SIMULTANEOUS PROGRAMMING OF TWO PARAMETERS IN MICRO- AND CAPILLARY-HPLC

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SUMMARY

The fundamental advantages of programmed elution in chromatography are the shortening of analysis times, narrowing of the chromatographic peak widths, and a consequent decrease in the minimum detectable amounts of solute in the chromatograph. In programmed mode, most often, only one parameter is varied during the analysis. The combination of several gradient modes (solvent, temperature, and flow programming) is rarely used in *HPLC* analysis. We have compared the analysis time and the resolution for separations performed under gradient elution (single parameter programming) and simultaneous programming of two parameters (temperature and flow rate or mobile phase) with corresponding isorheic, isothermal and isocratic separations. Simultaneous programming of several separation parameters offers new ways to optimize selectivity, improve resolution, and shorten analysis time. On the other hand, *simultaneous programming* of several parameters requires more complex instrumentation

than a single gradient elution mode, and method development can be difficult.

INTRODUCTION

In HPLC, there are many ways to modulate solute retention, optimize selectivity, improve resolution and shorten analysis time. Varying the ratio of aqueous to organic component in the mobile phase is the most common approach for altering selectivity in HPLC /1/. Though chromatography is essentially due to repeated partition equilibrium of the components between two phases, the environment in which it takes place can also influence the equilibrium /2/ The environmental conditions, such as column temperature and flow-rate, can influence the overall separation quality /3/. Temperature is an important parameter in HPLC. The importance is primarily the result of the marked temperature dependence of solute retention. Secondary effects such as the changes with temperature of mobile phase viscosity and solute diflusivities are also important. Changing temperature strongly affects mobile phase viscosity, and if the mobile phase contains a buffer, the degree of ionization of the buffer. The effect of increasing temperature on parameters such as retention factors, peak symmetry, and chromatographic efficiency in isothermal separation was investigated by Grushka *et al.* /4/ and Liu *et al.* /5/.

In chromatographic practice, we frequently find resolution many times larger than needed, particularly for an easy separation. Excess resolution can be traded for greater separation speed, which can be accomplished by increasing the flow rate of the eluent. While mobile phase flow is essential in a separation process, flow is not selective and cannot separate on its own. However, when combined with selective forces (phase distribution forces), flow becomes a powerful tool for altering separation quality /6/.

Real-life samples often confront us with the problem that the same conditions are not the best for all the components of the mixture. The more complex the system to be analyzed, the greater are the chances that its chromatography in a constant environment may lead to unsatisfactory results. If we change the conditions so as to increase the retention factors of the early eluting components, then the later eluting ones will tend to give rise to impractically high k' values.

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. Programmed analysis can be defined as chromatographic elution during which the operation conditions are varied. At present, there are four possible techniques for programming gradient formation in liquid chromatography: solvent programming, stationary phase programming (coupled columns), temperature programming, and flow programming. Snyder /7/ made a theoretical comparison of these various techniques, showing that separation quality, defined as resolution per unit time, decreases in the order:

solvent programming (best) > coupled columns > temperature programming ≈ flow programming > normal elution.

The main concerns found when selecting one programmed technique over another, are the relative resolution obtained per unit time provided by each programmed technique, and the ease of variation of the programmed quantity during a separation run. Furthermore, experimental considerations such as simplicity and convenience, additional equipment requirements for a given technique and the compatibility with a detection system should be taken into consideration when selecting a programming mode. The nature of the separation problem and sample complexity should be also considered when deciding on a programming approach.

Solvent strength programming is a typical programming method in LC to control the separation where fast and highly efficient analysis is desirable. Temperature programming is not often used in HPLC, mainly because it is so easy to manipulate the mobile phase composition. The effect of programmed temperature on separation in LC was compared with isothermal separation in liquid chromatography by McNair *et al.* /8/ and Scott *et al.* /9/. The optimization process does not end with achievement of the highest separation resolution. The time needed for the analysis must also be taken into account. The aim of using flow programming in liquid chromatography is to increase the migration rate of later eluting bands during separation, consequently reducing the time of analysis without worsening the resolution /10-13/.

In programmed mode, most often only one parameter is varied during the analysis. Very little attention has been given to the simultaneous programming of several parameters. The effects of two or several gradients

may be combined to obtain a better resolution and shorter run time /11,13/. Only recently has the issue of the combined use in liquid chromatography of temperature and solvent strength attracted attention /14-16/.

EXPERIMENTAL

The instrumental set-up is shown in Figure 1. A liquid chromatograph, the Ultra-Plus Model (consisting of two pumps, a pump controller, and a mixer), was from Micro-Tech Scientific, Sunnyvale, CA, USA. The injection valve model C14W.O6/5 with a 60 nl internal loop, from Valco (Zurich, Switzerland), was placed in the column oven. The T-piece splitter with a 0.02 cm bore was from Valco (Zurich, Switzerland). It was used to split the mobile phase flow after the sample injection. A thermometer model 8536-26 from Cole-Palmer Instruments (Vernon Hills, IL) was used to measure column temperature. The column oven (forced air heating) and an

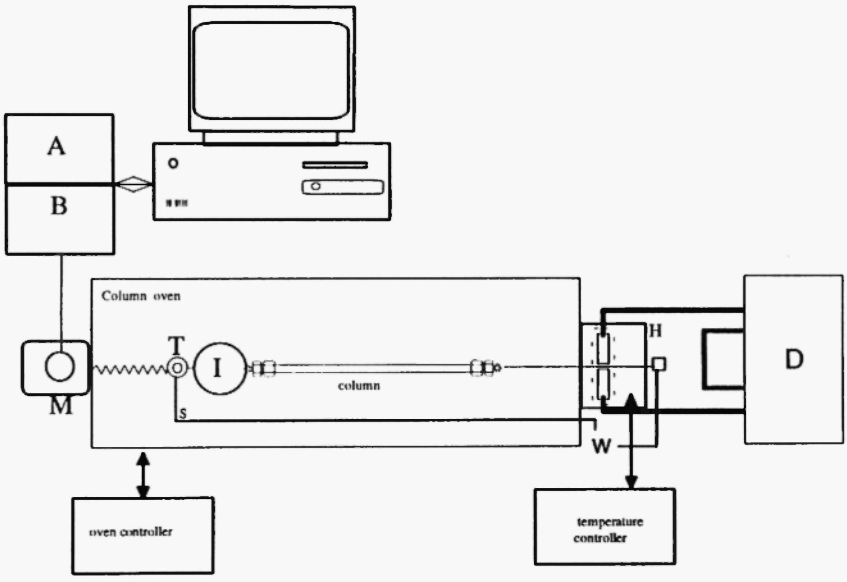

Fig. 1: Experimental set-up. A , B = HPLC pumps; M = mixer; T = split T-piece; I = injection valve; S = split capillary; H = detector cell heater; D = detector; W = waste.

HPLC microprocessor programmer 50 A/B (used as a temperature programmer) were from Knauer (Berlin, Germany). Chrom Perfect for Windows software, from Justice Innovations (Mountain View, CA) was used for data acquisition and processing on an IBM PC. The Spectroflow 783 UV detector (Kratos, Ramsey, NJ) was modified to allow the use of optical fibers. In order to avoid the baseline sloping during a temperature gradient, the detector cell is placed in a separate heating unit. The cell is kept at constant temperature, corresponding to the midpoint temperature of the gradient. Bare fused silica capillaries (75 μm i.d.), used either as restrictor (l = 25 cm) or as a splitting line (l = 80 cm), are from Polymicro Technologies (Phoenix, AZ, USA). The capillary column (50 mm x 0.180 mm) Hypersil ODS particle size 3μm, was from LC Packings (Amsterdam, The Netherlands). A micro-bore column (150 x 1.0 mm) Spherisorb ODS2 particle size 3μm, was from Waters Corporation (Milford, MA). All solvents were of HPLC grade from Merck (Darmstadt, Germany). The test compounds were obtained from Fluka (Buchs, Switzerland) or Aldrich-Chemie (Steinheim, Germany).

RESULTS AND DISCUSSION

Temperature and flow programming

The change in retention factor with temperature can be put in a form which represents the retention factor ratio k'_1/k'_2 at two different temperatures T_1 and T_2

$$k'_1/k'_2 = \exp [\Delta H(T_2-T_1)/RT_1T_2] \quad (1)$$

where k'_1 represents the retention factor for a solute at some temperature T_1, while ΔH denotes the enthalpy change associated with a solute molecule transfer from the stationary to the mobile phase. Differences in sorption behavior are characterized by the enthalpy changes. Solutes with higher entropy change (usually associated with stronger retention) will be more affected by temperature variations than weakly retained solutes. Figure 2 shows the separation of a test mixture, containing eleven compounds, utilizing a capillary column packed with Hypersil ODS reversed-phase material. The separation of all compounds contained in the test mixture was

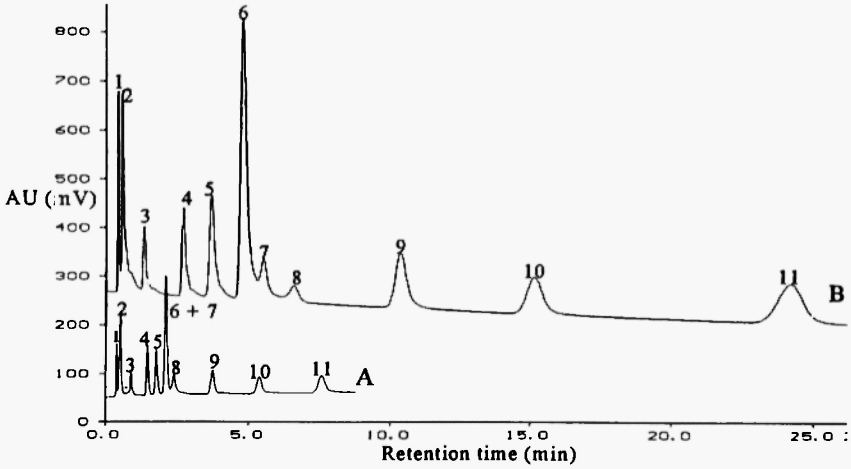

Fig. 2: Separation of a test mixture. Capillary column l = 5 cm, i.d. = 180 µm, Hypersil ODS particle size 3 µm; ACN/H_2O 30:70 (v/v), flow rate = 6 µl/min; UV detection at 210 nm. Peaks: 1) thiourea, 2) benzyl alcohol, 3) methyl benzoate, 4) toluene, 5) benzophenone, 6) naphthalene, 7) 1,4 di-chlorobenzene, 8) phenothiazine, 9) biphenyl, 10) 1,3,5 tri-chlorobenzene, 11) 1,2,4,5 tetra-chlorobenzene. Trace A: column temperature 100 °C. Trace B: column temperature 50 °C.

achieved in 25 min by using isocratic conditions (ACN/H_2O, 3:7 v/v) at 50°C and a 6 µl/min flow rate, Figure 2 (trace B). When the column temperature was increased to a 100°C, the analysis time decreased to about 8 min, Figure 2 (trace A). At this column temperature we did not separate all components of the mixture (naphthalene and 1,2 dichlorobenzene peaks were not resolved). However, this example clearly demonstrates the importance of temperature as an effective variable for optimizing a separation.

As a next step in optimizing separation, a temperature gradient was introduced in an attempt to reduce the run time while maintaining the quality of separation achieved at 50°C. In spite of the drawbacks associated with a programmed run (prolonged run time and possible "drift" of the

baseline during the gradient) it was necessary to use a programming mode for separation of a sample mixture with such a broad k' range. In linear temperature programming, the column temperature (T) at time t is given by

$$T=T_0+rt \quad (2)$$

where T_0 is the starting temperature and r the programming rate (°C/min). For separation of the test mixture, the initial and the final column temperatures were 50°C and 130°C, respectively, with a gradient rate of 6°C/min. As shown in Figure 3 trace B, the overall analysis time was

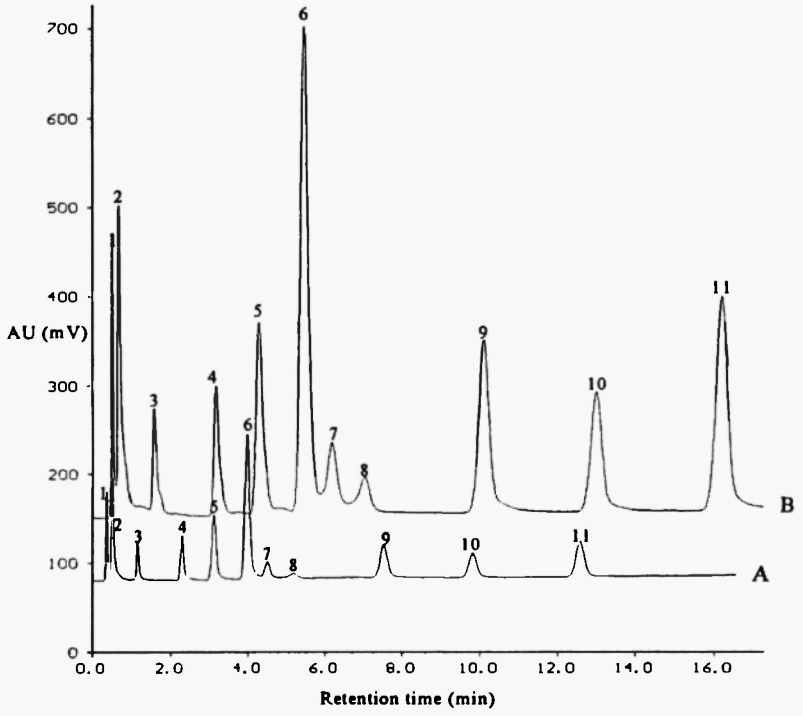

Fig. 3: Trace A: Temperature gradient from 50 °C to 130 °C, heating rate of 6 °C /min. Initial flow rate = 6 µl/min, final flow rate =13 µl/min; Trace B: Temperature gradient from 50 °C to 130 °C, heating rate of 6 °C /min, flow rate = 6 µl/min. Other conditions and peak identification as in Figure 2.

using a mobile phase composition of H_2O/ACN 30:70 (v/v) and the column temperature set at 40°C. All peaks in the mixture were separated but the overall analysis time was long (75 min), with late eluting peaks fading into the baseline. The mixture consisted of compounds having a very broad k' range and the spacing between the peaks is not optimal (equispacing). This is a typical example where a programming mode will help to significantly shorten the analysis time. If a number of different large molecular weight components are present in the sample, it may be almost impossible to find an isocratic composition that will give rise to optimum retention factors for all sample components, and hence use of gradient elution may be hard to avoid. In a mobile phase gradient mode, retention time t_r can be expressed as /20/.

$$t_r = (t_0/b) \log (2.3k'_0 b + 1) + t_0 + t_D \quad (8)$$

where k'_0 is the value of k' at the start of the gradient, b is gradient-steepness, t_0 is column dead-time and t_D the gradient dwell time. The gradient-steepness parameter b is given by /20/:

$$b = \Delta\Phi \, V_m \, s/(t_g F) \quad (9)$$

where t_g is gradient time, F is flow rate, V_m is the column dead volume, $\Delta\Phi$ is the change in volume fraction of organic modifier in the mobile phase from start to finish of the gradient.

By introducing a gradient elution the separation-quality was significantly improved, as shown in Figure 5. Isocratic conditions were maintained for 12 minutes (H_2O/ACN 30:70 v/v), followed by a mobile phase program producing a gradient from 70 % acetonitrile to 95 % in nine minutes. All other conditions were as in the separation depicted in Figure 4. Early eluting peaks were still well resolved and the run time was reduced by half (under 30 min). As can be seen, the peak sensitivity (the ratio of peak height to peak width) also significantly improved, especially for late eluting peaks. Nevertheless, there was some room for further method improvement. The late eluting peaks still lag behind in the chromatogram. By simply increasing the column temperature to 90°C, the analysis time decreased to around 20 minutes, but the resolution of early eluting peaks deteriorated, and several peaks coeluted, Figure 6. Further improvement in resolution could be obtained by fine-tuning the gradient range (mainly by changing the

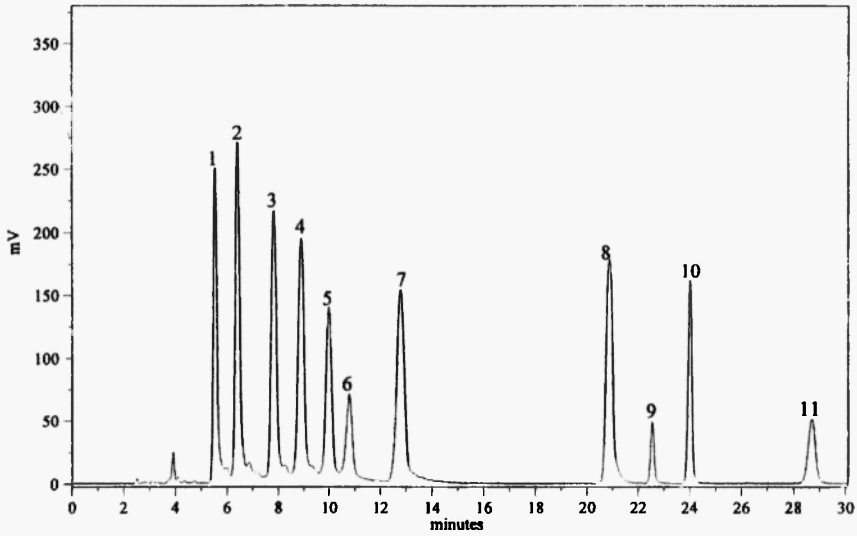

Fig. 5: Isocratic conditions were maintained for 12 minutes (H_2O/ACN 30:70 v/v) followed by a mobile phase gradient from 70 % ACN to 95 % ACN in 9 minutes. All other conditions as in Figure 4.

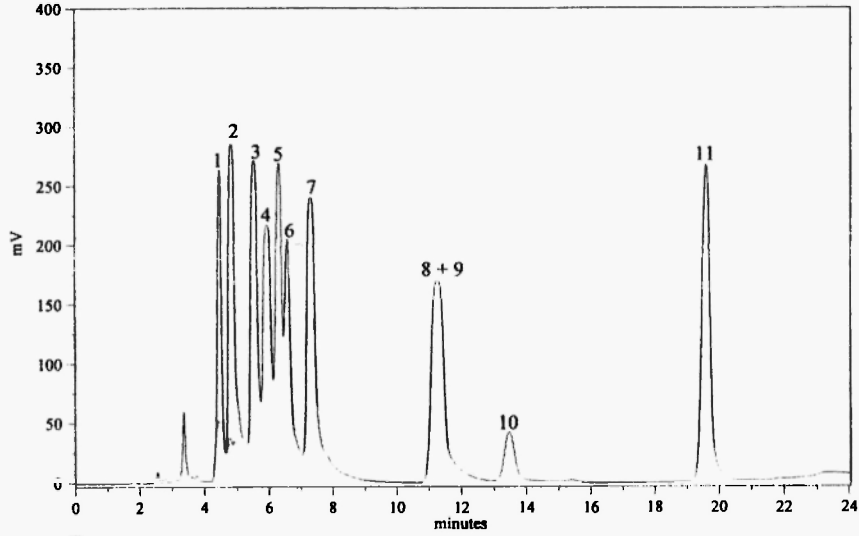

Fig. 6: Column temperature 90 °C. All other conditions as in Figure 5.

reduced to approximately 16 min, one third less than what was observed in isothermal separation at 50°C. All compounds were baseline separated in spite of this reduction in analysis time. The temperature gradient separation mode is easy to run, and its reproducibility is comparable to that of mobile phase gradient mode. The RSD of the solute retention times for five consecutive runs was less than 2%.

The applicability of the high flow rates is limited by the back pressure that different parts of the chromatographic system (pump, injector and column) can withstand. The equation describing the linear flow rate through a packed column in a pressure-driven system is given as /17/

$$u=d^2\Delta p/\phi\eta L \qquad (3)$$

where d stands for the particle size, ϕ is the pressure resistance factor for packed columns, Δp is the pressure drop across the column, L is the column length and η is the solvent viscosity. In LC, elevated column temperature can be used as a tool to overcome the flow rate problem associated with high back pressure and allows the use of flow rates that otherwise cannot be applied. Pressure reduction is due to a decrease in eluent viscosity with increasing temperature /3/. For a temperature range from the freezing point to around the normal boiling temperature, it is often a good approximation to assume the relationship between viscosity and temperature /18/

$$\ln \eta = a + (b/T) \qquad (4)$$

where a and b are empirically determined constants. Higher column operating temperature will result in a decrease in the mobile phase viscosity, that is, a decrease in the column inlet pressure required to maintain a given flow rate. As demonstrated by Liu *et al.* /5/, the optimum linear velocity for an unretained compound (k'=0) in open tubular capillary LC increased from 0.03 cm/s at 22 °C to 0.09 cm/s at 100 °C. At elevated temperatures, an appropriate increase of the mobile phase flow rate must accompany the temperature increase to maintain separation at the optimum efficiency.

In positive flow rate programming (flow rate increases with time), the decrease in retention time is proportional to an increase in mobile phase flow rate. The slope of the flow rate programming, S, can be written as

$$S = (F_f - F_i) / (t_f - t_i) \qquad (5)$$

where F_f and F_i are the final and the initial flow rates at times t_f and t_i, respectively. Flow rate programming in combination with temperature programming should make it possible to considerably reduce the retention time of very strongly retained compounds, without losing column performance.

When a micro-flow is generated by utilizing flow splitting, in micro- or capillary-LC, the flow programming mode requires very little additional modification to the instrumentation. The mobile phase viscosity changes, created when a temperature program is applied during a chromatographic run, can be used to generate a flow rate gradient. Temperature changes influence the mobile phase viscosity (equation 4) and as a result the column back pressure in the separation column and in the capillary split-line. A flow rate gradient in the separation column is generated when the split capillary is held at a constant temperature and only the separation column is exposed to a temperature gradient. Only mobile phase passing through the separation column experiences viscosity changes, which can be related to the mobile phase linear velocities (u_i) at two different temperatures by equation 6.

$$u_1\eta_1 = u_2\eta_2 \qquad (6)$$

Due to the changing viscosity of the mobile phase passing through the separation column, the constant change in the pressure difference between the split capillary and the separation column produces a flow gradient. The flow rate variations in the separation column were proportional to the viscosity changes induced by the temperature gradient. In order to further reduce the run time of the separation depicted in Figure 3, trace B, the flow gradient was superimposed on the temperature gradient while all other separation parameters were kept the same. This combination of temperature and flow programming led to an additional shortening of the separation time. The coupled effect of temperature and flow gradient produced more than a 30 % reduction in analysis run time with temperature programming alone, and 50 % when compared to isocratic, isothermal (50°C) and isorheic (constant flow) separation. During the run, the pump pressure of 210 bar (for a total flow rate of 0.7 ml/min) and the pressure drop across the split capillary were kept constant. Mobile phase passing through the column was exposed to a continual temperature variation (introduced by the temperature program) which produced proportional viscosity and flow rate changes. The

flow rate at the beginning and at the end of the temperature program was 6 µl/min and 13 µl/min, respectively. The RSD for retention times obtained for five consecutive separations under the temperature and flow programmed condition was less than 2%, clearly indicating that the reproducibility of this separation mode is sufficient for routine use.

Mobile phase gradient elution and temperature programming

The change in retention factor with variation of mobile phase composition (solvent strength variation) in isothermal and isorheic (constant flow) runs [19] is given by equation 7

$$\log k' = \log k'_w - s\Phi \qquad (7)$$

where k'_w is the solute retention factor for pure aqueous mobile phase, Φ is the volume-fraction of organic modifier in the mobile phase, and s is a constant for each solute. Figure 4 depicts an isocratic separation achieved

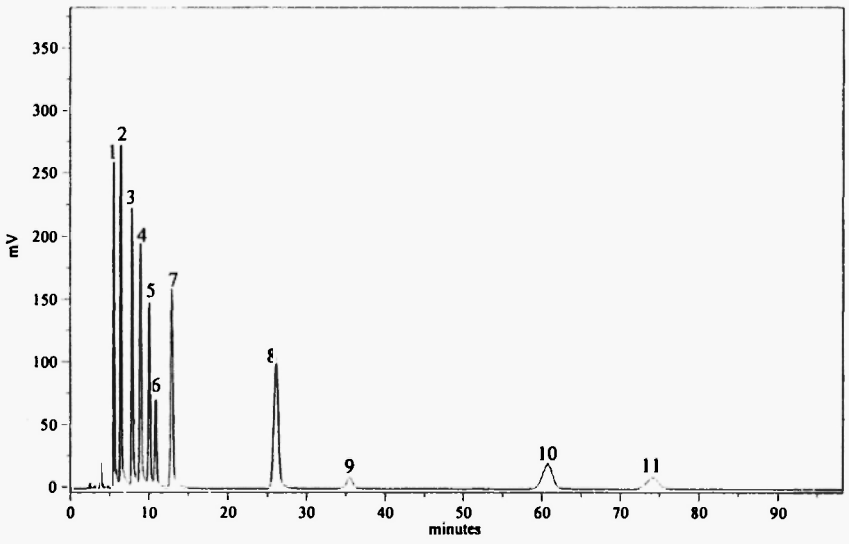

Fig. 4: Spherisorb ODS2, 3µm, 150 x 1.0 mm; mobile phase composition H_2O/ACN 30:70 (v/v); flow rate =33 µl/min; column temperature 40 °C; UV detection at 210 nm. Peak identification as in Figure 2.

mobile phase starting conditions), or by using nonlinear gradients (segmented gradients). For a complex sample mixture, selectivity optimization can be accomplished by designing a multisegment gradient, though this has its limitations. The optimization of multisegment programs can be complex and tedious.

A temperature gradient combined with the mobile phase gradient was used as an alternative to a multi-segment mobile phase program. Since the early eluting compounds were sensitive to the temperature changes, a temperature gradient was introduced. A temperature gradient from 40°C to 90°C in nine minutes was applied immediately after sample injection. The mobile phase composition was held constant (H_2O/ACN 30:70 v/v) for the duration of the temperature program. The mobile phase gradient started 12 minutes after the sample was introduced. The volume fraction of organic modifier was increased from 70 % ACN to 95 % ACN in nine minutes. The combined programs produced the separation shown in Figure 7. Baseline separation of the early eluting peaks was preserved while the elution time of strongly retained peaks was shortened and the total analysis time was reduced to around 20 minutes.

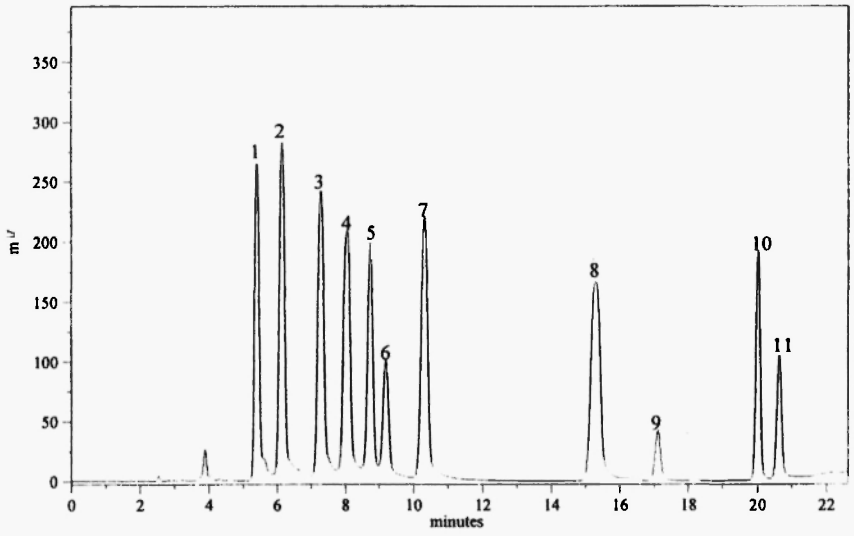

Fig. 7: Temperature gradient imposed immediately after injection from 40 °C to 90 °C in 9 min. Isocratic separation for 12 min, mobile phase composition H_2O/ACN 30:70 (v/v), followed by mobile phase gradient from 70 % ACN to 95 % ACN in 9 minutes.

CONCLUSION

It has been shown that in capillary- and micro-HPLC separations, concurrent programming of two parameters decreases analysis time without sacrificing high resolution and run-to-run reproducibility. Combined programming of two parameters may be used as an alternative to a segmented gradient, which can be tedious in development and result in run-to-run reproducibility which may be difficult to sustain. The combination of temperature and flow rate programming modes is particularly favorable as a substitute for solvent gradient elution in capillary-LC, because it bypasses the technical difficulties associated with solvent gradient elution in capillary columns.

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