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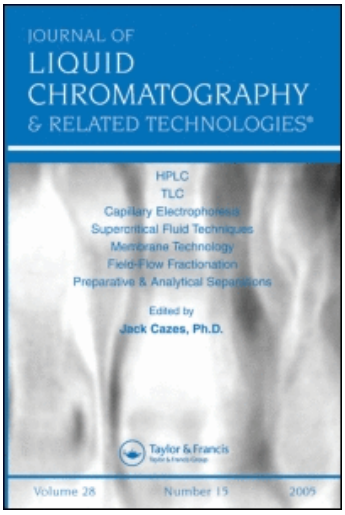

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TEMPERATURE PROGRAMMING IN OPEN TUBULAR LIQUID CHROMATOGRAPHY

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ABSTRACT

We have examined the separation of test mixtures by linear and step temperature gradients in open tubular capillary liquid chromatography, in reverse and normal-phase modes. Separations using temperature gradients with the upper temperature limit of 433 K were compared to isothermal runs in terms of chromatographic efficiency, analysis time and selectivity. When a temperature gradient (363 K to 433 K in 12 min) was utilized in reverse phase separation of chlorobenzenes, a 40 % reduction in analysis time, compared to an isothermal separation at 363 K, was accomplished.

INTRODUCTION

Liquid chromatography in capillary columns is a fast developing technique, offering the potential of increased performance in comparison to the well established wide-bore columns. In order to reach high column efficiency (one million theoretical plates) needed for the resolution of complex mixtures, open tubular columns are being developed for liquid chromatography.[1-8] While isocratic and isothermal separations are limited in their application range, the mobile phase gradient elution mode offers the tremendous advantage of tuning selectivity by eluent composition in the same separation.

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This elution mode also offers easy access to sample enrichment.[9] Control of chromatographic separation selectivity in HPLC by physical parameters such as temperature, is particularly interesting. The use of elevated temperatures for reverse phase HPLC has been advocated, as a means to increase column efficiency, shorten analysis time and alter separation selectivity.[3,4,10-16] The effect of temperature changes on separation may be compared with the effect of changing the composition of mobile phases. A change in column temperature can lead to significant changes in band position (selectivity) whenever the shape and the size of two compounds differs significantly. It can also affect peak shape, especially when adsorption effects are profound at low temperatures.

Temperature programming can be a practical alternative to gradient elution. Lawrence and Scott[15] applied temperature programming with conventional size LC columns for a normal phase separation. From the temperature dependence of k', the heats of solute transfer from the stationary to mobile phase were determined by Knox and Vasvari.[17] They have indicated the potential benefits of temperature gradient liquid chromatography (TG-LC). The effect of increasing temperature on analyte retention times, peak symmetry, and chromatographic efficiency in isothermal separation was investigated by Grushka.[18] Snyder[19] compared mobile phase gradient elution and temperature programming, while Snyder and Kirkland[20] elaborated on temperature programming. The use of temperature gradients to achieve reverse phase liquid chromatographic separations for systems detected by plasma techniques was demonstrated by W. R. Biggs,[21] while Renn and Sinovec[22] examined the effect of temperature on separation efficiency in size exclusion chromatography. McNair[23] evaluated the potential of temperature programming in conjunction with microbore HPLC columns, and concluded that this produces faster analyses and increased efficiency.

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The effects of temperature changes for achieving separation are complex, involving changes in thermodynamic parameters, efficiency and separation time. The relationship between the capacity factor, k', and the column temperature can be estimated by the equation:[13]

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \phi \qquad (1)$$

where R is the universal gas constant, T is the temperature in kelvin, while ΔH and ΔS denote enthalpy and entropy of a solute molecule transfer from the stationary phase to the mobile phase, respectively. The phase ratio of the column, ϕ, represents the stationary to mobile phase volume ratio. Differences in sorption behavior in HPLC are characterized by enthalpy changes. The change in capacity factor with temperature can be put in a form which represents the capacity factor ratio k'_1/k'_2 at two different temperatures T_1 and T_2:[24]

$$\frac{k'_1}{k'_2} = \exp\left[\frac{\Delta H(T_2 - T_1)}{RT_1T_2}\right] \quad (2)$$

As predicted by the above equation, the effect of temperature programming on the capacity factor ratio will strongly depend on the solute ΔH value. For the same temperature change, the solutes with large ΔH will be strongly affected, whereas for solutes with small ΔH the temperature change will not produce significant changes in the capacity ratio. When reduction in retention times of the late eluting solutes is desirable, changing the column temperature can provide performance similar to that of solvent gradient systems.

Using a temperature gradient, the retention of the peaks could be selectively influenced. This fact might contribute to the solution of some common chromatographic problems. Early eluted substances usually show a sharp peak-form, but often an insufficient resolution. On the other hand, the longer compounds are retained the broader the resulting peaks are. These peaks are difficult to integrate and in some cases, they may even be undetectable, due to their fusion with the base-line.

The aim of this work is to study the effect of temperature programming on retention and efficiency in open tubular liquid chromatography (OTLC).

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EXPERIMENTAL SECTION

The instrumental set-up utilized for step temperature gradients does not differ from the instrumental set-up used for isothermal high temperature separation, which has been described elsewhere.[9] When linear temperature gradients were performed, a laboratory-constructed column oven was replaced with a column oven (forced air heating) equipped with an HPLC microprocessor programmer 50 A/B from Knauer (Berlin, Germany). This set-up, depicted in Fig. 1, enabled control of the temperature gradient with a maximum heating rate of 6°/min and a maximum column temperature of 433 K. To prevent possible solute precipitation in the detector cell, the cell had to be heated. One problem encountered with the experimental set-up for step temperature gradients used in this study, was strong base-line sloping (UV detection) caused by changes of refractive index with temperature.[25,26] In order to avoid this baseline sloping, the detector cell was taken from the column oven and placed in a separate heating unit. The cell was kept at a constant temperature, which corresponded to the midpoint temperature of the linear gradient. The use of a restrictor to generate high pressure allows one to work well above the normal boiling point of the solvent, thus increasing the effective useful temperature programming range. A SB-methyl-100 column ($l = 5$ m, 50 μm i.d., film thickness $d_f = 0.25$ μm) was purchased from Lee Scientific (Salt Lake City, UT, U.S.A.), while an OV-1 7 column ($l = 20$ m, 50 μm i.d.,

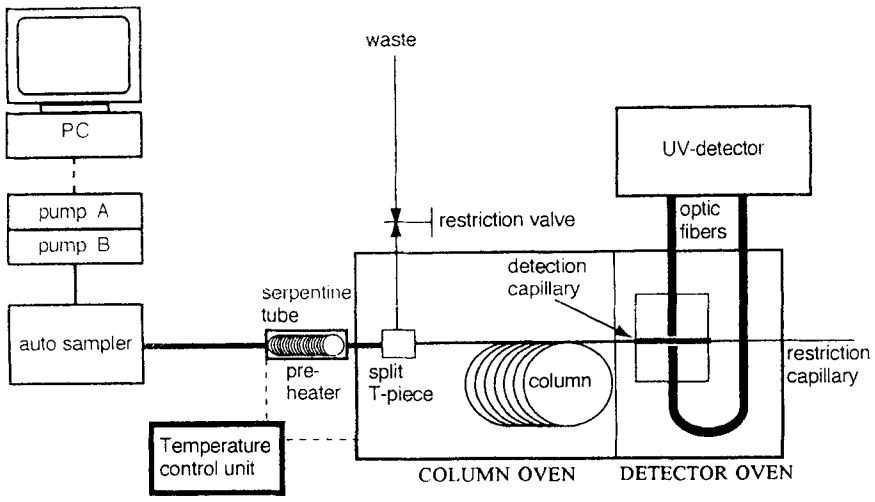

Figure 1. Experimental set-up for linear temperature programming.

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d_f = 0.1 μm) and an OV-1701 column (l = 5 m, 50 μm i.d., d_f = 0.4 μm) were obtained from Macherey-Nagel, Oensingen, Switzerland. Bare fused silica capillaries used either as restrictors, or as analytical columns (14.4 m, 50 μm i.d.) for normal-phase LC separation, were from Polymicro Technologies (Phoenix, AZ, U.S.A.). The testosterone-esters (acetate, cypionate, propionate, enanthate, and benzoate) were obtained from Sigma (Buchs, Switzerland). The chlorobenzenes were obtained from Aldrich-Chemie (Steiheim, Germany) or Fluka (Buchs, Switzerland). Methanol and n-hexane were of HPLC grade from Rathburn (Welkerburn, UK). Molecular sieve beads 0.3 nm , 10 mesh, from Merck (Darmstad, Germany) were used to dry n-hexane.

RESULTS AND DISCUSSION

Reversed-Phase Separation

OTLC is ideal for temperature programming in LC, due to a rapid heat transfer through thin silica walls (~ 100 μm) and low volumetric flow rates which promote fast heating and equilibration of the eluent. Different shapes of temperature programs in chromatography can be classified as: step, linear, convex, concave, and multisegment. The simplest program is a single step in which the column temperature is changed instantaneously at a certain time. In OTLC, the volume of mobile phase is much larger than the stationary phase volume. Because

Table 1

Column Plate Number at 373 K and 423 K and Apparent Plate Number for the Step Gradient (373-423 K). The Other Conditions as in Figure 1

Solute	373 K	423 K	Step Gradient
Testosterone-benzoate	6434	18767	16515
Testosterone-enanthate	6089	16530	14856

of the low flow rates of OTLC (the optimum flow rate for a 50 μm i.d. column is below 1 μL/min) the elution time of the unretained compound (t_o) is quite large. Therefore, the start of the temperature gradient should be delayed for the value of t_o. If the gradient was started immediately after injection, the system would reach the final temperature even before an unretained compound eluted from the column. In this case, the effect of the step temperature gradient would be as if the whole run was performed isothermally at the final step temperature. This should be taken into consideration when separation procedures using temperature gradients are optimized. All step gradients performed with the OV-17 column were started 40 min after sample injection. An increase in column temperature during the separation greatly decreases the retention time and increases the relative peak sensitivity. There are several mechanisms responsible for sample retention in reverse phase HPLC (hydrophobic interactions, adsorption on residual silanols and solute solubility in the mobile phase and stationary phase).[27] The relative contribution of these processes are altered when a temperature gradient run is performed. Furthermore, solute mass transfer in the mobile and stationary phases, a kinetic process, is also changed in the temperature gradient mode. Combined thermodynamic and kinetic effects in the temperature gradient mode lead to peak compression. In Table 1, the plate numbers obtained for testosterone-benzoateand testosterone enanthate in isothermal runs (373 K and 423 K) were compared with the apparent plate numbers for the same solutes, obtained by the step gradient 373-423 K. Clearly, only minimal efficiency (ten percent) has been sacrificed by the temperature programming when compared with the isothermal run at 423 K. The commercial capillary columns utilized in this work, were designed for application in supercritical fluid chromatography and have limited stability at elevated temperature when aqueous mobile-phases are used.[4] By running temperature programming, the analytical columns are exposed to the elevated temperatures for shorter periods of time then when isothermal runs are performed. A ten percent loss of efficiency is than negligible in comparison to the benefit of prolonging column lifetime. The separations of five testosterone-esters obtained under isothermal conditions at 373 K (chromatogram I) and with step gradients from 373 K to 383 K, and from 373 K to 393 K (chromatograms II and III) are shown in Fig. 2.

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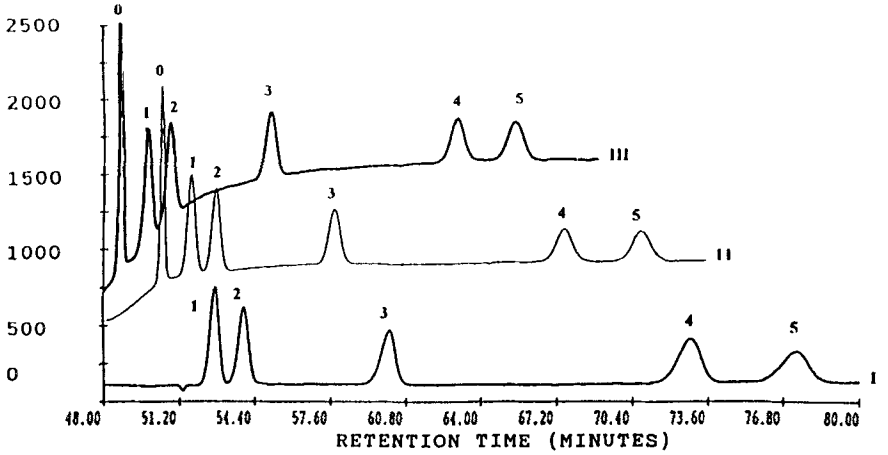

Figure 2 . Effects of step-temperature gradients on the separation of a mixture of testosterones. Column OV-17, 20 m x 50 μm i.d., d_f = 0.1 μm; MeOH/H_2O 30:70 (v/v), flow rate = 1.0 μL/min: UV detection at 241 nm. Peaks: 0) potassium iodide, 1) testosterone-acetate, 2) testosterone-propionate, 3) testosterone-benzoate, 4) testosterone-enanthate, 5) testosterone-17β cypionate. I = 373 K, II = isothermal 41 min at 373 K then step gradient to 383 K, III= isothermal 41 min at 373 K then step gradient to 393 K.

The separation of a test mixture of chlorobenzenes using linear temperature gradients in OTLC in reverse phase mode were examined. In Fig. 3, a comparison was made between an isothermal separation at 363 K (chromatogram I) and two linear step gradients from 363 K to 433 K at 2°/min and 6°/min, chromatograms II and III, respectively. Tropolone was used as unretained compound to determine t_o.

The linear temperature gradients were started 7 min after sample injection. As expected, early eluting solutes were not significantly affected by the temperature gradients. Shorter analysis times for the solutes with larger k' (peaks labeled 4, 5 and 6) and increased peak sensitivity (inversely proportional to peak width), were observed. There was a significant improvement in the relative peak sensitivity in the gradient mode compared with isothermal elution.

The excellent efficiency of the temperature-programmed separation can be attributed to initial concentration of analytes on the column due to the weak mobile phase, particularly for strongly retained components. As the temperature is raised, the analytes desorb from the stationary phase and migrate down the column with more favorable phase transfer kinetics.

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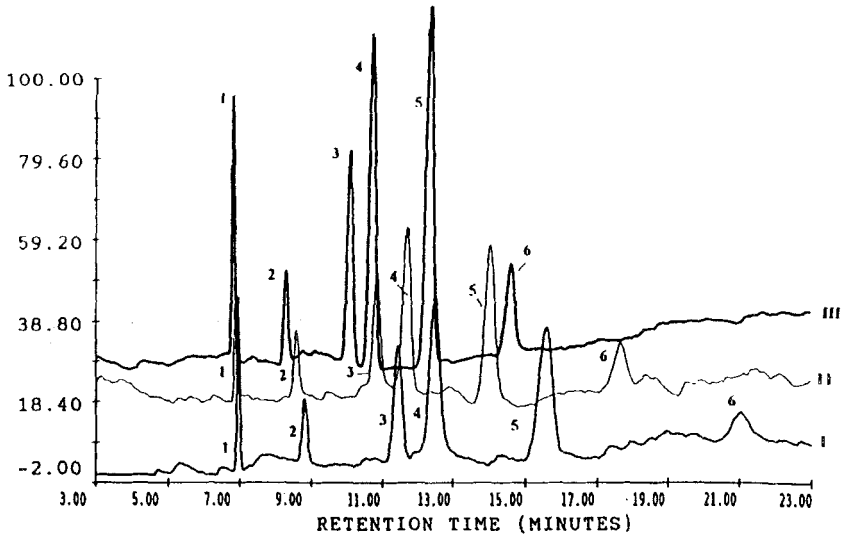

Figure 3. *Separation of a mixture of chlorobenzenes.* Column SB-methyl-100, 5 m x 50 μm i.d., d_f = 0.25 μm; MeOH/H_2O 25:75 (v/v), flow rate = 0.75 μL/min; UV detection at 210 nm. Peaks: 1) tropolone, 2) 1,4-dichlorobenzene,3) 1,2,4 tri-chlorobenzene,4) 1,2,4,5-tetrachlorobenzene, 5) pentachlorobenzene, 6) hexachlorobenzene. I = 363 K, II = isothermal 7 min at 363 K then gradient 2 °/min to 433 K, III = isothermal 7 min at 363 K then gradient 6 °/min to 433 K.

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Normal-Phase Separation

As expected, a pure untreated fused-silica column shows selectivity towards aromatic hydroxy compounds. The separations of the test compounds obtained on a 14.4 m x 50 μm fused-silica column, with n-hexane as mobile phase, are displayed in Fig. 4. The separations obtained at 301 K, 333 K, and by temperature programming: isothermal at 301 K for 20 min than to 373 K with a heating rate of 6°/min, are labeled as chromatograms I-III, respectively. A qualitative comparison of these separations shows several apparent changes in band spacing and band shape, due to this difference in temperature. For example, the two peaks, labeled 2 and 3, displayed a profound peak tailing at 301 K, caused by a strong solute adsorption on the silanol groups. At elevated temperatures (at 333 K and in the temperature gradient runs), the peak symmetry significantly improved. In normal-phase liquid chromatography, sample retention is governed by solute adsorption to the stationary phase. For retention to occur, a sample molecule must displace one or more solvent molecules from the stationary phase. In addition to this displacement effect, polar solute molecules can exhibit very strong interaction with particular sites on the stationary phase (localization effect).

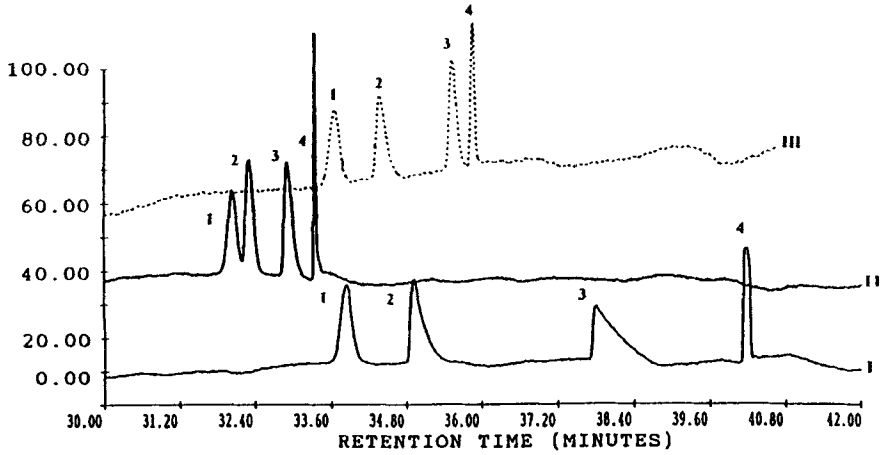

Figure 4. Fused silica capillary column, 14.4 m x 50 μm i.d., n-hexane, flow rate = 0.92 μL/min: detection UV at 210 nm. Peaks: 1) 2,6-di-tert-butyl-4-methylphenol,2) 2,6-dimethylphenol, 3) 1-naphthol, 4) 2-naphthol. I = 301 K, II = 333 K, III = isothermal 20 min at 301 K then gradient 6 °/min to 373 K.

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These two effects, displacement and localization, are the primary sources of mobile phase selectivity in normal-phase HPLC. To alter these affects, thus changing selectivity, temperature gradient can be utilized. In Fig. 5, a comparison between two gradient rates is depicted. Selectivity obtained with a heating rate of 3 °/min is displayed in chromatogram I, while the chromatogram labeled II corresponds to a separation accomplished with a temperature gradient of 6 °/min.

CONCLUSION

Temperature programming is a useful mode in liquid chromatography for extending the applicability of the isocratic mode, optimizing resolution, shortening analysis times and at the same time achieving a higher relative peak sensitivity. The concept of the temperature gradient separation in OTLC parallels the strategy for temperature programming in column liquid chromatography or microbore column liquid chromatography. When applying temperature programming, care must be taken to avoid a large temperature difference between the column wall and the mobile phase. The small radius and mass of open tubular capillary columns makes them more suitable than micro-bore or conventional bore columns for temperature programming in liquid chromatography. Temperature programming in HPLC may be a useful supplement to gradient elution. Instrument requirements are somewhat simpler for temperature programming than for gradient elution. It is much simpler to control the temperature of a mobile phase

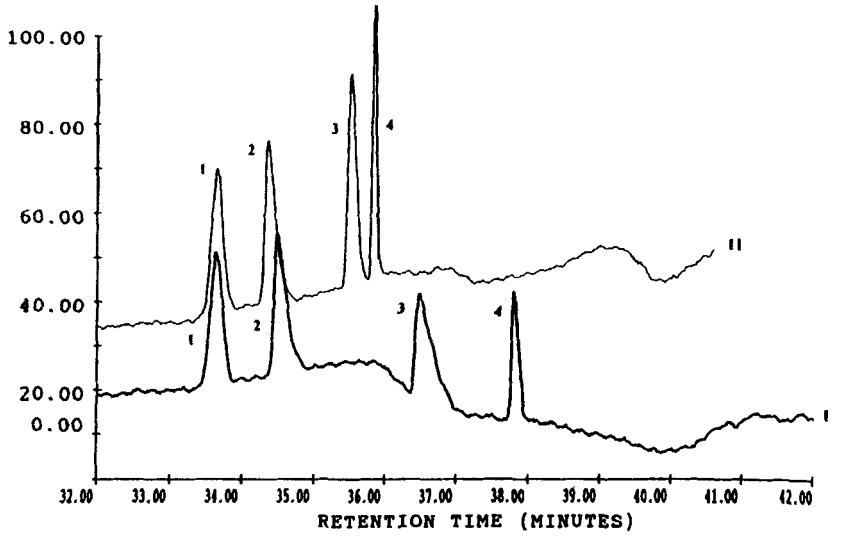

Figure 5. Temperature programming at 3 °/min (I) and 6 °/min (II). Condition as in Fig. 4.

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than to uniformly mix two mobile phases with the precision necessary for reproducible separation in open tubular capillary LC. Temperature programming in combination with gradient elution may be an interesting approach for future separations in micro column HPLC.

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