Anal. Chem. **2006,** *78,* 7186−7190

Scanning Temperature Gradient Focusing

Stacey J. Hoebel, Karin M. Balss,† Barbara J. Jones, Constantin D. Malliaris, Matthew S. Munson, Wyatt N. Vreeland, and David Ross*

National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, Maryland 20899

Temperature gradient focusing (TGF) is a recently developed technique for the simultaneous concentration and electrophoretic separation of ionic analytes in microfluidic channels. One drawback to TGF as it has previously been described is the limited peak capacity; only a small number of analyte peaks (∼2−3) can be simultaneously focused and separated. In this paper, we report on a variation of the TGF method whereby the bulk flow rate is varied over time so that a large number of analytes can be sequentially focused, moved past a fixed detection point, and flushed to waste. In addition to improved peak capacity, the detection limits of the scanning TGF method can be adjusted on-the-fly, as needed for different samples. Finally, scanning TGF provides a technique by which high-resolution, high-peak-capacity electrophoretic separations can be performed in simple, straight, and short microfluidic channels.

The most commonly used methods for analytical separations (gas chromatography, liquid chromatography, capillary electrophoresis (CE), etc.) all begin with the injection of a plug of mixed sample at the beginning of a separation column and proceed with the migration of the sample plug along the column. Separation occurs as each analyte in the sample migrates with a different speed and so can be individually detected at the end of the column. A common feature of these types of separations is that the widths of the analyte bands increase and their amplitudes decrease as they migrate along the column. Consequently, with CE or separations in a microfluidic chip format, because of the small geometries involved, achieving both high resolution and low concentration detection limits can be difficult. To address this issue, a number of methods have been developed for the injection of a large amount of sample and compression of the sample plug into the narrow band required for high resolution. For electrophoretic separations, the most common examples are sample stacking, sweeping, and transient isotachophoresis (for example reviews, see refs 1−3).

A different approach to analytical separations is the use of an equilibrium gradient focusing method.[4] In this approach, a gradient (in pH, electric field, conductivity, temperature, etc.) is formed along the length of the separation column and different analytes are separated as they focus or accumulate at different positions along the gradient. A major advantage of this type of method is that it combines the concentration and separation of analytes into one step; peaks become narrower and more concentrated as the separation progresses. Consequently, it is possible to achieve both high resolution and low detection limits. A second important advantage is that the width of the injected sample plug need not be carefully defined, since the analytes will move toward and be focused at their respective positions regardless of how they were introduced into the column. Currently, there is only one widely used equilibrium gradient focusing method suitable for use in capillary or microfluidic chip formats. That method, isoelectric focusing, has proven to be an extremely powerful tool for the analysis of proteins and peptides (for example reviews, see refs 5−7). However, it can only be used with analytes having an isoelectric point in the range approximately between pH 3 and pH 10, limiting its utility to proteins and peptides.

Recently, a number of new equilibrium gradient focusing methods have been described that can be applied to the separation of broader classes of analytes. These new methods include electric field gradient focusing (EFGF),[8−11] micellar affinity gradient focusing,[12] and temperature gradient focusing (TGF),[13−15] which is the subject of this paper.

Briefly, TGF works by balancing the electrophoretic motion of an analyte against the bulk flow of buffer through a microchannel or capillary. A buffer with a temperature-dependent ionic strength is used so that the application of a temperature gradient along the length of the channel will give rise to a corresponding gradient in the electrophoretic velocity of the analyte. The velocity of the bulk buffer flow can then be adjusted so that the total velocity of the analyte (the sum of the electrophoretic velocity and bulk flow velocity) will be equal to zero only at a single point

* To whom correspondence should be addressed. Email: david.ross@nist.gov.
† Current affiliation: Cordis, a Johnson and Johnson Company, Welsh and McKean Rds., Spring House. PA 19477.

(1) Lin, C. H.; Kaneta, T. *Electrophoresis* **2004**, *25*, 4058−4073.
(2) Quirino, J. P.; Terabe, S. *J. Chromatogr., A* **2000**, *902*, 119−135.
(3) Osbourn, D. M.; Weiss, D. J.; Lunte, C. E. *Electrophoresis* **2000**, *21*, 2768−2779.
(4) Giddings, J. C.; Dahlgren, K. *Sep. Sci. Technol.* **1971**, *6*, 345−356.
(5) Rodriguez-Diaz, R.; Wehr, T.; Zhu, M. D. *Electrophoresis* **1997**, *18*, 2134−2144.
(6) Kilar, F. *Electrophoresis* **2003**, *24*, 3908−3916.
(7) Shimura, K. *Electrophoresis* **2002**, *23*, 3847−3857.
(8) Huang, Z.; Ivory, C. F. *Anal. Chem.* **1999**, *71*, 1628−1632.
(9) Koegler, W. S.; Ivory, C. F. *J. Chromatogr., A* **1996**, *726*, 229−236.
(10) Petsev, D. N.; Lopez, G. P.; Ivory, C. F.; Sibbett, S. S. *Lab Chip* **2005**, *5*, 587−597.
(11) Humble, P. H.; Kelly, R. T.; Woolley, A. T.; Tolley, H. D.; Lee, M. L. *Anal. Chem.* **2004**, *76*, 5641−5648.
(12) Balss, K. M.; Vreeland, W. N.; Howell, P. B.; Henry, A. C.; Ross, D. *J. Am. Chem. Soc.* **2004**, *126*, 1936−1937.
(13) Balss, K. M.; Ross, D.; Begley, H. C.; Olsen, K. G.; Tarlov, M. J. *J. Am. Chem. Soc.* **2004**, *126*, 13474−13479.
(14) Balss, K. M.; Vreeland, W. N.; Phinney, K. W.; Ross, D. *Anal. Chem.* **2004**, *76*, 7243−7249.
(15) Ross, D.; Locascio, L. E. *Anal. Chem.* **2002**, *74*, 2556−2564.

10.1021/ac060934r
Published on Web 09/21/2006

along the gradient and all of the analyte will move toward that zero velocity point where it will accumulate or focus. Analytes with different electrophoretic mobilities will have a total velocity of zero at different positions along the channel and so will be simultaneously concentrated and separated. In the initial paper on TGF,[15] it was shown that it could be used with any charged analyte, ranging from small molecules and DNA to proteins and even micrometer-sized particles.

One drawback to TGF that is evident from previously published work is the limited peak capacity; only a small number of analyte peaks (~2−3) can be simultaneously focused and separated. For many applications, it is necessary to separate and quantitate a large number of components from a complex sample mixture. In a recent theoretical paper, it was suggested that the peak capacity of EFGF and other related methods, such as TGF, could be increased by using a nonlinear field (temperature) gradient and an applied voltage that is varied in time over the course of the separation.[16] We have chosen a different approach, whereby the applied voltage is left constant and the bulk flow rate is ramped or "scanned" over time so that analyte peaks sequentially enter the focusing gradient, are focused as they traverse the gradient, are detected at a fixed point near the end of the gradient, and are then rinsed to waste. The separation data are then presented in a format similar to a conventional chromatogram. With scanning TGF, separations with both high resolution and high peak capacity are possible. Additional advantages of scanning TGF are (1) because scanning effectively controls the time each analyte peak is allowed to focus, peak areas are both repeatable and quantitative; (2) detection limits can be adjusted as needed to suit the requirements of the sample by changing the rate at which the bulk flow is scanned; (3) scanning TGF can be done in simple, short, straight microchannels or capillaries with no need for voltage switching or channel structures to define an injection.

EXPERIMENTAL SECTION

Materials. All reagents were used as received, and all aqueous solutions were prepared using deionized ultrafiltered water (Fisher Scientific, Fair Lawn, NJ). Dansyl-amino acids, tris(hydroxymethyl)aminomethane (Tris), boric acid, L-phenylalanine, L-aspartic acid, and γ-cyclodextrin hydrate were purchased from Sigma (St. Louis, MO). Fluorescein, 5-(and-6)-carboxyfluorescein (FAM), and 5-carboxyfluorescein, succinimidyl ester (5-FAM, SE) were purchased from Invitrogen (Carlsbad, CA).

All TGF separations were performed in buffers composed of 1 mol/L Tris and 1 mol/L boric acid (1 mol/L TB, pH 8.5). Although 1 mol/L is an unusually high concentration, it should be noted that the conductivity of 1 mol/L TB (2 mS/cm) is similar to that of 20 mmol/L sodium carbonate buffer, pH 9.4.

Fluorescent Labeling Reactions. For the data shown in Figure 5, phenylalanine and aspartic acid were labeled with 5-FAM, SE according to the following procedure: Amino acid solutions were prepared (1 mmol/L) in 100 mmol/L sodium borate buffer (pH 8.0). A stock solution of 5-FAM, SE (100 mmol/L) in dimethyl sulfoxide was prepared and used the same day. A volume of 990 μL of amino acid solution was mixed with 10 μL of 5-FAM, SE solution and mixed overnight. Labeled amino acid concentrations given below assume quantitative yield for the labeling reactions.

(16) Tolley, H. D.; Wang, Q. G.; Lefebre, D. A.; Lee, M. L. *Anal. Chem.* **2002**, *74*, 4456−4463.

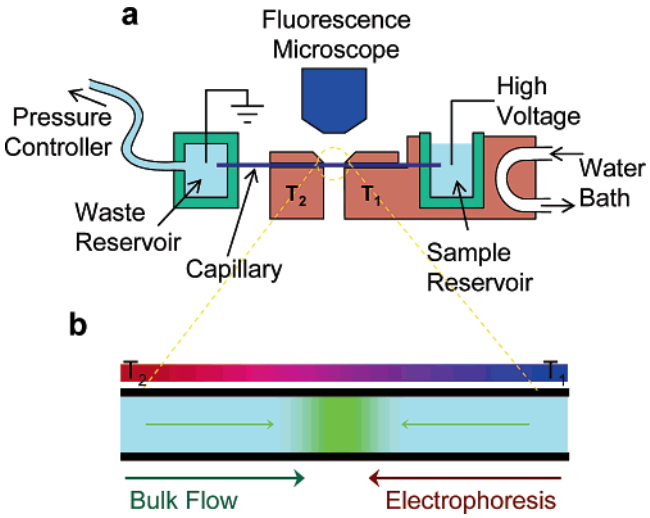

Figure 1. (a) Schematic of scanning TGF apparatus. The 3-cm-long capillary is connected to the sample reservoir on one end and a waste reservoir on the other end. The sample reservoir is open to ambient pressure, and the waste reservoir is sealed and connected to a pressure controller for precise control of the bulk flow rate through the capillary. High voltage is applied to the reservoirs via platinum electrodes. (b) Schematic of TGF. The electrophoretic motion of an analyte is opposed by bulk flow of buffer. The bulk flow velocity is primarily driven by electroosmosis but is precisely adjusted by application of a controlled pressure to the waste reservoir (see above). A buffer with a temperature-dependent ionic strength is used so that the application of a temperature gradient along the length of the capillary will result in a corresponding gradient in the electrophoretic velocity so that the total velocity (bulk + electrophoretic) will sum to zero at a single point along the gradient, and the analyte will be focused at that point.

Temperature Gradient Focusing Apparatus. The capillary device used in these experiments was a 3-cm-long, 30-μm-i.d., 360-μm-o.d. fused-silica capillary embedded between polycarbonate sheets. The device was prepared by sandwiching the capillary between two 380-μm-thick polycarbonate sheets (McMaster Carr, Atlanta, GA), placed in a hydraulic press at 180 °C at 4500 N for 10 s, and cooled to 120 °C before releasing the pressure. Metal shims (510 μm thick) were used in the press to define the final thickness of the device and to prevent crushing of the capillary. The capillary device was thermally and mechanically anchored to two copper blocks as shown in Figure 1. T_2 was regulated using a thermoelectric module, and T_1 (as well as the temperature of the sample reservoir) was set by the temperature of a recirculating water bath. The gap between the two blocks defined the temperature gradient zone and was set to 2 or 5 mm as described in the text and figure captions. The capillary was connected at one end to a polypropylene sample reservoir (150-μL volume) via a 360-μm hole drilled into the reservoir and on the other end to the waste reservoir via a PTFE-backed silicone rubber septum. The waste reservoir was sealed, and the pressure in the waste reservoir was precisely controlled as described below.

For the data of Figures 2, 3, 5, and 6, the waste reservoir was connected via nylon tubing to another reservoir anchored to a vertical translation stage (not shown in Figure 1). The waste reservoir, nylon tube, and the reservoir on the translation stage were all filled with buffer, and the pressure applied to the waste reservoir was precisely controlled by varying the height of the translation stage.

For the data of Figure 4, the waste reservoir was partially filled with buffer and was connected to a high-precision pneumatic pressure controller (Mensor Corp., San Marcos, TX) via (air-filled) nylon and polyetheretherketone (PEEK) tubing.

Fluorescence Microscopy. The experiments were performed with a fluorescence microscope equipped with a mercury arc lamp, long-working-distance 10× objective, and appropriate filter sets for the fluorescent labels. A color charge-coupled device (CCD) camera with frame grabbing software (Scion Corp., Frederick, MD) was used to acquire images. Image processing software (ScionImage or ImageJ) was used to emulate a single-point detector by computing the average fluorescence intensity at a detection spot near the end of the temperature gradient zone for each image. The detection spot was set to cover a short section of the capillary (~80 μm long) located ~450 μm from the outlet edge (the left edge in Figure 1) of the temperature gradient zone.

Capillary Electrophoresis. Capillary electrophoresis was performed on an HP 3D CE system. Detection was accomplished by monitoring UV absorbance at 220 nm. A 75-μm-i.d., 69.5-cm effective length (78 cm total length) bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ) was used and thermostated at 25 °C. Separations were carried out in 25 mmol/L pH 8.5 borate buffer at +17 kV. A solution of dansylated tryptophan, phenylalanine, serine, glycine, and aspartic acid was prepared by dilution in 12.5 mmol/L pH 8.5 borate buffer to facilitate sample stacking and then introduced into the capillary by pressure injection for 1 s at 3.5 kPa. The capillary was rinsed for 5 min with 25 mmol/L pH 8.5 borate buffer between each run.

RESULTS AND DISCUSSION

For a scanning TGF separation, 135 μL of sample is placed in the sample reservoir, high voltage is applied, and the pressure applied to the waste reservoir is slowly varied from high to low over the course of the separation. Sample injection is continuous, and there is no switching of voltages or reservoirs to define an initial injected plug of sample. Whether or not a given analyte ion will move into the capillary to be focused and detected is determined by the balance between electrophoresis of the analyte and the bulk flow of buffer. Note that the capillary is typically not treated to modify or eliminate electroosmotic flow. Consequently, the bulk flow is primarily driven by electroosmosis and the applied pressure is used as a convenient means to vary the bulk flow rate over time. Ideally, the electroosmotic mobility of the capillary or channel should be uniform and should match the electrophoretic mobility of the analytes to be focused in order to minimize the applied pressure required for focusing and the resultant effects of Taylor dispersion on the resolution of the separation.[17,18]

An example of a scanning TGF separation of five dansyl-amino acids is shown in Figure 2a. At the beginning of the separation, the pressure was set sufficiently high so that none of the amino acids would migrate into the capillary. As the pressure was reduced, the "fastest" (highest mobility) analyte, aspartic acid, was the first to enter the capillary and be focused on the inlet end of the gradient. As the pressure was further reduced, the focused aspartic acid peak moved across the gradient and was detected at the detection spot near the outlet (waste) end of the gradient.

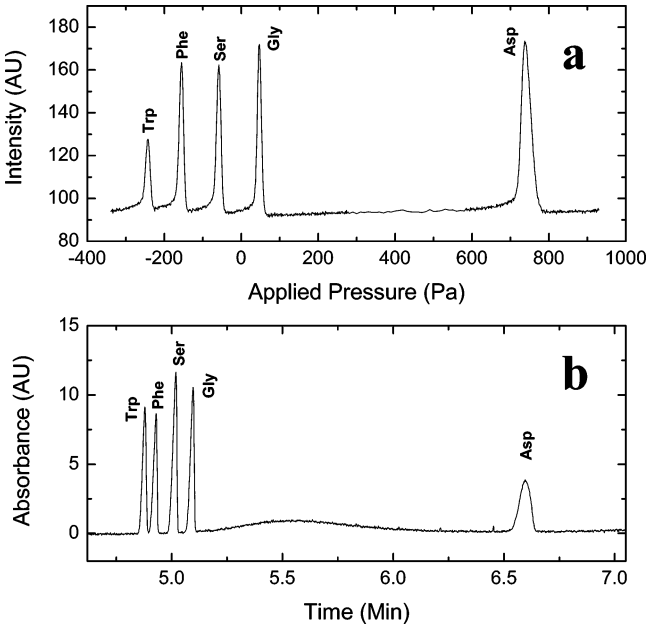

Figure 2. Comparison of scanning TGF with CE for dansyl-amino acids. (a) Scanning TGF. Sample, 5 μmol/L of each amino acid in 1 mol/L TB. Conditions: −3000 V, T1 = 20 °C, T2 = 80 °C, gradient length 5 mm. The pressure applied to the waste reservoir was varied at a rate of −25.4 Pa/min from 930 to 580 Pa, at a rate of −600 Pa/min from 580 to 280 Pa, and at a rate of −13.7 Pa/min from 280 Pa to −340 Pa. The three different scan rates were chosen to first scan at a moderate rate across the peak for aspartic acid, which focuses fairly rapidly. Second, a very rapid rate was used to quickly scan the blank space between aspartic acid and the other amino acids. Third, a relatively slow rate was chosen for the remaining amino acids, which focus more slowly. (b) Conventional CE. Conditions as given in the Experimental Section.

Upon further reduction of the pressure, the aspartic acid peak moved out of the gradient zone toward the waste reservoir. At this point, aspartic acid in the sample reservoir continued to enter the capillary, flow through the gradient zone, and to the waste reservoir contributing to a small increase in the baseline signal at the detection spot. The continuous injection and flow of the aspartic acid through the detection spot also gives rise to the tailing peak shape seen in Figure 2a. As the separation proceeded, each successively slower amino acid entered the capillary, was focused and detected, and then eluted to the waste reservoir. After each analyte peak is detected and eluted to waste, that particular analyte is added to the portion of the sample that flows (unfocused) through the detection spot. So, each peak has a tail similar to that found for aspartic acid, and each analyte, in turn, contributes to a small rise in the signal baseline.

Figure 2a shows that, by scanning the bulk flow rate, a set of analytes spanning a wide range of electrophoretic mobilities can be separated and detected in one experiment and that the peak capacity is significantly improved over the previously described static TGF method.[15] For comparison, a conventional CE separation of the same set of analytes is shown in Figure 2b. If not for the tailing of the peaks in the TGF separation, the resolution obtained with the TGF separation would be significantly better than that obtained with the CE separation. Even with the tailing, the resolution of the two separation techniques is comparable, which is noteworthy, given that the gradient zone length for the

(17) Ghosal, S.; Horek, J. *Anal. Chem.* **2005**, *77*, 5380−5384.
(18) Huber, D.; Santiago, J. G. Personal communication, 2006.

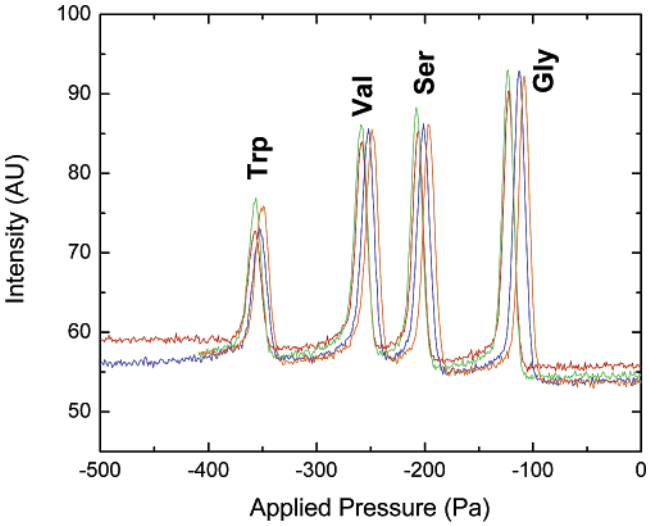

Figure 3. Repeatability of scanning TGF. Four repeated separations are shown for identical samples. Sample, 5 μmol/L of each amino acid in 1 mol/L TB. Conditions: −3000 V, T1 = 20 °C, T2 = 80 °C, gradient length 5 mm. The pressure applied to the waste reservoir was varied at a rate of −11.2 Pa/min.

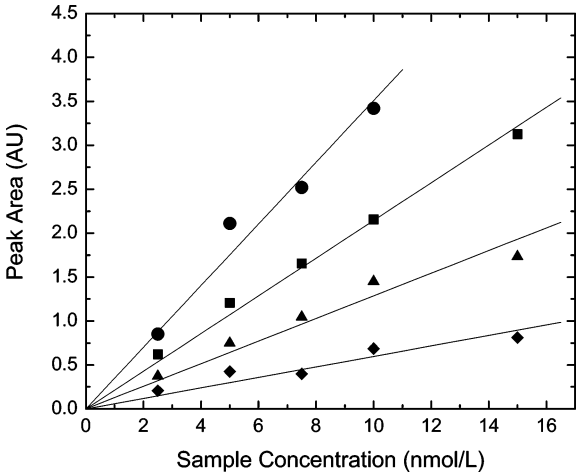

Figure 4. Calibration curves for scanning TGF of fluorescein and carboxyfluorescein (FAM). Conditions: −3000 V, T1 = 20 °C, T2 = 80 °C, gradient length 2 mm. The diamond and square point symbols are the data for fluorescein and carboxyfluorescein at a scan rate of −90 Pa/min, respectively. The triangle and circle point symbols are the data for fluorescein and carboxyfluorescein at a scan rate of −36 Pa/min, respectively.

TGF separation was more than 100 times less than the effective capillary length for the CE separation.

Another advantage to scanning the bulk flow rate during a TGF separation is that the time allowed for each analyte to focus is easily controlled and reproducible, so that peak heights or areas are also reproducible and can be used for quantitation. Figure 3 shows an example of four repeated scanning TGF separations of a set of four dansyl-amino acids. For the four separations shown in Figure 3 and four similar repeats run the following day, the variation of peak area was 8, 10, 12, and 15% relative standard deviation (RSD) for glycine, serine, valine, and tryptophan, respectively (peak height variation was 7, 11, 10, and 17% RSD). These values are a bit larger than what is typically obtained with conventional separation methods such as CE or HPLC. Much of the variation is probably due to the variability of the mercury arc

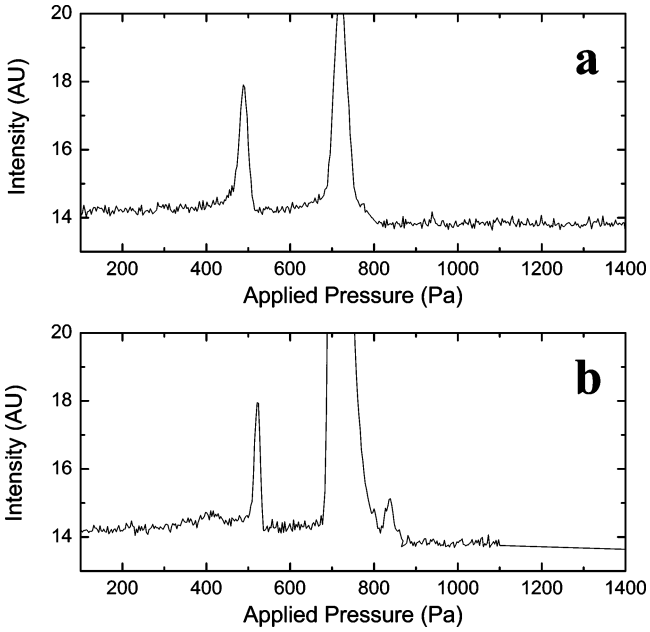

Figure 5. Example of adjusting detection limit with scanning TGF by changing the scan rate. Sample, amino acids labeled with 5-FAM, SE (see text), 90 nmol/L Phe, 10 nmol/L Asp in 1 mol/L TB. Conditions: −3000 V, T1 = 20 °C, T2 = 80 °C, gradient length 2 mm. (a) Constant scan rate of −36 Pa/min. (b) −3 Pa/min from 1100 to 800 Pa and then −36 Pa/min.

lamp source and the lack of repeatability of repositioning the capillary under the microscope from run to run. With greater care in the positioning of the capillary and the detection spot, run-to-run peak area variability for fluorescein is typically between 3 and 5%, which is more in line with that found with conventional techniques.

For the peak heights and peak areas to be repeatable, it is important that the starting pressure of a scan be sufficiently greater than the pressure at which the first peak is focused. Otherwise, the first peak will initially be focused somewhere in the middle of the temperature gradient rather than at the inlet edge of the gradient, and its focusing time will be consequently reduced. For the range of analytes and operating parameters used for the examples given in this paper, a sufficient pressure difference is between 150 and 400 Pa.

Figure 4 shows a plot of the peak area versus input concentration for a series of scanning TGF separations of FAM and fluorescein. In both cases, detection limits in the low nanomole per liter concentration range are easily achieved despite the disadvantages of the detection scheme used (mercury arc lamp, room temperature CCD camera, etc.). For both fluorescein and FAM, the peak areas are linearly proportional to input concentration up to 15 nmol/L input concentration. At higher input concentrations, the signal from the FAM peaks saturated the CCD detector. For each analyte, peak area versus input concentration curves are shown for two different scanning rates (−90 and −36 Pa/min). For both analytes, the slower scan rate allows for a longer focusing time and correspondingly greater peak areas. In other words, the sensitivity of the method can be adjusted, on-the-fly, as needed for a particular sample and limit of detection. It should be noted that depending on the intensity of the excitation source used and the portion of the capillary that is illuminated,

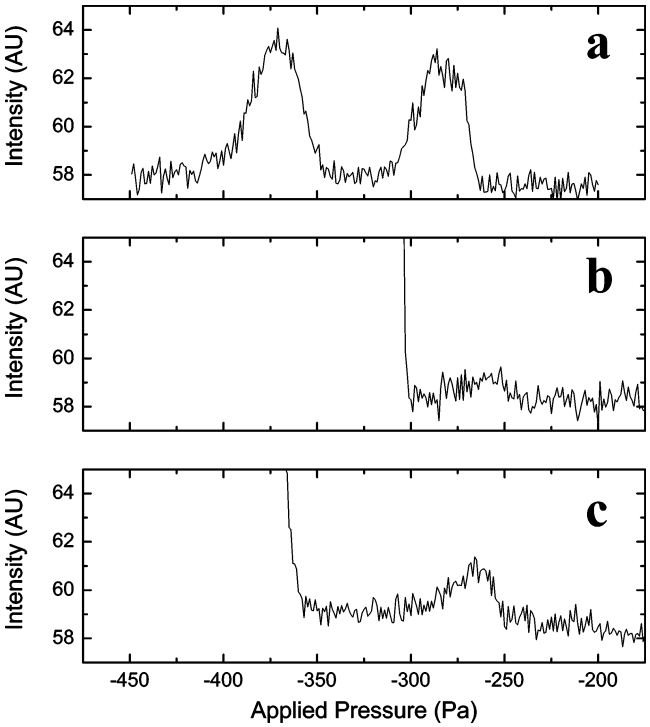

Figure 6. Scanning TGF for chiral separation of dansyl-glutamic acid. Conditions: +3000V, T1 = 30 °C, T2 = 10 °C, gradient length 2 mm, 26 mg/mL γ-cyclodextrin added to buffer. (a) Sample: 2.5 μmol/L D-glu, 2.5 μmol/L L-glu. Scan rate: −8.24 Pa/min. (b) Sample: 0.5 μmol/L D-glu, 1000 μmol/L L-glu. Scan rate: −8.24 Pa/min. (c) Sample: 0.5 μmol/L D-glu, 1000 μmol/L L-glu. Scan rate: −4.21 Pa/min from −150 Pa to −310 Pa and then −47.2 Pa/min.

photobleaching can result in peak areas that do not increase as rapidly as expected with reduced scan rate.

An example of the use of the adjustable sensitivity of scanning TGF is shown in Figure 5. The sample consisted of 90 nmol/L L-phenylalinine and 10 nmol/L L-aspartic acid, fluorescently labeled with 5-FAM, SE. Figure 5a shows the results of the first TGF scan run at a constant, fairly rapid scan rate (−36 Pa/min). The large peak at ~700 Pa is a free dye peak, and the peak at 500 Pa corresponds to phenylalanine. No aspartic acid peak was detectable. The scan was then repeated using the same sample, and the results are shown in Figure 5b. The first part of the scan (pressure >800 Pa) was run at a slow scan rate (−3 Pa/min), and the remainder of the scan was run at the original scan rate (−36 Pa/min). With the slower scan rate, the small aspartic acid peak became clearly evident at 840 Pa, while the height of the phenylalanine peak, which was scanned at the faster rate, remained the same as in the previous scan.

Scanning TGF is also applicable to chiral separations and to the detection of small amounts of enantiomeric impurities as shown in Figure 6. Figure 6a shows the scanning TGF separation of a sample made up of equal parts dansyl-D-glutamic acid and dansyl-L-glutamic acid. As in the case of static TGF,[14] the two enantiomers were easily baseline resolved. Figure 6b shows the result of a separation (using the same parameters) of a sample composed of a 2000-fold excess of the L enantiomer and only a trace amount of the D enantiomer. The D enantiomer peak (at ~ −260 Pa) was just barely evident over the noise with the relatively fast scan rate. Once again, however, the scan rate could be reduced to improve the sensitivity, making the D enantiomer peak more readily detectable over the noise as shown in Figure 6c.

CONCLUSIONS

The scanning TGF method, as described above, provides a simple way to improve the peak capacity of equilibrium gradient focusing methods such as TGF. In addition, it provides a route by which TGF can be used for repeatable and quantitative analysis of dilute analytes. More importantly, it makes it possible for TGF (and other counterflow gradient methods such as EFGF) to be used with the full range of conventional, single-point detectors that have been demonstrated with CE (laser-induced fluorescence, UV absorbance, conductivity, electrochemical, etc.). Finally, and perhaps most significantly, scanning TGF is a method whereby high resolution and high peak capacity separations can be performed in very simple, straight (with no injection structure), and very short (2−5 mm) microfluidic channels, many of which could easily be fit into the small footprint of a lab-on-a-chip device.

ACKNOWLEDGMENT

M.S.M., K.M.B., and W.N.V. acknowledge the National Research Council for financial support. S.J.H. and C.D.M. acknowledge the NIST Summer Undergraduate Research Fellowship program. Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Received for review May 19, 2006. Accepted August 2, 2006.

AC060934R