

microchannel, and consists of 20 mM SDS, and 5 mM carbonate buffer in 25% ethanol, 75% water. An initial concentration of rhodamine B of 0.2 μM is used.

[0038] FIG. 4 shows a plot of the focused peak concentration, inferred from the measured fluorescence intensities, as a function of time. After the voltage is switched on around 5 seconds, the peak concentration increased with time, reaching a value greater than 7 μM in about 3 minutes. About a 35-fold increase in concentration is achieved in three minutes.

[0039] Referring now to FIG. 5, in a further embodiment, the present method can be used for focusing and separating more than one species in a capillary. Building on the embodiment of FIGS. 2 and 3 from above for focusing rhodamine B, after the rhodamine B is focused for several minutes, identified as B, a solution of approximately 3 μM rhodamine 110 is introduced at the entrance to the capillary. After a few minutes of additional focusing, the rhodamine 110 was clearly visible as an additional focused peak as shown in FIG. 5 identified as 110. Note that in normal operation, a separation would be accomplished by injecting mixed sample into the separation channel rather than by sequential injection of different samples as was done for this demonstration.

[0040] In most cases the distribution coefficient is temperature-dependent, so that a retention factor gradient could be made by applying a temperature gradient, even if the CMC and the phase ratio were temperature-independent. In the above example, there is probably a combination of these two temperature-dependent effects that contribute to the focusing.

[0041] There have also been pseudostationary phases specifically developed to provide a temperature-dependent affinity for various analytes. These are used in "temperature-programming" EKC, where the temperature of the separation is changed over time to achieve improved separations. Use of these types of pseudostationary phases for focusing with a temperature gradient according to the methods described herein would be relatively straight forward and readily implemented by one skilled in the art. Examples include the use of a thermoresponsive polymer poly(*N*-isopropyl-acrylamide), which can be used to make pseudostationary phases that can be switched on and off at a transition temperature.

[0042] Both the distribution coefficient and phase ratio can be dependant upon the composition of the solution used, and so a retention factor gradient can be produced using a gradient in the solution composition.

[0043] Referring now to FIG. 6, in another embodiment, of a separation conduit, such as a microchannel or capillary for use in gradient focusing, where like elements to the embodiment of FIG. 1 are identified as reference numbers increased by 50, microchannel 60 is divided into two adjacent conduits, separation channel 60a and control channel 60b. The two channels over a certain portion of their length, the gradient region, are connected by a selectively-permeable membrane or other selectively permeable structure 61. The analyte and the pseudostationary phase are contained in the separation channel 60a. As in the embodiment of FIG. 1, the solution in the separation channel 60a is caused to flow from the region of low retention factor to the region of

high retention factor. The control channel 60b and the membrane 61 are used to form a gradient in one or more components of the buffer in the separation channel 60a and to thereby form the retention factor gradient. The control channel 60b contains a solution of a composition differing from that in the separation channel 60a. The difference between the two solutions will most often be in the concentration of one or more of the solution components. The membrane is chosen to be permeable to one or more of the components that are present in differing concentrations in the two solutions. The passage of the solution components through the membrane will cause the composition of the solution in the separation channel to change. Because the solution in the separation channel 60a is flowing from the region of low retention factor to the region of high retention factor, this change will be greater at the high retention factor end of the gradient region than at the low retention factor end. Thus, a steady-state gradient in the composition of the solution in the separation channel 60a will be established.

[0044] The composition gradient can be, for example, a gradient in the surfactant concentration in a micellar system, or a gradient in the concentration of an organic modifier, or a gradient in the concentration of a salt dissolved in the solution. Because the retention factor is a function of the composition of the solution used, this will result in a steady-state gradient of the retention factor in the separation channel. This retention factor gradient can then be used for focusing as in the previously described embodiments.

[0045] Note that the geometrical arrangement of the separation channel 60a and the control channel 60b need not be side-by-side as drawn in FIG. 6. They could also be arranged coaxially, annularly, or in any other arrangement that accomplished the desired solution composition gradient. In addition, the relative sizes of the two channels need not be equal as is shown in FIG. 6. The control channel could in fact be replaced with a much larger container of solution. For examples of devices using similar semi-permeable structures to separate two channels see Z. Huang and C. F. Ivory, *Analytical Chemistry* 71, 1628 (1999); Z. Huang and C. F. Ivory, *Abstracts of Papers of the American Chemical Society* 219, 208-BIOT (2000); and S. Song, A. K. Singh, T. J. Sheppard, and B. J. Kirby, *Analytical Chemistry* 2004, vol. 76, p. 2367, all herein incorporated by reference.

[0046] As previously noted, materials and strategies that have been developed for EKC methods could be modified and applied to the present method as would be now readily apparent to one of ordinary skill in the art. For example, it is common in EKC to vary the solution composition (e.g., salt concentration, fraction of alcohol used, surfactant concentration, etc.) as a function of time to produce a time variation of the retention factor. These strategies—referred to as "solvent programming"—could be combined with a spatial gradient of the solution composition to produce a spatial gradient of the retention factor that could be used for focusing.

[0047] A neutral micellar or other pseudostationary phase would always move with the bulk buffer. In this case, Equation (1) would become:

$$u_i = u_B + u_{EP} [1/(1+k)].$$

[0048] Because the electrophoretic mobility of the analyte changes when it moves from the mobile phase to the