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Temperature programming and gradient elution in reversed-phase chromatography with packed capillary columns

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Abstract

The two major anisocratic elution modes were compared in reversed-phase chromatography with 180 μm I.D. fused-silica capillary columns packed with 6 μm Zorbax SB ODS-silica. By evaluating the retention factors of alkylbenzenes at acetonitrile concentrations varying from 60 to 80% (v/v) in the aqueous eluent and in the temperature range of 30–80°C, it was found that a 5°C change in column temperature and a 1% change in acetonitrile concentration have almost the same effect on retention. This is illustrated by the almost identical chromatograms of an alkylbenzene sample obtained by temperature programming and by gradient elution under the same conditions otherwise and by simulation of the trajectories of the eluent peaks moving down the column. The results suggest that in reversed-phase HPLC with packed capillary columns temperature programming offers an alternative to gradient elution in a relatively narrow range of the required elution strength. Thermodynamic data from isocratic chromatographic measurements were used to predict the retention times of alkylbenzenes in reversed-phase chromatography with temperature programming at different heating rates and column inlet pressures. Temperature programming was used to separate β -lactoglobulins A and B by reversed-phase chromatography. It was also employed concomitantly with gradient elution to enhance the separation of a mixture of four standard proteins. The results indicate that temperature programming could serve as an adjunct to gradient elution by means of fine retention tuning to bring about or increase the resolution of closely related macromolecules. © 1997 Elsevier Science B.V.

Keywords: Temperature programming; Gradient elution; Alkylbenzenes; β -Lactoglobulins; Proteins

1. Introduction

In the chromatography of a mixture containing many components of widely different retention behavior, no equiresolutive chromatogram can be obtained by isocratic elution and this has been referred to as the “general elution problem” [1]. To resolve all the components without compromising analysis

time and sensitivity, anisocratic elution techniques, such as gradient elution in liquid chromatography (LC) and temperature programming in gas chromatography (GC), are used to diminish gradually and in a controlled fashion the retentive power of the column in the course of the chromatographic run [2].

Temperature programming involves a gradual raising of the temperature of the entire column during the run and it is extensively used in GC where the retention decreases strongly with increasing temperature [3]. Temperature programming has received only scant attention in LC [4–6], because

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retention and selectivity are less sensitive to temperature. Furthermore high-performance liquid chromatography (HPLC) columns of conventional dimensions have relatively high heat capacity and rapid change in temperature may result in radial temperature gradients in the column with concomitant loss of efficiency. In HPLC the retention is modulated upon changing the eluent strength of the mobile phase mostly by gradient elution, so that the composition of the eluent entering the column is changed in a gradual fashion with consequent spatial and temporal changes in the eluent composition inside the column [1]. A stepwise change in eluent strength is sometimes called "stepwise gradient", but it will not be discussed here. Recently the problem of combined use in liquid chromatography of temperature and solvent strength was addressed and certain advantages of this approach were pointed out [7,8].

Fused-silica capillaries were first introduced in GC [9]. They are finding increasing applications in LC as packed columns since they offer environmentally correct solvent consumption, high mass sensitivity in comparison to open tubular columns and easy conjugation and compatibility with the mass spectrometer [10–12]. Packed capillary columns are expected to be, due to their relatively low heat capacity and small diameter, more suitable for temperature programming than conventional HPLC columns. Nevertheless, there are practical difficulties associated with the generation of eluent gradients with short delay times accurately and reproducibly at the low volumetric flow-rates. Despite such impediments, we were encouraged by recent results, which revealed certain advantages of high column temperature in the separation of large molecules with high speed and high efficiency by HPLC [13–17]. Here we report on our results in exploring the promise of temperature programmed HPLC with packed capillary columns and compare both theoretically and experimentally the two major anisocratic elution modes, temperature programming and gradient elution, in HPLC.

2. Experimental

2.1. Chemicals

Acetonitrile (ACN) was HPLC grade and pur-

chased from Fisher (Springfield, NJ, USA). Trifluoroacetic acid was obtained from Baxter (Muskegon, MI, USA). Analytical grade benzene and toluene were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ethylbenzene and *n*-butylbenzene were also analytical grade and obtained from Chem-Service (West Chester, PA, USA). Ribonuclease A (bovine pancreas), cytochrome *c* (horse heart), lysozyme (chicken egg white), and β -lactoglobulins A and B (bovine serum) were purchased from Sigma (St. Louis, MO, USA).

2.2. Column preparation

Fused-silica capillaries of 180 μm I.D. \times 350 μm O.D. (Quadrex, Woodbridge, CT, USA) were used to pack the columns. A frit at 8 cm from the outlet end was prepared in situ by heating a thin (about 0.5 mm long) slug of 2 μm fused-silica beads (Glycotech, Hamden, CT, USA) which were kept in place by a tungsten wire of 150 μm diameter. Heat was applied locally by a Model 1000 microtorch (Microflame, Minnetonka, MN, USA) to sinter the particles and to burn off the polyimide outer coating of an approximately 4 mm segment used as the detection window. Zorbax SB, a 6 μm octadecyl silica with a mean pore diameter of 80 Å, was packed into fused-silica capillaries by a procedure described elsewhere [18].

2.3. Instrumentation

Two Model 100 DM syringe pumps and a syringe pump controller for both constant flow and constant pressure modes, on loan from Isco (Lincoln, NE, USA), were used as the eluent delivery system. The liquid chromatograph embodied two heat exchanger coils made from 0.5 m long No. 316 stainless-steel capillary tubing (0.5 mm O.D. \times 0.25 mm I.D.), a 3.2 μl static mixing tee (Upchurch Scientific, Oak Harbor, WA, USA), a Model R4CI4WE internal volume injector with injection volume of 60 nl (Valco Instruments, Houston, TX, USA), a laboratory-made holder for detection with capillary column at high temperature, a Model 2000 UV detector (Thermo Separation Products, Fremont, CA, USA) and a Chromatopac C-R3A integrator (Shimadzu, Columbia, MD, USA). As shown in Fig. 1A, the heat

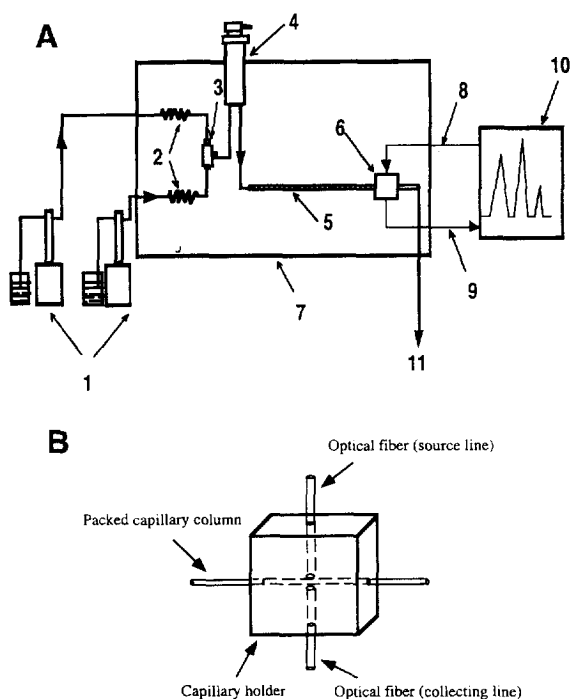


Fig. 1. Flow sheet of the HPLC unit (A) and schematic of the capillary holder (B) for use with packed capillary columns used in gradient elution and/or temperature programming. (1) Reservoirs and dual syringe pump, (2) heat exchangers, (3) static mixer, (4) injector, (5) packed capillary column, (6) capillary holder, (7) temperature programmable oven, (8) source line, (9) collecting line (10) UV detector, (11) waste.

exchangers, the mixer, the injector, the packed capillary column as well as the capillary holder were housed in the oven of a Model 8500 gas chromatograph (Perkin-Elmer, Norwalk, CT, USA) in order to allow heating of the eluent to the column temperature before entering the column which was thermostatted by the oven. The outlet end of the capillary column was connected to an open capillary (50 cm \times 50 μ m I.D.) by using a fused-silica union (Ohio Valley Specialty Chemical, Marietta, OH, USA). The open capillary was used to provide sufficient back pressure to the column and to direct the effluent waste out of the oven.

Two pieces of 40 cm long optical fibers (3M Specialty Optical Fibers, West Haven, CT, USA), the source line (from the light source to the column) and the collecting line (from the column to the photodiode), were used as light guides to monitor the

column effluent by letting the light pass through the detection window located downstream the retaining frit. One end of the source line was coupled to the light source of the detector via a focusing lens, whereas its other end was held in the capillary holder and aligned to the center of the detection window of the capillary as seen in Fig. 1B. The light transmitted through the detection window was collected and guided to the photocell by the collecting line. One end of the collecting line was positioned at the center of the detection window, whereas the other end was coupled to the photodiode. The respective core diameters of the source and collecting lines were 200 and 400 μ m (FG-200-UAT and FG-400-UAT) as they provided the highest signal-to-noise ratio.

2.4. Procedures

2.4.1. HPLC with temperature programming

Temperature programmed HPLC was mostly carried out under isobaric conditions, i.e., at constant column inlet pressure, and at heating rates of 10, 20 or 30°C per min. Acetone was used as the inert tracer for the measurement of the mobile phase hold-up times. Each data point is the average from at least three runs.

2.4.2. HPLC with gradient elution

In gradient elution with packed capillary columns, the dual syringe pumps were used under isobaric conditions. First the system was rinsed by the starting eluent using pump A, then pump A was turned off. To start gradient elution, pump B filled with the gradient former was brought to the operating column inlet pressure and began pumping in a constant pressure mode. Assuming perfect mixing of the two eluents in the mixer, the organic modifier concentration at time t , C_t , depends on the mixer volume, V , and flow-rate F as [19]

$$C_t = C_f - (C_f - C_0) \exp(-Ft/V) \quad (1)$$

where C_0 and C_f are the respective starting and final concentrations. Eq. (1) was used to calculate the ACN concentration at the column inlet as a function of time.

3. Results and discussion

3.1. Comparison of the two major anisocratic elution modes

The chromatograms of alkylbenzenes and polyaromatic hydrocarbons obtained in reversed-phase chromatography with a packed capillary column by isocratic elution and temperature programming are depicted in Figs. 2 and 3. Pairwise comparison of the chromatograms A and B reveals that temperature programming offers advantages with respect to isocratic elution that are similar to those obtained with

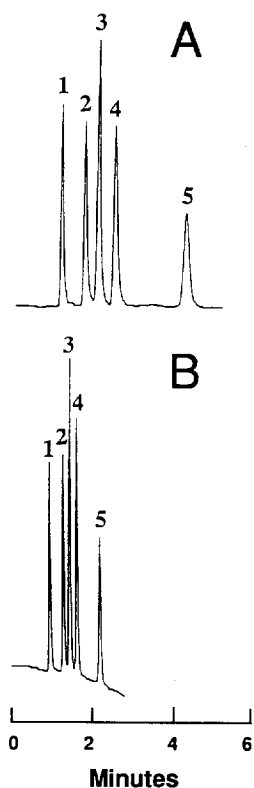


Fig. 2. Chromatograms of alkylbenzenes obtained by (A) isocratic elution at 30°C and (B) linear temperature programming under isobaric conditions from 30°C at a rate of 30°C/min. Column: 27 cm × 180 μm, packed with 6 μm Zorbax ODS-silica with mean pore diameter of 80 Å. Eluent: 75% (v/v) ACN in water; column inlet pressure: 100 bar; starting flow-rate: 4 μl/min. Detection wavelength: 254 nm. Order of peaks: (1) acetone, (2) benzene, (3) toluene, (4) ethylbenzene, (5) *n*-butylbenzene.

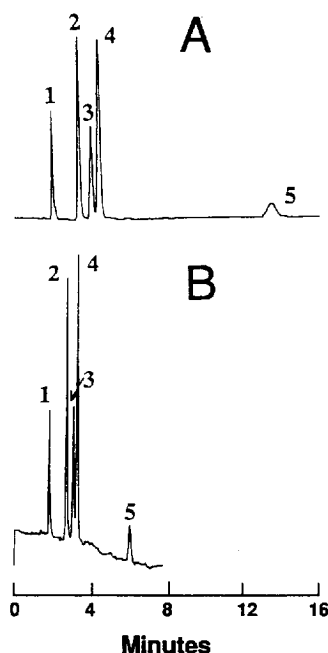


Fig. 3. Chromatograms of polyaromatic hydrocarbons obtained by (A) isocratic elution at 30°C and (B) linear temperature programming under isobaric conditions from 30°C at a rate of 10°C/min. Column: 24 cm × 180 μm, packed with 6 μm Zorbax ODS-silica with mean pore diameter of 80 Å. Eluent: 80% (v/v) ACN in water; column inlet pressure, 120 bar; starting flow-rate, 4 μl/min. Detection wavelength, 254 nm. Order of peaks, (1) naphthalene, (2) fluoranthene, (3) 2,3-benzofluorene, (4) 1,2-benzanthracene, (5) 9,10-diphenylanthracene.

gradient elution by reducing the time of analysis and by sharpening late eluting peaks.

In order to compare temperature programming and gradient elution in the reversed-phase chromatography of alkylbenzenes with a packed capillary column and ACN–water mixtures as the mobile phase, the retention factors were measured both isocratically and isothermally at ACN concentrations ranging from 60 to 80% (v/v) in the eluent and at column temperatures from 30 to 80°C in 10°C intervals. The pertinent van't Hoff plots of the data measured with the eluents rich in organic modifier are linear as exemplified by those obtained with 70% (v/v) ACN in the mobile phase and shown by the solid lines in Fig. 4. In these cases, i.e., in the absence of heat capacity effects [20], the dependence of the logarithmic retention factor, κ , on the absolute

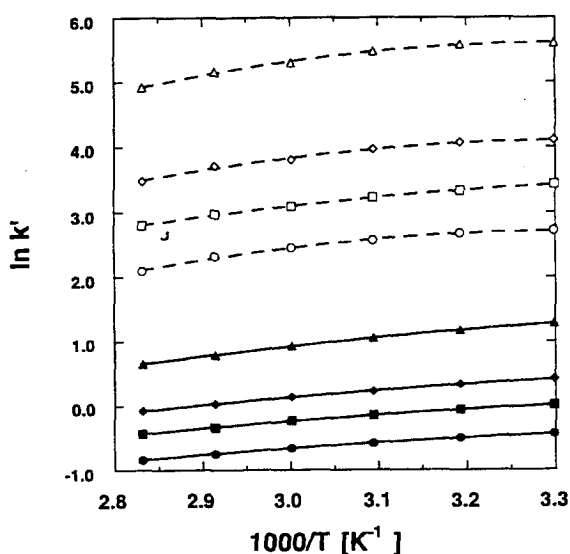


Fig. 4. Plots of the logarithmic retention factors at 70% ACN in the eluent (solid symbols) and the intercepts of κ vs. ϕ plots, κ_w , (open symbols) against the reciprocal absolute temperature. Parameter κ_w represents the logarithmic retention factor with the eluent containing no organic modifier when κ vs. ϕ plots are linear over entire ϕ range. Solid lines are linear fit to the solid symbols and dashed lines represent polynomial fits to the data shown by the open symbols. (● and ○) Benzene, (■ and □) toluene, (◆ and ◇) ethylbenzene, (▲ and △) *n*-butylbenzene.

temperature, T , at a given modifier concentration is given by

$$\kappa = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \phi \quad (2)$$

where R is the universal gas constant and ϕ is the

Table 2

Parameters \bar{s} and κ_w

Eluite	\bar{s}	κ_w at 30°C
Benzene	4.38	2.73
Toluene	4.72	3.44
Ethylbenzene	5.20	4.11
<i>n</i> -Butylbenzene	6.15	5.61

Parameter \bar{s} was obtained from the average slope of the κ vs. ϕ plots made at 10°C intervals in the temperature range from 30 to 80°C. Parameter κ_w was obtained from the intercepts of κ vs. ϕ plots at 30°C. Other experimental conditions as in Table 1.

phase ratio. Since the van't Hoff plots are almost linear, the retention enthalpies, ΔH^0 , were evaluated by using Eq. (2) and presented in Table 1. As the ϕ values are not available, instead of the retention entropies, the intercept of the van't Hoff plots are listed in Table 1. The quantities in Table 1 are commensurate with those reported in the literature for similar reversed-phase chromatographic systems using water lean eluents [21].

Plots of κ against the volume fraction of ACN in water, ϕ , were also linear and nearly parallel at any given temperature, they are believed to represent the relationship

$$\kappa = \kappa_w - s\phi \quad (3)$$

with s and κ_w as constants. Since the slopes of the plots decreased slightly with increasing temperature, average values, \bar{s} , were taken and listed in Table 2. Assuming Eq. (3) holds for the entire ϕ range, the intercept, κ_w , represents the logarithmic retention factor with the eluent containing no organic modifier.

Table 1

Retention enthalpies and the intercepts of the van't Hoff plots obtained with isocratic elution at different ACN concentrations

Eluite	$-\Delta H^0$ (kcal/mol)			$-\left(\frac{\Delta S^0}{R} + \ln \phi\right)$		
	ACN concentration (v/v) (%)			ACN concentration (v/v) (%)		
	60	70	80	60	70	80
Benzene	1.93	1.72	1.70	3.10	3.27	3.63
Toluene	2.05	1.90	1.85	2.81	3.11	3.45
Ethylbenzene	2.20	2.09	2.07	2.62	3.03	3.46
<i>n</i> -Butylbenzene	2.74	2.67	2.68	2.54	3.12	3.68

Column: 25 cm × 180 μ m, packed with 6 μ m Zorbax ODS-silica with mean pore diameter of 80 Å. Flow-rate: 4 μ l/min. Temperature: 30 to 80°C at 10°C intervals.

The κ_w values were obtained by extrapolation and plotted against the reciprocal of absolute temperature to obtain the curved dashed lines shown in Fig. 4. The non-linearity of these van't Hoff plots can be attributed to heat capacity effects that are observed with most processes based on the hydrophobic effect [22,23].

By differentiating Eq. (2) with respect to T and Eq. (3) with respect to φ , we can express the ratio of changes required in temperature and in organic modifier concentration to obtain the same infinitesimal change in the retention factor as follows

$$\frac{dT}{d\varphi} = -\frac{sRT^2}{\Delta H^0} \quad (4)$$

Eq. (4) is only valid at a fixed mobile phase composition and temperature where parameters ΔH^0 , ΔS^0 , s and κ_w are independent of φ and T . Under the experimental conditions stated in Tables 1 and 2, these parameters do not change significantly with T and φ , therefore we may use Eq. (4) and the data in Tables 1 and 2 to estimate the increase in organic modifier concentration required to bring about the same change in the retention factors as that occurs upon the increase in column temperature. We find that in reversed-phase chromatography of alkylbenzenes at temperatures ranging from 30 to 80°C an approximately 1% increase in the ACN concentration is equivalent to an approximately 5°C increase in temperature. The $dT/d\varphi$ values increase slightly with the temperature yet much more significantly with the organic modifier concentration. However, for large molecules, such as proteins, the $dT/d\varphi$ value is likely to be quite different. For instance, in reversed-phase HPLC of lysozyme, we estimated by using Eq. (4) and the retention data measured by isocratic elution [16] that the increase in the temperature corresponding to a 1% increase in the ACN concentration varies from 6 to 16°C with an average of 10°C at temperatures from 30 to 120°C and in the ACN concentration range between 24 to 35% (v/v).

By using the estimated average value of 5°C/1% ACN for $dT/d\varphi$, we could adjust the conditions for the separation of alkylbenzenes by reversed-phase chromatography using gradient elution and isobaric temperature programming so that the two anisocratic techniques yielded essentially the same elution pat-

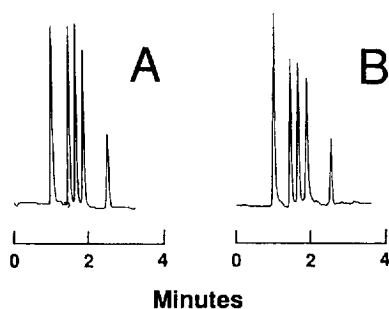


Fig. 5. Reversed-phase chromatography of alkylbenzenes obtained by (A) gradient elution and (B) isobaric temperature programming. The conditions were predicted by using 5°C/1% ACN for $dT/d\varphi$. Column: 27 cm \times 180 μ m, packed with 6 μ m Zorbax ODS-silica with mean pore diameter of 80 Å. Eluent: (A) gradient elution from 70 to 85% (v/v) ACN in water; (B) 70% (v/v) ACN in water. Column inlet pressure: 100 bar. Temperature: (A) 30°C; (B) 30 to 120°C at 30°C/min. Detection wavelength: 254 nm. Order of peaks as in Fig. 3.

tern. The chromatograms shown in Fig. 5 were obtained with a linear eluent gradient or temperature program with a 6% increase in the ACN concentration per min or a heating rate of 30°C per min, respectively. With gradient elution the sample was injected with a delay of 1 min to account for the about 4 μ l dwell volume between the gradient mixer and column inlet. Fig. 5 demonstrates that the two chromatograms are indeed very similar in terms of both the retention times and the peak widths.

The trajectories of the peaks in the column were calculated with the data in Tables 1 and 2 for both gradient elution and temperature programming. In the isocratic and the two anisocratic elution modes, the migration rate of the eluite at any axial distance in the column, $u_{e,z}$, can be expressed as [20]

$$u_{e,z} = \frac{dz}{dt} = \frac{L}{t_m(1+k'_t)} \quad (5)$$

where z is the migration distance of the peak center, t is the migration time, L is the total column length, k'_t is the local retention factor at position z at time t . The mobile phase hold-up time, t_m , is constant under isorheic conditions, i.e., at constant flow-rates. From Eq. (5) we obtain the dimensionless distance of the center of the eluite peak into the column, z/L , by the integral

$$\frac{z}{L} = \int_0^t \frac{1}{t_m(1+k'_t)} dt \quad (6)$$

In isocratic elution, the peak traverses the column at a constant velocity, thus Eq. (6) simplifies to

$$\frac{z}{L} = \frac{t}{t_m(1+k'_t)} \quad (7)$$

When Eq. (3) is valid for the chromatographic system with linear gradient elution at fixed column temperature, the logarithm of the local retention factor at time t , κ_t , can be written as

$$\kappa_t = \kappa_w - s(\varphi_0 + \dot{\varphi}t) \quad (8)$$

where φ_0 is the volume fraction of the organic modifier in the starting eluent and $\dot{\varphi}$ is the gradient steepness defined as $d\varphi/dt$ [1]. Combining Eqs. (6) and (8), we obtain [1,24,25] that

$$\frac{z}{L} = \int_0^t \frac{1}{t_m \{1 + \exp[\kappa_w - s(\varphi_0 + \dot{\varphi}t)]\}} dt \quad (9)$$

Eq. (9) describes the trajectory of the peak under consideration in the column during gradient elution under isothermal conditions.

When Eq. (3) holds and the column temperature is

uniform, in linear temperature programming with constant eluent composition, κ_t can be expressed as

$$\kappa_t = -\frac{\Delta H^0}{R(T_0 + rt)} + \frac{\Delta S^0}{R} + \ln \phi \quad (10)$$

where T_0 is the column temperature at the start of the program and r is the heating rate. The combination of Eqs. (6) and (10) yields the following expression

$$\frac{z}{L} = \int_0^t \frac{1}{t_m \left\{ 1 + \exp \left[\left(\frac{\Delta S^0}{R} + \ln \phi \right) + \frac{-\Delta H^0}{R(T_0 + rt)} \right] \right\}} dt \quad (11)$$

for the peak trajectory when temperature programming is employed under isorheic conditions and at constant mobile phase composition.

Eqs. (9) and (11) were solved numerically by the computer with the data in Tables 1 and 2, and the respective trajectories simulating the results of linear gradient elution and temperature programming in HPLC are illustrated in Fig. 6. It is seen that the peaks move with geometrically increasing velocities in both gradient elution and temperature programming and reach the outlet of the column at almost the same time under the conditions illustrated in Fig. 6.

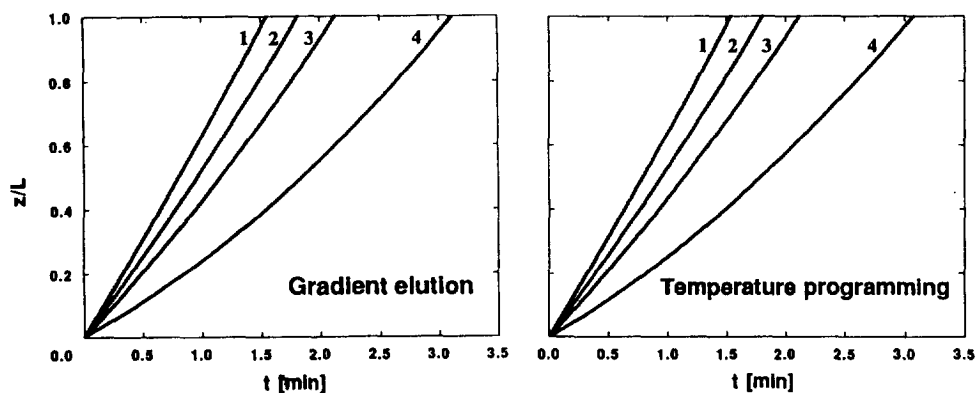


Fig. 6. Trajectories of the alkylbenzene peaks in the reversed-phase chromatography of alkylbenzenes with gradient elution and temperature programming under isorheic conditions. The gradient elution was from 70 to 85% ACN concentration with steepness of 6% ACN increment per min at 30°C. The heating rate in temperature programming was 30°C per min from 30 to 120°C. In both cases, t_m was set to 1.0 min. Order of curves: (1) benzene, (2) toluene, (3) ethylbenzene, (4) *n*-butylbenzene.

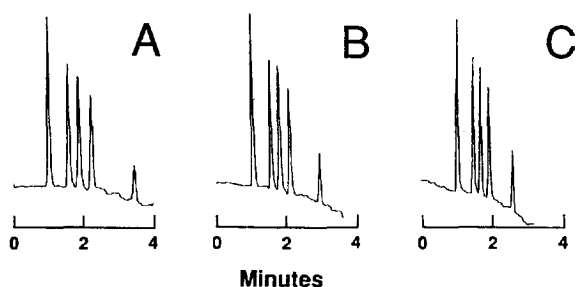


Fig. 7. Effect of the heating rate in temperature programmed HPLC of alkylbenzenes. Column: 25 cm \times 180 μ m, packed with 6 μ m Zorbax ODS-silica with mean pore diameter of 80 \AA . Eluent: 70% (v/v) ACN in water. Column inlet pressure: 94 bar. Starting temperature: 30°C; heating rate: (A) 10°C/min, (B) 20°C/min and (C) 30°C/min. Detection wavelength: 254 nm. Order of peaks as in Fig. 3.

3.2. Effect of heating rate and flow-rate in temperature programming

In HPLC the effect of operational variables are well understood with gradient elution [1], but not with temperature programming. In the following we shall examine the role of heating and flow-rates (or column inlet pressure) that are the major operational parameters in temperature programming. Their effect on the separation of alkylbenzenes is illustrated by the respective chromatograms in Figs. 7 and 8 which show that by using fast heating rate and high column

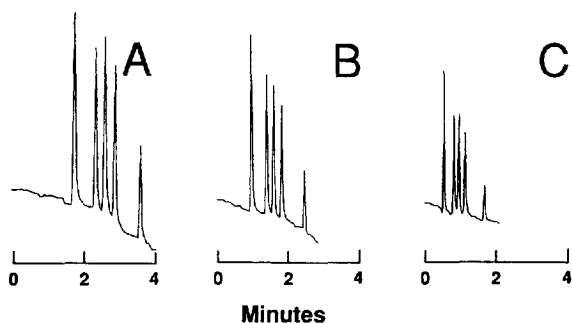


Fig. 8. Effect of column inlet pressure in temperature programmed HPLC of alkylbenzenes. Column: 25 cm \times 180 μ m, packed with 6 μ m Zorbax ODS-silica with mean pore diameter of 80 \AA . Eluent: 70% (v/v) ACN in water. Column inlet pressure: (A) 46 bar, (B) 94 bar and (C) 180 bar. Starting temperature: 30°C; heating rate: 30°C/min. Detection wavelength: 254 nm. Order of peaks as in Fig. 3.

inlet pressure the separation time can be shortened significantly.

In order to examine the effect of these parameters quantitatively on the chromatographic retention in temperature programming we shall use the thermodynamic data in Table 1 and Eq. (11) that is valid under isorheic conditions. However, in our experiments a syringe pump was used under isobaric conditions to obtain higher reproducibility than at constant flow-rate [26]. When the column inlet pressure is kept constant in HPLC, the flow-rate increases gradually due to a decrease in the viscosity of mobile phase during temperature programmed run and consequently the mobile phase hold-up time in Eq. (11) is no longer a constant. In order to account for the effect of changing viscosity we start with the ratio of the mobile phase hold-up time and the viscosity at a given temperature which is constant under isobaric conditions so that

$$t_{m,T} = t_{m,T_0} \frac{\eta_T}{\eta_{T_0}} \quad (12)$$

where η_{T_0} and η_T are the viscosities whereas t_{m,T_0} and $t_{m,T}$ are the mobile phase hold-up times at the starting temperature T_0 and temperature T , respectively. In turn the viscosity of the mobile phase at temperature T can be estimated by the Arrade equation [27]

$$\eta_T = \exp(A + B/T) \quad (13)$$

where A and B are constants that are available for water-ACN mixtures of chromatographic interest [16]. Upon combining Eqs. (11)–(13), the retention time of a peak, t_R , can be evaluated by the following expression

$$t = \int_{t_{m,T_0} \exp \left\{ B \left[\frac{1}{T_0} - \frac{1}{(T_0 + \tau)} \right] \right\}}^{t_R} \frac{dr}{1 + \exp \left[\left(\frac{\Delta S^0}{R} + \ln \phi \right) + \frac{-\Delta H^0}{R(T_0 + \tau)} \right]} \quad (14)$$

in linear temperature programmed HPLC under isobaric conditions.

By solving Eq. (14) for r and t_{m,T_0} using the data in Table 1, the retention times with temperature programming were calculated for different heating rates and column inlet pressures and the results were compared with the corresponding experimental data

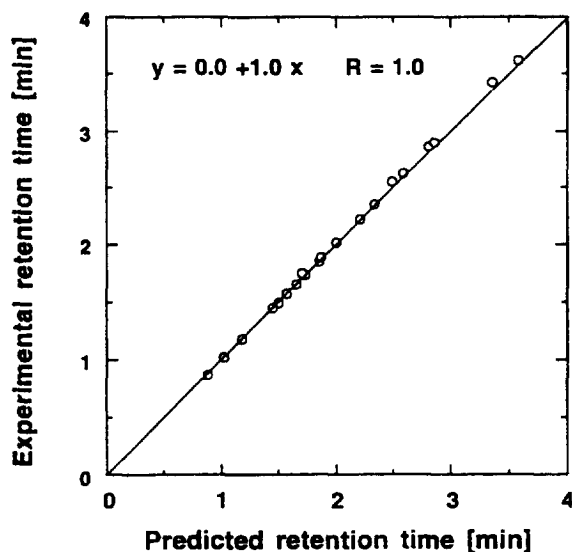


Fig. 9. Plot of experimental retention time vs. retention time calculated by Eq. (14) for the same conditions with linear isobaric temperature programming. The experimental conditions were the same as in Fig. 7. The data listed in Table 1 and $1.240 \cdot 10^3$ K for parameter B were used in the calculations.

by the correlation shown in Fig. 9. Each experimental data point is the average value of three measurements and the relative standard deviation is less than 1% which suggests that under the conditions employed temperature programming is an isocratic elution method of good reproducibility. The agreement between the calculated and measured data as shown in Fig. 9 confirms the validity of the predictions and also indicates that during programming there is no significant lag between the temperature of the oven and that of the packed capillary column. The rapid response of the capillary column to the change in temperature is likely due to the small diameter and low heat capacity of the column. The results further confirm that the thermodynamic data can be used to predict retention in temperature programming and thus to optimize the operational variables for a given separation. In turn the above results also imply that the retention enthalpy and entropy can be determined from temperature programmed runs with at least two different heating rates. This method is expected to be less time consuming than the series of isothermal measure-

ments conventionally employed in the measurement of data for van't Hoff plots.

3.3. HPLC of macromolecules by temperature programming

Unlike gradient elution, temperature programming has not been employed in the separation of large molecules by HPLC because of the relatively narrow range of elution strength. For the separation of closely related macromolecules, however, temperature programming may offer results similar to those obtained by a shallow gradient. This is illustrated in Fig. 10 with the separation of β -lactoglobulins A and B using isocratic elution at 50 and 80°C as well as temperature programming from 30°C at a heating rate of 30°C/min. Comparison of the chromatograms show that temperature has a strong effect on the retention of the proteins due to their high retention

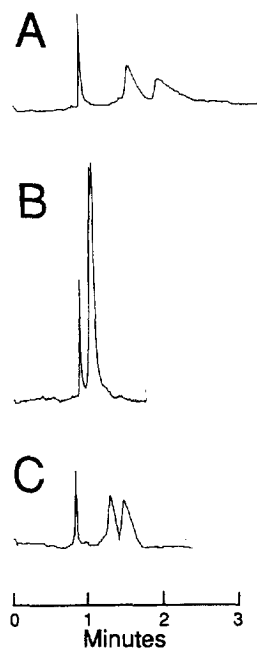


Fig. 10. Separation of β -lactoglobulins. Column: 25 cm \times 180 μ m, packed with 6 μ m Zorbax ODS-silica with mean pore diameter of 300 Å. Eluent: 48% (v/v) ACN in water containing 0.1% TFA. Column inlet pressure: 150 bar. Temperature: (A) 50°C, (B) 80°C and (C) programming from 30°C at 30°C/min. Detection wavelength: 214 nm. Order of peaks: (1) system peak, (2) β -lactoglobulin B, (3) β -lactoglobulin A.

enthalpies [16] and that the resolution of the two closely related proteins is higher with temperature programming than by isocratic elution.

Temperature programming and gradient elution were also used simultaneously for the separation of a mixture of four standard proteins. Chromatograms A and B in Fig. 11 were obtained isothermally at 30 and 80°C by gradient elution under isobaric conditions. Chromatogram C was obtained by a combination of temperature programming from 30 to 80°C and gradient elution from 0 to 70% (v/v) ACN in the eluent. The separation at 80°C gave the shortest analysis time whereas the combined use of the two

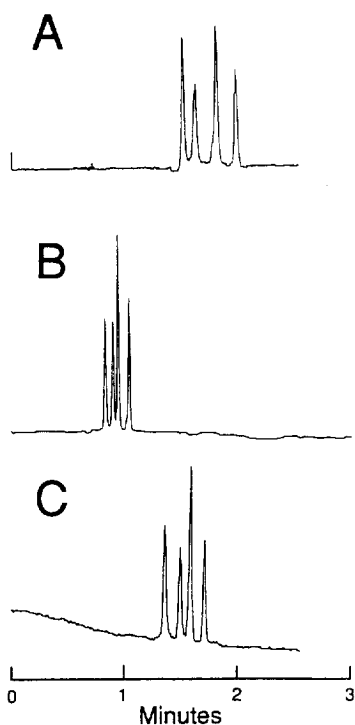


Fig. 11. Chromatograms of a standard protein mixture by gradient elution at two temperatures (A and B) and by combined temperature programming and gradient elution (C). Column: 25 cm \times 180 μ m, packed with 6 μ m Zorbax ODS-silica with mean pore diameter of 300 Å. Eluent: gradient elution from 0 to 70% (v/v) ACN in water containing 0.1% TFA. Column inlet pressure: 150 bar. Temperature: (A) 30°C, (B) 80°C and (C) programming from 30°C at 10°C/min. Detection wavelength: 214 nm. Order of peaks: (1) ribonuclease A, (2) cytochrome *c*, (3) lysozyme, (4) β -lactoglobulin B.

anisocratic elution modes offered the best compromise between resolution and analysis time.

4. Conclusions

The potential of temperature programming is demonstrated in reversed-phase chromatography with packed capillary columns. Temperature programmed HPLC was found to be a promising alternative to gradient elution in certain cases where the required range of elution strength is not too wide. In such cases temperature programming may replace gradient elution and may gain significance because of the technical problems associated with gradient elution at very low flow-rates. For the separation of multi-component mixture of large molecules where gradient elution is required, temperature programming used simultaneously could enhance the resolution of closely related macromolecules. Studies on operational variables in temperature programming revealed that it can be a highly reproducible method and by knowing the thermodynamic data of the chromatographic system, the retention of temperature programmed runs can be predicted accurately. Nevertheless, gradient elution has a great advantage by being capable of spanning a significantly wider eluent strength range than temperature programming can in the present chromatographic practice and will most likely still be the dominant anisocratic elution mode in HPLC with columns of conventional dimension. With packed capillary columns, temperature programming and gradient elution may complement each other to optimize separation speed and resolution of the components of interest.

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