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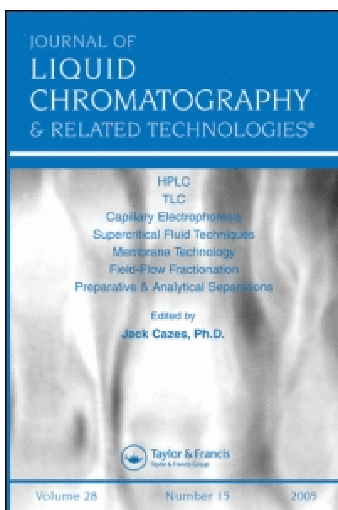

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Fast and Efficient Separations Using Reversed Phase Liquid Chromatography

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Fast and Efficient Separations Using Reversed Phase Liquid Chromatography

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Abstract: Liquid chromatography has seen a dramatic increase in speed and efficiency over the past decade. The advances in separation speed have been mostly related to the development of column technology and instrumentation. The column technology includes small uniform particles, monolithic columns, and thermally stable phases with various bonding chemistries. Relatively short columns packed with sub-2 μm particles provide high speed and efficient separations. The high porosity and small skeleton size of monolithic columns permit operation at high flow rates on relatively long columns, using a conventional LC system for high speed and high efficiency separation. The new instrumentation is associated with ultra-high pressure pump systems and high temperature systems. Ultra-high or very high pressure pump systems have been used to overcome the high pressure drop generated by small particles. High temperature liquid chromatography allows fast separation using high linear velocities and stable stationary phases. In this review, ultra-high pressure liquid chromatography, ultra-performance liquid chromatography, monolithic columns, and high temperature liquid chromatography are discussed as means for attaining fast and efficient separations. Minutes to sub-minute separations for various samples are demonstrated using these technologies.

Keywords: Fast separation, Reversed-phase liquid chromatography, Ultra-high pressure, Ultra-performance, Monolithic and packed columns, High temperature, High efficiency

INTRODUCTION

Over the course of the past two decades, an important trend in the development of liquid chromatographic technology has been towards the

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improvement of analysis speed.[1−6] High-throughput analyses are especially desired for rapid screening of numerous samples generated by combinatorial chemistry, particularly in drug discovery.[3,4,7] With an increase in the number of drug candidates, the demand to improve the productivity of analyses has significantly raised the level of interest in high speed liquid chromatography. In addition, real time quality control in the manufacturing of drug substances and products demands an improvement in analytical speed that is most often related to chromatographic separation. Fast separations are also desirable for applications involving environmental analysis, especially when quick answers are needed or a large number of samples are involved. The desire for on-line monitoring or field analysis by rapid analytical methods is increasing in various fields.[2] Finally, fast separations usually provide narrow peaks and, thus, high sensitivity.

No exact definition for a fast separation has been introduced in the literature, since separation time can be affected by many factors, such as sample complexity and column efficiency. The overall goal of analytical chromatography is to achieve sufficient resolution of analytes of interest within the shortest possible time. In this review, we emphasize applications of fast reversed-phase LC separation in which separation times are significantly reduced (e.g., 4–5 times) without significantly sacrificing column efficiency and resolution.

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In chromatography, the separation time can be defined as the retention time, t_R, of the most retained component in the sample. The separation time is given by:[8]

$$t_R = \frac{HN_{req}}{u}(1 + k) \quad (1)$$

where H is the theoretical plate height, N_{req} is the required plate number for a given separation, u is the linear velocity, and k is the retention factor. It can be seen from Equation (1) that, for a given separation (constant N_{req}), the separation time is proportional to the theoretical plate height at constant linear velocity and retention factor. A small plate height means high column efficiency per unit column length. For a column having a small plate height, a short column length can be used for a given separation (N_{req}) and, thus, a short separation time is obtained.

According to the plate theory,[9] the three major sources of band broadening in a column arise from flow phenomena, molecular longitudinal diffusion, and resistance to mass transfer. The relationship between plate height and these broadening processes in packed column chromatography is expressed by the van Deemter equation:[9,10]

$$H = A + \frac{B}{u} + Cu \approx 2\lambda d_p + \frac{2\gamma D_m}{u} + f(k')\frac{d_p^2 u}{D_m} \quad (2)$$

where A, B, and C are constants that account for contributions to band broadening from eddy diffusion, longitudinal diffusion, and mass transfer

resistance, u is the linear velocity, γ is a constant called the tortuosity or obstruction factor, D_m is the diffusion coefficient of an analyte in the mobile phase, d_p is the diameter of the packing material, and k' is the retention factor for an analyte. When bonded stationary phases or very thin stationary phase films are used in LC, the mass transfer resistance in the stationary phase is relatively small and, thus, the mass transfer resistance (C_m) in the mobile phase becomes dominant.[11]

In open tubular column LC, particle size d_p in Equation (2) is replaced by column internal diameter (i.d.), d_c and the A term in the equation does not exist. Open tubular LC column i.d. should be very small (usually less than 10 μm) to reduce the mass transfer resistance in the mobile phase, since solute diffusion coefficients are approximately 10,000 times smaller in liquids than in gases. However, columns of small internal diameter are very difficult to prepare, particularly with respect to uniformity of stationary phase coating on the capillary wall. Furthermore, small internal diameter columns have extremely low sample loading capacities, and sample injection and detection are challenging. Therefore, open tubular column LC is considered impractical for fast separations.[11,12]

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In fast packed column LC, the A and C terms in the van Deemter equation are more significant than the longitudinal diffusion (B term). A major factor affecting the A and C terms is particle size of packing material. Mass transfer resistance in the mobile phase can be decreased significantly using very small particles. Furthermore, small uniform particles produce a small eddy diffusion term. Thus, small uniform particles should be used for high efficiency and high speed separations in LC. However, small particles generate high pressures. The pressure drop across a column, ΔP, necessary to obtain a mobile phase linear velocity, u, is given by:

$$\Delta P = \frac{\phi \eta L}{d_p^2} u \quad (3)$$

where ϕ is the column resistance factor, η is the viscosity of the mobile phase, and L is the column length.[13]Note that ΔP is inversely proportional to the square of the particle diameter. In the case of a particle-packed column, H is roughly proportional to d_p and the column length for a required column efficiency is inversely proportional to the particle size. Thus, shorter columns packed with small particles can be used for fast separations, while maintaining column efficiency or resolution if the selectivity does not change with particle size. However, in practice the resulting column efficiency is sacrificed, since pressure drop increases more significantly than the total plate number of a column with decreasing particle size. Most conventional LC pumping systems have upper pressure limits of ~6,000 psi. Columns that are 15–25 cm long and packed with 3–5 μm particles, and that are typically used with these pumping systems can provide 12,000–20,000 plates. When using a column packed with 1.5 μm particles, only a column length of 3–5 cm can

be used with the same pumping system because the pressure drop per unit column length is high. This type of column can provide less than 7,000 plates, ultimately resulting in shorter analysis time, but decreased separation efficiency and resolution. Therefore, ultra-high pressure liquid chromatography (UHPLC) is the most straightforward approach to achieve high speed separations with high efficiency.[14–16] Higher pressures (>6,000 psi) allow for the use of longer column lengths with smaller particles to ensure high speed separations with high efficiency.

High efficiency can also be achieved with small particles by capillary electrochromatography (CEC), which is free from pressure drop problems. In addition, the contribution of the *A*-term is known to be less significant in CEC than in LC,[17,18] because of the plug-type flow profile of electroosmotic flow, which leads to high plate numbers. This approach, however, has not been widely accepted as routine yet, because of instrumental or operational difficulties.

Monolithic columns have provided another approach to fast LC separations without compromised efficiency or resolution. Although monolithic columns were reported as early as in the 1970s,[19,20] only recently has this technique become commercially available and practical.[21–25] The high permeability and large number of theoretical plates per unit pressure drop associated with monoliths are direct characteristics of their most important and distinguishing physical features (large through-pore size/skeleton size ratios and high porosities).[22] The short diffusion path length and high porosity supplied by the large through-pores not only lower the plate height, but also decrease the hydraulic resistance of the mobile phase flow, reducing the pressure drop. The lower pressure drop permits operation at high flow rates on relatively long columns, using a conventional LC system that is not viable with traditional packed columns.

Finally, the use of high column temperatures can improve separation speed and column efficiency. Equation (2) shows that, in addition to the particle size for packed columns, the diffusion of solutes in the mobile has a significant effect on plate height. Low viscosity and high diffusivity of a mobile phase at high temperatures produce much lower mass transfer resistance, which leads to flatter van Deemter curves. Therefore, high temperature LC can be faster and more efficient.[26,27] Equation (3) shows that the pressure drop decreases as the viscosity of the mobile phase decreases, which allows for higher linear velocities as the limit of pump pressure is approached. Therefore, the reduced mobile phase viscosity and consequent enhanced diffusivity allow for operation at high flow velocity and permit use of longer columns or smaller particles, both of which increase efficiency and speed.[27] Antia and Horvath predicted that a 20 fold improvement in analysis time would result when a column is operated at high temperatures (150–200°C).[27]

In this review, fast LC development in analytical scale during the last decade is discussed. Topics include fast LC using elevated pressures such

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as UHPLC and ultra-performance LC (UPLC), monolithic columns, high temperature LC, and some aspects that combine these technologies.

UHPLC SYSTEMS

Under isocratic conditions in LC, the retention time can be expressed as:[2]

$$t_R = \frac{(1+k)Nh}{D_m v} \cdot d_p^2 \tag{4}$$

where k is the retention factor of the last eluting peak, N is the number of theoretical plates, h is the reduced plate height, D_m is the diffusion coefficient of solute in the mobile phase, v is the reduced velocity, and d_p is the particle diameter. It can be seen from Equation (4) that an efficient way to reduce separation time is to use small particles as packing materials. Columns packed with small particles provide enhanced efficiency by virtue of the relatively small intra-particulate mass transfer resistance due to short diffusion distances and, to a lesser extent, the small contribution of eddy diffusion to plate height. The evolution of HPLC has dictated the reduction of the particle size of column packings.[28] The size of the packing material has been reduced to current 1.5–5 μm from that greater than 100 μm in the 1960s. In the mean time, the separation time has been decreased from hours to minutes and even seconds for a comparable separation. However, small particles stress the pressure limitations of conventional pumping systems, since they require increasingly greater pressures.

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Fast Separations

MacNair et al. introduced ultra-high-pressure capillary liquid chromatography (UHPLC) in order to overcome the pressure limitations of conventional pumping systems.[14,29] Using UHPLC, they were able to use long capillary columns to achieve very high total column efficiency (>200,000 plates) for relative complex samples.[29] A chromatogram of the TRITC-tagged peptides generated from the digest of ovalbumin is shown in Figure 1.[29] The analysis was performed on a 27 cm long capillary packed with 1.0 μm nonporous C_{18} particles. The pump flow rate was 33 μL min^{-1}, which resulted in an average column head pressure of 2,550 bar (37,000 psi). The peak capacity was determined to be 300 within a 30 min separation space, compared to 150 obtained in 45 min using conventional column HPLC for peptides from a tryptic digest of lysozyme.

Wu et al. demonstrated high speed separations with high efficiency, using shorter capillaries packed with 1.5 μm ODS particles and utilizing a home-built UHPLC/TOFMS system.[15] In order to prove the concept in Equation (4)

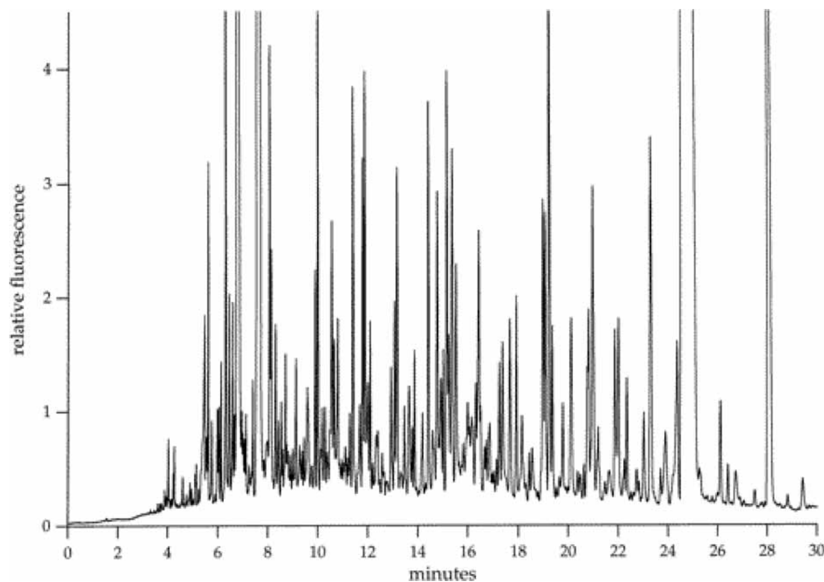

Figure 1. Chromatogram of TRITC-tagged peptides from a tryptic digest of ovalbumin. Conditions: 27 cm length × 33 μm i.d. fused silica capillary column packed with 1.0 μm nonporous octadecylsilane silica (ODS or C_{18}) particles; gradients started at 15% acetonitrile with 0.1% TFA/85% deionized water with 0.1% TFA and to 50% acetonitrile with 0.1% TFA/50% deionized water with 0.1% TFA; pump flow rate: 33 μL min^{-1}; room temperature; laser induced fluorescence detection at 543.5 nm. (Reprinted with permission from Ref. 29, Copyright © 2005 American Chemical Society).

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that separation time is proportional to the square of particle diameter of the packing material, they utilized two columns packed with 1.5 μm and 3.0 μm nonporous ODS particles, respectively. Figure 2 shows two chromatograms for parabens using the two columns in UHPLC. Nearly constant average column efficiency, retention factor, and reduced linear velocity were maintained for the two separations by adjusting column length, column inlet pressure, and mobile phase composition. It was assumed that the two mobile phases had the same diffusion coefficients and the two stationary phases had similar surface chemical structures. Comparing Figures (A) and (B), it can be seen that while the average resolution for the two separations is almost the same, the separation using 1.5 μm particles is approximately 4 times faster than for 3 μm particles. However, the pressure required for 1.5 μm particles was 40 Kpsi, compared to 5 Kpsi for 3 μm particles in order to achieve a similar separation. Therefore, short columns packed with small particles favored fast separations, but much higher pressures were required.

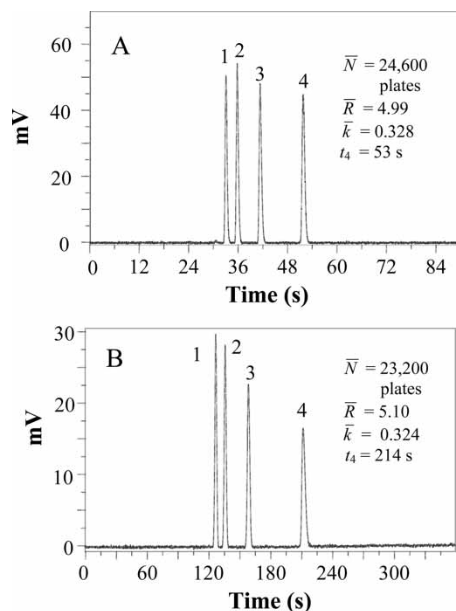

Figure 2. Chromatograms of parabens. Conditions: 254 nm UV detection, ascorbic acid as unretained marker; (A) 15 cm × 29 μm i.d. fused silica capillary packed with 1.5 μm nonporous Micra ODS particles, 40 Kpsi inlet pressure, 500 psi injection pressure, room temperature, water (20 mM NH_4Ac, pH = 3.5)/acetonitrile (60:40 v/v); $u = 0.49\,cm\,s^{-1}$; (B) 30 cm H 29 μm i.d. fused silica capillary packed with 3.0 μm nonporous Micra ODS particles, 5 Kpsi inlet pressure, 150 psi injection pressure, room temperature, water (20 mM NH_4Ac, pH = 3.5)/acetonitrile (70 : 30 v/v); $u = 0.25\,cm\,s^{-1}$. Peak identifications: (1) methyl paraben, (2) ethyl paraben, (3) propyl paraben, and (4) butyl paraben. (Reprinted with permission from Ref.15. Copyright © 2000 John Wiley & Sons, Inc.).

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Figure 3 shows a separation of parabens using 1.5 μm ODS particles at 20 Kpsi inlet pressure. By comparing Figures 2A and 3, it can be seen that the separation at 40 Kpsi was almost two times faster with a similar efficiency. This observation is attributed to the flat van Deemter curve for the 1.5 μm particles at the linear velocities beyond the optimum linear velocity ($\sim 0.16\,cm\,s^{-1}$) and at pressures higher than 20 Kpsi. It is important that fast separations be carried out with minimum compromise in resolution. It was observed that when the inlet pressure increased from 20 to 40 Kpsi, surprisingly, the average resolution increased slightly. It is expected that resolution will decrease with increasing pressure in conventional LC when linear velocities are higher than the optimum value. In this case, the effect of decreased efficiency on resolution was counteracted by slightly increased retention factor and selectivity. Subsequently, the overall resolution slightly increased with increasing pressure.

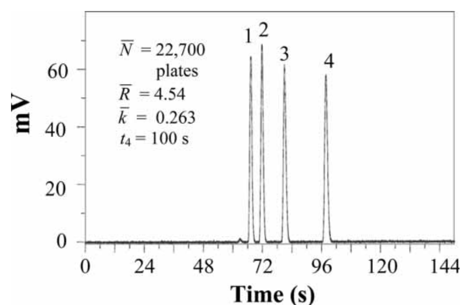

Figure 3. Chromatogram of parabens. Conditions: 20 Kpsi, other conditions are the same as in Figure 2A. (Reprinted with permission from Ref. 15, Copyright © 2000 John Wiley & Sons, Inc.).

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A combinatorial chemistry sample was separated by UHPLC using a 13 cm × 100 μm internal diameter (i.d.) column packed with 1.5 μm nonporous C_6 bonded particles with high speed TOFMS detection, as shown in Figure 4A. The separation was accomplished in 80 s with high efficiency. When a 25 cm × 75 μm i.d. column packed with 3 μm porous ODS particles was used, it took 35 min for the same separation, as shown in Figure 4B. In this case, UHPLC is 26 times faster than the conventional LC with an efficiency of 26,000 plates for the last two peaks. However, it must be reiterated that the pressure associated with UHPLC is approximately 10 times greater than that for conventional LC. The resolution for the last two peaks is different due to slight differences in selectivity for the two columns.

Xiang et al. used UHPLC for fast separations of enantiomers. Figure 5 shows a chromatogram for enantiomers of oxazepam, a chiral drug, by UHPLC.[30]Capillary columns were packed with 1.0 μm Kovasil S-H nonporous particles, and 2-hydroxypropyl-β-cyclodextrin was added to the mobile phase as modifier to produce transient diastereomeric complexes with the analytes. Pressures of up to 40,000 psi were applied, and efficiencies in excess of 50,000 plates were obtained for a high resolution separation that was accomplished in less than 1 minute.

Stationary Phases

Most stationary phases used for UHPLC are silica based nonporous particles. A major advantage of nonporous particles is the absence of sample diffusion in pores, leading to faster mass transfer than with porous particles. Columns packed with small nonporous particles can be very useful for the fast separation of proteins, since the mass transfer of large molecules is more significant in the mobile phase. Issaeva et al.[31] separated proteins and their digests using short columns packed with 1.5 nonporous particles with delicate gradients.

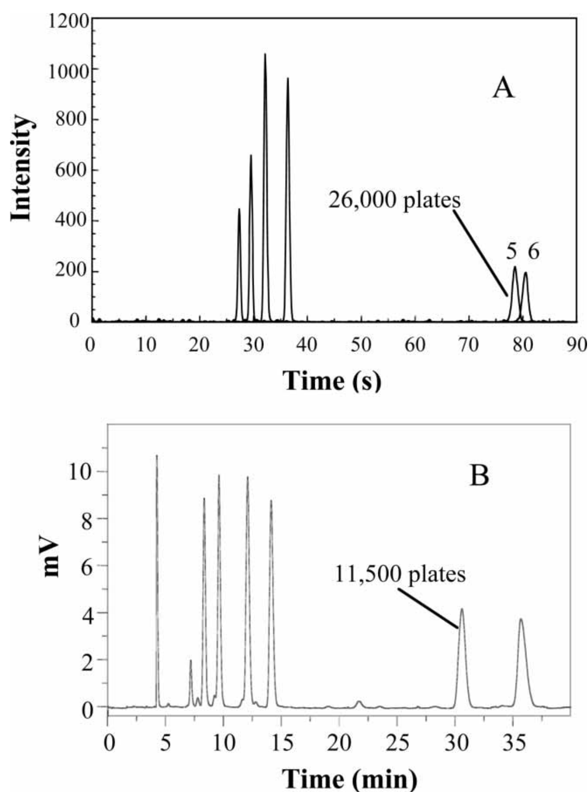

Figure 4. Chromatograms of a combinatorial chemistry sample. Conditions: (A) 13 cm × 100 μm i.d. capillary column packed with 1.5 μm nonporous Kovasil MS-H particles, water (0.2 mM $NaNO_3$)/acetonitrile (70 : 30 v/v), 40 Kpsi. TOFMS: 2.0 μL min^{-1} liquid sheath using water (0.2 mM $NaNO_3$)/methanol (70 : 30 v/v), 3500 V electrospray voltage, 1000 mL min^{-1} N_2 curtain gas (70°C); (B) 25 cm × 75 μm i.d. capillary column packed with 3 μm ODS1 Spherisorb particles, acetonitrile/water (70/30, v/v), 200 nm UV detection, 4.6 Kpsi inlet pressure. (Reprinted with permission from Ref. 15, Copyright © 2000 John Wiley & Sons, Inc.).

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Seven proteins could be separated in 30 s by a 15 mm column packed with 1.5 nonporous ODS particles. Figure 6 displays a separation of an old tryptic digest sample of hemoglobin on a Micra nonporous C_{18} column at room temperature. More than 60 components were resolved within *ca.* 3 min, demonstrating the high resolving potential of the nonporous packing for high molecular weight analytes.

Cintron and Colon synthesized uniform, spherical organosilica nanoparticles (670 nm) containing octadecyl moieties using a simple one-step process.[32] The retention characteristics of a column packed with the organo-nano-particles are stable under acidic (pH < 1) and basic (pH > 11)

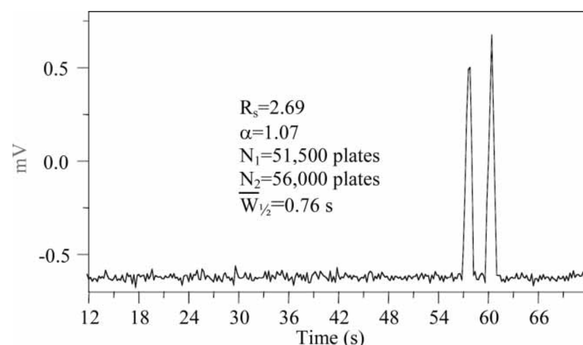

Figure 5. Chromatogram of Oxazepam. Conditions: water (0.1% TFA, pH = 4, 15 mM 2-hydroxypropyl-β-cyclodextrin)/acetonitrile (74 : 26, v/v). 40,000 psi inlet pressure; 13.5 cm × 29 μm i.d. fused silica capillary column packed with 1.0 μm Kovasil S-H nonporous particles; 215 nm UV detection; ascorbic acid as marker. (Reprinted with permission from Ref. 30, Copyright © 2004 Friedrich Vieweg & Sohn-Verlag GmbH).

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conditions in UHPLC. Fast analysis times and relatively high separation efficiencies were obtained under inlet pressures of ~50,000 psi. In addition, polybutadiene coated 1 μm nonporous zirconia particles were evaluated for high temperature UHPLC.[33] The particles could withstand both high

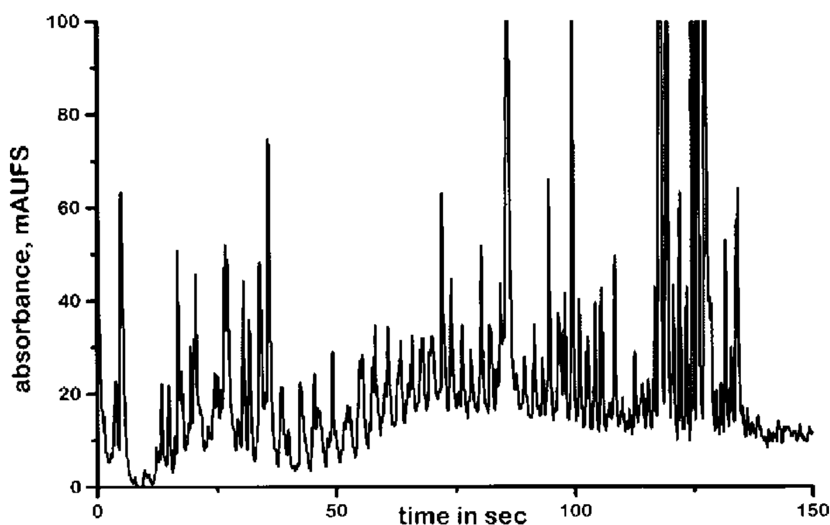

Figure 6. Separation of an old tryptic digest sample of hemoglobin. Conditions: column 33 × 4.6 mm 1.5 μm Micra C_{18}, flow rate 1 mL/min gradient of acetonitrile in water with 0.1% TFA according to program (% acetonitrile, time in seconds): 1% 0; 20% 20; 30% 90; 40% 100; 40% 120; 60% 130. 30°C. (Reprinted with permission from Ref. 31, Copyright © 1999 Elsevier B.V.).

temperatures and high pressures. A column efficiency of 420,000 plates m^{-1} was obtained at 90°C and 26,000 psi. The major limitation of nonporous particles is that they have very low sample loading capacity. More recently, 1.5 μm ethyl-bridged hybrid porous silica particles were evaluated in terms of its suitability for UHPLC.[34] The efficiency of these particles is similar to that of 1.0 μm nonporous silica particles. The mechanical strength of the ethyl-bridged hybrid material was evaluated by running a 15 cm long column at pressures up to 65,000 psi. No breakdown of the particles in the packed bed was found.

Practical Considerations

In UHPLC, capillaries (<100 μm i.d.) have been used for packing small particles, so that the frictional heat generated in the capillary columns under high pressures can be easily dissipated. In addition, relatively short capillary columns are used for fast separations, making the total column volume very small. This small void volume as well as high efficiency require that the sample volume and mass introduced into a UHPLC system be extremely small (e.g., <0.5 nL). Most UHPLC systems use a static split injection system to introduce such a small amount of sample under ultra-high pressures. Unfortunately, this type of injection is not very reproducible and requires large consumption of sample, although the actual injected amount onto the column is small. It also takes several minutes to complete one injection because sample loading must be performed at atmospheric pressure. Other injection modes have also been investigated for UHPLC. A so called pressure-balanced injection valve was evaluated for use with UHPLC at pressures up to 18,000 psi (1,200 bar). This valve was more reproducible, convenient, and required much less sample than the static split injection system. More recently, another injection system built from 6 sub-valves, each of which is automatically controlled, has been studied.[35] This injector with a specially designed column connector claims to withstand 30,000 psi without leaking. In addition, the injector provided very good reproducibility in terms of peak area, retention time, and plate number.

Another practical concern is that capillary columns in UHPLC provide low sample capacity. Wu et al. studied the effect of column diameter on efficiency and sensitivity in a UHPLC/UV system.[16] The 100 μm i.d. columns demonstrated approximately 40% lower efficiency but 10 fold higher sensitivity than the 29 μm i.d. columns. Therefore, there is a trade-off between column efficiency and sample capacity and, hence, sensitivity in UHPLC. Finally, gradient separation is challenging for a capillary UHPLC system, since the mixer should withstand ultra-high pressures and have an extremely small volume for a capillary column.

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ULTRA-PERFORMANCE LC (UPLC)

Instrumentation

A UPLC system (Acquity) was commercialized by Waters Corporation in 2004, based on the concept of UHPLC that the use of small packing particles can increase separation speed and efficiency. This new system utilizes sub-2 μm particles and pressures as high as 15,000 psi to increase separation speed and resolution.[36,37] Compared to the capillary UHPLC system, this commercial instrument can also provide higher sensitivity and reproducibility, and is more user friendly. The Acquity system uses columns with a much larger internal diameter (2.1 mm) packed with 1.7 μm porous particles to increase sample loading capacity and, thus, sensitivity. The column i.d. is smaller than the 4.6 mm for conventional LC in order to minimize the adverse effect that frictional heat generated within the column has on efficiency. In addition, this system is integrated with an automatic high-pressure injection system to ensure system's reproducibility. What's more, the system has minimized its dead volume by utilizing a filter as mixer, a small flow cell, and small i.d. connection tubings. This feature allows for the use of fast gradients, which is useful for the fast separations of complex samples. Finally, a high speed detector with fast data acquisition is used to ensure the optimal peak capture.

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Applications

UPLC is an ideal fast separation tool for complex mixture analysis in both isocratic and gradient modes. It has been increasingly used in the pharmaceutical industry, from drug discovery[38–42] to drug development.[43,44] Since pharmaceutical samples can be complex and the sample numbers are usually large, separations with high resolution and high sensitivity are often required.

Two isocratic separations of alkylbenzenes using HPLC and UPLC are shown in Figure 7. It can be seen that the two chromatograms provide comparable average efficiency and resolution. However, the separation time for UPLC is approximately 4 times shorter than that for conventional HPLC. In addition, the pressure used in UPLC is 5 times higher than in HPLC. Figure 8 is a chromatogram using a gradient for a crude drug substance.[45] The separation was accomplished in 4 min, in contrast to 20–40 min for conventional LC with the comparable separation.

Plumb et al. demonstrated a rapid 1.5 min separation to support high throughput metabonomics screening activities using UPLC-TOFMS.[38] The peak capacity and the number of peaks detected using fast UPLC gradients and TOF-MS was similar to, or better than, that generated with a 10 min LC separation. This fast approach to metabonomics allows an overall view of any variation or biochemical changes in the sample to be rapidly acquired.

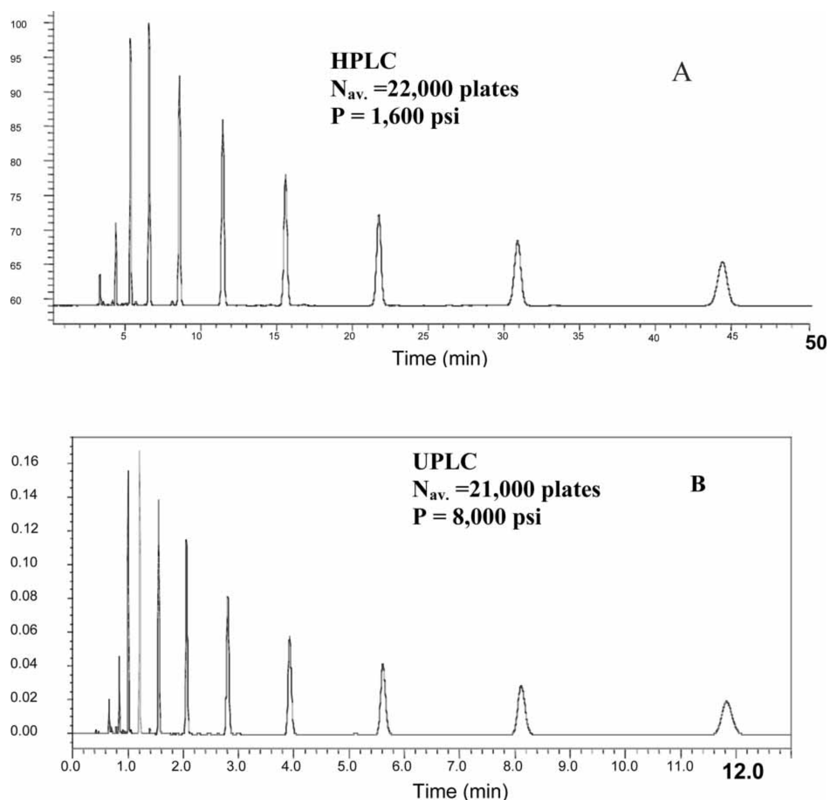

Figure 7. UPLC separations of alkylbenzenes. Conditions: (A) Agilent HPLC 1100 system; 70 : 30 ACN/H_2O mobile phase; 250 × 4.6 mm YMC-AQ, 5 μm porous C_{18} particles; 1.0 mL min^{-1}; UV 254 nm; 25°C. (B) Waters ACQUITY UPLC system; 70:30 ACN/H_2O mobile phase; 2.1 × 100 mm BEH C_{18} column, 1.7 μm porous particles; 0.5 mL min^{-1}, UV 254 nm; 25°C.

They applied the UPLC-MS approach to the analysis of urine samples from rodents, including normal and Zucker obese rats and three strains of mice (of both sexes), and demonstrated rapid discrimination between age, strain, gender, and diurnal variation. Johnson and Plumb[40] also compared fast separations by UPLC with those by monolithic LC. It was observed that the UPLC/MS approach was approximately three times more sensitive than the monolithic column LC/MS approach, along with the detection of significantly more metabolite peaks.

Wilson et al.[42] showed that the analysis of a Zucker rat urine sample by UPLC/MS with a 1 min run time enabled a total of some 1,000 peaks to be detected. This approach, therefore, allows similar results to be achieved to those obtained previously by conventional LC, but in one tenth of the time. Alternatively, a longer run (e.g., 5 min) can be employed and the number of

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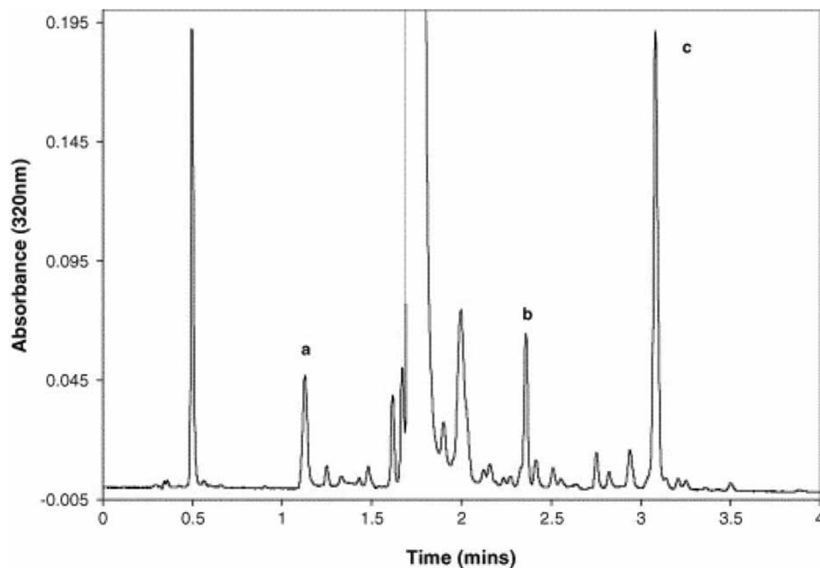

Figure 8. Chromatogram produced by using a 50 mm long column with a flow rate of 0.5 mL min^{-1}. Conditions: Waters Acquity system, 50 × 2.1 mm i.d. packed with 1.7 μm Acquity C_{18} BEH particles. The A solvent was 0.1% Tri Fluoroacetic Acid (TFA) in water (v/v) and the B solvent 0.1% TFA in acetonitrile (v/v). 10% B was increased to 40% B in 4 min, 40°C, UV 320 nm. The sample was a crude drug substance used as a System Suitability Test (SST) mixture. (Reprinted with permission from Ref. 45, Copyright © 2005 Elsevier B.V.).

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peaks detected increased to *ca.* 5000 as shown in Figure 9. King et al.[43] evaluated UPLC for the rapid analysis of pharmaceutical formulation samples. They investigated the instrumentation performance for isocratic and gradient separations as a function of flow rate using several commercially available drugs as model probe compounds. The use of high speed separations for dose formulation strength analysis was evaluated using two model drug compounds: mefenamic acid and chloramphenicol, in a dimethylacetamide/polyethyleneglycol-200 vehicle. Relatively high accuracy, precision, and resolution were demonstrated under high speed gradient conditions for the dose formulation analysis.

A typical LC assay can be transferred to and optimized for a Waters Acquity UPLC system to achieve both higher throughput and better assay sensitivity. Analysis of operation costs and sample throughput found UPLC cost advantageous over conventional LC. Yang and Hodges discussed some strategies to expedite method transfers.[46] Rapid gradients and high linear velocities are usually needed in UPLC to take advantage of its high speed with high resolution. Volumetric flows should, however, be appropriately decreased, since the smaller i.d. columns are used in UPLC compared to the

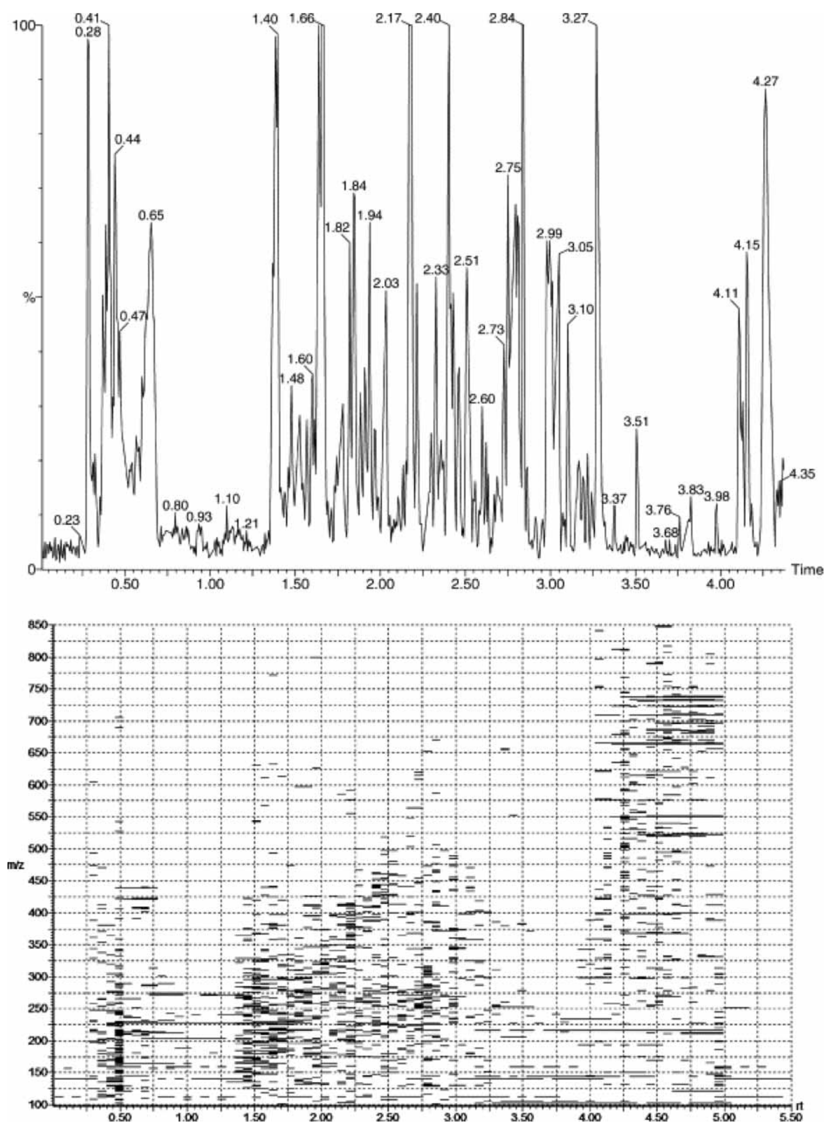

Figure 9. A UPLC-MS separation of rat urine. Conditions: a 30 × 2.0 mm 1.7 μm C_{18} bonded column using a 0–95% acetonitrile gradient over 5 min at 600 μL min and *ca.* 12,000 psi. Upper: trace, total ion current, lower: 2D mass chromatogram. (Reprinted with permission from Ref. 42, Copyright © 2005 Royal Society of Chemistry).

4.6 mm i.d. in conventional LC. For example, 0.3–0.6 mL min^{-1} for a 50–100 × 2.1 mm column packed with 1.7 μm particles can be used in UPLC, compared to 1.0–1.5 mL min^{-1} for a 150–250 × 4.6 mm i.d. column packed with 5 μm particles in conventional LC.

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There are some areas for development with UPLC. Columns available for UPLC include those packed with BEH C_{18}, Shield RP C_{18}, C_8, and phenyl-bonded packings. Columns with a greater variety of bonding chemistries, including chiral columns, are highly desirable. In addition, current UPLC systems only have binary pump systems and it may be difficult for such a system to separate those samples that requires ternary or tertiary mobile phases. Finally, a UPLC system that withstands pressures higher than 15,000 psi can further improve separation speed and resolution.

MONOLITHIC COLUMNS

Column Type

In chromatography, monolithic columns are those containing a single piece of continuous material that is porous and permeable, hence, can be percolated by a mobile phase, and has a sufficiently large surface area for analytes to exhibit significant retention. Several types of monolithic columns have been reported for LC or CEC, including silica polymer-based, organic polymer-based, and particle-based monolithics.

Silica Polymer-Based Monoliths

Most silica-based monoliths, or silica monoliths, are prepared by neutralizing an alkaline silicate solution to generate monosilisic acid, $Si(OH)_4$, which readily polymerizes, resulting in bulk or precipitated silica hydrogels. Silanes such as tetramethoxysilane (TMOS) or tetraethoxysilane undergo hydrolytic polymerization in aqueous acetic acid in the presence of polyethylene glycol (PEG) to form monolithic silica having network structures. Monolithic silica columns can be prepared either in a tube (6–9 mm i.d.) or in a fused-silica capillary, as described previously.[25,47,48] The preparation in a mold is accompanied by volume reduction of the entire structure. The resulting silica monoliths are covered with PTFE tubing or with PEEK resin to fabricate a column for LC. The column length is usually limited to less than 15 cm. This type of monolithic silica column, is commercially available at 5- or 10 cm length from Merck KGaA (Chromolith). To prepare a monolith in a fused silica capillary for capillary LC or CEC, the silica network structure must be attached to the capillary wall in order to prevent shrinkage of the skeletons. After the formation of the network structure of silica skeletons, mesopores are formed by treatment with ammonia. The total porosity of monolithic silica columns is much greater than that of a column packed with particles. This is mainly attributed to external porosities, 39% for a typical particle packed column, 65–70% for a Chromolith column. Similar to most silica-based particulate columns, a silica-based monolithic column has a limited pH range of 2–8.

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Organic Polymer-Based Monoliths

Organic polymer-based monoliths or polymer monoliths have been prepared by *in situ* polymerization in tubings up to 8 mm i.d. for HPLC and capillaries 20 to 500 μm i.d. for micro LC and CEC.[49] They have been shown to be effective for high speed separation of proteins, polypeptides, oligonucleotides, synthetic polymers, and some small molecules.[49] The preparation of an organic polymer monolith is similar to that used for polymer beads.[50] The starting solutions contain monomers, porogens, and an initiator. Polymerization usually proceeds with a nucleation and growth mechanism. The decomposition of an initiator to form radicals initiates the polymerization of monomers. The growing polymers produce nuclei (precipitates) to which solvating solvents and remaining monomers distribute. Continued polymerization in swollen globules increases the size, causing coalescence to form clusters, which, in turn, join to form an integrated structure. The interconnected clusters of globules form the monolith.[51,52] This type of organic polymer monolithic columns is commercially available from Teledyne Isco, Inc. (Isco Swift).

Monolithic polymer columns made of organic materials are capable of high speed separation of polypeptides and proteins in reversed-phase and in ion-exchange chromatography, but show relatively low efficiency for small solutes. Compared to polymer beads, organic polymer monoliths provide higher efficiency and less swelling problems. In addition, this type of column can tolerate extreme changes in pH, from 1–14 for reversed phase *Swift* columns. Limitations include their relatively low mechanical stability and the presence of micropores, which cause a large *C* term contribution for small molecules.

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Particle-Based Monolithics

Particle-based monolithic columns are prepared by sintering or embedding silica particles in a capillary or a tube for LC, CEC,[53–55] and even GC.[56] Solgel-bonded monolithic columns, employing an inert solgel matrix and supercritical CO_2 drying, are the most efficient columns of this type. Efficiencies of 220,000 plates m^{-1} and 175,000 plates m^{-1} were obtained from columns containing sol-gel bonded 7 μm, 1400ÅODS and columns containing sol-gel bonded 3 μm, 80ÅODS, respectively, in CEC. These are among the highest reported efficiencies for continuous-bed columns.[57] Monolithic columns from particles are expected to be stable without the need of end-frits and minimize the shrinkage of the continuous bed. Monolithic columns prepared from particles were designed to inherit the efficiency and selectivity of versatile LC packing materials, since the bonding chemistry for conventional monoliths is limited. However, this type of monolithic column has similar to or, even slightly higher, pressure drops than the columns packed with the same phases in conventional LC.

Many other types of monolithic columns have also been reported. Tennikova and Svec developed monolithic discs for fast LC separation of high molecular weight substances, particularly proteins.[58] Monoliths from compressed soft gels were used by Hjerten to separate proteins.[50] A graphitized carbon monolithic column was reported by Liang et al.[59] More recently, monoliths composed of ZrO_2 and HfO_2 were synthesized in situ inside capillary columns by Colon's group.[60] The material shows a globular like structure and through-pores. Separation of a simple sample demonstrated some potential for the new metal oxide monolithic columns. Li et al. incorporated single wall carbon nanotubes into an organic polymer monolith containing vinylbenzyl chloride and ethylene dimethacrylate to form a novel monolithic stationary phase for capillary LC and CEC.[61]

Performance Characteristics

Pressure Drop

The macropores in a monolithic can be seen as the equivalent of the interparticle void volume of a particle-packed column. Consequently, the pressure drop for a particle packed column is only determined by the diameter of sphere particles; while the pressure drop for a monolithic column is determined by through-pore size and skeleton size. Leinweber et al.[62] found that a Chromolith column exhibited a pressure drop equivalent to a column packed with 11 μm particles. This is in accordance with experimental studies where columns having different particle sizes but the same lengths were used.[47,63] It may be more practical to compare pressure drops between monolithic and particle packed columns which have the same total column efficiency. The linear relationship between pressure and linear velocity for packed and monolithic columns is demonstrated in Figure 10.[64] These three columns provided a similar total column efficiency (15,000–20,000 plates). A 150 × 4.6 mm Chromolith column provided ~3.5 times lower pressure drop than a 3 μm packed column of the same length, and 2.8 times lower pressure drop than a 250 × 4.6 mm, 5 μm packed column. Low back pressures for monolithic columns mean that long columns and high flow rates can be used to achieve fast and efficient separations.

Retention and Selectivity

The relationship between retention factor and mobile phase composition is shown in Figure 11.[64] The average retention factors for a C_{18} Chromolith column are approximately 2.5–3.1 times lower than those for a YMC packed C_{18} column using toluene and *N,N*-dimethyl aniline as solutes and mobile phases containing 35 to 70% acetonitrile. The lower retention factors for monolithic columns are also indicated by data obtained using

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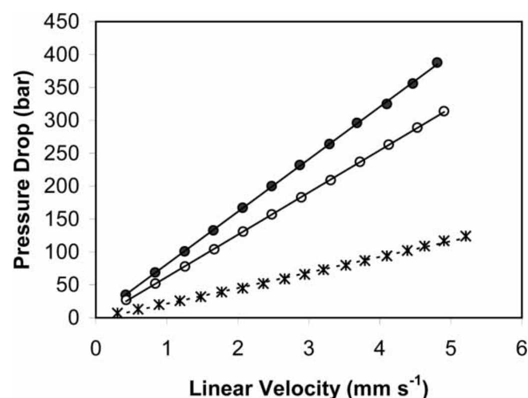

Figure 10. Relationship between pressure and linear velocity for packed and monolithic columns. Conditions: 70/30 (v/v) acetonitrile/water as mobile phase; uracil as unretained marker; 5 μL injection; flow rates: 0.25–3 mL min^{-1} for YMC C_{18} columns, 0.25–5 mL min^{-1} for E. Merck monolithic column. Keys: ● YMC C_{18} 150 × 4.6 mm, 3 μm packed column; ○ YMC C_{18} 250 × 4.6 mm, 5 μm packed column; ∗ 150 × 4.6 mm Chromolith column. (Reprinted with permission from Ref. 64 Copyright © 2004 Elsevier B.V.).

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methanol/water mobile phases.[21,65] The low retention of monolithic columns may be ascribed to its high porosity and low density. Although both Chromolith and packed YMC C_{18} columns claim to have a similar surface area per gram (300 m^2g^{-1}), Chromolith columns have higher

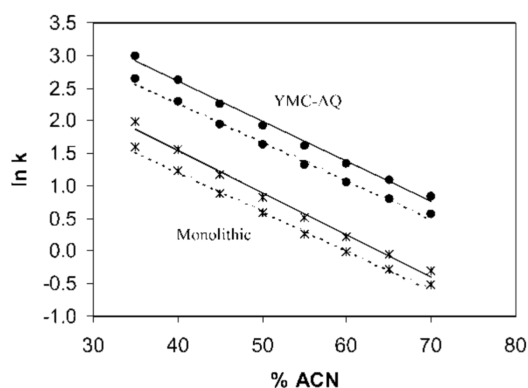

Figure 11. Comparison of retention factors for monolithic and packed columns. Conditions: 150 × 4.6 mm YMC C_{18}, 3 μm packed and Chromolith column; 5 μL injection; 1.5 mL min^{-1}; UV, 254 nm; 25°C. Keys: solid lines, toluene solute, acetonitrile/0.1% H_3PO_4 in water as mobile phase; dash lines: *N,N*-dimethyl aniline, acetonitrile/10 mM K_2HPO_4 (pH 7) in water as mobile phase. (Reprinted with permission from Ref. 64 Copyright © 2004 Elsevier B.V.).

porosity and, thus, lower density. Leinweber and Tallarek[62] reported that the density of the Chromolith bed is 0.26 g mL^{-1} and the density of the YMC C_{18} packing was determined as 0.76 g mL^{-1}.[64] The lower density means the lower surface area per unit volume or the lower phase ratio for the monolithic column, which can lead to the lower retention.

The comparison of selectivity factors between a monolith and a packed column was compared by Wu et al.[64] It was found that selectivities were comparable for both columns, even though the monolithic column is slightly higher for $\alpha_{toluene/phenol}$ and the packed column is slightly higher for $\alpha_{toluene/benzene}$. This implies that the separation mechanisms are similar for these solutes on both reversed-phase columns.

Efficiency

The optimum plate heights for the Chromolith columns reported in the literature are from 8–10 μm, which are equivalent to 3.5–4.0 μm particle packed columns.[22,62,64] The van Deemter curves for monolithic and packed columns are shown in Figure 12.[64] For fast separation, linear velocities higher than the optimum are usually used. It can be seen that the monolithic column provided flatter curves or lower plate heights than both packed columns at high flow rates. The flatter curve for the monolithic column demonstrates that the monolithic column provides higher efficiency at high flow rates compared to the two packed columns. For example, the plate height for the monolithic column at 7.5 mm s^{-1} is approximately the same

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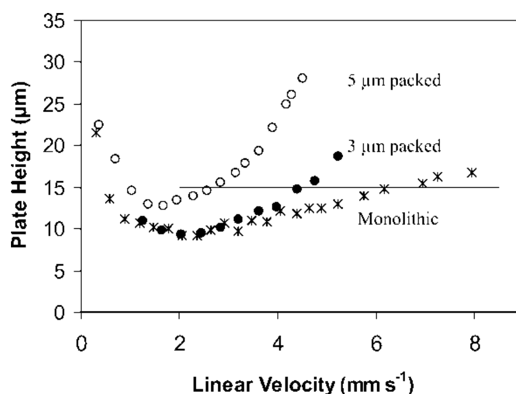

Figure 12. van Deemter curves for packed and monolithic columns. Conditions: 75/25 (v/v) acetonitrile/water as mobile phase for YMC C_{18} packed columns, 68/32 (v/v) acetonitrile/water for Chromolith column; uracil as unretained marker, *n*-propyl benzene as solute; 2 μL injection. Keys: ○ YMC C_{18} 100 × 4.6 mm, 5 μm packed column; • YMC C_{18} 100 × 4.6 mm, 3 μm packed column; * 100 × 4.6 mm Chromolith column. (Reprinted with permission from Ref. 64 Copyright © 2004 Elsevier B.V.).

(15 μm) as that for the 5 μm packed column at 2.5 mm s^{-1}, or as that for the 3 μm packed column at 5.0 mm s^{-1}. This suggests that the monolithic column can be approximately 3 times faster than the 5 μm or 1.5 times faster than 3 μm packed columns with comparable resolution and efficiency. Figure 13 shows the separation of five drugs by a Chromolith column at different flow rates of from 1 to 9 mL min^{-1}.[25] The five compounds were separated within one minute at 9 mL min^{-1}, while maintaining high resolution, thanks to the high permeability and the flatter van Deemter curve of the monolithic column.

Some Practical Aspects

In silica particle based columns, packing particles are chemically modified in large batches and, subsequently, packed into individual columns under regulated conditions. Column to column, they are relatively homogenous. The production of silica-based monolithic columns is more complicated. It involves successively preparing the silica monolith, bonding it to octadecylsilanes, and then to end capping groups, and enclosing it in a polyetheretherketone (PEEK) tubing envelop.[25,66] This individual preparation leads to concerns of potential reproducibility issues from batch to batch or column to column. Wu et al. compared the batch to batch reproducibility of both monolithic and packed columns in terms of retention time, retention factor,

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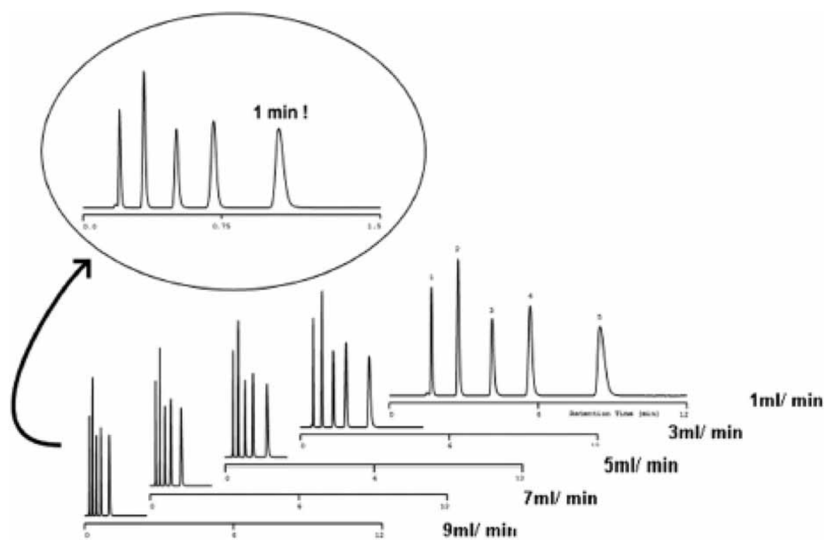

Figure 13. Separation of five β-blocking drugs using a monolithic silica column Chromolith Performance RP-18e, 100 × 4.6 mm at different flow rates leading to a separation time of 1 min. (Reprinted with permission from Ref. 25 Copyright © 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim).

and selectivity.[64] The data indicates that the batch to batch reproducibility of 100×4.6 mm Chromolith columns is highly comparable to that of 250×4.6 mm YMC C_{18} columns. In their studies, the columns were randomly ordered, which may be more representative to the real industrial laboratory situation. Kele and Guiochon conducted similar reproducibility performance examinations on monolithic and packed columns that were ordered from the consecutive batches.[65] The performance of monolithic columns consistently matches or exceeds that of conventional packed columns.

Many chromatographers have noticed tailing with monolithic columns.[23,64,67] It was observed that the tailing factors of monolithic columns are marginally higher than packed columns for acidic, basic, and even neutral compounds.[64] Kele and Guiochon suggested that the tailing of monolithic columns may be due to column-bed heterogeneity.[67] The peak shape could be improved by using gradients or elevated temperatures. Further research is needed to investigate the mechanisms that induce tailing on monolithic columns. Additionally, the lower density of monolithic columns, such as Chromolith, suggests that it have a lower phase ratio compared to particle-packed columns, which can lead to low sample loading capacity. Finally, although monolithic columns with various dimensions and bonding chemistries have been reported in the literature, the commercial availability of monolithic columns with these varieties is still limited on a routine basis.

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Applications

Monolithic columns have been used for fast analysis in various fields, including drugs and their metabolites, biological samples, environmental samples. Several reviews have been reported.[25,68–70]

Drugs and Metabolites

A fast method was developed for the simultaneous determination of a drug discovery compound and its metabolite in blood plasma using LC/MS/MS with a monolithic column.[71] It was shown that the monolithic column LC/MS/MS system offered shorter separation times by increasing flow rates, without sacrificing separation power for the drug candidate and its metabolite. The analysis results obtained by monolithic column methods were in good agreement with those by the conventional particle-packed column method.[71] The same group utilized an alkyl-bonded silica rod column combined with flow programming for fast macromolecule removal and chromatographic separation, without the need for significant sample preparation.[72] The matrix ionization suppression effect on the monolithic column LC-MS/MS system was investigated using the postcolumn infusion

technique. No significant deterioration of the column was observed in terms of column efficiency and retention time after 200 plasma injections on a 50×4.6 mm monolithic silica column. Dear et al. developed an LC/MS/MS method for the separation of six metabolites using a monolithic column.[73] They separated the six isomers in 1 min. The resolution and selectivity achieved with a 5 cm monolithic silica column were comparable to those obtained in conventional LC, while the analysis time per sample was reduced from 30 to 5 min.

Kennedy et al. developed a unique LC-TOFMS method for the ultra fast separation and identification of creatinine, hydroxyproline, and 17 amino acids.[74] Separation of 19 amino acids and related compounds was achieved in less than 3 min using a monolithic column, run at 2 mL min^{-1}, with the aid of ion-pairing reagent, perfluoroheptanoic acid. Fast data acquisition by TOFMS was especially suitable for detection of narrow peaks obtained by monolithic LC. Recently, Wu et al. demonstrated practical aspects of fast LC using 10 cm or coupled 15 cm Chromolith columns in pharmaceutical process development.[63,64] Applications of monolithic columns to typical complex samples were demonstrated for pharmaceutical process development, which include crude drug substances, reaction mixtures, and mother liquors. The analysis times were decreased by $3 \sim 7$ times on the monolithic column, while maintaining comparable resolution to the typical 5 μm particle-packed 250×4.6 mm column. Gerber et al. also showed the suitability of monolithic columns for development and production of pharmaceuticals working under current good manufacturing practice (GMP) conditions. They concluded that monolithic columns not only demonstrated repeatability and reproducibility comparable to packed columns, but were also easy to handle on conventional LC systems and exhibited very good stability.[75]

Biological Samples

Most biological samples are relatively complicated and have interactions between analytes and stationary phases, and thus, fast chromatographic separation of these samples is usually challenging. Svec and Frechet prepared continuous rods incorporating macropores for fast separations of proteins.[76] The column was prepared by *in situ* polymerization within the confines of the tube of a chromatographic column. The column could resolve several proteins in a significantly reduced time. Walcher et al.[77] used capillary poly(styrene-divinylbenzene) monoliths to separate peptides and proteins. Fast and efficient separation of nine proteins was achieved with good peak shapes at elevated temperatures.

Pham-Tuan et al. developed a fast LC method using a monolithic column and a rapid gradient with a high flow rate for rapid and detailed profiling of larger numbers of biofluids.[78] A rapid LC assay was developed using a monolithic C_{18} column for a mixture of 17 CBI-amino acids in a buffer.[79]

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The separation was accomplished within 10 min compared to 50 min for a conventional LC columns. This assay was successfully used for the measurement of amino acids in microdialysis samples from sub regions of the rat nucleus accumbens.

Hennessy et al. described a general procedure for the generation of peptide mapping of various cytochrome *c* species using monolithic silica based columns.[80] They demonstrated that the use of silica based monoliths could significantly reduce times for peptide mapping analysis, thus enabling increased sample throughput. Moreover, procedures developed for microparticulate columns could be readily transferred to monolithic column systems. Xiong et al. evaluated the efficiency of silica monolith columns for high speed profiling of peptides.[81] They used a 100×4.6 mm Chromolith column with an ODS stationary phase and a tryptic digest of cytochrome *c*. It was concluded that silica monolith reversed-phase chromatography columns could maintain the resolution of peptides ranging up to several thousand Daltons in molecular weight as the mobile phase linear velocity increased by 10 fold. Thus, silica monolith chromatography columns are of great value in the fast analysis of peptides. Minakuchi et al. demonstrated that continuous porous silica rods consisting of a mesoporous (14 or 25 nm) silica skeleton of approximately 1 μm size and through-pores of 1.7 μm provided much better separation at a high flow rate than that of conventional columns packed with 5 μm C18 silica particles having 12 and 30 nm pores, especially for high molecular weight species.[82]

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Environmentally Important Samples

Volmer et al. evaluated the performance of monolithic columns for high speed LC/MS analyses and for high resolution separations of several azaspiracid biotoxin analogs.[83] The chromatographic run times could be reduced to *ca.* 30 s without sacrificing the required resolution by increasing flow rate from 1 to 8 mL min^{-1}. An increase from 13,000 plates for a 10 cm column to 80,000 for a 70 cm column was demonstrated by connecting a series of columns. They showed the potential of the monolithic columns for fast and efficient separations for a complex biotoxin mixture using a 40 cm long column.

Hatsis et al. used a monolithic column to perform ultra fast separations of common inorganic anions using ion-interaction chromatography with tetrabutyl-ammonium–phthalate as the ion interaction reagent.[84] Analytes were monitored using either direct conductivity or indirect absorbance detection. The proposed method was validated *versus* standard chromatographic methods for the analysis of an industrial water sample. Koal et al. developed a rapid on line SPE-LC-MS-MS method for fast assays of trace level pesticides in drinking and surface water using a monolithic column.[85] This approach gives quantitative results for nearly 30 pesticide species in

less than 14 min and, thus, allows considerably increased sample throughput. They showed that the method was reproducible, robust, and sensitive.

HIGH TEMPERATURE LC

Column temperature has long been recognized as an important parameter for improving LC separation speed since the 1970's.[86,87] However, high temperature liquid chromatography has only had limited use, due to three major impediments. The stationary phase must be thermally stable. The temperature mismatch between the incoming eluent and the column must be minimized, because such a mismatch is a major cause of peak broadening, especially in high speed separation. Third, analytes that are exposed to high temperatures must be thermally stable on the time scale of the chromatographic run. Recently, the development of stable stationary phases and new instrumentation has made it possible to utilize high temperature LC for fast separation on a more routine basis.[88,89]

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Thermally Stable Stationary Phases

Most conventional silica-based stationary phases have a limitation of $\sim$60°C in reversed-phase LC, depending on mobile phase composition. It is critical to develop more stable stationary phases for high temperature LC. Currently, modified metal oxides, graphitic carbon, and modified silica particles have been used in high temperature LC.[89–91]

Great efforts have been made on development of metal oxide-based stationary phases by Carr's group.[89] Most often used metal oxides include zirconia and titania. There are three types of surface modifications: dynamic chemical modification, "permanent" covalent chemical modification, and physical screening or cladding.[89] The most common method for deposition of carbon on a surface is *via* the pyrolysis of a carbonaceous precursor at 700–1100 K.[92] Several zirconia-based columns are commercially available from ZirChrom Separations. They include PBD and polystyrene (PS) coated zirconia and carbon clad Zirconia (DiamondBond C_{18}). These polymer modified and carbon clad zirconia phases offer high thermal and pH stability. There was no evidence of degradation of the PBD-zirconia phase after exposure to 1 M NaOH or at 100°C, even during prolonged use.[93,94] Graphitic carbon is an absorbent, which is commercially available as HyperCarb phase. Hypercarb consists of fully porous, spherical carbon particles comprised of flat sheets of hexagonally arranged carbon atoms. The carbon atoms have a fully satisfied valence and offer completely different retention and selectivity to silica and polymer based phases. Hypercarb can be used for reversed phase and normal phase chromatography from pH = 0–14, and at high temperature.

Several approaches have been used for improving the thermal stability of silica particles in high temperature LC, since silica particles have some advantageous properties, such as lower Lewis acid activity.[90,91] An ethyl-bridged hybrid C_{18} silica phase was designed by the cocondensation of 1,2-bis (triethoxoxysilyl)ethane with tetra-ethoxysilane on the silica surface.[95] The phase could withstand higher pHs and temperatures than conventional C_{18} phases. Yong et al. reported that the retention factor for butylbenzene changed only by 10% after the column was constantly pumped a pure water mobile phase at 200°C and 5 mL min^{-1} for one month.[96] Another so called polydentate silica phase also provided high thermal stability.[90] It was reported that the durability of the stationary phase at high temperatures varied with the type of organic modifier used and the pH of the mobile phase. Coating on the silica surface with a polymer layer is another approach to generate thermally stable silica particles.[97] It was observed that PBD on silica tended to block more pore volume compared to PBD on zirconia, which can affect the performance of PBD-coated silica particles, such as column efficiency, stability, and peak symmetry.[89]

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Column Temperature Control

The importance of column temperature control is well known for its influence on retention, selectivity, and column efficiency.[98–101] Such influence becomes more significant, when column temperatures are higher than 40°C and/or flow rates are higher than 1.5 mL $min^{-1}$for a 4.6 mm i.d. column.[102] In addition, the thermal mismatch problem is also related to column diameter. Thompson et al.[102] compared a narrow bore column (2.1 mm i.d.) to a conventional bore column (4.6 mm i.d.) at elevated temperatures under conditions where thermal mismatch broadening is significant, and showed that narrow bore columns offered significant advantages in terms of efficiency and peak shape at higher linear velocities. Thus, the use of narrow bore columns at high temperature improves separation speed and efficiency over wider bore columns. They suggested that the temperature mismatch between the incoming eluent and the column should be less than 5°C, because such a mismatch could be a major cause of peak broadening, especially in high speed separations.

Column temperature in an LC system is typically controlled by block heaters, Peltier heaters, ovens with circulating air, or water baths. In fast LC, it is important to ensure that the mobile phase temperature at the column inlet matches the desired column temperature. This match can be achieved by using a length of preheating tubing upstream from the column. Ideally, this tubing should be put between the pump and injection systems to minimize the dead volume. Some commercial LC systems preheat the eluent by embedding the preheating tubing in a heated block. Preheaters used in laboratories can be made by coiling the preheat tubing and

clamping it to the heating source inside the oven.[98,102,103] Secondly, heading and cooling rates should be fast enough to reach the setting temperature, especially when a temperature program is used. Thirdly, the column temperature should be accurate and reproducible in order to get reproducible separation data. Finally, when the column temperature is higher than the boiling point of the eluent, the eluent at column outlet should be quickly cooled down to ambient so that analytes can be detected by a UV detector. Otherwise, the chromatography would behave like solvating gas chromatography.[103] Recently, it was reported that a commercial LC heater from Selerity Technologies could use temperature programming as quick as $30^{\circ}C\,min^{-1}$ and provide rapid column reequilibration between runs.[104]

On-Column Degradation

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On-column degradation of analytes is one of the major concerns for high temperature LC. Isomerization, oxidation, hydrolysis, and epimerization are the major types of chemical reactions that affect the stability of analytes and are considered undesirable.[105–108]In addition to column temperature, various other factors, such as solute residence time, eluent additives, pH, and sampler temperature also affect the stability of analytes. Horváth and coworkers found that temperature, column length, pH, and flow velocity could be adjusted to improve the peak profile and decrease broadening due to on-column reaction.[109] They also developed a theory to predict the effect that analysis time has on the extent of the on-column reaction broadening.[27,110,111]

Thompson and Carr[112] studied the ability of a number of pharmaceuticals to withstand super ambient temperatures on the time scale of fast separations. They proposed criteria by which a particular analyte might be rejected as a candidate for high temperature analysis, and demonstrated that complex molecules were amenable to quantitation, even at temperatures in excess of $100^{\circ}C$ in aqueous media. They concluded that a decrease in the column residence time of a thermally labile analyte may decrease the extent of the on-column reaction. The approach to shortening the resident time includes using high flow rates or short columns. In addition, area changes caused by eluent exposure to higher temperatures make no difference in the reliability of the calibration curve in quantitative analysis.[112]

Examples and Applications

Yan et al. used a home designed LC system for high temperature and high speed separation.[113] Several long chain alkylphenones were completely resolved at $150^{\circ}C$ and at a flow rate of $15\,mL\,min^{-1}$, with a $50 \times 4.6\,mm$ i.d. column packed with $3\,\mu m$ polystyrene-coated zirconia porous particles,

and the analytical time could be decreased by a factor of 50 compared to that at 25°C, and at a conventional flow rate (4 mL min^{-1}). The total column efficiency for these separations, however, is still quite low, and improved peak shapes are desired for some polar analytes such as phenols. Figure 14 demonstrated the effect of temperature on separation speed and resolution.[89]It can be seen that the separation time was reduced 5 fold from 25 to 80°C, while the resolutions were comparable for the two separations. Xiang et al.[33] used UHPLC for fast and efficient separations of parabens and herbicides at high temperatures, as shown in Figure 15. A column efficiency as high as 420,000 plates m^{-1} was obtained using polybutadiene-coated 1 μm nonporous zirconia particles. Separation of five herbicides was completed in 60 s using 26,000 psi and 90°C. It was shown that high temperature could further improve column efficiency and speed in UHPLC.

Neat water has been investigated as a mobile phase in high temperature LC since it is a "green" solvent. In addition, a more "universal" flame ionization detector (FID) can be used in place of a UV detector, since water does not respond to FID.[103,114] UV-"transparent" alkyl alcohols were successfully separated by using a neat water eluent and detected by an FID.[103] Yong

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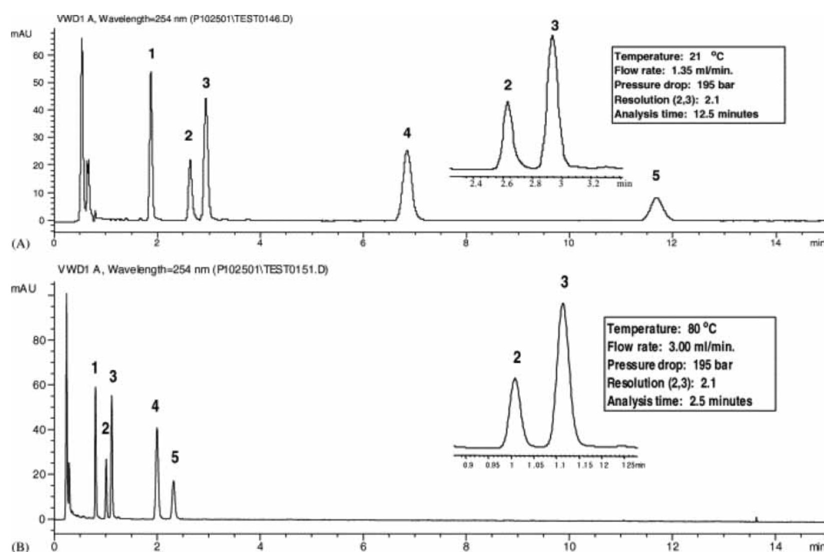

Figure 14. Fast separation of antihistamines. LC conditions: column, 100 mm × 4.6, ZirChrom-PBD; solutes: 1, doxylamine; 2, methapyrilene; 3, chlorpheniramine; 4, triprolidine; 5, meclizine. (A) Mobile phase; 29/71 ACN/50 mM tetramethylammonium hydroxide; pH at 12.2; flow rate, 1.35 mL/min; injection volume, 0.5 μL; 254 nm detection; column temperature = 21°C; pressure drop = 195 bar. (B) Same as (A), except mobile phase, 26.5/73.5 ACN/50 mM tetramethylammonium hydroxide; pH at 12.2; flow rate, 3.00 ml/min; column temperature = 80°C; pressure drop = 195 bar. (Reprinted with permission from Ref. 89 Copyright © 2004 Elsevier B.V.).

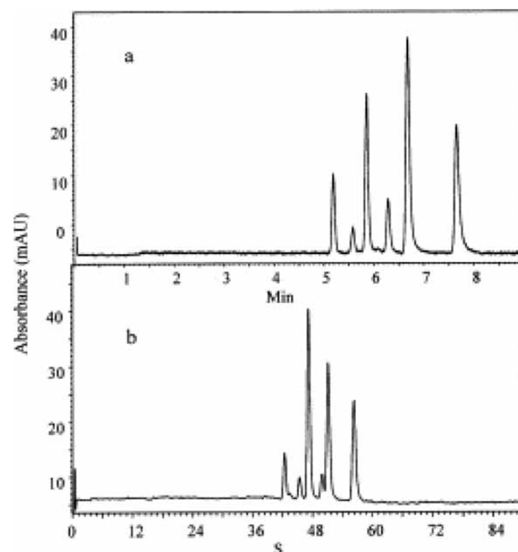

Figure 15. Fast separation of herbicides. Conditions: (a) 22°C; 10,000 psi inlet pressure; 13 cm × 50 μm i.d. fused-silica capillary column packed with 1.0 μm PBD coated nonporous zirconia particles; water (40 m*M* NaH_2PO_4, pH 7.0)–acetonitrile (55:45, v/v); 215-nm UV detection; uracil as marker. (b) 90°C; 26,000 p.s.i. inlet pressure; other conditions as in (a). (Reprinted with permission from Ref. 33 Copyright © 2003 Elsevier B.V.).

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et al.[96] separated alkyl benzenes in 5 min using a 150 mm long column packed with ethyl-bridged C_{18} silica based particles and a high aqueous mobile phase at 150°C (Figure 16). Their data showed that a phase transition might occur on this stationary phase at 90°C. Sanagi et al.[115] also observed irregularity of the Van't Hoff analysis over 80–140°C for a PBD-coated zirconia phase. The plot tended to deviate from linearity at higher operating temperatures (140 and 150°C).

Walcher et al.[77] studied the effect of temperature on protein separations in capillary poly(styrene-divinylbenzene) monolithic columns. All proteins, except the pair β-lactoglobulin A/B, were completely separated in 10 min at all temperatures, as shown in Figure 17. The average peak width at half height was decreased from 23 to 70°C. The best separation of peaks 6 and 7 was achieved at 70°C. It was noted that the elution order of trypsin inhibitor (peak 1 to other peaks) was shifted remarkably between 23 and 50°C, which may be attributed to the transition of two different conformations of the protein at the two temperatures. Yang et al.[116] developed a novel type of highly stable silica based stationary phase for protein separations at high temperatures. A dense monolayer of dimethyl-(chloromethyl-phenylethyl)-chlorosilane (DM-CMPES) on the silica surface was "hyper-crosslinked"

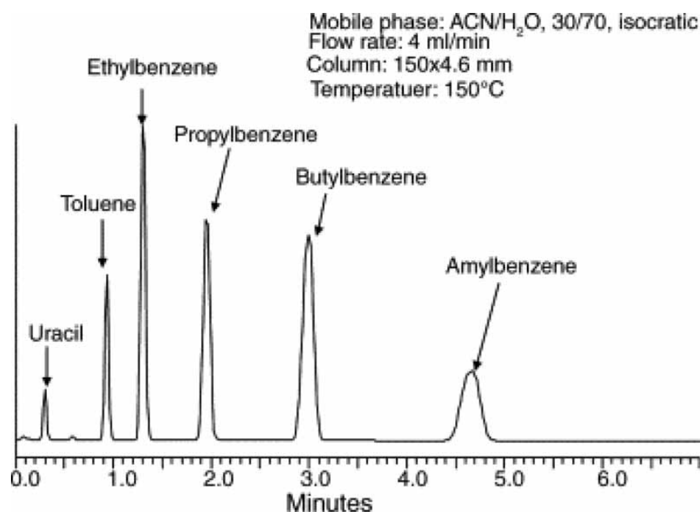

Figure 16. Chromatogram of alkyl benzenes with acetonitrile in the mobile phase. Conditions: flow rate: $4\,mL\,min^{-1}$; mobile phase: acetonitrile–water 30 : 70 (v/v); column temperature: 150°C; UV detection: 254 nm. (Reprinted with permission from Ref. 96 Copyright © 2005 Elsevier B.V.).

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with a polyfunctional aromatic crosslinker through Friedel-Crafts chemistry. The resulting stationary phases are extremely stable under highly acidic mobile phase conditions at a temperature as high as 150°C. In addition, the use of strong ion pairing reagents, such as HPF_6, can improve the separation efficiency. As shown in Figure 18, separation for three proteins was achieved within 20 s at 150°C using a flow rate of $5\,mL\,min^{-1}$. Furthermore, the stationary phase was stable even after 50 h of exposure to 0.1% TFA at 120°C. However, at conventional linear velocities the instability of proteins at high temperature becomes a problem, which establishes an upper temperature limit. The use of high flow rates and a narrow bore column (e.g., 2.1 mm i.d.) could significantly alleviate this problem by reducing the residence time of proteins in the hot column.

Most recently, Stoll and Carr[117] described fast comprehensive two-dimensional LC separation of tryptic peptides using high temperature LC. The conventional 2D-LC is time consuming, sometimes taking days per analysis. They used high temperature and fast gradient, along with the instrument modifications to reduce the analysis time and increase the peak capacity. Their system was able to generate a high peak capacity characteristic (~1350) of comprehensive 2D-LC in a relatively shorter analysis time (20 min). The great increase in the speed of reversed-phase gradient elution through the use of high temperature LC and instrument modifications led to a significant improvement in the performance of 2D-LC.

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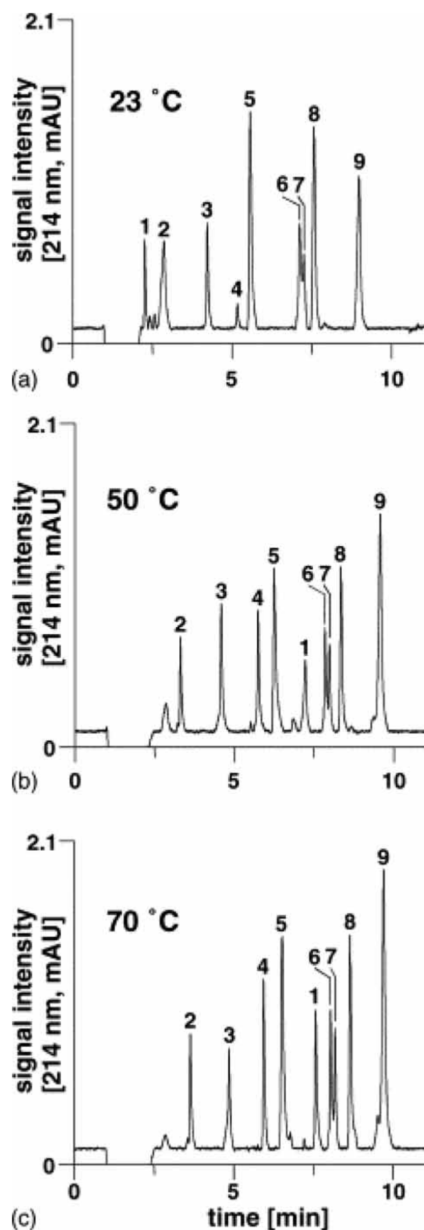

Figure 17. Separation of nine proteins using a monolithic capillary column at different temperatures. Column: PS–DVB monolith, 60 × 0.20 mm i.d.; mobile phase: (A) 0.05% trifluoroacetic acid in water, (B) 0.05% trifluoroacetic acid, 80% acetonitrile in water; linear gradient: 20–70% B in 10 min; flow rate: 1.7–2.3 μL/min; temperature: (a) 23°C, (b) 50°C, (c) 70°C; detection: UV, 214 nm; sample: mixture of nine proteins, 10–60 fmol of each protein. (Reprinted with permission from Ref. 77 Copyright © 2004 Elsevier B.V.).

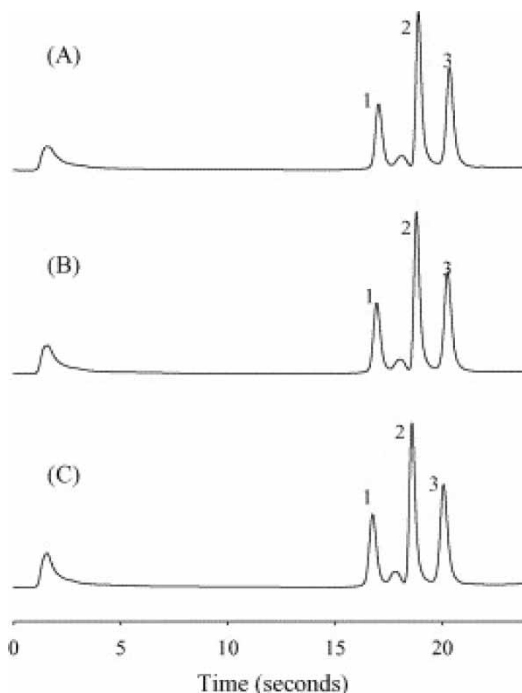

Figure 18. Chromatograms of a protein mixture separated on the HC-C_8 phase at 120°C. The column was kept at 120°C throughout the experiment (about 50 h). (A) Injection was made right after the column was equilibrated at 120°C; (B) after 24 h; (C) after 50 h. Conditions: 120°C, 5 mL/min, 1 μL, 220 nm. Solvent (A) 0.1% TFA in water; solvent (B) 0.067% TFA in ACN. Linear gradient from 10 to 90% B in 0.3 min (dwell time is 0.2 min). Solutes: (1) ribonuclease A; (2) insulin; (3) α-chymotrypsin. (Reprinted with permission from Ref. 116 Copyright © 2005 Elsevier B.V.).

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Clark[118] compared temperature programming and eluent gradient in fast LC in terms of analyte retention and eluent flow profile. It was believed that temperature programming could mimic solvent gradients, since temperature can change the polarity and viscosity of the mobile phase and interactions with the stationary phase. Thompson and Carr[119] optimized fast separation by adjusting eluent composition and column temperature simultaneously. Sub minute separations were expected to be readily achieved when a hot column was used at the maximum system backpressure. The best way to facilitate such an optimization, assuming constant selectivity, is to use a very retentive column so that one can work at both high temperature and high volume fraction of organic modifier to achieve the lowest possible eluent viscosity. The authors also determined the effect that key extra column contributions have on column selection and system design.

Marin et al.[88] compared six commercially available analytical columns (4.1 or 4.6 mm i.d.) in high temperature LC under temperature programming conditions to evaluate their stability and performance at extreme temperatures. They found that the Selerity Blaze C_8, Hamilton PRP-1, and Thermo Hypersil-Keystone HyperCarb columns could be used at the maximum temperatures between 100 and 200°C with temperature programming, without evidence of column degradation. Acidic and basic pH conditions combined with high column temperatures did not appear to cause any collapse of the stationary phase. Calculation of peak capacities indicated that comparable peak quality was attained when a temperature program and an isocratic mobile phase were used in place of a solvent gradient to perform a separation. Although zirconia based stationary phases are routinely used isothermally at temperatures up to 200°C, they are not good candidates for temperature programmed high temperature LC due to a significant rise in the baseline during temperature programming.

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CONCLUSIONS

The development of new instrumentation and novel column technology has allowed significant improvement in separation speed and efficiency of modern HPLC in the last decade. The commercialization of these technologies, which include Acquity UPLC, monolithic columns, thermally stable stationary phases, and high temperature LC systems, has accelerated advances in fast LC. These innovative technologies have made it possible to achieve 5–10 fold faster separations compared to conventional LC systems, while maintaining or providing even higher resolution. Future directions will likely include the development and refinement of these technologies. A greater variety of columns packed with sub 2 μm particles, such as chiral columns, are highly desirable. In addition, monolithic columns with various bonding chemistries and dimensions are also needed for routine industrial laboratories. Commercial UPLC systems with higher pressures (e.g. >15,000 psi) and with ternary or tertiary pumps will open up more areas of application for high speed separations. Finally, integrated high temperature LC systems will provide greater feasibility for high speed analyses.

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