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Advanced high-performance liquid chromatography method development
Discovering unexpected choices in chromatography

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Abstract

The influence of some important experimental parameters on the resolution of compounds as well as the validity of widely used rules of thumb and of common expectations about how to improve resolution is discussed. It will be shown, on the basis of selected examples, that the general expectations about how the experimental parameters have to be adjusted for better resolution does not cover all chances for resolution improvement. The tool for understanding the method and to discover all chances for increasing selectivity is the resolution map of a method.

Keywords: Method development; Optimization; Computer simulation; Resolution; Robustness testing; Liquid chromatography

1. Introduction

Chromatography, especially reversed-phase high-performance liquid chromatography (RP-HPLC), has become one of the most important analytical methods over the last 30–35 years. There exists, therefore, a great amount of experience with this technique and a thorough theoretical understanding of the retention processes, to a good extent due to the work of Snyder (see, e.g., Refs. [1,2]).

But even with great experimental experience and a good understanding of the chromatographic mechanisms is it often difficult to predict the retention behaviour of the substances of a given sample. It is also difficult to calculate or even estimate the retention or resolution from characteristics of the column material or from the chemical structure of the sample compounds. What is well known and has been proved experimentally, is the dependence of peak movements from eluent parameters or the temperature. For example, a linear relationship of the logarithm of the retention factor k can be assumed for parameters like solvent composition (percentage of organic eluent, % B), gradient time (t_G) or reciprocal temperature ($1/T$) [3–7] within limits.

A discussion about these limits and possible sources and amounts of errors using special relationships for peak movements caused by varying experimental conditions can be found in Ref. [8]. For other parameters like pH, buffer concentration or ternary eluent composition the dependence is more complex, but a statistical fit can be used to describe the experimentally evaluated dependencies of retention time on the experimental parameter.

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This situation led quite early to a concept for computer simulation of chromatographic RP retention [9] where a few experimental runs were used to calibrate the retention behaviour of a given sample. In these calibration runs some experimental parameters like % B, t_G, T, pH, etc., or combinations, were varied in a regular manner. From the results of these runs and from the known dependency of log k from the varied experimental parameters a simulation of chromatograms for each value of the experimental parameter can be performed.

The accuracy of predicted retention with experiments have been evaluated for a wide range of substances [3–8,10].

The advantages of such computer simulations for practical work are obvious:

(i) A lot of ''experiments'' to obtain chromatographic informations can be performed much faster and cheaper at the computer instead of at a HPLC apparatus.

(ii) The overview about changes in selectivity caused by varying different experimental parameters is much better due to the possibility to gradually change parameter values by simulation.

(iii) The influence of parameters of column dimensions (length or inner diameter), particle diameter or flow-rate can be easily evaluated without the need to change columns or make experiments at the HPLC apparatus.

(iv) Computer simulations are a fast and cost effective way to learn chromatography. The influence of various parameters on the chromatogram can be modelled by a computer without the need to perform experiments.

Examples how to use computer simulation for method development and method control can be found elsewhere [11–13].

The benefits of computer simulations are especially obvious when simulation results are unexpected compared with experiences or rules of thumb [14]. The knowledge of such unexpected variations in chromatographic behaviour prevents the user from trying optimizations in the wrong direction. This article mainly focuses on undiscovered chances for resolution improvement of critical bands. The evaluation of a great number of HPLC methods has shown, that such unexpected results are not as unusual as one might think. The best tool for discovering these additional chances is the map of critical resolution for a method.

2. Experimental

Most chromatograms shown in this work are computer simulated chromatograms, based on a few experimental results. For these input runs, it is important that only the variable under investigation is changed, whereas all other variables that could influence the chromatogram (including column type and column dimensions as well as equipment characteristics such as dwell volume) are held constant.

For experimental parameters which show, after mathematical conversion, a linear relationship on retention time, only two experiments, performed at two different values of that experimental parameter, are necessary. For some other experimental parameters, where no reliable mathematical dependence on retention time has been found, at least three experiments should be performed for a good simulation. In that case, a non-linear statistical fit can be used to describe the experimentally determined relationship on retention time.

The input runs for the computer models shown in this work were performed with typical RP phases, C_8 or C_{18}, with column dimensions of 15 or 25×0.46 cm and 5 μm material. Further experimental details, where necessary, will be given later together with the discussion of the resolution maps. The DryLab software (LCResources, USA; in Europe: Molnar-Institut für Angewandte Chromatographie, Berlin, Germany) was used to simulate model chromatograms.

Long experience with computer simulation of HPLC measurements has shown that the simulated chromatograms correspond very well with experimental results. Since the simulation is based on extrapolation of experimental results, the accuracy of the simulation will be best in the range of the experiments and will become less accurate the more the simulated conditions deviate from the conditions used for the experiments. In many cases, the differences between simulation and experiments are in the range of experimental variance.

A comparison of simulation and experiment is given in Fig. 1. This example also demonstrates

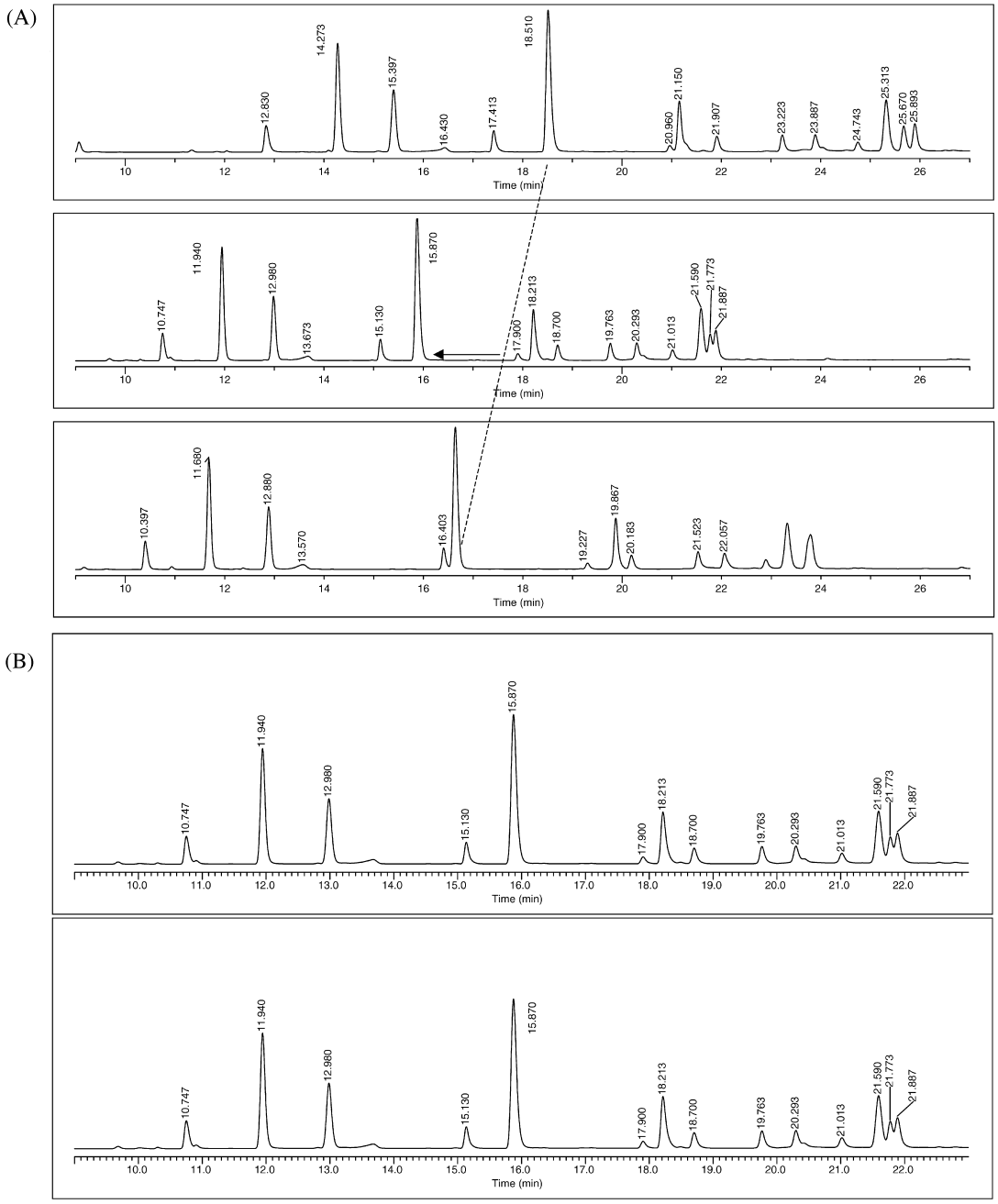

Fig. 1. (A) Unexpectedly short retention times at 55 °C due to error in gradient programming: chromatograms recorded at 40 °C (top), 55 °C (middle), 70 °C (bottom). Linear gradient was supposed to be programmed from 5→80% B in 30 min, however, in the middle chromatogram the peak at 15.87 min is eluted by ca. 1.7 min too early. The reason for the early elution was an error in gradient range from 5→95% B. Eluent B: acetonitrile, eluent A: 5 m*M* phosphate buffer, pH 2.4. Column: 150×3 mm ODS3, 3 μm; detection: 210 nm. (B) Discovering the error in gradient programming by DryLab simulation: it was assumed that the gradient range is going from →95% B instead from 5→80% B. The comparison of the experimental (top) and the simulated (bottom) chromatogram.shows, that the assumption about the wrong gradient range (5→95% B) was correct. With that DryLab model we could detect a programming error in the gradient method. Experimental conditions as in (A), except for gradient, which was 5→95% B in 30 min.

another aspect of this paper, which is concerning about the proper treatment and discovery of ''unexpected'' chromatographic results. Fig. 1A shows chromatograms recorded at three different temperatures (30, 55 and 70 °C). It was assumed that retention times will continuously decrease with increasing temperatures. But the run at 55 °C shows ''unexpected'' behaviour: the elution times were not only shorter than at 40 °C but also shorter than at 70 °C. Looking for the reason, the gradient was suspected to run up to 95% B instead of only up to 80% B in the same gradient time. So the steeper gradient would result in shorter retention times. Now, from the two runs at 40 and 70 °C a computer model could be calculated and a run with a gradient up to 95% B could be simulated. The results are shown in Fig. 1B. The deviations are now remarkably low. This means, that the application of computer modelling helped to discover programming errors in the gradient range by the laboratory technician.

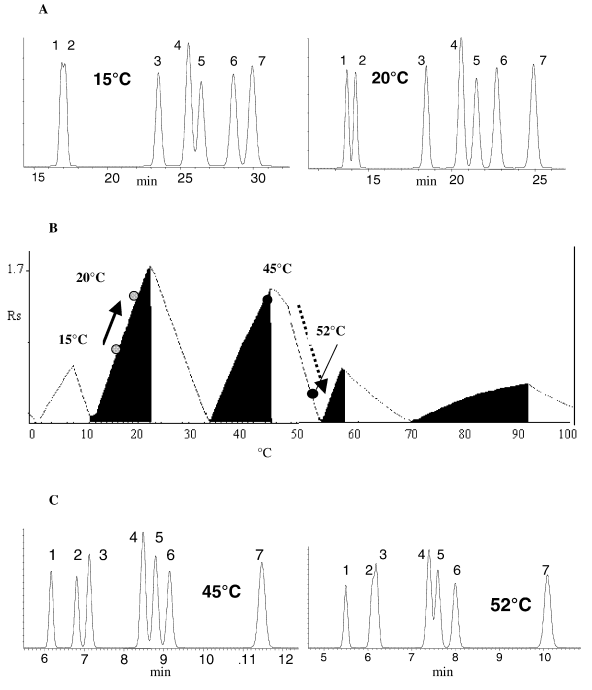

Fig. 2. (A) ''Unexpected'' increase in resolution with higher temperatures. Changing from 15 to 20 °C, the peak pair 1 and 2 is the critical peak pair, but 4 and 5 is another pair of peaks which are better separated at the higher temperature. (B) Inside of the dark ranges an increase in temperature 15→20 °C (see the → arrow) results in an ''unexpected'' increase in R_s, as long in the range 45→52 °C (see the ----> arrow) an increase in temperature is connected to an ''expected'' decrease in resolution. As we can see, there are ca. 50% ''unexpected'' choices for improvements in resolution available, using the tool ''resolution map''. (C) ''Expected'' decrease in resolution with increasing temperature: Changing from 45 to 52 °C the critical peaks 2–3 and 4–5 overlap increasingly.

3. Results and discussion

A DryLab model of a chromatographic method consists mainly of a so-called critical resolution map. These maps show the critical resolution, i.e., the smallest value of the resolution of any two peaks in the chromatogram, as a function of the varied experimental parameter. For each value of the experimental parameter, displayed on the *x*-axis, one can read the critical resolution at the *y*-axis.

The perhaps most important eluent parameters in RP-HPLC are temperature, % B and t_G.

We will take a look at each of these variables.

3.1. Temperature

Regarding temperature, the expectation is a decrease in resolution with increasing temperature. Fig. 2 shows the resolution map of a separation of food additives as a function of the column temperature (separation conditions: isocratic elution with 50% acetonitrile, C_8 column). The map (Fig. 2B) suggests, that there are as many ranges of temperature, where indeed the resolution decreases with higher temperature (dotted arrow) as there are ranges, where the contrary is true: resolution increases with higher temperature (solid arrow). This is demonstrated by the simulated chromatograms for different values of temperatures in Fig. 2

3.2. % B (isocratic separation)

In isocratic experiments, higher % B values mean shorter run times. From that, it is a common suggestion that resolution can only become better by decreasing the % B value and making the run time longer. Fig. 3 shows the resolution as a function of the % B value for a separation of some antidepressants. The map shows again regions of % B values,

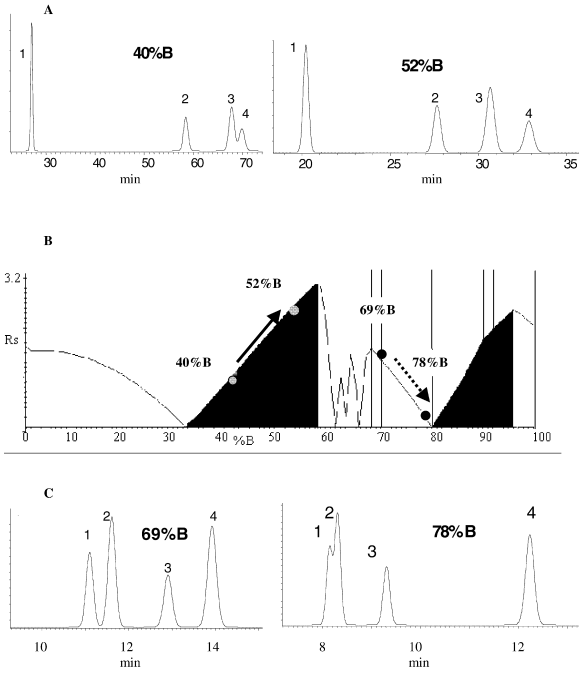

Fig. 3. (A) ''Unexpected'' increase in resolution with higher % B, i.e., in a shorter run time. Changing from 40 to 52%, the peak pair 3 and 4 is the critical peak pair and is better separated unexpectedly at a higher % B. (B) Inside of the dark ranges an increase in % B 40→52% (see the → arrow) results in an ''unexpected'' increase in R_s, whereas in the range 69→78% (see the ----> arrow) an increase in % B is connected to an ''expected'' decrease in resolution. As we can see, there are ca. 50% ''unexpected'' choices for improvements in resolution available, using the tool ''resolution map''. (C) ''Expected'' decrease in resolution with higher % B, i.e., shorter run time. Changing from 69 to 78% the peaks 1 and 2 are more overlapping.

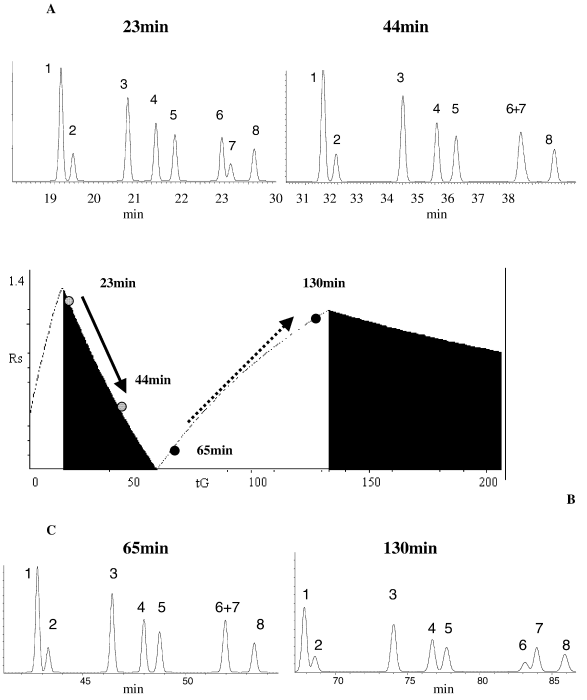

Fig. 4. (A) ''Unexpected'' decrease in resolution with longer t_G. Changing from 23 to 44 min, the critical peak pair 6 and 7 will be a double peak. (B) Inside of the dark ranges an increase in t_G 23→44 min (see the → arrow) results in an ''unexpected'' decrease in R_s for the critical peak pair, whereas in the range 65→130 min (see the ----> arrow) an increase in t_G is connected to an ''expected'' increase in resolution for the critical peak pair. As we can see, there are ca. 50% ''unexpected'' choices for improvements in resolution available, using the tool ''resolution map''. (C) ''Expected'' increase in resolution with longer t_G. Changing from 65 to 130 min the double peak 6/7 is beginning to separate into two peaks.

where the resolution increases with lower expected % B and regions were, unexpectedly, resolution decreases with lower % B. Separation were with mixtures of water–acetonitrile (35 °C) on a C_8 column.

3.3. Gradient time (gradient separation)

In gradient separation experiments, resolution is expected to decrease with steeper gradients, because a steeper gradient means a shorter run time. But again, both increasing and decreasing gradient time can improve resolution. The resolution map of Fig. 4 clearly shows the regions for this method, where resolution becomes as expected better or worst (unexpected) with longer run times. The chromatograms shown here are from samples of hydrocarbons, separated using a gradient from 5% B to 100% B with the indicated gradient times. Eluent A was water, eluent B acetonitrile, temperature was 35 °C, flow-rate 2.0 ml/min. The dwell volume of the apparatus was 5.50 ml.

3.4. Two-dimensional t_G/T optimization

In Figs. 5 and 6, two-dimensional resolution maps are shown. Here, the resolution (scaled by the gray code) is plotted as a function of two simultaneously

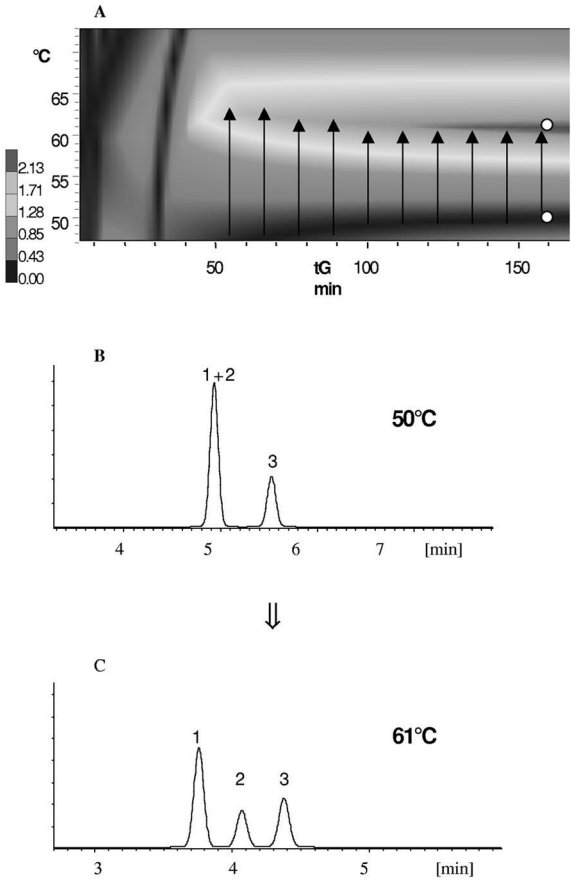

Fig. 5. (A) The two-dimensional resolution map of temperature and gradient run time, showing the critical resolution (scale on the left) between $R_s=0$ (dark blue) and $R_s=2.13$ (red). All experiments, characterized by an arrow, start from the dark blue area (peak overlap) and move in direction of higher temperatures (by 10–15 °C) leading to a good baseline separation. These experiments are against expectations, even from experienced HPLC experts, and represent ''unexpected'' options for resolving critical peak pairs. A large part of the critical resolution map shows such regions, where ''unexpected'' improvements are possible. For better understanding, we select one movement which is shown in the resolution map. By $t_G=160$ min we move from a dark blue area (50 °C; white lower circle) to the red area (62 °C; white upper circle). We can see a better baseline separation of a group of three peaks, showing in the two chromatograms below (Fig. 4B and C). (B, C) ''Unexpected'' increase in resolution with increasing temperature. Changing from 50 to 61 °C, the overlapping peaks 1 and 2 are baseline separated at 61 °C.

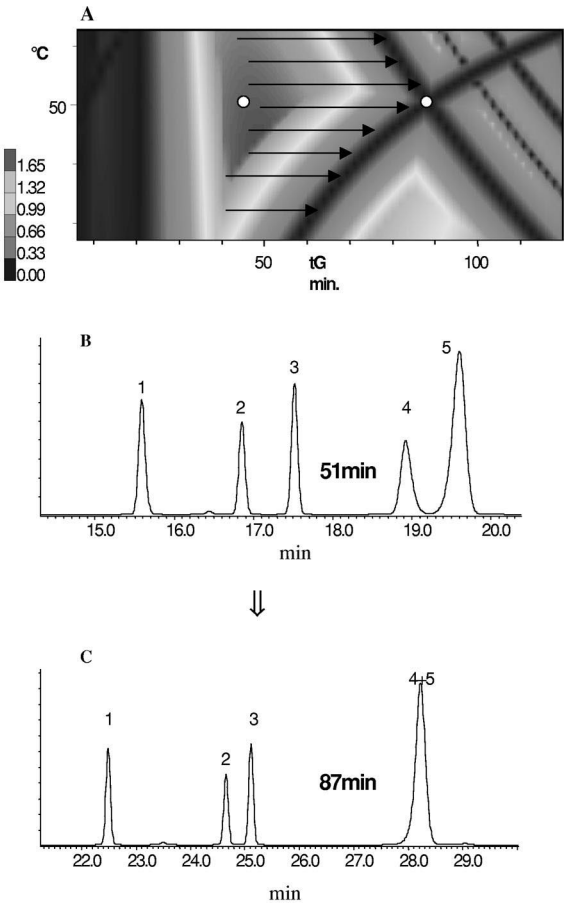

Fig. 6. (A) The two-dimensional resolution map of temperature and gradient run time showing the resolution of critical peak pairs (scale on the left) between $R_s=0$ (dark blue) and $R_s=1.65$ (red). All experiments, characterized by an arrow, which start from the red–orange area and move in direction of longer gradient run times (by 20–30 min) lead to an overlap. For better understanding, we select one movement, which is shown in the resolution map. By $T=51$ °C we move from an orange area (51 min; white left circle) to the blue area (87 min; white right circle). We can see a decreased resolution of peaks 4 and 5. (B, C) ''Unexpected'' decrease in resolution with longer gradient run times. Changing from $t_G=51$ to 87 min, the peak pair 4 and 5 overlap.

varied experimental parameters, i.e., gradient time and temperature. From these resolution maps, combinations of both parameters can be found, which will increase resolution. Inspections of the two-dimensional resolution maps allows one again to find regions with expected and unexpected improvement of selectivity.

A closer inspection of the resolution maps show that from a point where the resolution is zero (two

peaks overlap) there are always choices for an increase in resolution, regardless of the direction an experimental parameter is changed.

The resolution maps show immediately the regions where two peaks overlap. This is a valuable information for the analyst because near these points he can expect increasing resolution by changing the parameter values in both directions.

4. Conclusions

The influence of the most important experimental parameters used to adjust resolution in chromatographic separations on selectivity has been evaluated. It has been demonstrated by computer calculated resolution maps that common expectations about how these parameters have to be varied for a better resolution are only partly true. There are often as many chances to improve resolution by varying experimental parameters in the ''unexpected'' direction as by varying them in the ''expected'' direction. This extends the possibilities for method development, providing the dependence of retention on the parameter is known over a sufficiently wide range. The resolution maps, as shown in this work, are assumed to be the best way of learning about these dependencies. Furthermore, it was shown in this work, that errors in programming of gradient ranges could be discovered, after a DryLab model was available, showing that the same results as shown in the experiment could be generated with altered settings in elution conditions.

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