Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 8, pp. 611–618, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00

The use of a computer to select optimized conditions for high-performance liquid chromatography separation*

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Abstract: Computer simulation allows the convenient prediction and optimization of HPLC separation as a function of various separation conditions. The use of retention and bandwidth relationships that have been validated for a broad range of chromatographic systems minimizes the number of experimental runs needed, especially for the new technique of restricted multi-parameter optimization. The chromatographer is free to use these procedures in a trial-and-error mode, or alternatively use can be made of resolution maps and other data summaries. "Gridding" experiments, based on the automated collection of chromatographic data, can be used to supplement predictions obtained from computer simulation.

Keywords: *Computer; conditions; DryLab®; HPLC; optimization; simulation.*

Introduction

Within the past decade the use of the computer to facilitate HPLC method development has grown rapidly [1–3]. This has led to a number of different approaches [3] which are potentially useful for the practising chromatographer. Commercial software based on the pioneering work of Glajch *et al.* for the optimization of reversed-phase HPLC conditions [4] was first introduced by Du Pont in the early 1980s [5]. Starting with experimental runs for seven different mobile phases, sample retention and resolution could be optimized by varying the proportions of methanol, acetonitrile or tetrahydrofuran in the mobile phase. Similar computer programs for HPLC method development have since been offered by other companies.

Whilst these software tools were greeted by practical workers with initial enthusiasm, several problems with this approach to method optimization were soon apparent: (1) a large number of initial experimental runs were required for most samples because of a need for "peak tracking" [1, 2, 6], i.e. matching bands for the same compound between different runs; (2) these method development procedures were somewhat arbitrary and rigid, with the result that the experimental approach often represented "overkill" for samples that proved fairly easy to separate; (3) the necessary compromise between run time and the quality of the separation (resolution R_s) was ignored, i.e. the possibility of changing column conditions (column length, particle size or flow rate) as a means of trading an increase in run time for an increase in resolution (and vice versa) was not taken into account; (4) these computer programs were presented as a kind of "automatic method development", rather than just one of many tools to be used by an experienced chromatographer.

A few years later the technique of "computer simulation" was described as an alternative for HPLC method development [7, 8]. This approach allows method development to begin with just two experimental runs. The effects of solvent strength (%B) and column conditions on the separation can then be explored via computer. Computer simulation was soon extended to include separations by gradient elution [9]. Commercial software (DryLab® I and G; LC Resources) for computer simulation allows predicted separations to be viewed as chromatograms, tabulations or summaries (resolution maps) of up to 35 separate runs. Computer simulation can also

*Presented at the Symposium on "Chemometrics in Pharmaceutical and Biomedical Analysis", November 1990, Stockholm, Sweden.

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be used in combination with software that allows the further exploration of other separation variables (e.g. PESOS from Perkin–Elmer [10]).

The present paper will summarize an effective strategy for the use of computer simulation as part of a more comprehensive approach to HPLC method development (as described in ref. 11).

Experimental

A nine-component mixture was used as the sample; it included the following benzoic acid derivatives: 2-nitro, 3-nitro, 2-fluoro, 3-fluoro, 3-cyano, 2-chloro and 2,6-dimethyl benzoic acid, phthalic acid and an unknown acidic impurity. A 25 × 0.46-cm Zorbax® C8 column was used with methanol–buffer mobile phases at 1 ml min^{-1}. The 25 mM sodium acetate buffer was adjusted to give pH values between 2.7 and 3.2. Equipment, materials and procedures are described in further detail in ref. 12.

Discussion

Computer simulation to optimize retention

The nine-component mixture of benzoic acids described in the Experimental section was used as an example of the present approach to HPLC method development. Initial separations were carried out with mobile phases having a pH of 2.7 and two different methanol concentrations, 35 and 45%. The resulting chromatograms are shown in Fig. 1. While only eight distinct bands are observed in each chromatogram, peak tracking based on relative retention and band size indicated the presence of nine components — as expected. Peak tracking for changes in mobile phase composition (%B) as described here is seldom a problem, since changes in relative retention between runs are often minor, and a third run can be used to confirm the accuracy of peak matching for the two initial runs. When other variables (pH, type of organic solvent) are changed between two runs, peak tracking can be more difficult.

Run conditions plus the retention times and band areas from Fig. 1 were used as input for computer simulation. Figure 2 shows a plot of sample resolution versus mobile phase composition (predicted by DryLab I) for the other conditions of Fig. 1. This "resolution map" tracks the separation of the least-resolved band-pair (see numbers in Fig. 2) as a function of % methanol; it is similar to a "window diagram" [13]. It is noted that the resolution map covers a range (30–65 %B) that exceeds that bracketed by the experimental data used as input (35–45 %B). Accurate predictions outside the experimental range are possible, because a reliable relationship between k' and %B is assumed ($\log k' = A - B[\%B]$). When values of %B are extrapolated outside of the experimental range (35–45%), DryLab advises the user of the probable error in predicted separations as a result of excessive extrapolation.

Maximum resolution ($R_s = 0.6$) is found for a mobile phase of 31% methanol; the predicted chromatogram is shown in Fig. 1. There is obviously insufficient resolution for these conditions (note the expansion of bands 1 + 2 as inset). The run time (≈30 min) is also excessive, meaning that a change in column

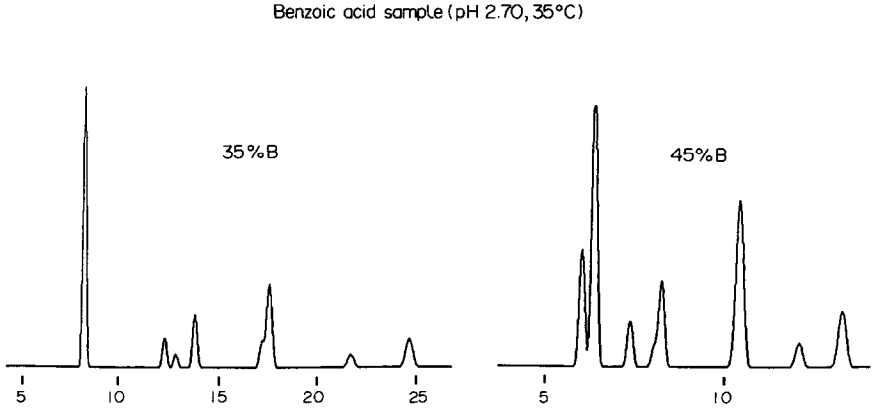

Figure 1
Experimental chromatograms for the separation of a mixture of benzoic acids. Conditions: mobile phase pH 2.70, 35 or 45% methanol; other conditions given in Experimental section.

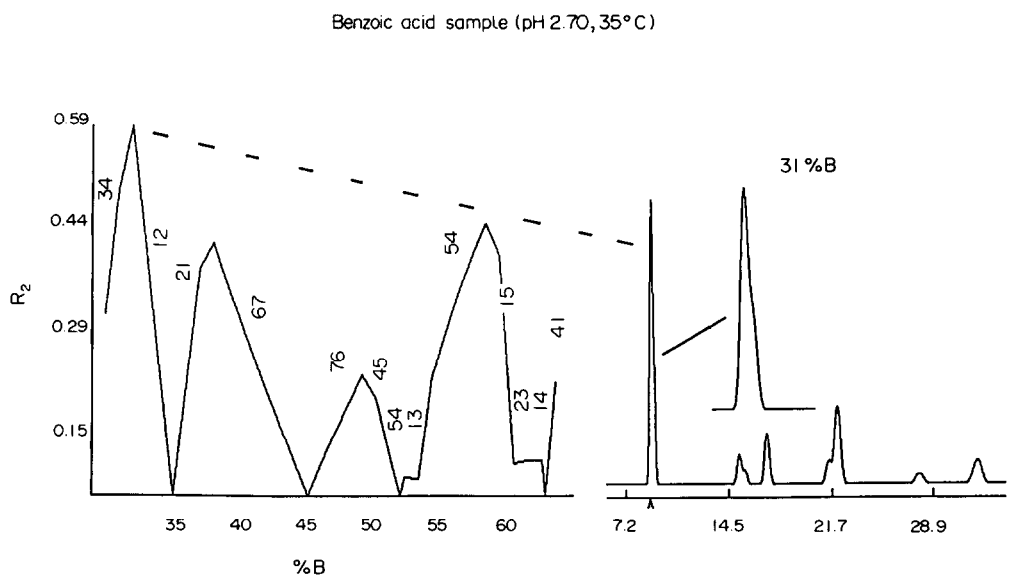

Figure 2
Computer simulations based on separations of Fig. 1 (DryLab I). See text for details.

conditions (increase in column length, decrease in flow rate) is not a useful option for improving resolution.

When computer simulation shows that adequate resolution is unobtainable for any value of %B, a change in some other variable can be tried and computer simulation (variation of %B) repeated as in the examples of ref. 8. In the present case, a change in pH was made. Because the pK_a values (in water) of these substituted benzoic acids are in the range of 4–6, at pH 2.7 it is expected that these compounds will be largely in the non-ionized form. A change in band spacing as a result of pH variation is therefore most likely for an increase in pH.

Figure 3 shows two new experimental runs for a pH of 3.2 and mobile phases of 35 and 45% methanol. The chromatogram for 35 %B exhibits nine fairly well resolved bands, suggesting that the further optimization of %B will lead to a successful separation. Figure 4 shows the resolution map that results from the use of the runs of Fig. 3 for computer simulation. The optimum mobile phase composition (31% methanol) provides a sample resolution of $R_s = 1.6$. The predicted separation for 31 %B is also shown in Fig. 4. The run time is still somewhat long (28 min), but baseline separation of all bands has been achieved. Further improvements in this separation could be attempted, by fine-tuning pH and repeating computer simulation. An alternative approach based on "gridding" experiments is described below.

"Gridding" experiments (PESOS)

Once the four experiments of Figs 1 and 3 have been completed, the further study of separation as a function of pH, %B and possibly other variables can be carried out via gridding experiments. "Gridding" refers to the repeated separation of a sample while varying two or more variables in small steps. The PESOS software supplied by Perkin–Elmer as part of its Analyst HPLC system allows this to be done in an efficient, automated fashion [10]. Computer simulation (DryLab I) was used first to predict a range of %B values for pH 2.70 and 3.20, such that sample retention falls within a useable range of k'-values: 31–43 %B, so that $1 < k' < 20$ for every component.

Figure 5 illustrates the application of gridding in this way, where %B is varied in increments of 3%, and pH is varied in increments of 0.1 unit. Resolution (R_s) is shown in Fig. 5 for each chromatogram. If desired, a resolution map as a function of both pH and %B can be generated by PESOS from these gridding experiments. The separations of Fig. 5 yield sample resolutions that vary from 0.1 to 1.9, with the maximum resolution for each value of pH circled for easy recognition. Maximum overall resolution ($R_s = 1.9$) is seen

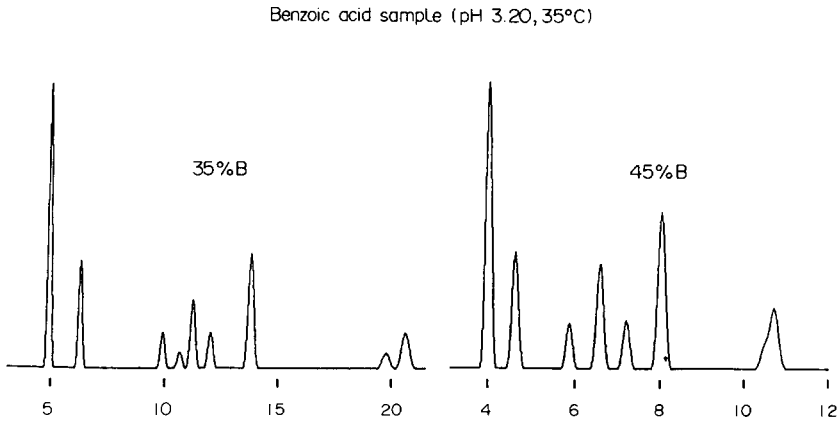

Figure 3
Experimental chromatograms for the separation of a mixture of benzoic acids. Conditions as in Fig. 1, except pH 3.20.

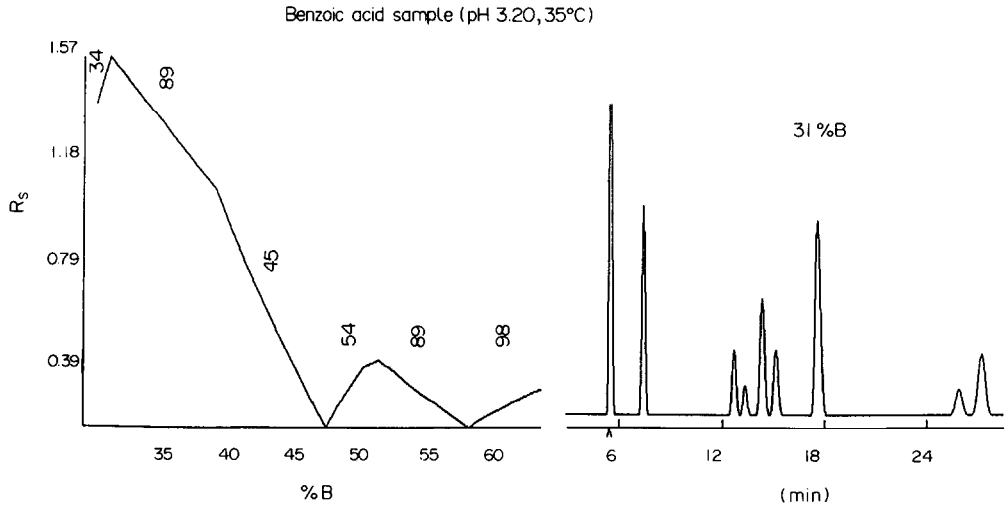

Figure 4
Computer simulations based on separations of Fig. 3 (DryLab I). See text for details.

to occur for pH 3.1 and 34 %B (shown in Fig. 6). This is clearly the best separation achieved so far (cf. Figs 2 and 4).

The gridding experiments of Fig. 5 also serve to define method ruggedness, i.e. changes in separation as a result of small errors in mobile phase formulation. It is seen that errors of ±0.1 unit in pH or ±3% in methanol concentration do not lead to R_s values lower than 1.4, which still provides near-baseline separation; i.e. errors this large will still allow an acceptable separation of the sample. A worst-case separation (R_s = 1.2) would occur for a simultaneous error in pH of +0.1 unit and in %B of +3%, but this is considered unlikely.

It should be emphasized that "gridding" as described above is not an "efficient" experimental design nor does it utilize the computer to predict separations other than those actually measured. Nevertheless, it can have important advantages in many situations. Thus no assumptions are made concerning the variation of retention or bandwidth with conditions, and peak tracking is not required. The greater number of runs required in this approach represents an added cost to method development, but this is offset by the automated collection of data — usually during the night shift.

Computer simulation to optimize column conditions

A remaining problem with the separation of Fig. 6 is a rather long run time, although many workers would not object to a 25-min separation for a sample as complex as this.

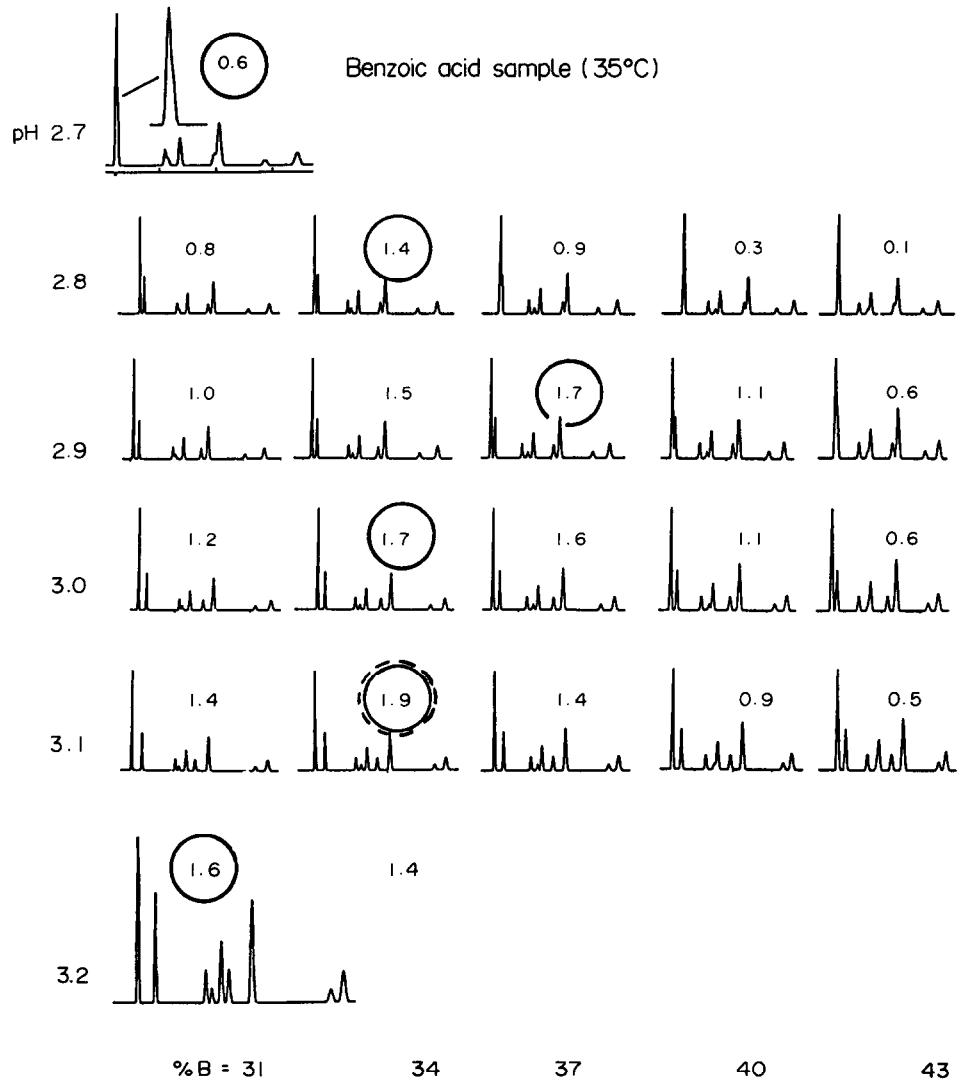

Figure 5
Gridding experiments starting with the computer simulations of Figs 3 and 5. Separation of benzoic acid sample as a function of pH and %B. Other conditions as in Fig. 1.

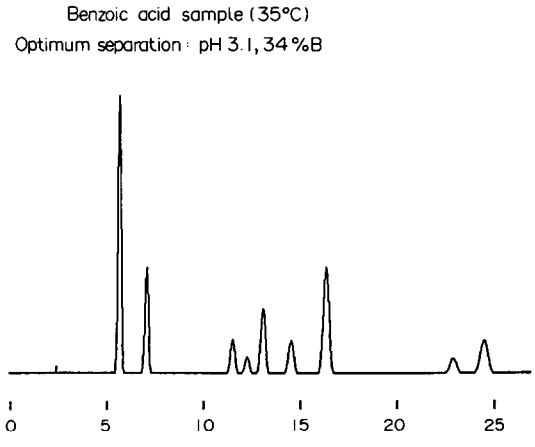

Figure 6
Best separation of benzoic acid sample as a function of pH and %B. Conditions: 34 %B and pH 3.10; other conditions as in Fig. 1.

Computer simulation can be used at this point to study the effects of a change in column conditions. When varying column conditions, our general goal is to achieve adequate resolution, acceptable column pressure and minimum run time. Baseline resolution ($R >$ 1.5) is desirable for maximum accuracy in the measurement of band size, since this removes any doubt as to how the baseline is to be drawn under the band. The column pressure should be maintained <2000 psi for a new column, since higher operating pressures tend to increase system wear and downtime.

Computer simulation was used to explore the effect of column length, particle size and flow rate on the separation of Fig. 6. Retention times and run conditions were used as input for

DryLab I; these allow the computer to estimate the column plate number and resolution. The predicted resolution was $R_s = 2.0$, versus an actual value of $R_s = 1.9$. This small discrepancy was corrected by an empirical adjustment, prior to further computer simulations.

The simplest expedient for reducing run time is an increase in flow rate while holding other conditions constant. The column pressure for the separation of Fig. 6 is 1600 psi, which limits an increase in flow rate to about 1.3 ml min^{-1} for a pressure <2000 psi. The simulated separation for this flow rate is shown in Fig. 7(A), where run time is reduced to 18 min and resolution is now $R_s = 1.8$. An alternative is to use a shorter column; column lengths of 8, 15 and 25 cm are available for this particular column (Zorbax C8). Figure 7(B) shows the simulation for a 15-cm column and a flow rate of 0.8 ml min^{-1}. Resolution is only $R_s = 1.5$, with a run time of 18 min, i.e. this separation is inferior to that of Fig. 7(A). Further changes in flow rate for the 15-cm column give either longer run times or reduced resolution; they are therefore unattractive.

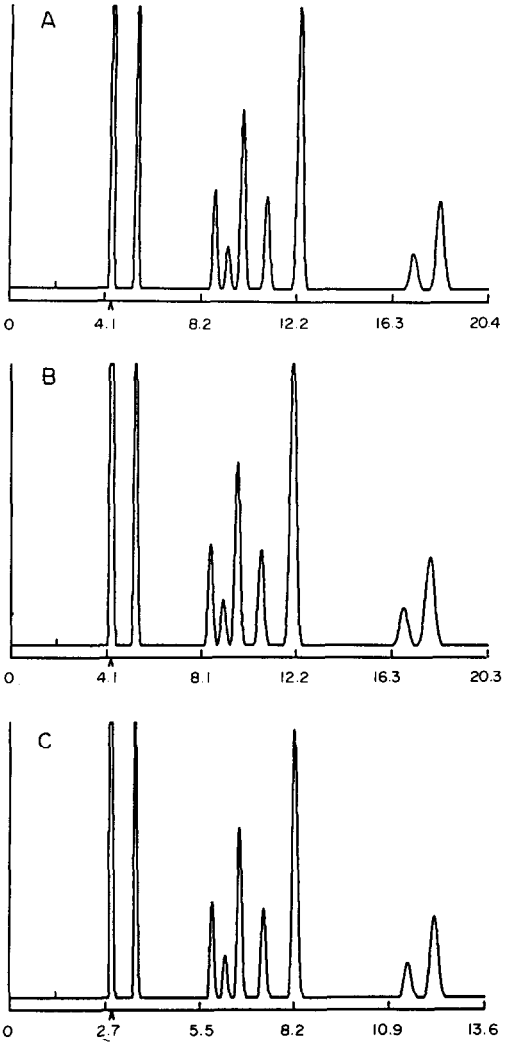

Figure 7
Predicted separation of benzoic acid sample as a function of column conditions; other conditions as in Fig. 6. (A) 25 × 0.46-cm 5-μm column, 1.3 ml min^{-1}; (B) 15 × 0.46-cm 5-μm column, 0.8 ml min^{-1}; (C) 12 × 0.62-cm 3-μm column, 1.7 ml min^{-1}.

A reduction in particle size from 5 to 3 μm can be advantageous in terms of resolution versus run time and pressure, so this option was also explored. Column lengths of 4 and 8 cm are available for the 3-μm Zorbax C8 packing. The best choice of conditions was found to be a 12-cm column length (a 4- and 8-cm column in series) with a flow rate of 1.7 ml min^{-1} [Fig. 7(C)]. This yielded a resolution of $R_s = 1.9$, a run time of 12 min and a pressure of 2000 psi. Because columns of 3-μm particles tend to plug more easily, the latter column might not be the best choice for a routine laboratory. However, the final decision would depend on the importance of run time for this assay.

A reviewer has asked: "Why not change temperature instead of column conditions in order to reduce run time?" Once band spacing has been optimized, in this case by varying pH and %B, a change in column conditions will not affect values of k' and α', i.e. the optimized band spacing will be retained when changes are made in column length, particle size or flow rate. This would not be the case if temperature is varied, since k' and α are functions of temperature.

Restricted multi-parameter optimization

A potentially more efficient use of experimental runs in HPLC method development has recently been described [12]: restricted multi-parameter optimization (RMPO). This computer-simulation procedure allows the simultaneous variation of several experimental conditions, e.g. %B, pH, temperature, concentrations of buffer, ion-pair reagent or additives, etc. Only one or two additional experimental runs are required for each new variable, which means that interaction effects (the effect of a change in one variable on the dependence of retention on a second variable) are ignored. While this approach would normally lead to significant errors in predicted retention times, two features of RMPO work

to minimize the effects of such errors. Firstly, errors of, for example, ±10–20% in predicted retention times are *per se* of minor significance in method optimization. Rather, it is the effect of such errors on *resolution* which are of critical significance. Thus a 10% error in retention time *could* result in a much larger relative error in resolution. However, we have found for a wide range of HPLC systems that errors in resolution are usually *smaller* than expected, due to compensation in the errors of the retention times for two adjacent bands.

A second feature of RMPO is the use of general rules which can predict the approximate error in predicted resolution as a function of simultaneous change in two or more variables (with interaction effects ignored). By restricting the allowed variation of different separation conditions (according to these rules), it is possible to limit the error in predicted values of resolution to an acceptable degree. The net result is that separation for a broad range of conditions can be reliably predicted, with far fewer runs than are normally required for a factorial design involving three or more parameters.

In addition to a more complete optimization of experimental conditions, multi-parameter optimization provides valuable information on method ruggedness and facilitates the modification of conditions to restore adequate resolution when a new column exhibits differences in sample retention, i.e. problems with column reproducibility. This new computer-simulation option is expected to be particularly useful for more complex samples, especially mixtures of acids and/or bases. Its application to the present sample allowed the selection of conditions that provide excellent separation even for errors in pH of ±0.1 unit; see Fig. 8, which can be compared with the separations of Figs 5 and 6.

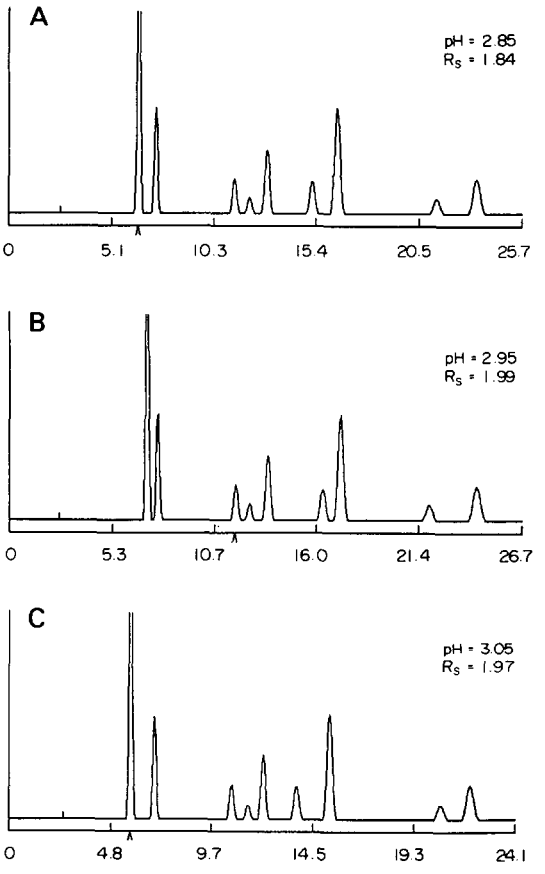

Figure 8
Development of a separation of the benzoic acid sample that is insensitive to errors in mobile phase pH. Conditions: pH 2.95, 36 %B, 32°C (shown in B); (A) separation for error of −0.1 unit in pH; (C) separation for error of +0.1 unit in pH.

Conclusions

A variety of computer software packages are now available as tools for HPLC method development. Computer simulation (DryLab) and "gridding" (PESOS) are especially attractive techniques for the experienced chromatographer. Computer simulation allows a few initial experiments to be used for selecting the best experimental conditions: %B, column length, particle size and flow rate. Gridding permits the automatic collection of data to fine-tune results suggested by computer simulation, without the need for any assumptions (models). An example of this approach is given for a nine-component mixture of substituted benzoic acids.

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[*Received for review 26 November 1990; revised manuscript received 20 March 1991*]