

Liquid Chromatographic Separation Using a 2 μm i.d. Open Tubular Column at Elevated Temperature

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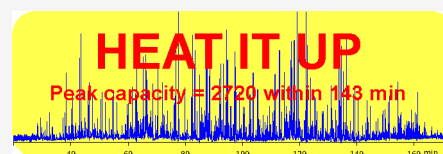


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Supporting Information

ABSTRACT: We have experimentally demonstrated the extraordinarily high resolving power of liquid chromatography (LC) using a narrow open tubular (OT) column. In this work, we show that we can further increase its efficiency, peak capacity, and separation speed by elevating the operation (or column) temperature; all of these three numbers can be improved without mutual compromises. We use a mixture of five amino acids as a sample and show that we can increase the efficiency by 34%–260% and the separation speeds by 7%–10% by raising the operation temperature from 30 to 70 °C. When we use a 2 μm i.d. \times 80 cm in length OT column coated with OTMS at a temperature of 70 °C, we can frequently obtain peak capacities of 700–800 within 20–30 min for separating cytochrome C digests. By increasing the column length to 160 cm, we can obtain a peak capacity of 2720 within 143 min for separating a complex peptide sample. This peak capacity is the highest peak capacity to date for one-dimensional LC separations. Importantly, heating the column is easy to implement and does not cost much, and many commercial LC systems already have compartments to control column temperatures. Running LC using a narrow OT column at an elevated temperature should broaden the applications of OT-LC in chemical and biochemical analyses.



INTRODUCTION

Ultrahigh efficiency,¹ ultrahigh peak capacity,² and ultrafast liquid chromatographic separations³ have been obtained using open tubular (OT) columns having an inner diameter (i.d.) of 2 μm . OT columns were initially employed in gas chromatography (GC) by Golay⁴ in 1957, and these columns rapidly replaced packed columns in GC because of their improved performances. OT columns have also been predicted to generate maximized efficiencies in liquid chromatography (LC) according to chromatography theoretical studies^{5–8} with a condition that very narrow (e.g., 1–2 μm i.d.) columns must be used.⁸ We have recently experimentally validated these predictions;⁹ efficiencies of multimillion plates per meter¹ and one-dimensional peak capacities of more than 2000 within less than 3 h¹⁰ have been frequently achieved.

It has long been recognized that elevated operation or column temperatures can increase efficiencies and enhance separation speed.^{11,12} At an elevated temperature, the mass transfer rates within both mobile and stationary phases increase, and the solute-stationary phase interaction kinetics accelerates. This often leads to reduced peak tailing and therefore improved efficiencies. Elevating the operation temperature can also decrease the mobile phase viscosity, resulting in an increased flow rate and consequently an enhanced separation speed. In this technical note, we present the ultrahigh performances of running LC using 2 μm i.d. OT columns at elevated temperatures. The numbers of theoretical plates are increased by 34%–260%, while the separation speeds increased by 7%–10% for five amino acids as the temperature is increased from 30 to 70 °C. Efficiencies of over 10 million theoretical plates are frequently obtained. We also

show that we can routinely obtain one-dimensional LC peak capacities of 700–800 within 20–30 min for cytochrome C digests. The peak capacities can be improved conveniently by increasing the column length and gradient time. In this work, we exhibit a new one-dimensional LC peak capacity record of 2720 within 143 min using an OT column having an i.d. of 2 μm and a length of 160 cm (155 cm effective) and an operation temperature of 70 °C. Because a laser-induced fluorescence (LIF) detector was used for this work, a drawback of operating at elevated temperatures was the reduced fluorescence intensity presumably due to fluorescent analyte photobleaching and thermal decompositions, especially after 70 °C.

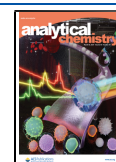
EXPERIMENTAL SECTION

Materials and Reagents. Fluorescein, amino acids, cytochrome C, myoglobin, sodium hydroxide, ammonia bicarbonate, acetonitrile, toluene, and octadecyltrimethoxysilane (OTMS) were purchased from Sigma-Aldrich (St. Louis, MO). An ATTO-TAG FQ amine-derivatization kit was purchased from Thermo-Fisher Scientific (Waltham, MA). Trypsin was purchased from Promega (Madison, WI). Pepsin was purchased from MP Biomedicals (Santa Ana, CA). All

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solutions were prepared using ultrapure water (nanopure ultrapure water system, Barnstead, Dubuque, IA) and filtered through a 0.22 μm filter (VWR, Sugar Land, TX), degassed before use. Fused-silica capillaries were purchased from Polymicro Technologies, a subsidiary of Molex (Phoenix, AZ). A Peltier thermoelectric plate with a temperature controller was purchased from DigiKey (Thief River Falls, MN). Thermal conductive silicone grease was ordered from Insigina. (Minneapolis, MN).

Preparation of OT Columns. The coating procedure has been described in detail previously.¹⁰ Briefly, a 2 μm i.d. capillary with a certain length (80, 140, or 160 cm) was cut, and about 1 cm of polyimide coating at one end was removed. A 25 G X 7/8 in. hypodermic needle was used as a guide to facilitate the insertion of this capillary through a septum into a pressure chamber holding a vial containing 50 μL of 1 M NaOH solution. The other end (with polyimide coating) of the capillary was inserted into a vial containing DDI water. Nitrogen at a pressure of 1000 psi was delivered to the pressure chamber to pressurize the NaOH solution through the capillary at 100 $^{\circ}\text{C}$ for 2 h. The NaOH solution in the pressure chamber was then replaced with DDI water to rinse the capillary for 1 h under the same pressure. After the capillary was moved out of the oven, it was rinsed with acetonitrile for about 30 min at ambient temperature and dried with nitrogen overnight. A coating reagent consisting of 70 μL of OTMS and 30 μL of toluene was prepared in a dry glovebox and placed inside the pressure chamber. The polyimide-removed end of the capillary was dipped into the reagent. The opposite end of the capillary was merged into a vial containing toluene. Pressurized nitrogen was delivered to the pressure chamber, and the coating reagent was flushed through the capillary to derivatize the capillary wall. After this reaction proceeded for 18 h, the coating reagent was then replaced with toluene to rinse the capillary for 1 h. The column was ready for use after it was dried with nitrogen.

Preparation of Fluorescently Labeled Amino Acids. The amino acids were fluorescently labeled with the ATTO-TAG FQ amine-derivatization kit according to the instructions from the company. Briefly, a 10 mM ATTO-TAG FQ stock solution was prepared by dissolving 5.0 mg of ATTO-TAG FQ in 2.0 mL of methanol. A 10 mM KCN solution was prepared by diluting a 0.2 M KCN stock solution with a 10 mM borax solution (pH 9.2). Amino acid stock solutions (each with a concentration of 1 mM) were prepared by dissolving individual amino acid in DDI water and filtered with a 0.22 μm filter. A volume of 1.0 μL of the amino acid stock solution was mixed with 10 μL of the 10 mM KCN working solution and 5 μL of the 10 mM FQ solution in a 0.25 mL vial. This mixture was maintained at room temperature for 1 h in the dark before it was ready for the test.

Preparation of Fluorescently Labeled Peptides. *Escherichia coli* (*E. coli*) cells and MCF-7 cells were cultured as sources of peptides. To prepare tryptic digests, 1 mL of the cell lysate solution (estimated to contain ~ 10 mg total protein per mL) was mixed with 5 μL of 1 M NaAc/HAc buffer (pH 4) and 1 μL of pepsin (1 $\mu\text{g}/\text{mL}$) and incubated at 37 $^{\circ}\text{C}$ for 1 h. The above solution (100 μL) was diluted with 900 μL of 25 mM NH_4HCO_3 and mixed with 1 μL of 1 M DTT at room temperature for at least 1 h. Then, 10 μL of 0.2 mg/mL trypsin solution was added into the above mixture, and the mixture was incubated at 37 $^{\circ}\text{C}$ for 24 h. The digests were stored at -80 $^{\circ}\text{C}$ after being freeze dried.

The cytochrome C or myoglobin solution (1 mg/mL) was prepared by dissolving 1 mg of protein with 1 mL of 10 mM NH_4HCO_3 . Then, 100 μL of filtered solution was mixed with 1 μL of 1 M DTT. After a 1 h incubation at 37 $^{\circ}\text{C}$, 10 μL of 0.2 mg/mL trypsin solution was added. After incubation at 37 $^{\circ}\text{C}$ for 24 h, the mixture is ready for storage (-80 $^{\circ}\text{C}$) or fluorescent labeling.

To label the digested peptides, 10 μL of the peptide solution was mixed with 10 μL of the 10 mM KCN working solution and 10 μL of the 10 mM ATTO-TAG FQ solution. After a 1 h reaction in the dark at room temperature, the peptides were ready for dilution (with 10 mM NH_4HCO_3) and separation.

Apparatus. Figure 1 describes the experimental setup used in this work. An Agilent 1200 quaternary pump (Agilent, Santa

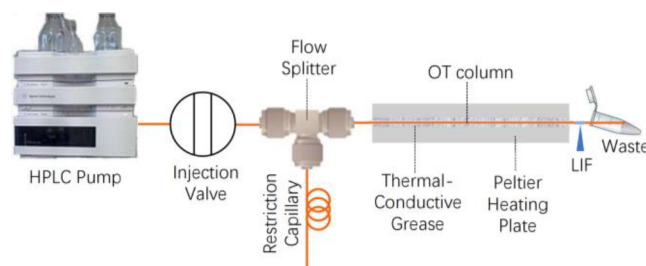


Figure 1. Schematic diagram of the experimental setup. See the Apparatus section for details.

Clara, CA) was utilized as the eluent delivery system. A six-port valve (Valco Instrument, Houston, TX) was connected to the pump and a flow splitter via 150 μm i.d. and 10 cm long capillaries. A 20 μm i.d. capillary with a preset length was utilized as the restriction capillary. Inside the flow splitter, the head of the OT column was inserted ~ 1 mm deep into the connecting capillary after the polyimide coating on the column head was removed. A detection window was formed at ~ 5 cm from the exit end of the column by removing ~ 3 mm of the polyimide coating. The detection end of the column (~ 10 cm) was affixed to a capillary holder on an x-y-z translation stage for the detection window alignment,¹³ and a confocal LIF detector was employed to monitor the resolved analytes. The majority of the OT column (e.g., 60 cm out of 80 or 140 cm out of 160 cm) was placed on the top surface of a Peltier heating plate, and a thermal conductive grease was applied. Another copper plate was used to cover the greased column gently. Once the temperature controller indicated a stabilized temperature (usually within ± 0.05 $^{\circ}\text{C}$), the separation could be started.

RESULTS AND DISCUSSION

Effect of Temperature on Efficiency and Elution Velocity. Figure 2A presents five chromatograms for separating a mixture of five amino acids at different temperatures. As can be seen, the retention times decreased significantly as the temperature increased from 30 to 70 $^{\circ}\text{C}$. The plot in Figure S1 exhibits the effect of temperature on full widths at half-maximum (fwhm); the widths were narrowed from 21% (for glycine) to 51% (for phenylalanine). The plot in Figure S2 displays the effect of temperature on separation speed; the elution velocities were increased from 7% (for leucine) to 10% (for histidine). Figure 2B presents the effect of temperature on efficiencies; the numbers of theoretical plates increased from 34% (for glycine) to 260% (for phenylalanine).

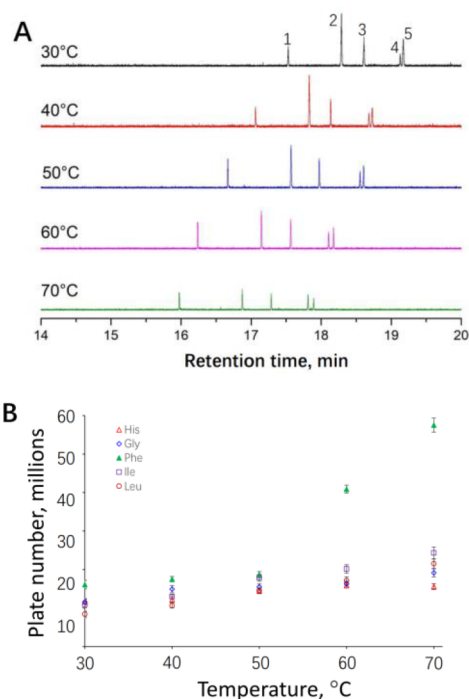


Figure 2. Effect of temperature on efficiency and elution velocity. The column was a 2 μm i.d. \times 140 cm in length (135 cm effective) capillary coated with OTMS. The majority (120 cm out of the 140 cm) of the column was sandwiched between an electric Peltier thermal plate and a copper cover plate, and silicone thermal conductive paste was placed between the plates to ensure adequate thermal conduction. (A) Chromatograms for amino acid separation at different operation temperatures. The mobile phase was 20% (v/v) acetonitrile in 10 mM NH_4HCO_3 . Sample contained histidine (1), glycine (2), phenylalanine (3), isoleucine (4), and leucine (5), each at 0.1 μM . The restriction capillary had an i.d. of 20 μm and a length of 20 cm. The sample volume injected into the OT column was estimated to be \sim 100 pL. The pump flow rate was set as 0.2 mL/min, and the pressure meter indicated a pressure of 1200 psi. (B) Effect of temperature on efficiency.

The highest plate number obtained was 58 million plates per meter, while more than 10 million plates per meter were regularly obtained. Although isocratic elution was used for these tests, a focusing effect, induced from the mismatch between the sample matrix and the eluent,¹⁴ was involved and could have boosted the efficiency numbers. One should be reminded that running an isocratic separation under this mismatched condition is common, although the focusing effect may falsely inflate the plate numbers. Nevertheless, an elevated operation temperature is beneficial in improving the resolving power and increasing the separation speed.

Effect of Temperature on Performance for Separating Complex Sample. Figure 3 presents the effect of temperature on peak capacity, separation time, and fluorescence intensity for separating a complex peptide sample. The sample was a mixture of peptides from pepsin and trypsin-digested *E. coli* cells (40%), pepsin and trypsin-digested MCF-7 (40%), trypsin-digested cytochrome C (5%), and trypsin-digested myoglobin (5%). The sample was estimated to contain \sim 0.03 mg proteins/mL with a bicinchoninic acid method. The blue line in Figure 3A presents the effect of temperature on peak capacity, and the red line shows the effect on separation time. Peak capacities increased from 1500 within 158 min to 2720 within 143 min, while separation time was

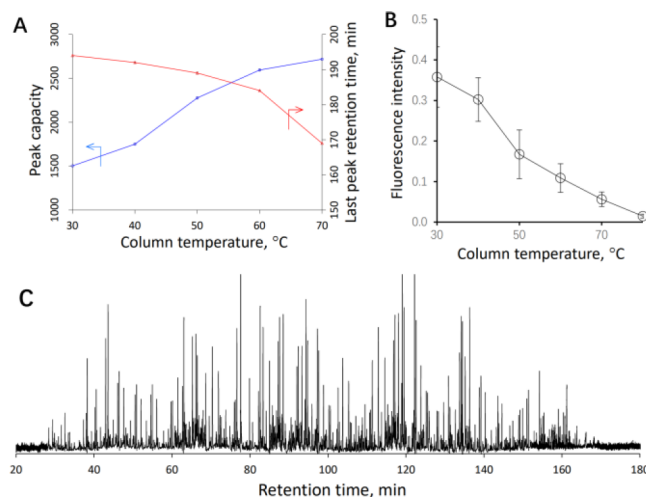


Figure 3. Effect of temperature on peak capacity, separation time, and fluorescence intensity. The column was a 2 μm i.d. \times 160 cm in length (155 cm effective) capillary coated with OTMS. The majority (140 cm out of the 160 cm) of the column was sandwiched between an electric Peltier thermal plate and a copper cover plate. The restriction capillary had an i.d. of 20 μm and a length of 20 cm. The sample was a mixture of digests from *E. coli*, MCF-7, cytochrome C, and myoglobin, and its concentration was estimated to contain \sim 0.03 mg proteins/mL. The sample volume injected into the OT column was estimated to be \sim 100 pL. Mobile phase A was 10 mM NH_4HCO_3 in DDI water, and mobile phase B was 80% acetonitrile in 10 mM NH_4HCO_3 . The gradient was as set as mobile phase B increases from 10% to 80% within 180 min. The elution pressure was \sim 600 psi. (A) Effect of temperature on peak capacity and separation time. (B) Effect of temperature on fluorescence intensity. (C) Chromatogram obtained at 70 °C. The chromatogram had a peak capacity of 2720 within 143 min.

decreased from 194 to 169 min as the column temperature was raised from 30 to 70 °C. [Note: the column temperature and the operation temperature are interchangeably used in this work.] Again, we gained both the peak capacity and separation speed. However, one should be aware that an elevated temperature can accelerate analyte photobleaching and decomposition. As presented in Figure 3B, fluorescence intensity (the average signal of the 10 highest peaks) decreased quickly as the column temperature increased. The intensity dropped by more than a half as the temperature changed from 30 to 50 °C, and most of the peaks disappeared at 80 °C. Figure S3 presents the chromatograms obtained at 30, 40, 50, 60, 70, and 80 °C. The peak capacity for the chromatogram obtained at 80 °C was not calculated because too many peaks disappeared. Figure 3C exhibits a single-panel presentation, and Figure S4 presents a three-panel presentation of the chromatogram obtained at 70 °C. This chromatogram has a peak capacity of 2720 within 143 min. The method utilized to calculate the peak capacity was described previously.^{2,15}

Effect of Gradient Time on Performance. Gradient time is a key condition for a LC method. A steep profile can accelerate the separation but reduce the resolutions (too many peaks are compressed together within a short period of time), and often a compromise is made between speed and resolution. To make an appropriate compromise, one needs to have a sense of the trade between the loss and gain. Figure S5 presents chromatograms obtained under different gradient times. Figure 4A presents the effect of gradient time on peak capacity, and from these results, we see that the peak capacity

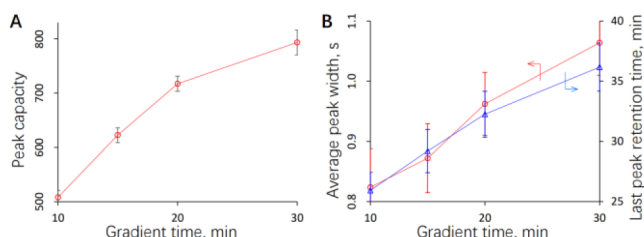


Figure 4. Effect of gradient time on peak capacity and resolution. The column was a 2 μm i.d. \times 80 cm in length (75 cm effective) capillary coated with OTMS. The majority (60 cm out of the 80 cm) of the column was heated at 70 $^{\circ}\text{C}$. The sample was a peptide sample from trypsin-digested cytochrome C (0.5 μM). The gradient was mobile phase B increasing from 10% to 80% within 10, 15, 20, or 30 min and remaining at 80% for 3 min. All other conditions are the same as in Figure 3. (A) Effect of gradient time on peak capacity. (B) Effect of gradient time on resolution.

increased from 510 within 12 min to 790 within 24 min as the gradient time increased from 10 to 30 min. Peak capacities of 700–800 were regularly obtained with gradient times between 20 and 30 min. The average peak widths in Figure 4B were calculated by averaging the fwhm of the highest 10 peaks, and the retention time of the last peak was considered the time required to complete the separation. On the basis of the data presented in Figure 4B, the average peak width increased by 29% (from 0.82 to 1.06 s), while the separation time increased by 39% (from 26 to 36 min) as the gradient time extended from 10 to 30 min. Resolutions were subsequently improved because peak widths increased at a higher rate than retention times.

CONCLUSIONS

LC with a narrow OT column has extraordinarily high resolving power, and we have demonstrated that we can further increase its efficiency, peak capacity, and separation speed simultaneously by elevating the operation temperature. Using a 2 μm i.d. \times 160 cm in length OT column coated with OTMS and running the separation at 70 $^{\circ}\text{C}$, we have achieved a peak capacity of 2720 within 143 min, the highest number so far for one-dimensional LC separations. Heating the column is easy and inexpensive to implement, and many commercial LC systems already have compartments to control column temperatures. Potential issues associated with elevated column temperature include analyte decomposition and coating degradation. For applications requiring fluorescence detection, photobleaching can occur. On the basis of the experience obtained from this research, column temperatures of higher than 70 $^{\circ}\text{C}$ are not recommended for separating fluorescently labeled peptides. Mass spectrometry can be an ideal detector under this circumstance because it is sensitive, and an elevated temperature can help improve ionization. We expect that OT-LC will have a wide range of applications in chemical and biochemical analyses because of its remarkable resolving power and high separation speed.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications Web site. The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.1c00296>.

Figure S1 (effect of temperature on fwhm), Figure S2 (effect of temperature on separation speed), Figure S3 (chromatograms obtained at different temperatures), Figure S4 (three-panel display for the chromatogram having a peak capacity of 2720 within 143 min), and Figure S5 (chromatograms obtained under different gradient times) (PDF)

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Notes

The authors declare no competing financial interest.

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