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Capillary Array High-Performance Liquid Chromatography of Nucleic Acids and Proteins

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An array of monolithic poly(styrene/divinylbenzene) capillaries with individual column thermostats was constructed to demonstrate its utility for the separation of nucleic acids, proteins, and tryptic digests in combination with UV absorbance detection. Because of polymerization-related variation in surface area of monolithic columns, the concentration of acetonitrile required for elution of DNA fragments in denaturing HPLC may vary sufficiently to affect the degree of denaturation. Modulation of column temperature offers a convenient way to harmonize elution profiles among columns. Individual regulation of column temperature also provides the means to determine rapidly in a single parallel run the optimum temperature for resolution of biomolecules. Given the high reproducibility of separations among columns and the ease with which poly(styrene/divinylbenzene)-based stationary phases can be modified to accommodate different modes of chromatography, such arrays will find broad applicability in proteogenomics.

Traditionally, high-performance liquid chromatography has been carried out on single-column instruments. Advances, particularly, in the synthesis of combinatorial libraries, have created a tremendous demand for increased throughput for the purpose of purity control that cannot any longer be met by mere increases in mobile phase velocity in combination with either nonporous[1,2] or perfusive[3] separation media. The first four-channel liquid chromatography array of packed-bed columns with an internal diameter of 2.1 mm, which was interfaced with a time-of-flight mass spectrometer fitted with a four-way multiplexed electrospray interface, was introduced in 1999 for quality control of the automated synthesis of pharmaceuticals.[4] The same instrumental setup was subsequently applied to the direct determination of a novel isoquinoline drug in plasma employing both narrow-bore (1-mm i.d.) and capillary (180-μm i.d.) large-particle-size columns.[5] An array of four monolithic octadecylated silica columns with 4.6-mm i.d. was applied to the high-speed gradient parallel liquid chromatography/tandem mass spectrometry of oxazepam in plasma with a turnaround time of 2 min, which allowed the analysis of 1152 samples/day.[6] An eight-channel parallel liquid chromatography system with on-line UV absorbance detection and mass spectrometry was applied to the fast characterization of intact proteins with the eight 2.1-mm-i.d. reversed-phase packed-bed columns being used primarily for sample desalting rather than for refined separation.[7] On the preparative end, an eight-channel device with on-line UV absorbance detection has been described for high-throughput fractionation of natural product extracts.[8]

The aforementioned studies clearly established the feasibility of bundling narrow-bore and capillary packed-bed as well as 4.6-mm-i.d. continuous-bed HPLC columns into arrays of 4−8 separation channels with solvent delivery accomplished by a single pump. However, the use of these arrays was limited for the most part to the mere desalting of samples prior to mass spectrometry. In contrast, arrays of monolithic poly(styrene/divinylbenzene) capillary columns with 0.2-mm i.d. have allowed separations of nucleic acids with a degree of refinement matching that of capillary array electrophoresis.[9,10] The performance of these monoliths had exceeded that of capillary columns packed with micropellicular, octadecylated poly(styrene/divinylbenzene) particles by as much as 40%.[11] Arrays of 4 and 16 columns, respectively, have been used in conjunction with laser-induced fluorescence detection to screen for mutations by denaturing HPLC[9] and to resolve the allelic products of single-nucleotide extension reactions.[10] The use of laser-induced fluorescence detection carried the additional advantage that multiple polymerase chain reaction and single-

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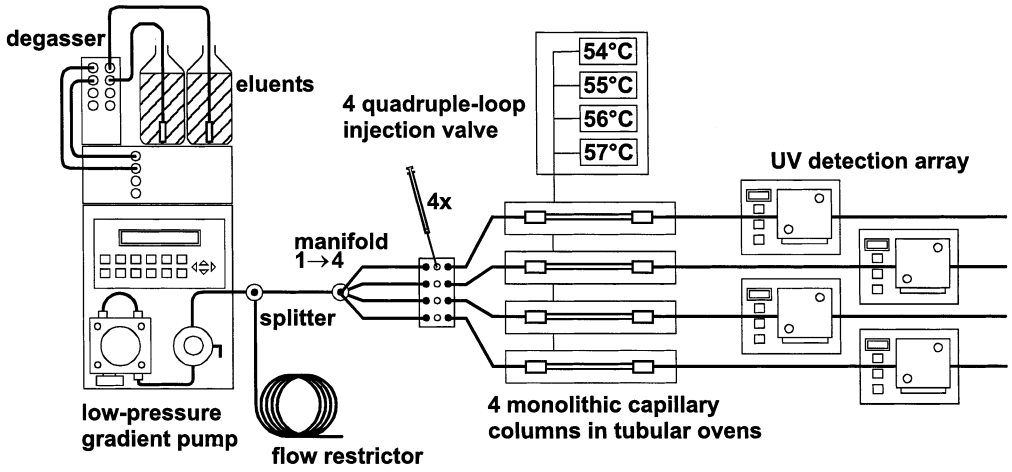

Figure 1. Scheme of the instrument setup used for the parallel analysis of unlabeled nucleic acids, proteins, and peptides.

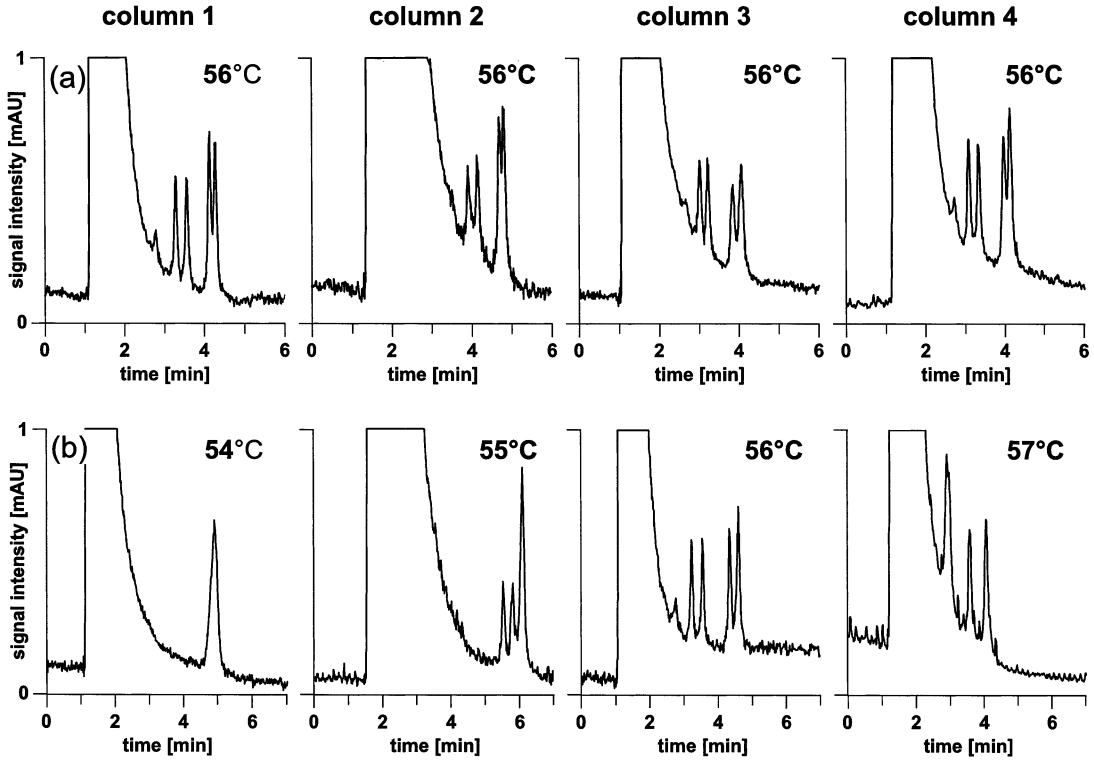

Figure 2. Detection of a single base-pair mismatch in a 209-bp DNA fragment by parallel partially denaturing HPLC. (a) The upper row of chromatograms shows the similarity of elution profiles following modulation of individual column temperatures to compensate for the different acetonitrile concentrations required for elution due to polymerization-related differences in surface area among the four columns. (b) The lower row shows the changes that occur in elution profiles of hetero- and homoduplexes as a function of column temperature. Columns: 4× monolithic poly(styrene/divinylbenzene), 60 × 0.2 mm i.d. Mobile phase: 100 mM triethylammonium acetate, 0.1 mM Na_4EDTA, pH 7.0. Linear gradient: (a) 15.5−20.5% acetonitrile in 10 min, (b) 14.3−19.3% acetonitrile in 10 min. Secondary flow rate: 2−3 μL/min. Effective column temperature: (a) 56 °C on all four columns; (b) 54, 55, 56, and 57 °C. Detection: UV, 254 nm. Injection volume: 1 μL each. Sample: 209 bp amplicon from Y chromosome, heterozygous (168 A > G).

nucleotide extension reactions labeled with different fluorescent dyes commonly used in DNA sequencing could be pooled and, hence, allowed a further increase in throughput. Given the multitude of detection methods and the wide selection of stationary phases available for the separation of both low- and high-molecular-weight compounds whose chemical properties range from nonpolar to ionic, HPLC appears to be positioned well to eclipse capillary electrophoresis in the post-genome era. One such example is its increasing use as a multidimensional separation platform for proteins and peptides prior to mass spectrometry.[12]

The present study expands the utility of monolithic poly(styrene/divinylbenzene) capillary column arrays to the separation of not only unlabeled nucleic acids but also proteins and tryptic digests by replacing laser-induced fluorescence with UV absorbance detection. In addition, it is demonstrated that variation of individual column temperature in conjunction with an array

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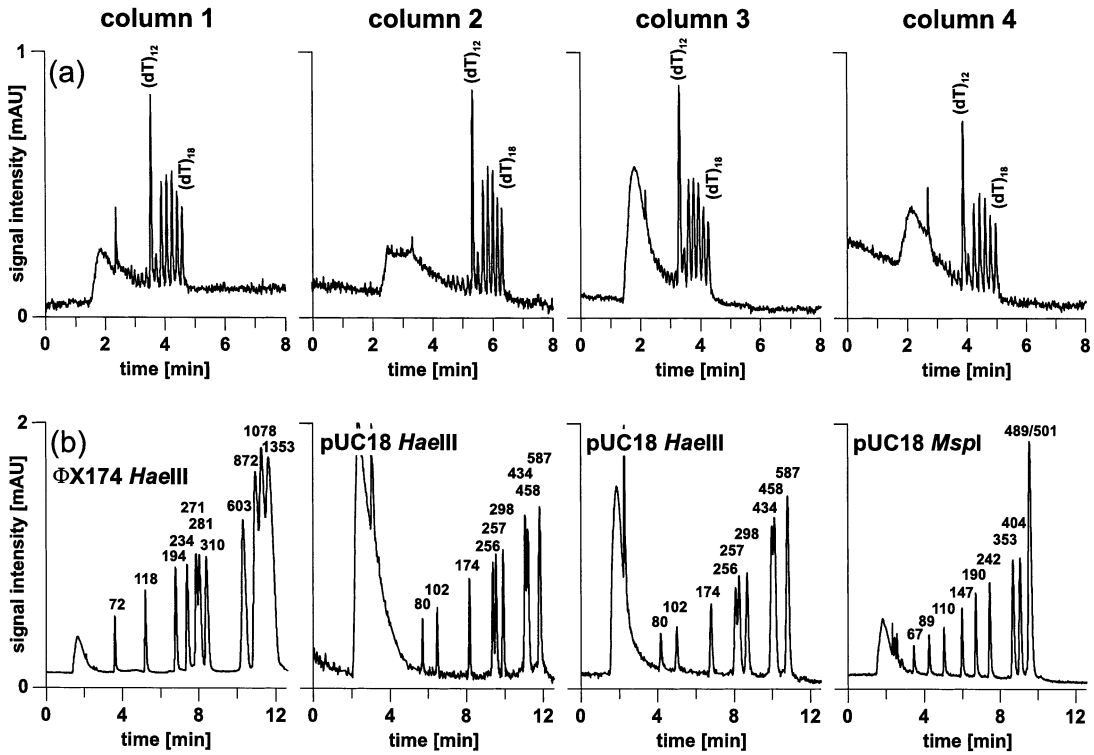

Figure 3. Parallel separation of (a) single-stranded oligonucleotides and (b) double-stranded DNA restriction digests. Columns: 4× monolithic poly(styrene/divinylbenzene), 60 × 0.2 mm i.d. Mobile phase: 100 mM triethylammonium acetate, 0.1 mM Na_4EDTA, pH 7.0. Linear gradient: (a) 10.0−17.5% acetonitrile in 10 min, (b) 11.3−14.5% acetonitrile in 3 min, 14.5−18.8% acetonitrile in 7 min. Secondary flow rate: 2−2.5 μL/min. Column temperature: (a) 55 °C, (b) 40 °C. Detection: UV, 254 nm. Sample: (a) mixture of oligodeoxythymidilic acids $(dT)_{12-18}$, 50 fmol each; (b) column 1, ΦX174 *Hae*III, 35 fmol of each fragment; column 2, 3, pUC18 *Hae*III, 50 fmol of each fragment; column 4, pUC18 *Msp*I, 15 fmol of each fragment.

constitutes a practical means of rapidly determining the optimum column temperature for the separation of biopolymers and other compounds whose resolution is affected by column temperature.[13]

EXPERIMENTAL SECTION

Instrumentation. The chromatograph consisted of a degasser (DG-1210, Uniflows Co., Tokyo, Japan), a solvent organizer (WellChrom K-1500, Knauer Co., Berlin, Germany), a pump (WellChrom K-1001), four K-200 UV absorbance detectors, a universal chromatography interface (UCI-100, Dionex, Sunnyvale, CA), and a PC-based data system (Chromeleon, version 6.40, Dionex). A T-piece was used to split the primary flow of 140 μL/min (back-pressure, 140 bar) to control the secondary low-flow stream to the columns. The individual column heaters and the 16-channel heater control system were purchased from Xeragen, Inc. (San Luis Obispo, CA). A custom-made electrically actuated injector with four internal 1-μL sample loops was obtained from Valco Instruments Co. (Houston, TX). The injection valve was connected upstream to a stainless 1/16-in. manifold (model no. Z4M1, Valco) with one inlet and four outlets. The synthesis of the monolithic poly(styrene/divinylbenzene) columns has been described elsewhere.[11]

Samples. Dephosphorylated oligodeoxythymidylic acids, DNA restriction digests, and protein standards were obtained from Sigma (St. Louis, CA), and the tryptic digests were purchased from Michrom BioResources, Inc. (Auburn, CA). The 209-bp amplicon containing either an A or a G at nucleotide position 168 was generated as described previously.[9]

RESULTS AND DISCUSSION

Figure 1 shows a scheme of the instrument configuration used. It is essentially identical to the array applied to the detection of DNA sequence variation by means of denaturing HPLC,[9] except that the laser-induced fluorescence scanner was replaced with four UV absorbance detectors.

Previously, it had proven necessary to vary column temperature of the individual separation channels in the array by as much as 2 °C to obtain highly similar elution profiles in partially denaturing HPLC.[9] The necessity to modulate column temperature was attributed to differences in the degree of polymerization. As a consequence, differences in pore size and surface area between columns necessitated different concentrations of acetonitrile to elute DNA fragments. Particularly under partially denaturing conditions, slight changes in the concentration of acetonitrile, which facilitates the denaturation of DNA, will lead to significantly different elution profiles. In the most extreme case, a mutation may be missed, because the combined effect of column temperature and acetonitrile concentration in the mobile phase at the time of elution will cause either insufficient or complete denaturation of the DNA. In such cases, modulation of individual column temperature constitutes an effective way to compensate for differences in retention behavior.

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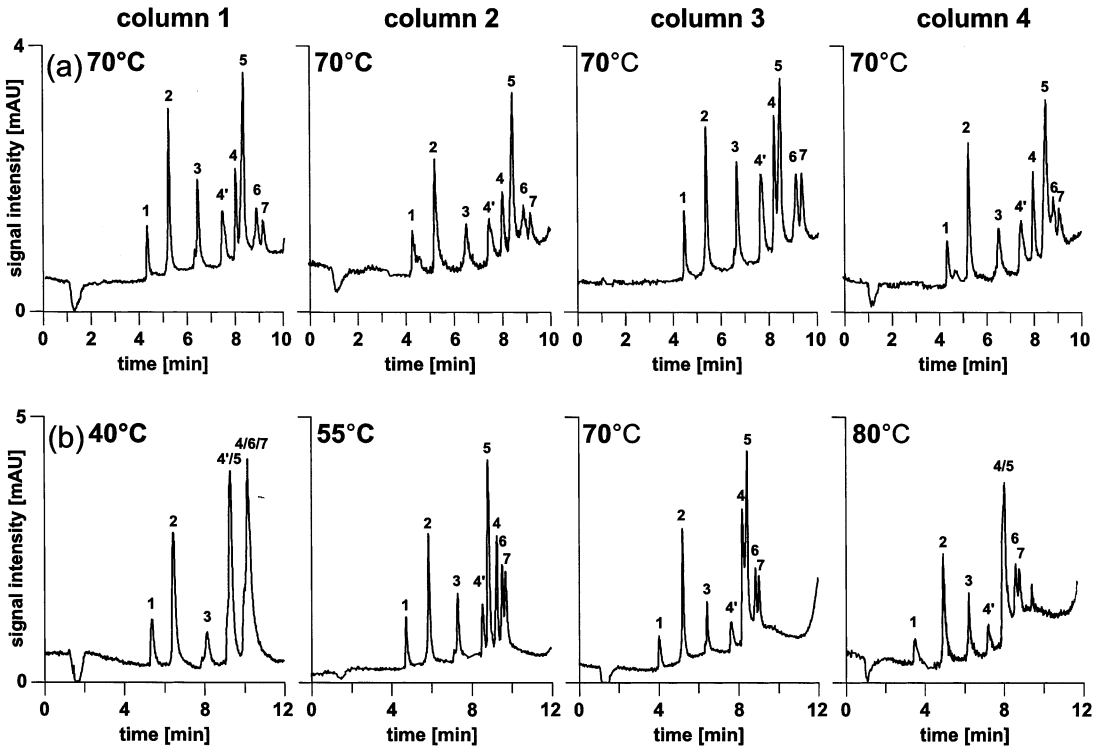

Figure 4. Parallel separation of a protein mixture by reversed-phase HPLC: (a) comparability of chromatographic elution profiles obtained on the four columns of the array and (b) fast method optimization by simultaneous acquisition of chromatograms at four different temperatures. Columns: 4× monolithic poly(styrene/divinylbenzene), 60 × 0.2 mm i.d. Mobile phase: 0.5% acqueous formic acid. Linear gradient: 5.9−44.9% acetonitrile in 10 min. Secondary flow rate: 2−3.5 μL/min. Column temperature: (a) 70, (b) 40, 55, 70, and 80 °C. Detection: UV, 254 nm. Sample: mixture of 7 proteins. Peak identification: 1, ribonuclease A; 2, cytochrome *c*; 3, lysozyme; 4, myoglobin; 5, α-lactalbumin; 6, β-lactoglobulin B; 7, β-lactoglobulin A; 25−50 ng each (1.5−3.5 pmol).

In the present study, individual column temperature had to be varied only by as much as 0.5 °C to obtain high similarity of elution profiles in partially denaturing HPLC (Figure 2a). This was attributed to an improved reproducibility of the in situ synthesis of monolithic poly(styrene/divinylbenzene) capillaries because of increased experience with the procedure, the high-precision preparation of polymerization mixtures using an analytical balance, and improved temperature control during polymerization. Further, care was taken to bundle only capillary columns with similar back-pressure values. Column temperatures were calibrated with the use of a mutation standard that yields characteristic elution profiles at specific temperatures.[14] Following successful calibration, readings of the individual thermocouples are offset to the same value. Subsequent changes in column temperature will then have identical effects on peak patterns of all columns.

As described previously,[9] the parallel analysis of one and the same DNA fragment at different column temperatures optimizes the ability of DHPLC to detect mutations (Figure 2b). The mutation detection sensitivity of DHPLC has been shown to approach 100% provided that DNA fragments containing multiple melting domains differing by more than 5 °C are analyzed at multiple column temperatures.[15] Most mutations can be detected over a temperature window spanning at least 4 °C.[16] A few cases, however, have been reported in which the temperature range was as narrow as 2 °C.[17] Furthermore, the use of UV absorbance rather than laser-induced fluorescence detection eliminates the need to label DNA fragments prior to DHPLC analysis. This constitutes a cost advantage, particularly when a fragment of interest is analyzed only a few times, as is the case in fine structure mapping of mutants, which has become one of the most powerful applications of DHPLC.[18−20]

The excellent reproducibility of elution profiles among columns is demonstrated in Figure 3. The upper row of chromatograms (Figure 3a) shows the parallel analysis of a mixture of oligodeoxythymidylic acids 12−18 nucleotides in length. The lower row of chromatograms (Figure 3b) depicts the simultaneous separation of three different DNA restriction digests. The *Hae*III digest of the *pUC* vector was injected twice onto different columns run in parallel to provide further evidence for the excellent column-to-column reproducibility of separation performance. Differences in retention time of nucleic acids of identical size that can be observed between columns are primarily due to differences in

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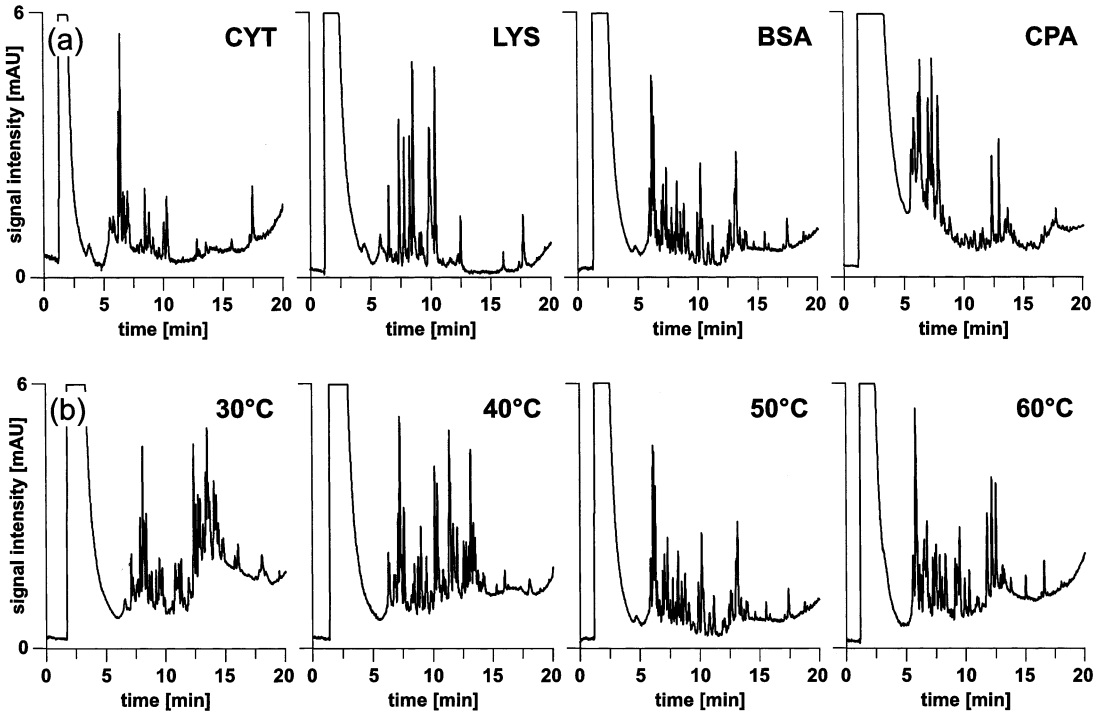

Figure 5. Parallel separation of peptides from tryptic digests by reversed-phase HPLC: (a) simultaneous analysis of four different tryptic digests and (b) fast method optimization by simultaneous acquisition of chromatograms at four different temperatures. Columns: 4× monolithic poly(styrene/divinylbenzene), 60 × 0.2 mm i.d. Mobile phase: 0.2% acqueous trifluoroacetic acid, 2.0% acetonitrile for 3 min. Linear gradient: 2.0−33.2% acetonitrile in 12 min, 33.2−64.4% acetonitrile in 5 min. Secondary flow rate: 2.0−3.0 μL/min. Column temperature: (a) 50 °C, (b) 30, 40, 50, and 60 °C, respectively. Detection: UV, 215 nm. Sample: (a) tryptic digest of cytochrome *c*, lysozyme, bovine serum albumin, and carboxypeptidase A, respectively, 2 pmol each; (b) tryptic digest of bovine serum albumin, 2 pmol.

back pressure. Such differences can be minimized by integrating only columns that generate very similar back pressures into the array. If accurate measurement of the size of unknown fragments is desired, controls of known sizes have to be spiked into the sample as described previously.[21] The array can be also used for preparative isolation of DNA fragments, yielding sufficient material for direct cloning or sequencing.[22] This may constitute an attractive alternative to capillary electrophoresis-based fraction collection, particularly because there is no need to take precautions to avoid interruption of the electric current.[23]

The use of UV absorbance instead of laser-induced fluorescence detection enables the ready application of the array to the separation of biomolecules other than nucleic acids. The utility of poly(styrene/divinylbenzene) monoliths for high-resolution separations of proteins and peptides was demonstrated recently in conjunction with electrospray ionization mass spectrometry.[24] Figure 4 shows the separation of ribonuclease A, cytochrome *c*, lysozyme, myoglobin, α-lactalbumin, β-lactoglobulin B, and β-lactoglobulin A. At a column temperature of 70 °C, all seven proteins were resolved with similar efficiency on the four columns of the array, including a contaminant in the myoglobin sample that may represent apomyoglobin. Although protein separations do not require the same sophistication of temperature control, column temperature does affect selectivity, as shown in the lower panel of the chromatograms in Figure 4 that were obtained in parallel. This demonstrates the utility of the array in combination with individual column thermostats for the purpose of rapidly optimizing the separation of a complex sample of biomolecules. This is even more apparent in Figure 5, which shows the separation of tryptic digests of cytochrome *c*, lysozyme, bovine serum albumin, and carboxypeptidase A, respectively. Using a uniform gradient and column temperature of 50 °C, a total of 29, 25, 46, and 62 peptide fragments could be resolved (Figure 5a). To investigate the impact of column temperature on the separation of peptides, aliquots of the tryptic digest of bovine serum albumin were injected onto the same column at increasing temperatures to exclude slight differences in separation efficiency among columns as a confounding factor. At temperatures of 30, 40, and 50 °C, the number of peptides resolved varied only slightly with the number of peaks, ranging from 43 to 46. However, distinct changes in elution profiles could be noticed. At a temperature of 60 °C, the number of peaks dropped to 39, indicating a loss of selectivity.

In the present study, we used inexpensive single-wavelength UV detectors. However, it is conceivable, provided that the peak concentrations of the analytes are sufficiently high, to accomplish monitoring by means of a photodiode array UV absorption detector that was originally developed for parallel detection capillary array electrophoresis.[25]

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CONCLUSIONS

Individual column temperature control constitutes an effective mean to compensate for differences in retention of DNA homo- and heteroduplexes in denaturing HPLC due to polymerization-related variations in the surface area of the individual columns in an array. It may also be used for rapidly determining the optimum temperature for the separation of peptides, proteins, and other biomolecules that are amenable to chromatographic separation. The number of columns per array can be easily increased to 48 using a single analytical HPLC pump. This will require improvements in commercially available autosamplers that currently allow only parallel handling and injection of samples onto arrays made of eight columns.

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