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High temperature and temperature programming in capillary electrochromatography

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Abstract

In electrochromatography, solvent electrophoretic mobility and solute partitioning are temperature dependent processes. If temperature variations are controlled, solute selectivity and analysis times can be tailored. In this study the feasibility of temperature programming in capillary electrochromatography (CEC) was demonstrated using a reversed-phase CEC mode. The outcome of programmed separations was compared with isothermal, isocratic and isorheic (constant flow) separations. The combined effects of column temperature and mobile phase flow-rate changes during the separation run, resulted in up to a 50% reduction in the separation run time, without adversely affecting the quality of separation. For capillary electrochromatography, temperature programming may be a valuable alternative to solvent programming modes because of the great technical difficulties associated with carrying out solvent gradient elution.

Keywords: Temperature effects; Temperature programming; Electrochromatography

1. Introduction

At present, there is a great deal of interest in capillary electrochromatography (CEC), in particular, in exploring the prospect of high efficiency separations and versatile selectivity, for both neutral and charged analytes. The theory covering retention in CEC has been refined [1–4] and the applicability of the technique has been evaluated [4–12]. In addition, several reviews on CEC have already appeared in the literature [13–16]. For CEC to develop into a practical analytical technique, that could compete with pressure-driven high-performance liquid chromatography (HPLC), several problems associated with column technology and instrumentation have to be resolved [16]. For CEC to be accepted as a widely applicable separation technique, suitable for the separation of complex mixtures, a gradient mode, which could be reliably and routinely performed on commercial equipment, is essential.

The idea of programmed analysis is to vary the operating conditions during the analysis, so that all components of the sample may be eluted under optimum conditions and to achieve shorter analysis time. In spite of the few gradient elution schemes developed for mobile phase programming in CEC [17–21], the separations in CEC have been restricted to isocratic elution mode.

When fast and highly efficient analyses are desirable, solvent gradient elution is the first method of choice in liquid chromatography and probably should

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also be in CEC. However, solvent gradient elution mode in current CEC systems has a big disadvantage; it requires complex home built instruments since no simple and reliable commercial instruments are available for performing this type of gradient. Apart from varying the ratio of aqueous to organic component in the mobile phase, there are some additional parameters which are suitable for programming in electrochromatographic separations. Tsuda [22] and Bocek et al. [23] experimented with pH gradients in CE, while Sudor et al. [24] developed an ionic matrix composition programming method. Voltage programming has been demonstrated as a useful approach to shorten overall run time in micellar electrokinetic chromatography (MEKC) [25] and CEC [26].

Although separation in CEC is essentially due to the partition equilibrium of the solutes between two phases, the environment in which the equilibrium takes place can also influence the separation. Environmental conditions, such as column temperature, can influence the partition process and therefore the overall separation quality [27]. In electrochromatography and capillary electrophoresis, temperature is often regarded as a parameter that has an adverse effect on the separation efficiency and promotes the formation of gas bubbles in the separation column. Until recently, the primary aim of controlling capillary temperature was to remove Joule heat; nevertheless, temperature adjustment can also be used as a means of optimizing selectivity and run times in electrochromatographic separations [3,25]. Recently, a temperature gradient was utilized to optimize separation in capillary gel electrophoresis [28] and MEKC [25].

The importance of temperature stems from the marked influence of temperature on solute partitioning. In addition, secondary effects such as the changes with temperature of mobile phase viscosity [29], solute diffusivities [29], the degree of ionization of the buffer [29] buffer pH [30] and stationary phase transition [31] are also important. All of these parameters influence either the analysis time and efficiency (through their effect on electroosmotic flow) or separation selectivity (by influencing solute partitioning). If temperature variations are controlled, solute selectivity and analysis time in CEC can also be altered. In this work, we explore temperature programming as a means for adjusting selectivity and accelerating the elution of highly retained compounds. The experiments were performed on commercially available instrumentation without any alteration.

2. Experimental

The experiments were performed on a $HP^{3D}CE$ system from Hewlett-Packard (Waldbronn, Germany). The system incorporates an $HP^{3D}CE$ ChemStation for instrument control, data acquisition and data analysis. An external supply of nitrogen (Carba Gas, Basel, Switzerland) was required to equally pressurize (approximately 8 bar) the outlet and the inlet vial. Packed capillary columns used in the experiments were obtained from Hewlett-Packard. The columns were packed with Hypersil C_{18} (packed bed length 25 cm, total length 33.5 cm) and Spherisorb ODS-1 (packed bed lengths 16.5 cm and 25 cm, total length 33.5 cm). The shorter packed Spherisorb column, used for fast separations, was packed so that all of the stationary phase was enclosed within the column cartridge, i.e., there was an 8.5 cm section of empty column before the frit on the inlet side. The average particle size of the packing materials was 3 μm and the internal diameter of the columns was 100 μm. Immediately prior to experimental use, each column was conditioned using an HPLC pump, Rheos 4000 (Flux Instruments, Switzerland), with several column volumes of mobile phase.

Throughout this work, electrokinetic sample injection was utilized (5 kV or 10 kV over 2 s). A test mixture labeled A consisted of: thiourea, hydrocortisone, hydrocortisone 17-butyrate, hydrocortisone 21-acetate, hydrocortisone 17-valerate, hydrocortisone 21-caprylate, hydrocortisone 21-cypionate and hydrocortisone 21-hemisuccinate. A mixture labeled B contained: thiourea, benzyl alcohol, toluene, methyl benzoate, 1,4-dichlorobenzene, naphthalene, biphenyl, 1,3,5-trichlorobenzene, phenothiazine, 1,2,4,5-tetrachlorobenzene and benzophenone. The sample mixtures A and B were made from neat substances obtained from Aldrich (Steinheim, Germany) or Fluka (Buchs, Switzerland). All solvents were of HPLC grade from Merck (Darmstadt, Ger-

many). Mixing, in different proportions, acetonitrile (ACN) and phosphate buffer at pH 7 made the running buffer composition. UV detection was at 210 nm or 241 nm.

3. Discussion

In CEC as in liquid chromatography (LC), there are several ways to optimize selectivity, improve resolution and shorten analysis time. When selecting the mobile phase and the stationary phase in CEC, care should be taken that both high selectivity and high electroosmotic flow (EOF) can be achieved [4]. Mobile phase parameters commonly used to adjust selectivity and generate sufficient EOF include: the type of aqueous buffer components and organic solvent, proportion of organic modifier, pH and ionic strength. Column temperature has hardly been used as a parameter to optimize CEC separation, even though, nearly all of the physical parameters that play a role in CEC separation are a function of temperature. Temperature optimization is particularly complicated because the effect of temperature changes on separations are complex, involving changes in solute retention, band broadening and analysis time.

The particular advantage of utilizing temperature as a parameter to enhance separation is that temperature has a greater overall outcome on the chromatographic (distribution) process than any other single variable. In CEC, the separation of the analytes is based primarily on their different distribution ratios between the stationary phase and the mobile phase. The rate of advance of a solute band through a column depends on the distribution ratio K (the ratio of the concentration of the solute in the stationary phase to its concentration in the mobile phase). A solute is mobile (moves through the column) only when it is dissolved in the mobile phase. The partition ratio is proportional to the solubility of the solute in the liquid phase and solute sorption in the stationary phase. It decreases with increasing temperature according to the Van't Hoff equation:

$$\ln K = -\Delta H^0/RT + \Delta S^0/R \quad (1)$$

where ΔH^0 is the enthalpy change associated with the transfer of the solute from mobile phase to the stationary phase, ΔS^0 is the corresponding entropy change, R is the gas constant, and T is absolute temperature. The change in solute distribution with temperature can be put in a form which represents the distribution factor ratio K_1/K_2 at two different temperatures T_1 and T_2.

$$K_1/K_2 = \exp\left[\Delta H^0 (T_2 - T_1)/RT_1T_2\right] \quad (2)$$

The equation indicates that the magnitude of retention can be easily modulated by changing the column temperature. The effect of temperature variation on the partitioning process will strongly depend on the solute enthalpy. For the same temperature change, a solute with a large ΔH^0 will be strongly affected, whereas a solute with a small ΔH^0, temperature change will not produce as significant a variation in solute distribution between the stationary and mobile phases. If the solute is not sorbed by the stationary phase its retention time is solely the dead-space time, t_0, for the particular column. In chromatography, the distribution coefficient is related to the solute retention factor k,

$$k = K\beta \quad (3)$$

where $\beta = V_{st}/V_{mo}$ is the phase ratio, and V_{st} and V_{mo} are volumes of the stationary and the mobile phase, respectively. The solute retention factor is a measure of the relative time spent by the solute in the stationary and mobile phases and is calculated according to Eq. (4):

$$k = (t_r - t_0)/t_0 \quad (4)$$

where t_r is the solute elution time.

Fig. 1 depicts the separation of a test mixture labeled A under isothermal (30°C), isocratic (ACN–5.0 m*M* phosphate buffer, pH 7.0, 60:40) and isorheic (linear velocity 1.6 mm/s) conditions. The overall run time was 9 min. The retention factors were in the range from 0.23 for the early eluting hydrocortisone (2) to 2.51 for the late eluting hydrocortisone 21-hemisuccinate (8). In order to further speed up the run time, the column temperature was raised to 55°C, Fig. 2. All peaks remained baseline separated and the overall run time was reduced to 5 min. The retention factors were in the

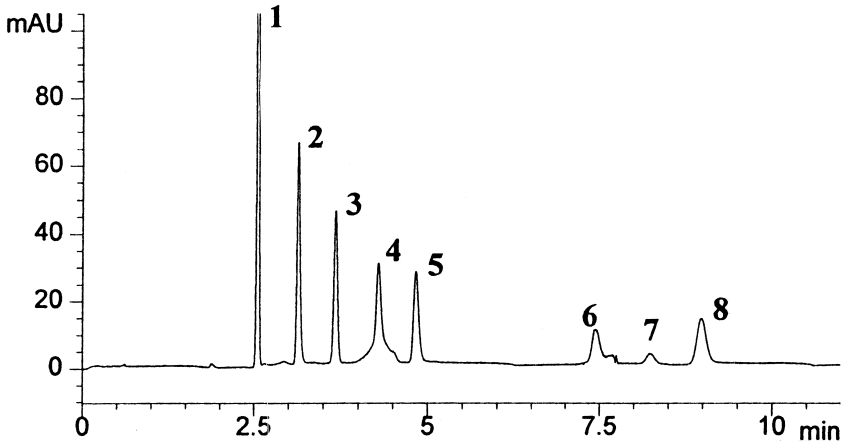

Fig. 1. Column: Spherisorb ODS-1 (packed bed length 16.5 cm, total length 33.5 cm). The running buffer was acetonitrile–5 m*M* phosphate buffer at pH 7 (60:40, v/v); column temperature = 30°C; applied voltage = 25 kV; detection wavelength = 241 nm. Peak identification: (1) thiourea, (2) hydrocortisone, (3) hydrocortisone 17-butyrate, (4) hydrocortisone 21-acetate, (5) hydrocortisone 17-valerate, (6) hydrocortisone 21-caprylate, (7) hydrocortisone 21-cypionate, (8) hydrocortisone 21-hemisuccinate.

range from 0.2 to 1.75. A 25°C increase in column temperature almost halved the analysis run time.

Temperature increase also had a profound influence on the EOF. At 55°C linear velocity was 2.3 mm/s. Most of the observed increase in EOF can probably be ascribed to the decrease in viscosity of the buffered mobile phase, although, contribution of temperature induced pH changes should not be ignored; it has been reported that the EOF increased with the increase in pH of the buffer [32] and with the reduction of the ionic strength [1].

The electroosmotic flow, u_{EOF}, generated in a packed capillary column when the electric field is applied, can be represented by the following equation:

$$u_{EOF} = \epsilon_0 \epsilon_r \zeta E / \eta \quad (5)$$

where ϵ_0 is the permittivity in a vacuum, ϵ_r is the relative permittivity or dielectric constant of the mobile phase, η is the viscosity of the fluid, E is the electric field strength, and ζ is the zeta potential. From this equation it follows that the u_{EOF} is

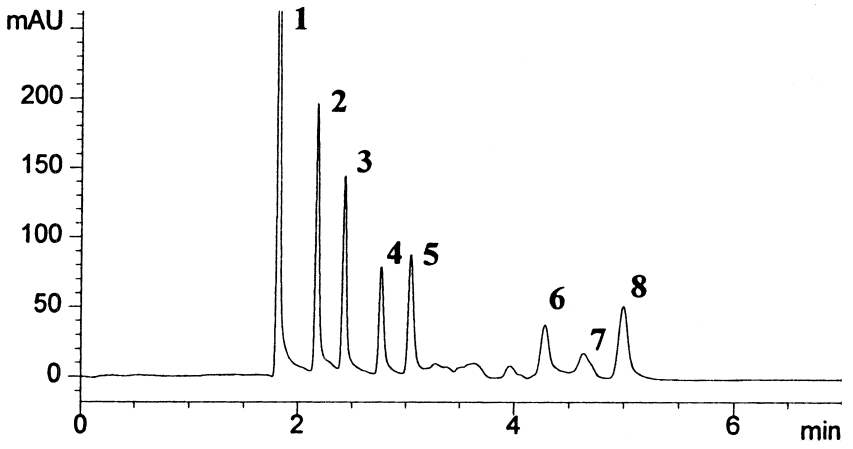

Fig. 2. Column temperature 55°C. Other conditions and peak identification as in Fig. 1.

proportional to the electric field strength and inversely proportional to the mobile phase viscosity. Temperature changes will alter viscosity according to Eq. (6)

$$\ln \eta_L = A + B/T \quad (6)$$

where A and B are constants. If extremely short run times are required, one should not only work at high field strengths, but also at elevated temperature, as the mobility increases when the viscosity decreases as predicted by the Eqs. (5) and (6).

In some instances temperature increases will yield faster separation times but a co-elution of early eluting peaks with low k values will occur. Fig. 3a depicts a separation of the sample mixture labeled B at 60°C. The last peak in the chromatogram eluted after 18.5 min. Reduction of analysis time of more than 50% was achieved when compared with a chromatographic run obtained at 20°C, Fig. 3b.

Although separation was faster at 60°C, not all constituents in the mixture were separated; the bands labeled 6 and 8 co-eluted. By examining solute retention factors at four different temperatures, a correlation between temperature and solute retention was established. It was evident from the plot of ln k

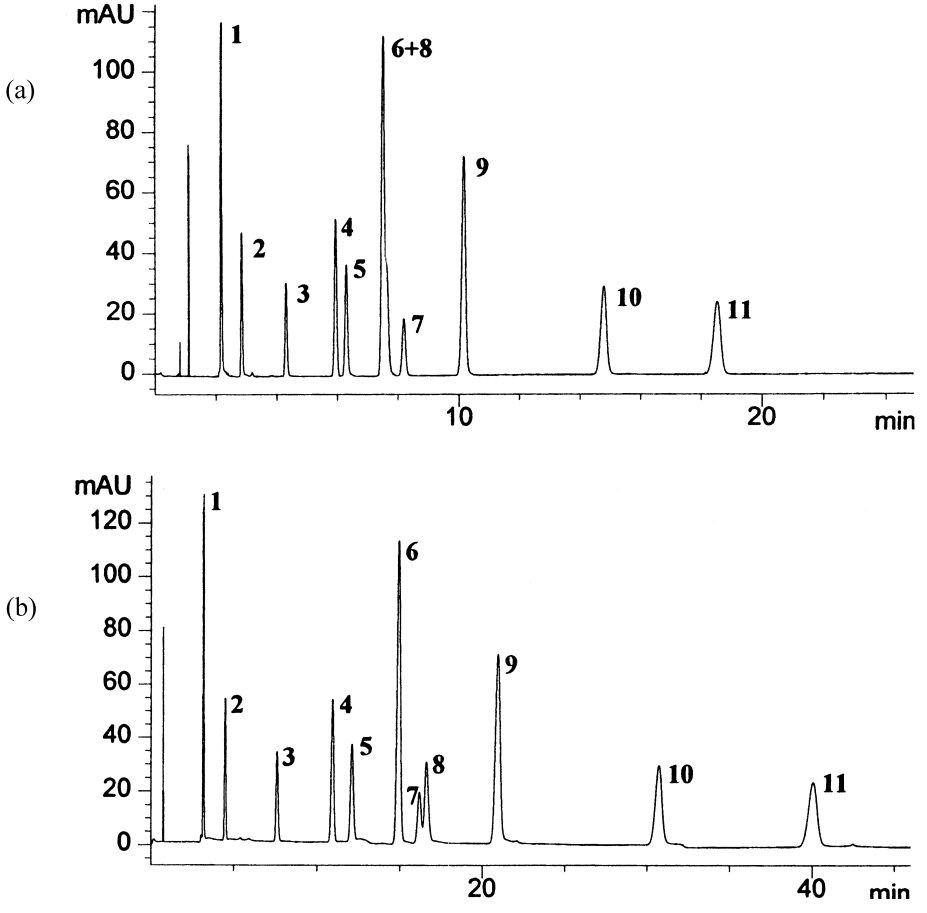

Fig. 3. (a) Column: Hypersil C_{18} (packed bed length 25 cm, total length 33.5 cm). The running buffer was acetonitrile–5 m*M* phosphate buffer at pH 7 (50:50, v/v); applied voltage = 25 kV; detection wavelength = 210 nm. Peaks: (1) thiourea, (2) benzyl alcohol, (3) methyl benzoate, (4) toluene, (5) benzophenone, (6) naphthalene, (7) 1,4-dichlorobenzene, (8) phenothiazine, (9) biphenyl, (10) 1,3,5-trichlorobenzene, (11) 1,2,4,5-tetrachlorobenzene. Column temperature: 60°C. (b) Column temperature: 20°C. Other conditions and peak identification as in (a).

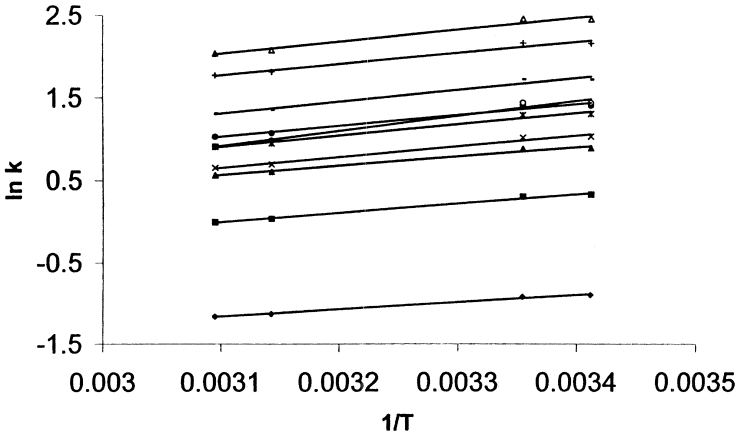

Fig. 4. Plot of ln k vs. $1/T$ (K^{-1}) for the solutes: ♦=benzyl alcohol, ■=methyl benzoate, ▲=toluene, ×=benzophenone, ★=naphthalene, ●=1,4-dichlorobenzene, ○=phenothiazine, —=biphenyl, +=1,3,5-trichlorobenzene, and △=1,2,4,5-tetrachlorobenzene.

vs. $1/T$, Fig. 4, that temperature did not have the same influence on all the constituents in the mixture.

Phenothiazine has the highest slope, which means that its retention is the most prone to temperature effects than the retention of other solutes in the mixture. In the graph, the line representing the retention of phenothiazine intersected with the line of 1,4-dichlorobenzene and naphthalene at 25°C and at 60°C, respectively, which indicates that these peaks will co-elute under these conditions. To optimize the separation of this complex mixture (with a broad range of retention factors $0.41<k<11.65$ at 20°C) a gradient (programmed separation parameter) approach is needed.

Varying the ratio of aqueous to organic component in the mobile phase is the most common approach for altering selectivity in LC and until now the only available gradient mode in CEC. Temperature programming has not been used in CEC, although it is easier to manipulate temperature than the mobile phase (buffer) composition. Because selectivity as well as the analysis time of the components in the mixture were very responsive to temperature change, a temperature gradient mode was selected to reduce the run time and achieve a complete separation of all solutes.

The separation procedure, in a temperature programmed run, has to be optimized in a such manner that by applying the appropriate temperature changes, elution of strongly retained compounds will be accelerated while a complete baseline separation of earlier eluting compounds is secured. Temperature programmed separation in CEC should normally start at a low temperature where less retained compounds will elute; the column temperature should than be increased to elute more strongly retained compounds. This temperature increase, influences solute partitioning and will also help to increase the EOF. When the elution process is started at a relatively low temperature, the hydrophobic solutes will spend most of their time in the stationary phase (due to their low solubility in mobile phase). Meanwhile, components with higher solubility in the mobile phase will move along the column. In effect, for each compound in the mixture there is an optimum elution temperature (for a given mobile and stationary phase).

In programmed-temperature operation, the temperature of the column is increased during analysis. The column temperature (T) at time t is given by:

$$T = T_0 + rt \tag{7}$$

where T_0 is the starting temperature and r the programming rate (°C/min). The difference between the theoretical and actual program is usually given as the time lag of the column temperature behind the theoretical temperature. The system temperature time lag (0.5 min) together with procedure how to determine system temperature lag has been previously described [25].

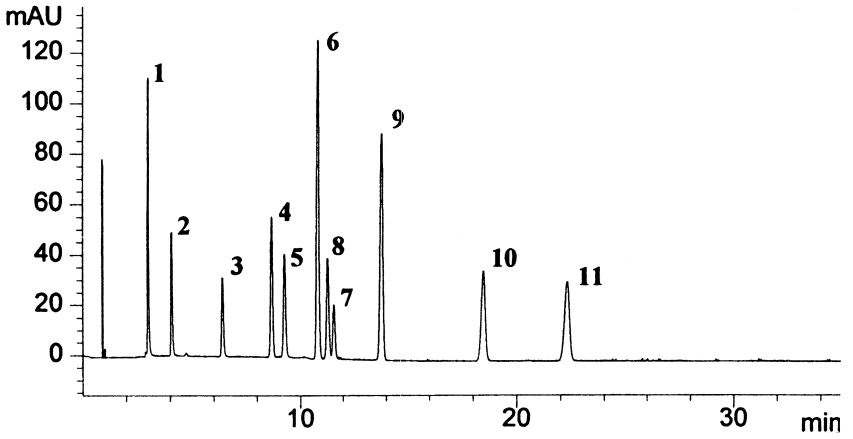

Fig. 5. Separation of a test mixture. Linear temperature gradient from 25°C to 60°C with a rate of 3°C/min. Other conditions and peak identification as in Fig. 3a.

The initial and the final column temperatures were 25°C and 60°C, respectively, with a gradient rate of 3°C/min. As shown in Fig. 5, the overall analysis time was reduced to approximately 22 min, or 45% less than the isothermal separation at 20°C. In spite of this significant reduction of analysis time, all compounds were baseline separated.

Flow-rate variations introduced by temperature programming also helped to speed up the analysis time. Flow-rate programming mode is seldom used in chromatography. In electrically-driven chromatography, it can be generated either by voltage or temperature programming. Voltage programming will not affect solute partitioning, but this programming mode would require large changes in the electric field strengths to achieve a meaningful reduction in the analysis time of strongly retained compounds. Temperature programming during the separation run induced changes in the mobile phase viscosity and as a consequence electroosmotic flow was increased. The changes of u_{EOF}, for the stationary phase and mobile phase used, have been related to temperature by the empirical relationship:

$$u_{EOF} = a + bT \quad (8)$$

For the system employed in this work parameters a and b were 0.0155 and 1.0041, respectively. Parameters were determined by a linear fit (correlation coefficient 0.99998) of measured u_{EOF} at four different temperatures (20°C, 25°C, 55°C and 60°C). Flow rate variation in combination with temperature programming makes it possible to considerably reduce the retention of very strongly retained compounds without having a detrimental affect on the column performance. The separations obtained under combined temperature and flow gradients show high reproducibility comparable to the reproducibility normally seen with a mobile phase gradient.

4. Conclusion

Temperature programming is a practical and useful method of shortening the analysis time and optimizing selectivity for complex mixtures. This programming mode is reproducible and simple to realize on commercially available equipment. The selectivity variation in conjunction with the EOF change in the course of temperature programming makes this mode applicable for development of selective and fast CEC separations.

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