

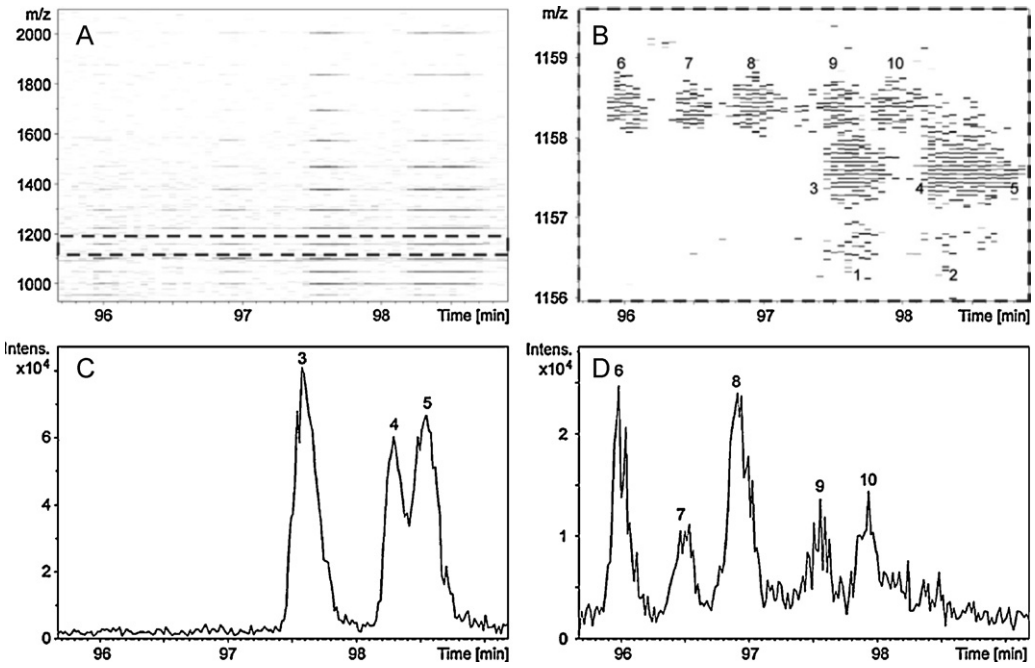

Fig. 11. Zoom-in density views (A and B) and extracted ion chromatograms (C and D) showing protein isoforms of peroxiredoxin 1 that differ in their oxidation state and place where oxidation occurs. Native peroxiredoxin 1 is represented by peaks 1–2; peaks 3–5 represent singly oxidized peroxiredoxin 1 protein isoforms; peaks 6–10 contain doubly oxidized peroxiredoxin 1.

Adapted from [230] with permission.

Fig. 10, where 22 196 peptides (2602 proteins) of *Escherichia coli* were successfully identified using a 350 cm × 100 μm I.D. silica-based monolithic column with a gradient time of 41 h [229].

Except for the analysis of very complex samples, which are often encountered in proteomics, RPLC–MS is also the method of choice for determining the intact masses of large biomolecules, protein heterogeneity, and post translational modifications (PTMs). However, the MS instrument should offer high resolution, high sensitivity and high mass accuracy over a wide mass range, which is the case for the TOF, Orbitrap and FTICR MS instruments. The separation of the oxidized (oxidation generally occurred in cysteine) form of a native protein using RPLC with an organic polymer monolithic capillary column combined with TOF/MS was recently published [230]. To reach a sufficient resolution for the oxidized protein isoforms, a 250-mm long column was heated to 60 °C, and the gradient time was extended to 2 h. In these conditions, a peak capacity of around 600 was attained for intact proteins. Fig. 11 shows the obvious complementarity between high-resolution RPLC and high-resolution MS. Fig. 11A corresponds to the 2D map of the RPLC–TOF/MS separation between 96 and 99 min (elution time of peroxiredoxin 1 protein) and a mass range between 1000 and 2000. The zoomed-in map presented in Fig. 11B corresponds to a mass range between 1156 and 1159 (mass range of peroxiredoxin 1 with 19 charges). Finally, the chromatograms of Fig. 11C and D show various singly oxidized forms of peroxiredoxin 1 and doubly oxidized forms of peroxiredoxin 1, respectively. This example highlights that high-resolution RPLC or high-resolution MS is insufficient for separating the native and all the oxidized forms of peroxiredoxin 1. Thus, there is a need to combine RPLC with MS to clearly separate and identify the different experimentally observed forms.

It is particularly informative to identify the solvent-accessible surfaces of a protein, revealed by solution-phase hydrogen/deuterium (H/D) exchange. H/D exchange [231,232] is especially powerful when combined with high-resolution nuclear magnetic resonance NMR [233,234], because one can simultaneously monitor H/D exchange at each assigned amide hydrogen resonance, in an experiment tuned to detect only those protons directly bonded to nitrogen. More recently, the H/D exchange approach has been adapted to mass spectrometry [235–237]. A dilute protein in H_2O solution is suddenly diluted with D_2O buffer. The exchangeable hydrogens (namely the backbone amide hydrogens and various side-chain hydrogens) are then replaced by deuteriums over time. The rate of uptake of deuterium is experimentally determined by quenching the exchange (by reducing the pH and freezing the sample), cleaving the protein with pepsin, and then measuring the increase in mass of each of the peptic peptides by LC–MS. Advantages of this approach are that only a small amount (typically 1 mg per sample) of protein is needed, and the protein need not be low molecular mass, highly soluble, or crystallisable [238].

4. Conclusion

As shown in the present paper, it is possible to analyze therapeutic peptides and proteins using RPLC. Of course, there are a number of problems related to elevated molecular mass, low diffusion coefficients, multiple conformations and protein isoforms that could lead to additional distortion, broadening or tailing of the chromatographic peaks.

However, most of these issues are fully or at least partially resolved with the latest generation of RPLC stationary phases because of the improved mass transfer characteristics and reduced silanol activity. Indeed, the columns packed with wide-pore core–shell particles or fully porous sub-2 μm particles of 300 Å are particularly promising in terms of achievable kinetic performance. Organic monoliths also represent a viable alternative for proteins, particularly for MW greater than 50 kDa because the mass transfer is mostly driven by convection rather than diffusion. Finally, PLOT columns also have significant potential for peptide and protein analysis but have not yet been sufficiently developed by the manufacturers.

As demonstrated throughout this review, the mobile phase temperature plays a key role for improving the peak shapes of proteins. Indeed, an elevated temperature improves the diffusion coefficients and reduces secondary ionic interactions. Currently, various wide-pore silica-based stationary phases withstand temperatures up to 80–100 °C and can be used routinely for peptide and protein analyses, provided that the latter are not subjected to thermal degradation (relationship with the temperature and residence time).

Regarding method development for protein separation, the classical approach consists of maximizing selectivity by changing the nature and concentration of the organic modifier and the ion-pairing reagent. However, in real-life applications, proteins that need to be separated share very similar molecular weights and nearly identical structures. Therefore, there is little chance of improving the selectivity, and the kinetic efficiency becomes more relevant for increasing the overall resolution. In this context, the quality of the stationary phase is of prime importance, and the mobile phase temperature and column dimensions could also play a crucial role.

Finally, the main interest for using RPLC in peptides and proteins analysis is that this technique is directly compatible with mass spectrometry, using either the ESI or MALDI ionization source. Thus, MS is able to provide an additional "dimension" to the separation in which the unresolved proteins by RPLC can be separated based on their m/z ratios.

References

[1] G. Walsh, Biopharmaceucals: an overview, in: G. Walsh, B. Murphy (Eds.), Biopharmaceuticals: An Industrial Perspective, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1999, pp. 1–34.
[2] G. Walsh, Pharmaceuticals, biologics and biopharmaceuticals, in: Pharmaceutical Biotechnology: Concepts and Applications, Wiley, West Sussex, England, 2007, pp. 1–11.
[3] www.imshealth.com (consulted in November 2011).
[4] http://www.portfoliomanagementsolutions.com/the-organization-of-pharmaceutical-rd/small-molecule-drugs-versus-biomolecular-drugs-biologics/ (consulted in November 2011).
[5] I.S. Krull, A. Rathore, T.E. Wheat, Current applications of UHPLC in biotechnology. Part I. Peptide mapping and amino acid analysis, LC–GC N. Am. 29 (2011) 838–848.
[6] A. Staub, D. Guillarme, J. Schappler, J.L. Veuthey, S. Rudaz, Intact protein analysis in the biopharmaceutical field, J. Pharm. Biomed. Anal. 55 (2011) 810–822.
[7] R. Haselberg, G.J. de Jong, G.W. Somsen, Capillary electrophoresis–mass spectrometry for the analysis of intact proteins, J. Chromatogr. A 1159 (2007) 81–109.
[8] F. Zhou, M.V. Johnston, Protein profiling by capillary isoelectric focusing, reversed-phase liquid chromatography, and mass spectrometry, Electrophoresis 26 (2005) 1383–1388.
[9] R. Haselberg, G.J. de Jong, G.W. Somsen, Capillary electrophoresis–mass spectrometry for the analysis of intact proteins 2007–2010, Electrophoresis 32 (2011) 66–82.
[10] M. Gilar, P. Olivova, A.E. Daly, J.C. Gebler, Orthogonality of separation in two-dimensional liquid chromatography, Anal. Chem. 77 (2005) 6426–6434.
[11] R.I. Boysen, M.T.W. Hearn, HPLC of peptides and proteins, unit 8.7, in: Current Protocols in Protein Science, John Wiley & Sons, Inc., 2001, pp. 3–23.
[12] M. Swartz, I. Krull, Analytical method validation for biotechnology proteins, peptides, and antibodies, LC–GC (2009), http://chromatographyonline.findanalytichem.com/lcgc/ (Chromatographyonline).
[13] ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2(R1), www.ich.org.
[14] ICH Harmonised Tripartite Guideline, Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products Q6B, www.ich.org.
[15] M.I. Aguilar, M.T.W. Hearn, High resolution reversed phase high performance liquid chromatography of peptides and proteins, Meth. Enzymol. 270 (1996) 3–26.
[16] C.T. Mant, R.S. Hodges, Analysis of peptides by high performance liquid chromatography, Meth. Enzymol. 271 (1996) 3–50.
[17] M.I. Aguilar, HPLC of Peptides and Proteins, Methods and Protocols, Human Press, Totowa, NJ, 2004, pp. 9–22.
[18] I. Molnár, Cs Horváth, Separation of amino acids and peptides on non-polar stationary phases by HPLC, J. Chromatogr. 142 (1977) 623–640.
[19] K.K. Unger, Silica as a support, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules: Methods and Applications, Dekker, New York, 1990, pp. 3–24.
[20] M. Henry, Design requirements of silica-based matrices for biopolymer chromatography, J. Chromatogr. 544 (1991) 413–443.
[21] N.E. Zhou, C.T. Mant, J.J. Kirkland, R.S. Hodges, Comparison of silica-based cyanopropyl and octyl reversed phase packings for the separation of peptides and proteins, J. Chromatogr. 548 (1991) 179–193.
[22] F.J. Moy, Y.-C. Li, P. Rauenbeuhler, M.E. Winkler, H.A. Scheraga, G.T. Montelione, Solution structure of human type-α-transforming growth factor determined by heteronuclear NMR spectroscopy and refined by energy minimisation with restraints, Biochemistry 32 (1993) 7334–7353.
[23] M.A. Chlenov, E.I. Kandyba, L.V. Nagornaya, I.L. Orlova, Y.V. Volgin, High performance liquid chromatography of human glycoprotein hormones, J. Chromatogr. 631 (1993) 261–267.
[24] B.S. Welinder, H.H. Sorenson, B. Hansen, Reversed-phase high performance liquid chromatography of insulin. Resolution and recovery in relation to column geometry and buffer components, J. Chromatogr. 361 (1986) 357–363.
[25] B.S. Welinder, Use of polymeric reversed-phase columns for the characterisation of polypeptides extracted from human pancreata. II. Effect of the stationary phase, J. Chromatogr. 542 (1991) 83–99.
[26] Amersham Pharmacia Biotech, Source 5RPC ST 4.6/150 High Performance Reversed Phase Chromatgoraphy Data File 18-1132-36, 1999, Amersham Pharmacia Biotech, Purification of Amyloid-β1-42 at High pH Using a Polymer Stationary Phase Application Note 18-1130-92, 1998.
[27] H.J. Wirth, K.O. Eriksson, P. Holt, M.I. Aguilar, M.T.W. Hearn, Ceramic based particles as chemically stable chromatographic supports, J. Chromatogr. 646 (1993) 129–141.
[28] L. Sun, P.W. Carr, Chromatography of proteins using polybutadienecoated zirconia, Anal. Chem. 67 (1995) 3717–3721.
[29] S.K. Paliwal, M. deFrutos, F.E. Regnier, Rapid separations of proteins by liquid chromatography, Meth. Enzymol. 270 (1996) 133–151.
[30] A. Premstaller, H. Oberacher, W. Walcher, High-performance liquid chromatography–electrospray ionization mass spectrometry using monolithic capillary columns for proteomic studies, Anal. Chem. 73 (2001) 2390–2396.
[31] J.W. Dolan, L.R. Snyder, M.A. Quarry, Computer, Simulation as a means of developing an optimized reversed-phase gradient separation, Chromatographia 24 (1988) 261–271.
[32] J.W. Dolan, L.R. Snyder, Developing a gradient elution method for reversed-phase HPLC, LC–GC 5 (1988) 970–978.
[33] B.F.D. Ghrist, L.R. Snyder, Design of optimized high-performance liquid chromatographic gradients for the separation of either small or large molecules. III. An overall strategy and its application to several examples, J. Chromatogr. 459 (1988) 43–63.
[34] I. Molnar, R.I. Boysen, V.A. Erdmann, High-performance liquid chromatography of *Thermus aquaticus* 50S and 30S ribosomal proteins, Chromatographia 28 (1989) 39–44.
[35] W. Hancock (Ed.), New Methods in Peptide Mapping for the Characterization of Proteins, CRC Press, Boca Raton, FL, 1996, pp. 31–56.
[36] C. Horvath, W. Melander, I. Molnar, Solvophobic interactions in liquid chromatography with nonpolar stationary phases, J. Chromatogr. 125 (1976) 29–156.
[37] C. Horvath, W. Melander, I. Molnar, Liquid chromatography of ionogenic substances with nonpolar stationary phases, Anal. Chem. 49 (1977) 142–154.
[38] L.R. Snyder, Gradient elution, in: C. Horvath (Ed.), HPLC: Advances and Perspectives, vol. 1, Academic Press, New York, 1980, pp. 208–316.
[39] R.I. Boysen, V.A. Erdmann, M.T.W. Hearn, Systematic, computer-assisted optimization of the isolation of Thermus thermophilus 50S ribosomal proteins by reversed-phase high performance liquid chromatography, J. Biochem. Biophys. Methods 37 (1998) 69–89.
[40] W. Hancock, C.A. Bishop, R.L. Prestidge, D.R. Harding, M.T.W. Hearn, Reversed phase, high-pressure liquid chromatography of peptides and proteins with ion-pairing reagents, Science 200 (1978) 1168–1170.
[41] L.R. Snyder, Classification of the solvent properties of common liquids, J. Chromatogr. 92 (1974) 223–230.
[42] L.R. Snyder, Classification of the solvent properties of common liquid solvents, J. Chromatogr. Sci. 16 (1978) 223–234.
[43] P.J. Schoenmakers, H.A.H. Biliet, L.D. Galan, Influence of organic modifiers on the retention behaviour in reversed-phase liquid chromatography and its consequences for gradient elution, J. Chromatogr. 185 (1979) 179–195.
[44] H.B. Patel, T.M. Jefferies, Eluotropic strength of solvents. Prediction and use in reversed-phase high-performance liquid chromatography, J. Chromatogr. 389 (1987) 21–32.
[45] M.T. Hearn, B. Grego, W. Hancock, Investigation of the effects of pH and ion-pair formation on the retention of peptides on chemically-bonded hydrocarbonaceous stationary phases, J. Chromatogr. 185 (1979) 429–444.
[46] A.P. Goldberg, A.E. Nowakowska, P.E. Antle, L.R. Snyder, Retention-optimization strategy for the high-performance liquid chromatographic ion-pair separation of samples containing basic compounds, J. Chromatogr. 316 (1984) 241–260.
[47] C.T. Mant, R.S. Hodges, The effects of anionic ion-pairing reagents on the peptide retention in reversed-phase chromatography, in: C.T. Mant, R.S. Hodges (Eds.), High-performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, vols. 327–341, CRC Press, Boca Raton, FL, 1991.
[48] J.W. Dolan, D.C. Lommen, L.R. Snyder, DryLab computer simulation for high-performance liquid chromatographic method development. II. Gradient elution, J. Chromatogr. 485 (1989) 91–112.

[49] B.F.D. Ghrist, L.R. Snyder, Design of optimized high-performance liquid chromatographic gradients for the separation of either small or large molecules. II. Background and theory, J. Chromatogr. 459 (1988) 25–41.
[50] B.F.D. Ghrist, B.S. Coopermann, L.R. Snyder, Design of optimized high-performance liquid chromatographic gradients for the separation of either small or large molecules. Minimising errors in computer simulations, J. Chromatogr. 459 (1988) 1–23.
[51] H. Eghbali, K. Sandra, F. Detobel, F. Lynen, K. Nakanishi, P. Sandra, G. Desmet, Performance evaluation of long monolithic silica capillary columns in gradient liquid chromatography using peptide mixtures, J. Chromatogr. A 1218 (2011) 3360–3366.
[52] F. Detobel, K. Broeckhoven, J. Wellens, B. Wouters, R. Swart, M. Ursem, G. Desmet, S. Eeltink, Parameters affecting the separation of intact proteins in gradient-elution reversed-phase chromatography using poly(styrene-co-divinylbenzene) monolithic capillary columns, J. Chromatogr. A 1217 (2010) 3085–3090.
[53] V. Bonfatti, L. Grigoletto, A. Cecchinato, L. Gallo, P. Carnier, Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants, J. Chromatogr. A 1195 (2008) 101–106.
[54] M. Umrethia, V.L. Kett, G.P. Andrews, R.K. Malcolm, A.D. Woolfson, Selection of an analytical method for evaluating bovine serum albumin concentrations in pharmaceutical polymeric formulations, J. Pharm. Biomed. Anal. 51 (2010) 1175–1179.
[55] R.F. Loureiro, J.E. Oliveira, P.A. Torjesen, P. Bartolini, M.T.C.P. Ribela, Analysis of intact human follicle-stimulating hormone preparations by reversed-phase high-performance liquid chromatography, J. Chromatogr. A 1136 (2006) 10–18.
[56] B.E. Almeida, J.E. Oliveira, C.M. Carvalho, S.L. Dalmora, P. Bartolini, M.T.C.P. Ribela, Analysis of human luteinizing hormone and human chorionic gonadotropin preparations of different origins by reversed-phase high-performance liquid chromatography, J. Pharm. Biomed. Anal. 53 (2010) 90–97.
[57] A. Zarrin, M. Foroozesh, M. Hamidi, S.M. Samani, A simple and rapid HPLC method for quantitation of interferon-beta-2b in dosage forms and delivery systems, J. Chromatogr. B 833 (2006) 199–203.
[58] H. Liu, H.C. Pan, L. Peng, S.X. Cai, RP-HPLC determination of recombinant human interferon omega in the *Pichia pastoris* fermentation broth, J. Pharm. Biomed. Anal. 38 (2005) 734–737.
[59] P. Moslemi, A.R. Najafabadi, H. Tajerzadeh, Rapid and sensitive method for simultaneous determination of insulin and A21-desamido insulin by high-performance liquid chromatography, J. Pharm. Biomed. Anal. 33 (2003) 45–51.
[60] N.V.S.R. Mamidi, V.V.S.S. Kumar, K. Katneni, M.R. Chaluvadi, S. Shreeram, S. Gangadhar, B. Nataraj, P.H. Kishore, V. Bhushan, S. Subramaniam, Validated HPLC method for determination of PAT-5A, an insulin sensitizing agent, in rat plasma, J. Pharm. Biomed. Anal. 22 (2000) 251–255.
[61] D. Gross, A. Skvorak, G. Hendrick, G. Weir, L.V. Komaroff, P. Halban, Oxidation of rat insulin II, but not I, leads to anomalous elution profiles upon HPLC analysis of insulin-related peptides, FEBS Lett. 241 (1988) 205–208.
[62] G. Karlsson, P. Gellerfors, A. Persson, B. Norén, P.O. Edlund, C. Sandberg, S. Birnbaum, Separation of oxidized and deamidated human growth hormone variants by isocratic reversed-phase high-performance liquid chromatography, J. Chromatogr. A 855 (1999) 147–155.
[63] H. Yokota, H. Saito, K. Masuoka, H. Kaniwa, T. Shibanuma, Reversed phase HPLC of Met^{58} oxidized rhIL-11: oxidation enhanced by plastic tubes, J. Pharm. Biomed. Anal. 24 (2000) 317–324.
[64] Y. Sadakane, T. Yamazaki, K. Nakagomi, T. Akizawa, N. Fujii, T. Tanimura, M. Kaneda, Y. Hatanaka, Quantification of the isomerization of Asp residue in recombinant human alpha-A-crystallin by reversed-phase HPLC, J. Pharm. Biomed. Anal. 30 (2003) 1825–1833.
[65] F.C. Rubio, M.L. Marina, C. Garcia, Perfusion reversed-phase high-performance liquid chromatography/mass spectrometry analysis of intact soybean proteins for the characterization of soybean cultivars, J. Chromatogr. A 1170 (2007) 34–43.
[66] S. Heinisch, J.-L. Rocca, Sense and non-sense of high-temperature liquid chromatography, J. Chromatogr. A 1216 (2009) 642–658.
[67] G. Vanhoenacker, P. Sandra, Elevated temperature and temperature programming in conventional liquid chromatography—fundamentals and applications, J. Sep. Sci. 29 (2006) 1822–1835.
[68] D. Guillarme, S. Heinisch, Detection modes with high temperature liquid chromatography: a review, Sep. Purif. Rev. 34 (2005) 181–216.
[69] D. Guillarme, S. Heinisch, J.L. Rocca, Effect of temperature in reversed phase liquid chromatography, J. Chromatogr. A 1052 (2004) 39–51.
[70] D. Guillarme, J. Ruta, S. Rudaz, J.L. Veuthey, New trends in fast and high resolution liquid chromatography: a critical comparison of existing approaches, Anal. Bioanal. Chem. 397 (2010) 1069–1082.
[71] J. Ruta, D. Guillarme, S. Rudaz, J.L. Veuthey, Comparison of columns packed with porous sub-2 μm and superficially porous sub-3 μm particles for peptides analysis at ambient and high temperature, J. Sep. Sci. 33 (2010) 2465–2477.
[72] H. Chen, Cs Horváth, High speed high-performance liquid chromatography of peptides and proteins, J. Chromatogr. A 705 (1995) 3–20.
[73] D. Guillarme, R. Russo, S. Rudaz, C. Bicchi, J.L. Veuthey, Chromatographic performance of silica-based stationary phases in high temperature liquid chromatography: pharmaceutical applications, Curr. Pharm. Anal. 4 (2007) 221–229.
[74] E. Grata, D. Guillarme, G. Glauser, J. Boccard, P.A. Carrupt, J.L. Veuthey, S. Rudaz, J.L. Wolfender, Metabolite profiling of plant extracts by high temperature UHPLC-TOF/MS, J. Chromatogr. A 1216 (2009) 5660–5668.
[75] S. Heinisch, G. Puy, M.P. Barrioulet, J.L. Rocca, Effect of temperature on the retention of ionizable compounds in reversed-phase liquid chromatography: application to method development, J. Chromatogr. A 1118 (2006) 234–243.
[76] A. Staub, D. Zurlino, S. Rudaz, J.L. Veuthey, D. Guillarme, Analysis of peptides and proteins using UHPLC and fused core technologies, J. Chromatogr. A 1218 (2011) 8903–8914.
[77] T. Teutenberg, H.J. Goetze, J. Tuerk, J. Ploeger, T.K. Kiffmeyer, K.G. Schmidt, W.G. Kohorst, T. Rohe, H.D. Jansen, H. Weber, Development and application of a specially designed heating system for temperature-programmed high-performance liquid chromatography using subcritical water as the mobile phase, J. Chromatogr. A 1114 (2006) 89–96.
[78] T. Teutenberg, K. Hollebekkers, S. Wiese, A. Boergers, Temperature and pH-stability of commercial stationary phases, J. Sep. Sci. 32 (2009) 1262–1274.
[79] D.T.T. Nguyen, D. Guillarme, S. Heinisch, M.P. Barrioulet, J.L. Rocca, S. Rudaz, J.L. Veuthey, High throughput liquid chromatography with sub-2μm particles at very high pressure and high temperature, J. Chromatogr. A 1167 (2007) 76–84.
[80] X. Yang, L. Ma, P.W. Carr, High temperature fast chromatography of proteins using a silica-based stationary phase with greatly enhanced low pH stability, J. Chromatogr. A 1079 (2005) 213–220.
[81] J.D. Thompson, P.W. Carr, A study of the critical criteria for analyte stability in high-temperature liquid chromatography, Anal. Chem. 74 (2002) 1017–1023.
[82] W.W. Yau, J.J. Kirkland, D.D. Bly, Modern Size-Exclusion Liquid Chromatography, Wiley, New York, 1979 (Chapter 2).
[83] C. Horvath, B.A. Preiss, S.R. Lipsky, Fast liquid chromatography: an investigation of operating parameters and the separation of nucleotides on pellicular ion exchangers, Anal. Chem. 39 (1967) 1422–1428.
[84] C. Horvath, S.R. Lipsky, Column design in high pressure liquid chromatography, J. Chromatogr. Sci. 7 (1969) 109–116.
[85] J.J. Kirkland, Controlled surface porosity supports for high speed gas and liquid chromatography, Anal. Chem. 41 (1969) 218–220.
[86] G. Guiochon, F. Gritti, Shell particles, trials, tribulations and triumphs, J. Chromatogr. A 1218 (2011) 1915–1938.
[87] J.J. Kirkland, F.A. Truszkowski, C.H. Dilks Jr., G.S. Engel, Superficially porous silica microspheres for fast high-performance liquid chromatography of macromolecules, J. Chromatogr. A 890 (2000) 3–13.
[88] S. Fekete, J. Fekete, K. Ganzler, Shell and small particles: evaluation of new column technology, J. Pharm. Biomed. Anal. 49 (2009) 64–71.
[89] F. Gritti, G. Guiochon, The mass transfer kinetics in columns packed with Halo-ES shell particles, J. Chromatogr. A 1218 (2011) 907–921.
[90] E. Olah, S. Fekete, J. Fekete, K. Ganzler, Comparative study of new shell-type, sub-2 μm fully porous and monolith stationary phases, focusing on mass-transfer resistance, J. Chromatogr. A 1217 (2010) 3642–3653.
[91] J.O. Omamogho, J.P. Hanrahan, J. Tobin, J.D. Glennon, Structural variation of solid core and thickness of porous shell of 1.7 μm core–shell silica particles on chromatographic performance: narrow bore columns, J. Chromatogr. A 1218 (2011) 1942–1953.
[92] S.A. Schuster, B.M. Wagner, B.E. Boyes, J.J. Kirkland, Wider pore superficially porous particles for peptide separations by HPLC, J. Chromatogr. Sci. 48 (2010) 566–571.
[93] S.A. Schuster, B.E. Boyes, B.M. Wagner, J.J. Kirkland, Fast high performance liquid chromatography separations for proteomic applications using Fused-Core® silica particles, J. Chromatogr. A 1228 (2012) 232–241.
[94] S. Fekete, R. Berky, J. Fekete, J.L. Veuthey, D. Guillarme, Evaluation of a new wide pore core–shell material (Aeris™ WIDEPORE) and comparison with other existing stationary PHASES FOR THE analysis of intact proteins, J. Chromatogr. A, (2012) doi:10.1016/j.chroma.2012.03.018.
[95] C. Viklund, F. Svec, J.M.J. Fréchet, K. Irgum, Monolithic, molded, porous materials with high flow characteristics for separations, catalysis, or solid-phase chemistry: control of porous properties during polymerization, Chem. Mater. 8 (1996) 744–750.
[96] A. Greider, L. Trojer, C.W. Huck, G.K. Bonn, Influence of the polymerisation time on the porous and chromatographic properties of monolithic poly(1,2-bis(*p*-vinylphenyl))ethane capillary columns, J. Chromatogr. A 1216 (2009) 7747–7754.
[97] P.A. Levkin, S. Eeltink, T.R. Stratton, R. Brennen, K. Robotti, H. Yin, K. Killeen, F. Svec, J.M.J. Fréchet, Monolithic porous polymer stationary phases in polyimide chips for the fast high-performance liquid chromatography separation of proteins and peptides, J. Chromatogr. A 1200 (2008) 55–61.
[98] Y.Y. Li, H.D. Tolley, M.L. Lee, Poly[hydroxyethyl crylate-co-poly(ethylene glycol) diacrylate] monolithic column for efficient hydrophobic interaction chromatography of proteins, Anal. Chem. 81 (2009) 9416–9424.
[99] E.J. Sneekes, J. Hun, M. Elliot, J. Ausio, C. Borchers, Accurate molecular weight analysis of histones using FFE and RP-HPLC on monolithic capillary columns, J. Sep. Sci. 32 (2009) 2691–2698.
[100] G. Holzl, H. Oberacher, S. Pitsch, A. Stutz, C.G. Huber, Analysis of biological and synthetic ribonucleic acids by liquid chromatography–mass spectrometry using monolithic capillary columns, Anal. Chem. 77 (2005) 673–680.
[101] H. Oberacher, W. Parson, P.J. Oefner, B.M. Mayr, C.G. Huber, Applicability of tandem mass spectrometry to the automated sequencing of long-chain oligonucleotides, J. Am. Soc. Mass Spectrom. 15 (2004) 510–522.

[102] A.R. Ivanov, L. Zang, B.L. Karger, Low Attomole ESI-MS/MS analysis of tryptic peptides using 20-μm I.D. monolithic capillary columns, Anal. Chem. 75 (2003) 5306–5316.
[103] S. Eeltink, L. Geiser, F. Svec, J.M.J. Fréchet, Optimization of the porous structure and polarity of polymethacrylate-based monolithic capillary columns for the LC–MS separation of enzymatic digests, J. Sep. Sci. 30 (2007) 2814–2820.
[104] S. Eeltink, S. Dolman, R. Swart, M. Ursem, P.J. Schoenmakers, High-efficiency liquid chromatography–mass spectrometry separations with 50 mm, 250 mm, and 1 m long polymer-based monolithic capillary columns for the characterization of complex proteolytic digests, J. Chromatogr. A 1217 (2010) 6610–6615.
[105] M. Petro, F. Svec, I. Gitsov, J.M.J. Fréchet, Molded monolithic rod of macroporous poly(styrene-co-divinylbenzene) as a separation medium for HPLC of synthetic polymers: on-column precipitation–redissolution chromatography as an alternative to size exclusion chromatography of styrene oligomers and polymers, Anal. Chem. 68 (1996) 315–321.
[106] A. Vellaichamy, J.C. Tran, A.D. Catherman, J.E. Lee, J.F. Kellie, S.M.M. Sweet, L. Zamdborg, P.M. Thomas, D.R. Ahlf, K.R. Durbin, G. Valaskovic, N.L. Kelleher, Size-sorting combined with improved nanocapillary-LC–MS for identification of intact proteins up to 80 kDa, Anal. Chem. 82 (2010) 1234–1244.
[107] M. Kubin, P. Spacek, R. Chromecek, Gel permeation chromatography on porous poly(ethylene glycol methacrylate), Collect. Czech. Chem. Commun. 32 (1967) 3881–3887.
[108] L.C. Hansen, R.E. Sievers, Highly permeable open-pore polyurethane columns for liquid chromatography, J. Chromatogr. 99 (1974) 123–133.
[109] S. Hjerten, J.L. Liao, R. Zhang, High performance liquid chromatography on continuous polymer beds, J. Chromatogr. 473 (1989) 273–275.
[110] F. Svec, J.M.J. Frechet, Continuous rods of macroporous polymer as high performance liquid chromatographic media, Anal. Chem. 64 (1992) 820–822.
[111] Q.C. Wang, F. Svec, J.M.J. Frechet, Rod of macroporous polymeric stationary phase as continuous separation medium for reversed phase chromatography, Anal. Chem. 65 (1993) 2243–2248.
[112] I. Gusev, X. Huang, C. Horvath, Capillary columns with in situ formed porous monolithic packing for micro high-performance liquid chromatography and capillary electrochromatography, J. Chromatogr. A 855 (1999) 273–290.
[113] M. Petro, F. Svec, J.M.J. Frechet, Molded continuous poly(styrene-co-divinylbenzene) rod as a separation medium for the very fast separations of polymers: a comparison of the chromatographic properties of the monolithic rod with columns packed with porous and non-porous beads in precipitation–redissolution chromatography of polystyrenes, J. Chromatogr. A 752 (1996) 59–66.
[114] A. Premstaller, H. Oberacher, C.G. Huber, High performance liquid chromatography–electrospray ionization mass spectrometry of single and double stranded nucleic acids using monolithic capillary columns, Anal. Chem. 72 (2000) 4386–4393.
[115] H. Oberacher, A. Premstaller, C.G. Huber, Characterization of some physical and chromatographic properties of monolithic poly-(styrene-co-divinylbenzene) columns, J. Chromatogr. A 1030 (2004) 201–208.
[116] M. Merhar, A. Podgornik, M. Barut, M. Zigon, A. Strancar, Methacrylate monoliths prepared from various hydrophobic and hydrophilic monomers—structural and chromatographic characteristics, J. Sep. Sci. 26 (2003) 322–330.
[117] F. Svec, Preparation and HPLC applications of rigid macroporous organic polymer monoliths, J. Sep. Sci. 27 (2004) 747–766.
[118] D. Lee, F. Svec, J.M.J. Frechet, Photopolymerized monolithic capillary columns for rapid HPLC separation of proteins, J. Chromatogr. A 1051 (2004) 53–60.
[119] F. Sinner, M.R. Buchmeiser, A new class of continuous polymer supports prepared by ring-opening metathesis polymerization: a straightforward route to functionalized monoliths, Macromolecules 33 (2000) 5777–5786.
[120] T.J. Causon, R.A. Shellie, E.F. Hilder, High temperature liquid chromatography with monolithic capillary columns and pure water eluent, Analyst 134 (2009) 440–442.
[121] S. Eeltink, S. Dolman, F. Detobel, G. Desmet, R. Swart, M. Ursem, 1 mm ID poly(styrene-co-divinylbenzene) monolithic columns for high-peak capacity one- and two-dimensional liquid chromatographic separations of intact proteins, J. Sep. Sci. 32 (2009) 2504–2509.
[122] J. Mohr, R. Swart, M. Samonig, G. Bohm, C.G. Huber, High-efficiency nano- and micro-HPLC-high-resolution Orbitrap-MS platform for top-down proteomics, Proteomics 10 (2010) 3598–3609.
[123] L. Trojer, S.H. Lubbad, C.P. Bisjak, G.K. Bonn, Monolithic poly(*p*-methylstyrene-co-1,2-bis(*p*-vinylphenyl)ethane) capillary columns as novel styrene stationary phases for biopolymer separation, J. Chromatogr. A 1117 (2006) 56–66.
[124] J.W. Cooper, Y. Wang, C.S. Lee, Recent advances in capillary separations for proteomics, Electrophoresis 25 (2004) 3913–3926.
[125] N. Lion, T.C. Rohner, L. Dayon, I.L. Arnaud, E. Damoc, N. Youhnovski, Z. Wu, C. Roussel, J. Josserand, H. Jensen, J.S. Rossier, M. Przybylski, H.H. Girault, Microfluidic systems in proteomics, Electrophoresis 24 (2003) 3533–3562.
[126] J. Hardouin, M. Duchateau, R. Joubert-Caron, M. Caron, Usefulness of an integrated microfluidic device (HPLC–Chip-MS) to enhance confidence in protein identification by proteomics, Rapid Commun. Mass Spectrom. 20 (2006) 3236–3244.
[127] J.C. McDonald, G.M. Whitesides, Poly(dimethylsiloxane) as a material for fabricating microfluidic devices, Acc. Chem. Res. 35 (2002) 491–499.
[128] J. Wang, M. Pumera, M.P. Chatrathi, A. Escarpa, R. Konrad, A. Griebel, W. Doerner, H. Loewe, Towards disposable lab-on-a-chip: poly(methylmethacrylate) microchip electrophoresis device with electrochemical detection, Electrophoresis 23 (2002) 596–601.
[129] S.K. Sia, G.M. Whitesides, Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies, Electrophoresis 24 (2003) 3563–3576.
[130] T.B. Stachowiak, F. Svec, J.M.J. Frechet, Chip electrochromatography, J. Chromatogr. A 1044 (2004) 97–111.
[131] H. Yin, K. Killeen, R. Brennen, D. Sobek, M. Werlich, T. Van de Goor, Microfluidic chip for peptide analysis with an integrated HPLC column, sample enrichment column, and nanoelectrospray tip, Anal. Chem. 77 (2005) 527–533.
[132] H. Yin, K. Killeen, The fundamental aspects and applications of Agilent HPLC-Chip, J. Sep. Sci. 30 (2007) 1427–1434.
[133] A.J.P. Martin, R.L.M. Synge, A new form of chromatogram employing two liquid phases, Biochem. J. 35 (1941) 1358–1368.
[134] U.D. Neue, HPLC, Columns, Chapter Column Design, Wiley-VCH, New York, 1997, pp. 41–53.
[135] M.E. Swartz, B. Murphy, New frontiers in chromatography, Am. Lab. 37 (2005) 22–27.
[136] M.E. Swartz, UPLC: an introduction and review, J. Liq. Chromatogr. 28 (2005) 1253–1263.
[137] D.J. Phillips, M. Capparella, U.D. Neue, Z. El Fallah, A new small particle packing for faster analysis with high resolution, J. Pharm. Biomed. Anal. 15 (1997) 1389–1395.
[138] F. Gerber, M. Krummen, H. Potgeter, A. Roth, C. Siffrin, C. Spoendlin, Practical aspects of fast reversed-phase high-performance liquid chromatography using 3 μm particle packed columns and monolithic columns in pharmaceutical development and production working under current good manufacturing practice, J. Chromatogr. A 1036 (2004) 127–133.
[139] J.H. Knox, M. Saleem, Kinetic conditions for optimum speed and resolution in column chromatography, J. Chromatogr. Sci. 7 (1969) 614–622.
[140] J.E. MacNair, K.C. Lewis, J.W. Jorgenson, Ultra high pressure reversed phase liquid chromatography in packed capillary columns, Anal. Chem. 69 (1997) 983–989.
[141] J.E. MacNair, K.D. Patel, J.W. Jorgenson, Ultra high pressure reversed-phase liquid chromatography: isocratic and gradient elution using columns packed with 1.0 μm particles, Anal. Chem. 71 (1999) 700–708.
[142] N. Wu, J.A. Lippert, M.L. Lee, Practical aspects of ultrahigh pressure capillary liquid chromatography, J. Chromatogr. A 911 (2001) 1–12.
[143] A. de Villiers, H. Lauer, R. Szucs, S. Goodall, P. Sandra, Influence of frictional heating on temperature gradients in ultra-high-pressure liquid chromatography on 2.1 mm I.D. columns, J. Chromatogr. A 1113 (2006) 84–91.
[144] F. Gritti, G. Guiochon, Measurement of the axial and radial temperature profiles of a chromatographic column: influence of thermal insulation on column efficiency, J. Chromatogr. A 1138 (2007) 141–157.
[145] F. Gritti, G. Guiochon, Complete temperature profiles in ultra-high-pressure liquid chromatography columns, Anal. Chem. 80 (2008) 5009–5020.
[146] J.S. Mellors, J.W. Jorgenson, The use of 1.5 micron porous ethyl-bridged hybrid particles as a stationary phase support for reversed-phase ultra-high pressure liquid chromatography, Anal. Chem. 76 (2004) 5441–5450.
[147] J.J. Kirkland, Ultrafast reversed-phase high-performance liquid chromatographic separations: an overview, J. Chromatogr. Sci. 38 (2000) 535–544.
[148] T.J. Barder, P.J. Wohlman, C. Thrall, P.D. DuBois, Fast chromatography and nonporous silica, LC–GC 15 (1997) 918–926.
[149] N. Wu, D.C. Collins, J.A. Lippert, Y. Xiang, M.L. Lee, Ultra-high pressure liquid chromatography/time-of-flight mass spectrometry for fast separations, J. Microcol. Sep. 12 (2000) 462–469.
[150] N. Wu, Y. Liu, M.L. Lee, Sub-2 μm porous and nonporous particles for fast separation in reversed-phase high performance liquid chromatography, J. Chromatogr. A 1131 (2006) 142–150.
[151] T. Issaeva, A. Kourganov, K. Unger, Super-high-speed liquid chromatography of proteins and peptides on non-porous Micra NPS-RP packings, J. Chromatogr. A 846 (1999) 13–23.
[152] R.M. Seifar, J.C. Kraak, W.Th. Kok, H. Poppe, Capillary electrochromatography with 1.8-μm ODS-modified porous silica particles, J. Chromatogr. A 808 (1998) 71–77.
[153] A.M. Goetze, Z. Zhang, L. Liu, F.W. Jacobsen, G.C. Flynn, Rapid LC–MS screening for IgG Fc modifications and allelic variants in blood, Molec. Immun. 49 (2011) 338–352.
[154] S. Wang, R. Ionescu, N. Peekhaus, J. Leung, S. Ha, J. Vlasak, Separation of post-translational modifications in monoclonal antibodies by exploiting subtle conformational changes under mildly acidic conditions, J. Chromatogr. A 1217 (2010) 6496–6502.
[155] U.D. Neue, H.B. Hewitson, T.E. Wheat, The determination of the plate count for large molecules under reversed-phase gradient conditions, J. Chromatogr. A 1217 (2010) 2179–2181.
[156] R.A. Everley, T.R. Croley, Ultra-performance liquid chromatography/mass spectrometry of intact proteins, J. Chromatogr. A 1192 (2008) 239–247.
[157] C. Esteve, C.D. Rio, M.L. Marina, M.C. Garcia, Development of an ultra-high performance liquid chromatography analytical methodology for the profiling of olive (*Olea europaea* L.) pulp proteins, Anal. Chim. Acta 690 (2011) 129–134.
[158] K. Gohlin, A. Buskhe, M. Larsson, Kinetic performance of open-tubular and packed columns in LC using the same stationary phase: immobilized polymethyloctadecylsiloxane, Chromatographia 39 (1994) 729–739.
[159] T. Tsuda, K. Hibi, T. Nakanishi, T. Takeuchi, D. Ishii, Studies of open-tubular microcapillary liquid chromatography. II. Chemically bonded octadecylsilane stationary phase, J. Chromatogr. 158 (1978) 227–232.

[160] J.W. Jorgenson, E.J. Guthrie, Liquid chromatography in open-tubular columns: theory of column optimization with limited pressure and analysis time, and fabrication of chemically bonded reversed-phase columns on etched borosilicate glass capillaries, J. Chromatogr. 255 (1983) 335–348.
[161] G. Hulthe, M.A. Petersson, E. Fogelqvist, Coupling of open tubular liquid chromatography to electrospray mass spectrometry with a nanospray interface, Anal. Chem. 71 (1999) 2915–2921.
[162] G. Yue, Q. Luo, J. Zhang, S.-L. Wu, B.L. Karger, Ultratrace LC/MS Proteomic analysis using 10 μm i.d. porous layer open tubular (PLOT) polystyrene-divinylbenzene capillary columns, Anal. Chem. 79 (2007) 938–946.
[163] Q. Luo, G. Yue, A. Valaskovic Gary, Y. Gu, S.-L. Wu, L. Karger Barry, On-line 1D and 2D porous layer open tubular/LC–ESI-MS using 10-microm-i.d. poly(styrene-divinylbenzene) columns for ultrasensitive proteomic analysis, Anal. Chem. 79 (2007) 6174–6181.
[164] M. Rogeberg, S.R. Wilson, T. Greibrokk, E. Lundanes, Separation of intact proteins on porous layer open tubular (PLOT) columns, J. Chromatogr. A 1217 (2010) 2782–2786.
[165] C.T. Mant, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991.
[166] C.T. Mant, R.S. Hodges, Analysis of peptides by high-performance liquid chromatography, Methods Enzymol. 271 (1996) 3–50.
[167] C.T. Mant, R.S. Hodges, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules, Marcel Dekker, New York, 2002.
[168] Y. Chen, C.T. Mant, R.S. Hodges, Temperature selectivity effects in reversed-phase liquid chromatography due to conformation differences between helical and non-helical peptides, J. Chromatogr. A 1010 (2003) 45–61.
[169] C.T. Mant, Y. Chen, R.S. Hodges, Temperature profiling of polypeptides in reversed-phase liquid chromatography. I. Monitoring of dimerization and unfolding of amphipathic α-helical peptides, J. Chromatogr. A 1009 (2003) 29–43.
[170] C.T. Mant, B. Tripet, R.S. Hodges, Temperature profiling of polypeptides in reversed-phase liquid chromatography. II. Monitoring of folding and stability of two-stranded α-helical coiled-coils, J. Chromatogr. A 1009 (2003) 45–59.
[171] Y. Chen, C.T. Mant, R.S. Hodges, Selectivity differences in the separation of amphipathic α-helical peptides during reversed-phase liquid chromatography at pHs 2.0 and 7.0. Effects of different packings, mobile phase conditions and temperature, J. Chromatogr. A 1043 (2004) 99–111.
[172] S.A. Cohen, K. Benedek, Y. Tapuhi, J.C. Ford, B.L. Karger, Conformational effects in the reversed phase liquid chromatography of ribonuclease A, Anal. Biochem. 144 (1985) 275–284.
[173] X.M. Lu, K. Benedek, B.L. Karger, Conformational effects in the high-performance liquid chromatography of proteins. Further studies of the reversed-phase chromatographic behavior of ribonuclease A, J. Chromatogr. 359 (1986) 19–29.
[174] P. Oroszlan, S. Wicar, G. Teshima, S.-L. Wu, W.S. Hancock, B.L. Karger, Conformational effects in the reversed-phase chromatographic behavior of recombinant human growth hormone (rhGH) and N-methionine recombinant human growth hormone (Met-hGH), Anal. Chem. 64 (1992) 1623–1631.
[175] A.W. Purcell, M.I. Aguilar, M.T.W. Hearn, Probing the conformational states of proteins with reversed phase high performance liquid chromatography, Anal. Chem. 71 (1999) 2440–2451.
[176] S.U. Sane, S.M. Cramer, T.M. Przybycien, Protein structure perturbations on chromatographic surfaces, J. Chromatogr. A. 849 (1999) 149–159.
[177] S. Wicar, M.G. Mulkerrin, G. Bathory, L.H. Khundkar, B.L. Karger, Conformational changes in the reversed phase liquid chromatography of recombinant human growth hormone as a function of organic solvent: the molten globule state, Anal. Chem. 66 (1994) 3908–3915.
[178] K. Sawicka, T. Sahota, M.J. Taylor, S. Tanna, Development of a reversed-phase high-performance liquid chromatography method for the analysis of components from a closed-loop insulin delivery system, J. Chromatogr. A 1132 (2006) 117–123.
[179] A.W. Purcell, M.I. Aguilar, M.T.W. Hearn, Conformational effects in reversed-phase high-performance liquid chromatography of polypeptides. I. Resolution of insulin variants, J. Chromatogr. A 711 (1995) 61–70.
[180] W. Lee, D. Cho, B.O. Chun, T. Chang, M. Ree, Characterization of polystyrene and polyisoprene by normal-phase temperature gradient interaction chromatography, J. Chromatogr. A 910 (2001) 51–60.
[181] J.C. Ford, J.A. Smith, Synthetic peptide purification by application of linear solvent strength gradient theory, J. Chromatogr. 483 (1989) 131–143.
[182] M.R. Sussman, Purification of integral plasma membrane proteins by reverse-phase high performance liquid chromatography, Anal. Biochem. 169 (1988) 395–399.
[183] Por-Hsiung, T. Strickland, Erythropoetin purification, U.S. Patent No. 04,667,016.
[184] E.L. Kovrigin, S.A. Potekhin, On the stabilizing action of protein denaturation: acetonitrile effect on stability of lysozyme in aqueous solution, Biophys. Chem. 83 (2000) 45–59.
[185] A.W. Purcell, G.L. Zhao, M.I. Aguilar, M.T.W. Hearn, Comparison between the isocratic and gradient retention behaviour of polypeptides in reversed-phase liquid chromatographic environments, J. Chromatogr. A 852 (1999) 43–57.
[186] R.I. Boysen, A.J.O. Jong, M.T.W. Hearn, Binding behaviour and conformational properties of globular proteins in the presence of immobilised non-polar ligands used in reversed-phase liquid chromatography, J. Chromatogr. A 1079 (2005) 173–186.
[187] A.J. Sadler, R. Micanovic, G.E. Katzenstein, R.V. Lewis, C.R. Middaugh, Protein conformation and reversed-phase high-performance liquid chromatography, J. Chromatogr. A 317 (1984) 93–101.
[188] H. Zhang, K. Kaneko, J.T. Nguyen, T.L. Livshits, M.A. Baldwin, F.E. Cohen, T.L. James, S.B. Prusiner, Conformational transitions in peptides containing two putative alpha-helices of the prion protein, J. Mol. Biol. 250 (1995) 514–526.
[189] A.I. Arunkumar, T.K.S. Kumar, T. Sivaraman, C. Yu, Acetonitrile-induced conformational transitions in poly-L-lysine, Int. J. Biol. Macromol. 21 (1997) 299–305.
[190] D.M.A.M. Luykx, S.S. Goerdayal, P.J. Dingemanse, W. Jiskoot, P.M.J.M. Jongen, HPLC and tandem detection to monitor conformational properties of biopharmaceuticals, J. Chromatogr. B 821 (2005) 45–52.
[191] A. Apffel, S. Fisher, G. Goldberg, P.C. Goodley, F.E. Kuhlmann, Enhanced sensitivity for peptide mapping with electrospray liquid chromatography–mass spectrometry in the presence of signal suppression due to trifluoroacetic acid-containing mobile phases, J. Chromatogr. A 712 (1995) 177–190.
[192] M.C. McCroskey, V.E. Groppi, J.D. Pearson, Separation, Purification of S49 mouse lymphoma histones by reversed-phase high performance liquid chromatography, Anal. Biochem. 169 (1987) 427–432.
[193] J. Rivier, R. McClintock, R. Galyean, H. Anderson, Reversed-phase high performance liquid chromatography: preparative purification of synthetic peptides, J. Chromatogr. 288 (1984) 303–328.
[194] C. Hoeger, R. Galyean, J. Boublik, R. McClintock, J. Rivier, Preparative reverse phase high performance liquid chromatography: effects of buffer ph on the purification of synthetic peptides, Biochromatography 2 (1987) 134–142.
[195] C.A. Hoeger, R. Galyean, R.A. McClintock, J.E. Rivier, Practical aspects of preparative reversed-phase chromatography of synthetic peptides, in: HPLC of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991, pp. 753–764.
[196] F.R. Huebner, J.A. Bietz, B.D. Webb, B.O. Juliano, Rice cultivar identification by high-performance liquid chromatography of endosperm proteins, Cereal Chem. 67 (1990) 129–135.
[197] D. Guo, C.T. Mant, R.S. Hodges, Effects of ion-pairing reagents on the prediction of peptide retention in reversed-phase high performance liquid chromatography, J. Chromatogr. 386 (1987) 205–222.
[198] The effects of anionic ion-pairing reagents on the peptide retention in reversed-phase chromatography, in: C.T. Mant, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991, pp. 327–341.
[199] R.L. Cunico, K.M. Gooding, T. Wehr (Eds.), HPLC and CE of Biomolecules, Bay Bioanalytical Laboratory, Richmond, VA, 1998.
[200] C.T. Mant, R.S. Hodges, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules, Marcel Dekker, New York, NY, 2002, pp. 433–511.
[201] D. Guo, C.T. Mant, A.K. Taneja, J.M.R. Parker, R.S. Hodges, Prediction of peptide retention times in reversed-phase high-performance liquid chromatography. II. Correlation of observed and predicted peptide retention times factors and influencing the retention times of peptides, J. Chromatogr. 359 (1986) 519–532.
[202] J.L. Glajch, J.J. Kirkland, J. Kohler, Effect of column degradation on the reversed-phase high-performance liquid chromatographic separation of peptides and proteins, J. Chromatogr. 384 (1987) 81–90.
[203] J.J. Kirkland, J.L. Glajch, R.D. Farlee, Synthesis and characterization of highly stable bonded phases for high-performance liquid chromatography column packings, Anal. Chem. 61 (1989) 2–11.
[204] J.L. Glajch, J.J. Kirkland, Stable, sterically protected, monofunctional-silane bonded-phase columns for high performance liquid chromatography, LC–GC 8 (1990) 140–150.
[205] B.E. Boyes, J.J. Kirkland, Rapid, high-resolution HPLC separation of peptides using small particles at elevated temperatures, Pept. Res. 6 (1993) 249–258.
[206] M. Shibue, C.T. Mant, R.S. Hodges, Effect of anionic ion-pairing reagent concentration (1–60 mM) on reversed-phase liquid chromatography elution behaviour of peptides, J. Chromatogr. A 1080 (2005) 58–67.
[207] R. Ohmacht, B. Boros, Effect of pressure on Solute capacity factor in HPLC using a non-porous stationary phase, Chromatographia 51 (2000) S205–S210.
[208] A. Felinger, B. Boros, R. Ohmacht, Effect of pressure on solute capacity factor in HPLC using a non-porous stationary phase II, Chromatographia 56 (2002) S61–S68.
[209] X. Liu, D. Zhou, P. Szabelski, G. Guiochon, Influence of pressure on the retention and separation of insulin variants under linear conditions, Anal. Chem. 75 (2003) 3999–4009.
[210] X. Liu, P. Szabelski, K. Kaczmarski, D. Zhou, G. Guiochon, Influence of pressure on the chromatographic behavior of insulin variants under nonlinear conditions, J. Chromatogr. A 988 (2003) 205–218.
[211] M.M. Fallas, U.D. Neue, M.R. Hadley, D. McCalley, Investigation of the effect of pressure on retention of small molecules using reversed-phase ultra-high-pressure liquid chromatography, J. Chromatogr. A 1209 (2008) 195–205.
[212] M.A. Stadalius, H.S. Gold, L.R. Snyder, Optimization model for the gradient elution separation of peptide mixtures by reversed-phase high-performance liquid chromatography: verification of retention relationships, J. Chromatogr. 296 (1984) 31–59.
[213] I. Kiss, I. Bacskay, F. Kilar, A. Felinger, Comparison of the mass transfer in totally porous and superficially porous stationary phases in liquid chromatography, Anal. Bioanal. Chem. 397 (2010) 1307–1314.
[214] F. Gritti, C.A. Sanchez, T. Farkas, G. Guiochon, Achieving the full performance of highly efficient columns by optimizing conventional benchmark

high-performance liquid chromatography instruments, J. Chromatogr. A 1217 (2010) 3000–30012.
[215] K.J. Fountain, U.D. Neue, E.S. Grumbach, D.M. Diehl, Effects of extra-column band spreading, liquid chromatography system operating pressure, and column temperature on the performance of sub-2-μm porous particles, J. Chromatogr. A 1216 (2009) 5979–5988.
[216] S. Fekete, J. Fekete, The impact of extra-column band broadening on the chromatographic efficiency of 5 cm long narrow-bore very efficient columns, J. Chromatogr. A 1218 (2011) 5286–5291.
[217] P.C. Sadek, P.W. Carr, L.D. Bowers, L.C. Haddad, A radiochemical study of irreversible protein loss on high-performance liquid chromatography column frits, Anal. Biochem. 144 (1985) 128–131.
[218] G.L. Glish, R.W. Vachet, The basics of mass spectrometry in the twenty-first century, Nat. Rev. Drug Discov. 2 (2003) 140–150.
[219] A. Makarov, Electrostatic axially harmonic orbital trapping: a high-performance technique of mass analysis, Anal. Chem. 72 (2000) 1156–1162.
[220] A.G. Marshall, C.L. Hendrickson, Fourier transform ion cyclotron resonance detection: principles and experimental configurations, Int. J. Mass Spectrom. 215 (2002) 59–75.
[221] A.P. Kafka, T. Kleffmann, T. Rades, A. McDowell, The application of MALDI TOF MS in biopharmaceutical research, Int. J. Pharm. 417 (2011) 70–82.
[222] M.L. Vestal, The future of biological mass spectrometry, J. Am. Soc. Mass Spectrom. 22 (2011) 953–959.
[223] J. Schappler, R. Nicoli, D. Nguyen, S. Rudaz, J.L. Veuthey, D. Guillarme, Coupling ultra high pressure liquid chromatography with single quadrupole mass spectrometry for the analysis of a complex drug mixture, Talanta 78 (2009) 377–387.
[224] D. Guillarme, J. Schappler, S. Rudaz, J.L. Veuthey, Coupling ultra high pressure liquid chromatography with mass spectrometry, Trends Anal. Chem. 29 (2010) 15–27.
[225] A. Staub, J. Schappler, S. Rudaz, J.L. Veuthey, CE-TOF/MS Fundamental concepts, instrumental considerations and applications, Electrophoresis 30 (2009) 1610–1623.
[226] T. Wehr, Top-down versus bottom-up approaches in proteomics, LC–GC 24 (2006) 492–497.
[227] M.H.M. van de Meent, G.J. de Jong, Novel liquid-chromatography columns for proteomics research, Trends Anal. Chem. 30 (2011) 1809–1818.
[228] J. Rozenbrand, W.P. Van Bennekom, Silica-based and organic monolithic capillary columns for LC: recent trends in proteomics, J. Sep. Sci. 34 (2011) 1934–1944.
[229] M. Iwasaki, S. Miwa, T. Ikegami, M. Tomita, N. Tanaka, Y. Ishihama, One-dimensional capillary liquid chromatographic separation coupled with tandem mass spectrometry unveils the *Escherichia coli* proteome on a microarray scale, Anal. Chem. 82 (2010) 2616–2620.
[230] S. Eeltink, B. Wouters, G. Desmet, M. Ursem, D. Blinco, G.D. Kemp, A. Treumann, High-resolution separations of proteins isoforms with liquid chromatography time-of-flight mass spectrometry using polymer monolithic capillary columns, J. Chromatogr. A 1218 (2011) 5504–5511.
[231] S.W. Englander, A.J. Wand, Main-chain-directed strategy for the assignment of 1H-NMR spectra of proteins, Biochemistry 26 (1987) 5953–5958.
[232] S.W. Englander, N.R. Kallenbach, Hydrogen exchange and structural dynamics of proteins and nucleic acids, Q. Rev. Biophys. 16 (1984) 18–21.
[233] C.J. Craven, N.M. Derix, J. Hendriks, R. Boelens, K.J. Hellingwerf, R. Kaptein, Probing the nature of the blue-shifted intermediate of photoactive yellow protein in solation by NMR: hydrogen-deuterium exchange data and pH studies, Biochemistry 39 (2000) 14392–14399.
[234] K.H. Gardner, L.E. Kay, The use of ^{2}H, ^{13}C, ^{15}N multidimensional NMR to study the structure and dynamics of proteins, Annu. Rev. Biophys. Biomol. Struct. 27 (1998) 357–406.
[235] Z. Zhang, D.L. Smith, Determination of amide hydrogen exchange by mass spectrometry: a new tool for protein structure elucidation, Protein Sci. 2 (1993) 522–531.
[236] D.L. Smith, Y. Deng, Z. Zhang, Probing the non-covalent structure of proteins by amide hydrogen exchange and mass spectrometry, J. Mass Spectrom. 32 (1997) 135–146.
[237] A. Miranker, C.V. Robinson, S.E. Radford, R.T. Aplin, C.M. Dobson, Detection of transient protein folding populations by mass spectrometry, Science 262 (1993) 896–900.
[238] T.T. Lam, J.K. Lanman, M.R. Emmett, C.L. Hendrickson, A.G. Marshall, P.E. Prevelige, Mapping of protein:protein contact surfaces by hydrogen/deuterium exchange, followed by on-line high-performance liquid chromatography–electrospray ionization Fourier-transform ion-cyclotron-resonance mass analysis, J. Chromatogr. A 982 (2002) 85–95.