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Review

Computerized design of separation strategies by reversed-phase liquid chromatography: development of DryLab software

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Abstract

The development of DryLab software is a special achievement in analytical HPLC which took place in the last 16 years. This paper tries to collect some of the historical mile stones and concepts. DryLab, being always subject to change according to the needs of the user, never stopped being developed. Under the influence of an ever changing science market, the DryLab development team had to consider not just scientific improvements, but also new technological achievements, such as the introduction of Windows 1.0 and 3.1, and later Windows NT and 2000. The recent availability of new 32-bit programming tools allowed calculations of chromatograms to be completed more quickly so as to show peak movements which result for example from slight changes in eluent pH. DryLab is a great success of interdisciplinary and intercontinental cooperation by many scientists.

Keywords: Method development; Resolution; Computer simulation; Resolution map; DryLab; Reviews; Quality control

Contents

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1. Introduction

Progress in methodology is progress in science. Reducing the time needed to understand how a mixture of substances is composed, will increase the speed to generate new scientific results. This was the main reason why DryLab was developed. The other scientific reason to develop this tool was simply to produce *more reliable methods* and *more reliable results* for scientific work. The third was a more economic reason: to save time and money using this tool.

The fourth important reason for using the computer besides data handling was to teach beginners to orient themselves in this highly complex matter [1]. According to the saying: ''One picture tells more than thousand words'', the idea of showing chromatograms instead of equations is expected to help to transmit complicated contexts more easily to the novice in HPLC.

A product in the chemical, pharmaceutical and food industries has to be controlled very precisely. One way to do this is by using HPLC, where the quality of a product will be proven by an HPLC method. But who takes care of the quality of the method?

We continually learn more about the composition of our products. With the increasing number of components to be controlled, the demand for satisfactory separation is also increasing. Gradient elution is replacing isocratic work. This is one of the reasons why scientists were looking for computerized ways in method development and in routine applications. One of these tools is DryLab. It is a valuable help in research and development of new drugs and in the process of making *product quality* in the pharmaceutical, chemical and food industry *safer*.

The start of the method development for DryLab software goes back to 1986.

2. Theoretical concepts

The theoretical background of judging column performance in gradient elution, in particular, how band spreading changes with flow-rate and column dimension, how resolution will be altered at another k^*-value of a solute molecule, was an important issue in the development of software for modeling the separation [2]. On the other hand, the development of a model that predicts plate number and bandwidth as a function of conditions for small-molecule samples, and a model for predicting large-molecule separations by gradient elution, particularly for reversed-phase LC, was needed for many HPLC users in life science. The broad theoretical background of reversed-phase chromatography, considering hydrophobic or more general ''solvophobic'' retention forces, has been studied and explained in detail by Horváth, Melander and Molnar, investigating the thermodynamics of the free energy in the chromatographic process [3,47,48]

$$\ln k = A + BD + C\Delta A + D(\kappa^e - 1)V^{2/3}\gamma + E + \ln(RT/P_oV)$$

This equation describes the influence of the eluent based on the surface tension γ, which is proportional to the value of (100 − %B = %A), on the influence of the temperature T, the influence of molecular properties (ΔA) of both the sample and the chemically bonded ligand, and on electrostatic properties, such as buffer concentration [47,48].

The book on automated optimization in HPLC by Berridge in 1985 [4] and the work of Schonmakers on optimization of chromatographic selectivity in 1986 [5] added further great interest towards the computerized search for better methods among the members of the HPLC community. The use of resolution maps (Window-Diagram), originally in-

vented by Purnell in GC, was a valuable tool to judge the influence of a chromatographic parameter on critical resolution (Fig. 1a–f).

At the center of interest were separations in life science. For this purpose computer-simulation and applications of reversed-phase gradient elution for many related substances seemed to be of great general interest [6–13]. Stationary phase properties were carefully studied and the results were introduced into software, which was named ''DryLab'' and which was to support the calculation of band spreading effects using proper chain lengths of the chemically bonded ligands and the actual pore size. Column performance was characterized by the *A*-value of the Knox equation or later by changing the plate number. Individual peak widths and peak asymmetry factors were introduced later to be able to adjust DryLab models to the real experiments even better and to mimic peak shapes and resolution more precisely [14–18].

3. Isocratic versus gradient methods

Isocratic methods are, as it is well known, fairly robust, they have few problems in method transfer from instrument to instrument and are the preferred way to work in routine analytical work in quality control. Not so in gradient elution, where instrument dwell volume can change retention times and selectivity, if the method is used on instruments from different manufacturers. The disadvantage of isocratic work on the other hand was to be unable to elute some of the strongly adsorbed components. Gradient scouting runs give a more complete picture about the total sample composition and are used today more and more to ensure that no components remain on the column.

Therefore it was desirable to predict isocratic

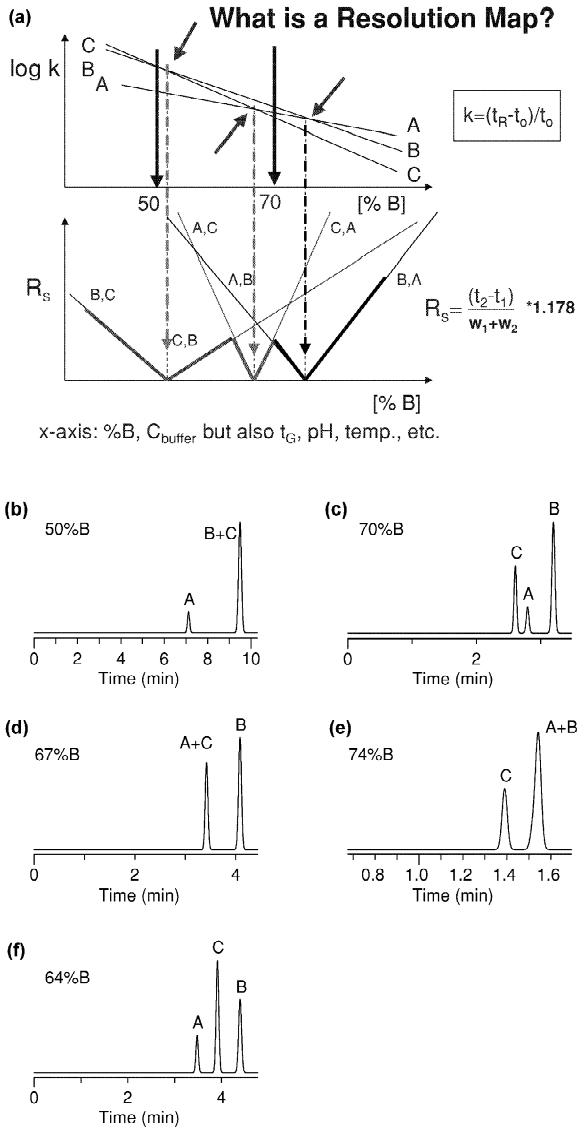


Fig. 1. (a) What is a Resolution Map? From measured retention times t_R and the column dead time to the retention factor k are calculated and logarithms plotted against one of the eluent properties, here against the amount of the organic modifier %B. However, other resolution maps for the same mixture are also possible, such as %B, C_{buffer} but also t_G, pH, temperature, etc. The critical resolution is by definition the lowest resolution value of the three peaks, shown in the lower figure as a bold line. As %B changes, the critical peak pair is changing too: On the left peaks B and C, in the middle, peaks A and C and on the right side, peaks B and A are the critical peak pair. (b) Eluent composition 50%B (left black arrow in upper part of: (a) The order of elution is first peak A, followed by a double peak from B+C. (c) Eluent composition 70%B. Order of elution is changed, as the first peak is now C, followed by A and the last peak is B. (d) Eluent composition 67%B. Two peaks, A and C overlap here. The resolution is zero. (e) At eluent composition 74%B, A and B overlap completely, $R_{s,crit}=0$. (f) The best separation can be obtained at 64%B. These conditions are characterized by maximized distances between the critical bands, also called ''equal band spacing''.

models from gradient data. Already in early days, this occupied the attention of many researchers. Dolan studied the selectivity in reversed-phase gradient elution as a function of gradient conditions [19]. Also predicting bandwidths in the HPLC separation of large biomolecules was reported by Stadalius and colleagues. They developed a general model for predicting retention for the four common HPLC methods [20].

However, one had to be careful with the precision of such computer-generated results. Therefore it was crucial to set the proper limits, between which reasonable modeling of ''virtual experiments'' is possible. This requires the collection and evaluation of a large number of real-life data and their comparison with computer models. This started with detailed analysis of separation phenomena that can limit the accuracy of gradient retention data.

The first description of DryLab 1, the ancestor of the column optimization part of the present DryLab software, as applied to a steroid sample and the first description of DryLab 4, the ancestor of the binary isocratic reversed-phase module of DryLab as applied to a mixture of nitro-aromatic compounds was published in 1986. A few years later DryLab 1, 2 and 3 were combined with DryLab 4 to generate DryLab I (I for isocratic), while DryLab 1, 2 and 3 were combined with DryLab 5 to generate the new mode DryLab G (G for gradient) in 1987.

4. Predictions in gradient elution

In gradient elution, it is often difficult to predict how resolution and retention will change, when we introduce a new gradient step. Today, it is done easily, after Snyder's gradient elution theory was put into DryLab, enabling chromatographers to find the optimum in much a shorter time than was possible previously.

The development of the basic theory relating gradient and isocratic separations, essential to work on DryLab I and DryLab G, was published in Horvath's sophisticated compendium on ''HPLC—Advances and Perspectives'', where Snyder wrote the basics, which explained, what was going on in the column in gradient elution and how to calculate retention values, peak widths, depending on experimental parameters [2].

5. Eluent influence—DryLab isocratic multiparameter version

DryLab started with modeling RPC data, but it was soon extended also for normal-phase chromatography and for GC. For the separation of macromolecules, other techniques, such as ion-exchange and hydrophobic interaction chromatography should also be considered to work with.

It was therefore a logical step to extend the calculation of the critical resolution in isocratic work, besides %B, also for other parameters such as temperature, using two basic experiments. Three basic runs each were needed for basic compounds, for the pH, for the ionic strength in eluent A, for normal-phase chromatography and for the concentration of additives, such as ion pairing agents. This was realized in the version called DryLab I/mp (i.e. ''isocratic multiparameter version''), which could help to reinvestigate the influence of the above mentioned experimental factors in method validation.

The incorporation of the well-known principle of Snyder's ''solvent triangle'' into DryLab was a challenging task, but it also could be solved using three experiments. A gradient multi-parameter version was not yet possible at that time. One had to use different plate numbers in different areas in DryLab I/mp. Later, peak widths were introduced, which allowed a big step forward, namely to use gradient runs in the computerized modelling process.

6. Column influence

The influence of column properties on the separation pattern was also examined in a different context, more often from the point of the stationary phase and less often from the influence of other parameters like temperature or eluent pH. The major problem in method optimization using different columns is the change in critical peak pairs from column to column. Which is better: to have all peaks resolved using appropriately mixed stationary phases or to adjust resolution continuously with pH or

temperature? Evidently the latter, because of the difficulties of tailoring the stationary phase to the particular mixture to be separated.

The problem of column-to-column reproducibility and the consequent adjusting conditions to minimize retention differences were published by Dolan and colleagues as early as 1987 using DryLab 4 and 5, the first DryLab programs to model retention effects in gradient elution [21] followed by a general discussion of the potential of band-spacing changes via a change in solvent strength to new samples [22–26].

7. Multi-step gradients

In parallel with this development, further exploration of the simulation of multiple steps in gradient elution was brought forward by Jupille [27] based on the gradient elution theory of Snyder. This part of DryLab was one of the strongest features of the software, often thought to be ''unbelievable'' by those, who were working for months to optimize a certain specific gradient method. Predictions in gradient elution were very valuable in protein separations but also for small molecules, as shown by Dolan and Ghrist [28–30].

Molnar et al. found in research on ribosomes, that the predictions of DryLab were highly reliable even for 54 different ribosomal proteins of *Thermus aquaticus* from the 30*S* and the 50*S* subunits. The proteins, which maintain the biological activity of the bacterium *Thermus aquaticus* at 80 °C, are held together by strong ionic and hydrophobic forces between themselves and the r-RNA, and could be precisely studied with DryLab [31].

Further investigations of the details of isocratic modelling were shown by Snyder et al. and Shaw [32,33]. This approach also served in the development of an isocratic HPLC assay to estimate synthetic intermediates of a leukotriene inhibitor, by Fulper [34]. Stuart showed that the separation of mixtures of OPA-derivatized amino acids by RP-gradient elution could be predicted by computer simulation with high accuracy [35] as long as selected nitro derivatives of polyaromatic hydrocarbons, fluoroxypyr herbicides and their metabolites were separated by computer supported gradient elution [36,37].

8. Peak tracking

Peak tracking remained a difficult task in the routine application (Fig. 2). However, unlike instrumental and therefore expensive peak matching procedures, which has inhibited the broad distribution and application of other excellent software in the past, peak tracking using peak areas turned out to be a rather simple and surprisingly accurate technique, as long as injection volume of the sample was kept constant between the necessary injections for the basic runs [38]. DryLab is now able to apply automated peak tracking between two runs, provided the mixture is clean and does not contain too many components.

In 1990, Snyder and Glajch initiated the edition of a book on computer-assisted method development, which appeared in the *Journal of Chromatography* as volume no. 485, consisting of 43 papers in a broad scientific context. The vigorous participation of many groups working in this field was a great success, as was shown in this volume, representing the great general interest to improve the understanding of chromatographic phenomena, to improve the separation and to reduce analysis time [39–43].

A critical question, was raised by chemometricians: how far could chromatographic parameters in HPLC influence each other? Snyder and colleagues could show however that multiple variables may be treated independently, if the range of variation is kept sufficiently narrow. This meant 15–20% change in %B, a factor of three in gradient elution time, 20–30 °C difference in temperature studies and 0.5–0.6 pH units of eluent A, between runs in the investigation of pH effects [44,45].

9. pH influence

In 1976, it was found that in RPC, the retention of some weak acids and bases is strongly dependent on the pH of the aqueous mobile phase using nonpolar stationary phases and neat aqueous eluents [46]. Horváth studied the underlying theory and used

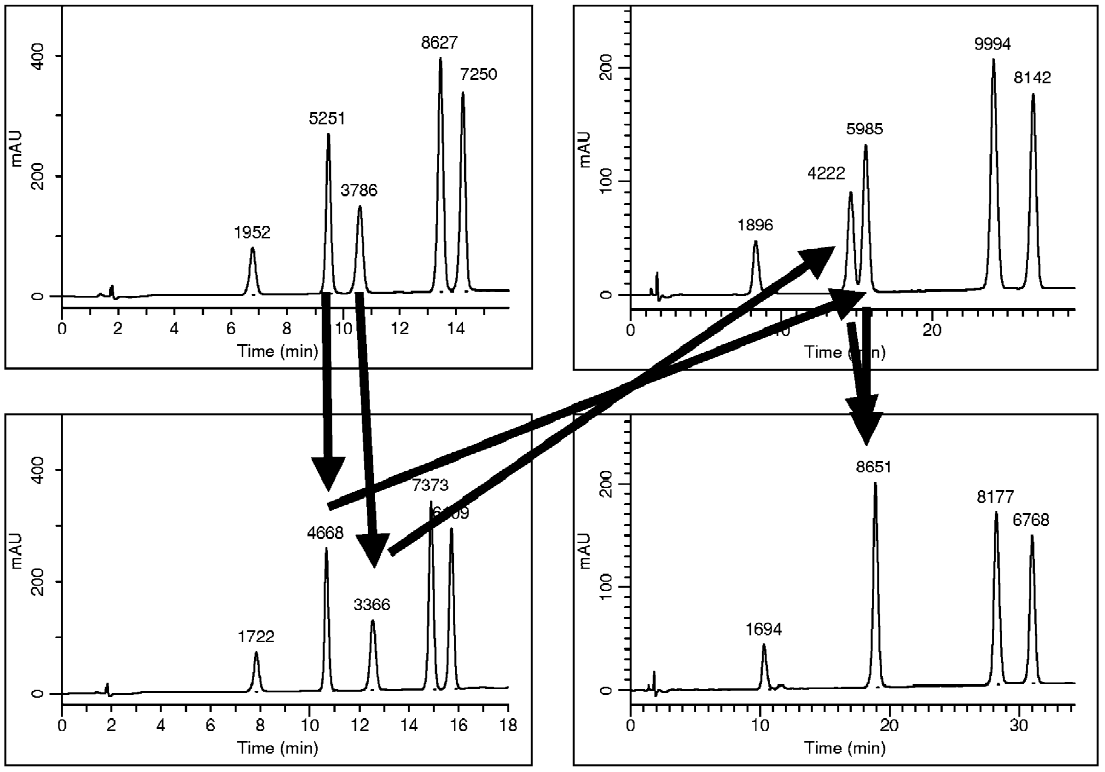


Fig. 2. Peak tracking: in most methods, we can change the selectivity, i.e. the relative position of a peak between its two neighbouring bands by changing elution conditions. In the above example, t_G and the column temperature were varied. Bottom left: t_G: 40 min, temp.: 30 °C; top left: t_G: 40 min, temp.: 60 °C; bottom right: t_G: 120 min, temp.: 30 °C; top right: t_G: 120 min, temp.: 60 °C. The numbers on top of the peaks are the corresponding peak areas: Their differences are in most cases sufficient to identify a peak or to discover a peak overlap.

computer calculations to explain the observed retention phenomena of mono-, di- and oligoprotic acids, which could change retention times in some cases as much as up to a factor of five. It became clear that already small pH-changes will change method robustness dramatically (Fig. 3) [47,48].

Predictions were in good correlation with the theory applied and it became obvious that for a certain separation *without* basic experiments, there would be *no reliable prediction* possible (i.e. with better than 99% accuracy), for corresponding retention times and resolution values (Fig. 3). This recognition helped to apply a design, which would not be based on structural elements of the sample molecules for software development, but which would use a limited number of experiments, namely in the case of pH, only three runs. After a careful look at the peak tracking situation, it was suggested that the three runs should have not more pH-distance than 0.5–0.6 pH units.

Quality control in the pharmaceutical industry has a high standard and needs the strict fulfillment of high method quality requirements [49]. Bilke investigated stability tests of pharmaceutical products, carried out with three runs and have shown that the effect of the eluent pH in the same chromatogram can be different to different substances: with a certain pH change a peak group can merge together, at the same time other peaks are drifting apart. Basic compounds move with increasing pH to higher retention times, as long acidic molecules are moving in the opposite direction. The results can be many undesirable coelutions, which often remain undiscovered, if a pH study of the total retention effects over a range of at least 1.5–2.0 pH units was not conducted in detail [50].

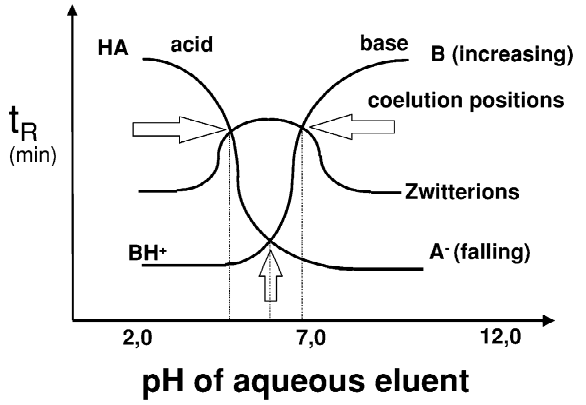


Fig. 3. Schematic visualization of changes in retention time of some peaks with the eluent pH. Peak movements due to pH changes affect the retention and the resolution of weak acids and bases due to opposite-directed movements in the chromatogram, which often results in coelution (arrows). Zwitter-ions, like peptides can additionally complicate the quantitative measurement of coeluting compounds, influencing the quality of information of HPLC dramatically in life science. In this area of science, predictions from molecular structure are less precise—only measured data can give satisfactory results in predictions.

Routine analysis in pharmaceutical quality control was often disturbed by unrecognized pH changes, as shown in Fig. 4. Here small changes in pH caused severe problems in quantitation of a major impurity. Several revalidations were necessary before the source of the problem could be addressed and a solution could be found (Fig. 4a–h).

At this time, using DryLab I/mp, only isocratic pH models could be predicted. In cases of gradient elution, it helped to subdivide the chromatogram into three ranges: front, middle and final third and using different plate numbers in these regions [88]. The demand for pH modelling in gradient elution could be first satisfied after the introduction of measured peak widths as input data and cubic spline modelling functions became available.

There is still a discussion about where to measure the pH, in eluent A or in the mixture of both. As the measurement of pH in organic solvents is difficult and the ion product of water is not 10^{-14} anymore, the pH as such loses its meaning to be the negative decadic logarithm of the hydronium-ion (OH_3^+) activity. Therefore it is necessary to measure the pH in the aqueous eluent A, before mixing to the organic modifier B takes place.

10. Gradient editing

Gradient editing requires certain prerequisites in viewing the elution process. As the eluent composition determines the retention time of a substance, it is important to know where to measure %B: at the mixer outlet, at the column top, or in the detector cell? One of the logical answers here is that we have to measure eluent composition where we detect the peaks, namely in the detector cell. Only then is it possible to set the gradient points correctly, which are necessary for the correct separation and elution of the total sample. In this process, it also becomes clear that the eluent profile in the elution process of the sample will contain an isocratic ‘‘pre-elution’’ step, which is different from instrument to instrument. The corresponding eluent volume was named by Snyder the ‘‘dwell volume’’.

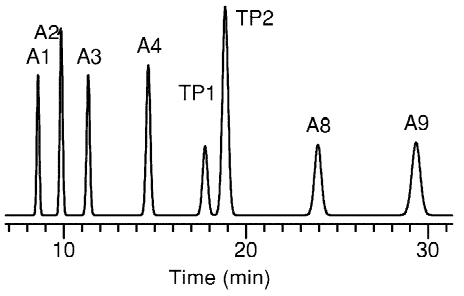
Method transfer can only be carried out correctly if the dwell volume has been measured carefully and is indicated in the method description of the validated gradient method. (In the case of isocratic methods, the meaning of dwell volume is not significant.) Furthermore, the dwell volume range, in which the method is applicable, should be indicated. The smaller the column volume, for example in LC–MS, in relation to a larger dwell volume of the HPLC-system and the smaller the flow-rate, the more dramatic this effect will be (Fig. 5a–d). The usual consequences are long discussions and several meetings between the participants of the method transfer process, which is also expensive, if the locations of the groups are in different countries.

Snyder and Dolan have shown, in cases where gradient separations do not reproduce well on different HPLC systems, how DryLab can help to solve these problems [51].

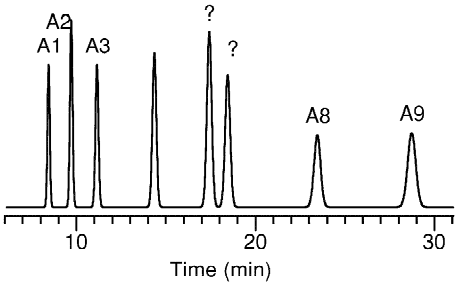
11. DryLab for gas chromatography (DryLab GC)

Multisegmented gradients were developed for the separation of some polyphenolic pollutants by Markowski et al. using DryLab [52] in liquid phase separations. Volatile substances, on the other hand, are the subject for separation in the gas phase by GC. The development of the GC version of DryLab was

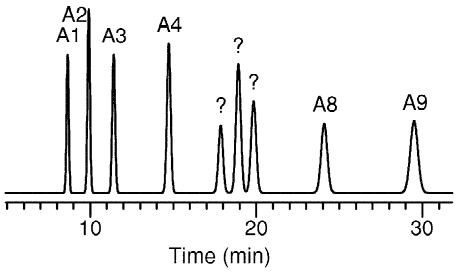
a. Validated method at pH 4.70



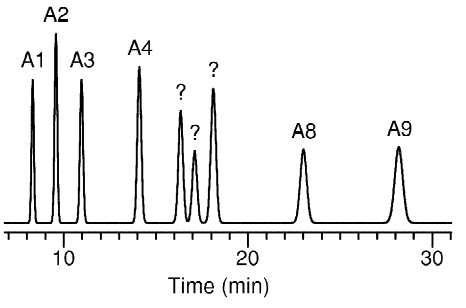
b. "which looked on some days..."



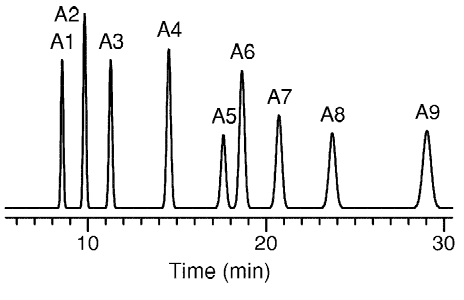
c. "and on some other days..."



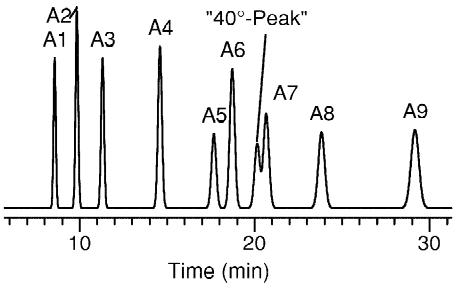
d. "and which looked only sometimes..."



e. After new validation at pH 4.40

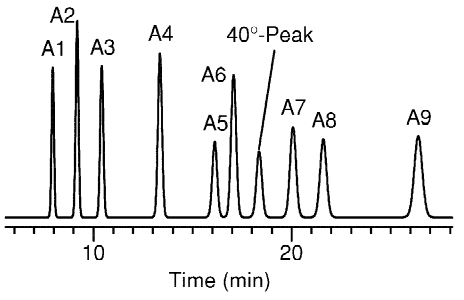


f. Trouble in 40°-sample: A new peak!!!

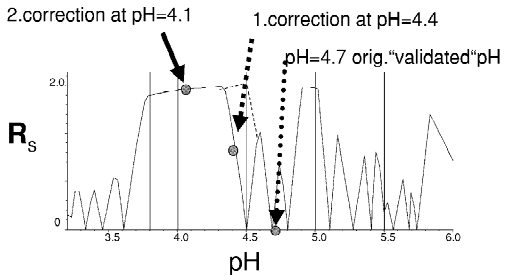


pH-influence in QC

g. Final validated method at pH 4.10



h. Optimum found only after 2.correction



the consequent next step to enhance the yield of information, which was available mainly on peak movements in GC. Computer simulation, based on a Linear-Elution-Strength (LES) approximation of Snyder was used as an aid for optimizing separations by programmed-temperature GC by Bautz et al., who developed the basic theory and assumptions underlying DryLab GC with a preliminary experimental validation of the model [53].

Several successful applications of DryLab GC to a wide range of environmental, pharmaceutical, and process samples describe a new way to solve GC separation problems, where different column materials might not be able to separate a critical peak pair. Using special resolution maps with a totally different temperature gradient could offer possibilities here [53–58]. Grob et al. have shown several applications of DryLab GC to chromatography instruction and teaching in the GC laboratory [59].

12. Other currently available optimization software for use in HPLC

A brief review of currently available expert systems and simulation software approaches and their comparison was discussed in several articles, in particular, a comparison of commercially available simulation software packages at the Pittsburgh Conference in 1991 [60,61].

Also here has to be mentioned the software ''Metabolexpert'', which was developed by Valko and colleagues in Hungary and which helped to obtain a picture of the metabolic pathway of a pharmaceutically active drug substance.

The search was started with the molecular structure of possible metabolites, using a computer-assisted metabolism prediction (CAMP), than assigning retention times to the structure using QSRR. The difference between predicted and experimental retention times was always less than 8 min, and the average deviation was an acceptable 1.8 min [62]. A further product for this approach, using structural information of the sample molecules was marketed under the name of ''Eluex''.

Later the ''Eluex''-approach was applied by Galushko, who marketed his product as ''ChromDream'' through Knauer. The commercial success however remained in a dormant state. Later, Merck took over the product under the new name ''Chrom Sword'' [63], which also starts with asking for molecular structure of the sample molecules of interest and is able to make predictions about the order of elution. Columns of different manufacturers are included in the database with their retention behaviour and the software aims to achieve method transfer from column to column. In the latest version, ChromSword claims to be able to run separations fully automated overnight under the name of ''AutoChromSword''. The software initiates up to 30–40 injections onto the column overnight, with isocratic or gradient methods chosen according to a proprietary process. The user can select in the morning the best looking chromatogram for further work.

Fig. 4. (a) Chromatogram in routine QC, validated at pH 4.7. Smooth operation was the case most of the time, but there were two peaks giving trouble, TP1 and TP2. (b) On some days, however, TP1 and TP2 seemed to turn over: Now the left peak was larger than the right one. What happened here? Did the peaks had a positional exchange? (c) On some other days, an additional new peak TP3 appeared in the group, so one had three peaks, the largest in the middle. (d) On some other days, one had three peaks in the group with the smallest in the middle. (e) Finally, a systematic investigation at three different pH values of eluent A (pH 4.0, 4.5 and 5.0) was carried out (s, in (h)), revealing that TP2 in (a) was composed of two peaks: A6 and A7, as long the left ''?''-peak in (b) was composed of A5 and A7. In (c) the order of elution was in the group: A5–A6–A7, as long in (d) the order of elution was: A7–A5–A6. All this could be explained by the fact that peak A7, a weak acid, changed its position quite dramatically with changing pH in the eluent and moved with increasing pH to shorter retention times. So far, the true pH was in the case of (b) ca. 4.8, in (c) ca. 4.6, only ± 0.1 pH unit off from the validated value of 4.7 in (a). In (d), the true eluent pH must have been 4.9, only 0.2 greater than the ''validated'' value of pH 4.7. After evaluating the pH influence with the resolution map (h), the eluent pH was established to become pH 4.40. (f) In stability studies at 40 °C, however, suddenly a new decomposition product (40°-Peak) turned out to be formed in the mixture. This peak was unfortunately strongly overlapping with the critical peak A7—so a new validation had to be carried out. (g) At this time, the validation was less time consuming: As shown in the resolution map (h), a fairly robust region for the pH could be found at pH 4.1 ± 0.2. The wider pH tolerance has been included in the protocol for the method for eventual later necessary pH adjustments, occurring at other locations or with columns from other batches, etc. (h) Resolution Map of pH versus critical resolution R_s.

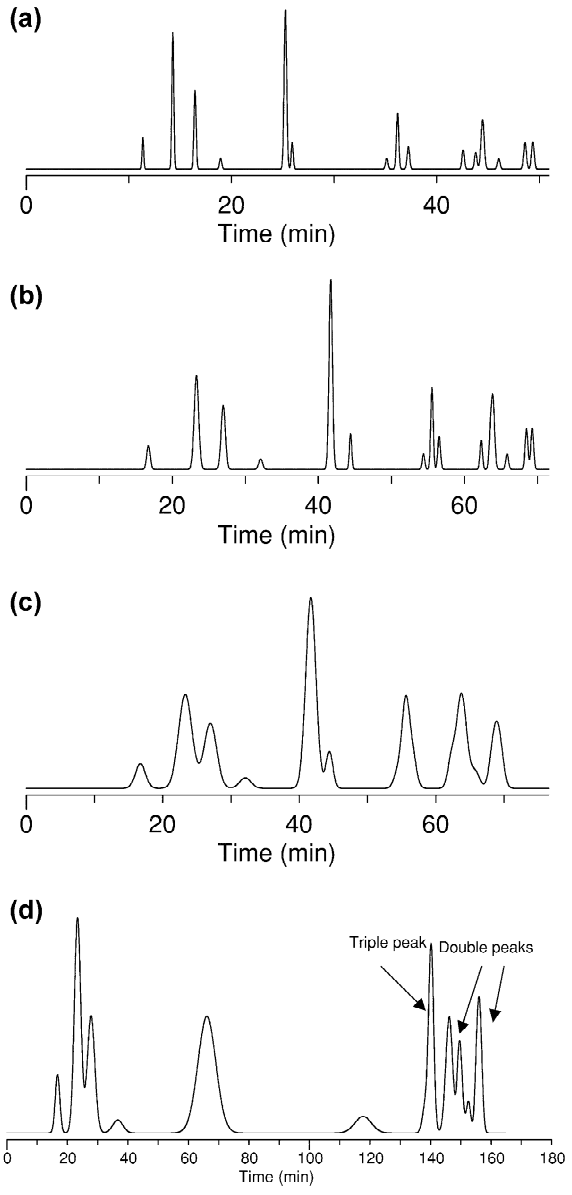


Fig. 5. Method transfer: Comparison of methods for LC–MS on two different columns and three different instruments. (a) 3 mm I.D. and at flow-rate: 0.50 ml/min, V_d: 1.05 ml, $V_{ext.col}$: 16 μl; (b) 1 mm I.D. and at flow-rate: 0.05 ml/min, V_d: 1.05 ml, $V_{ext.col}$: 4 μl; (c) 1 mm I.D. and at flow-rate: 0.05 ml/min, V_d: 1.05 ml, $V_{ext.col}$: 16 μl; (d) 1 mm I.D. and at flow-rate: 0.05 ml/min, V_d: 5.50 ml, $V_{ext.col}$: 16 μl. Column: C_{18}, 150 mm long, dp: 3 μm. Operating conditions: t_G: 80 min, temperature: 47 °C.

Another group in Canada, Advanced Chemistry Development (ACD), works on a similar principle as ChromSword, namely on the prediction of separations, based on molecular structure. Both programs ChromSword and ACD are aimed at research in pharmacology in the phase of early studies. ACD has developed a database which is used to link generic methods to the sample types, for which they are best suited. The software uses a structure similarity search combined with retention time prediction—called ''Chromatographic Smart Search'' (CSS) to choose between generic methods, passing the most promising method back to the instrument control software with the expected retention time, reducing the number of injections that are required to process a large number of samples, as well as easing the burden of data interpretation after the experiment.

Kaliszan et al. recently carried out a comparison of some segments of DryLab and ChromSword [64] mainly in isocratic RPC of neutral compounds. They pointed out that the accuracy of predictions based on molecular structure is much less accurate than expected, in comparison to measurements of the retention behaviour. The limited success of ChromSword in this area is attributed to the QSRR model, applied by Merck.

A similarly specific software was ''Peptide Digest'' of Hodges et al. from Edmonton, Alberta, Canada, in the field of structure-related peptide analysis in different HPLC modes, which could—as long as the gradient system remained unchanged—fairly precisely predict peptide retention, based on amino acid composition [65].

13. Technological progress

Adjustments of the software to new hardware requirements were quite difficult, as microprocessors were continuously being developed to higher and higher performance. Throughout the development of DryLab, which started in a DOS mode, several changes were necessary, such as rewriting the program to Windows 1.0, than 3.1 and finally from 16 bit to 32 bit (DryLab 2000). However, this changes also enabled the program to carry out calculations, which were not possible before. Such a progress was the development of an algorithm for the calculation

of resolution maps which were reduced from all to just one individual band or for a subgroup of single peaks. This was an important development, providing new ways for the separation of components in larger amounts, by maximizing peak distances to the adjacent bands.

Another major problem in routine analysis is *moving peaks*. They are an important reason for a necessary change in method validation. This changes cause serious losses in time, as the chromatographer is trying to correct with considerable expenditure of trial and error experimentation. Such ''method repair''-procedures might take days or even weeks without finally recognizing, what the reason for the trouble was. Here the computer could help to understand correlations better and to transfer the experience with a better description of the strength and the weaknesses of the method. ''Method development reports'' are today commonly used in communication between laboratories using the same method. In such reports, one comes quick to the understanding where the weak points are in the method and how to handle critical situations, using software support.

14. Progress in applications

Plant extracts are especially complicated to separate due to the large number of components. Such a product was Ginkgo Biloba, where Molnar et al. simulated the equivalent of 50 chromatographic runs in less than an hour. The entire method was developed in less than 8 h [66].

Separation and detection of oxidation products in neurolite raw material using DryLab G/plus by Ryan et al. helped to determine the optimum gradient separation conditions [67].

The computer-aided optimization of HPLC-analysis of flavonoids from some species of the genus *Althaea* was applied by Dzido and Soczewinski [68]. Stuart demonstrated the simulation of isocratic retentions of alkylketones using gradient data, which also worked well, with an average error in the 3–5% range [69]. In environmental analysis, DryLab G/plus was used by Liu and Robbat to determine the optimum gradient separation conditions for nitrated polycyclic aromatic hydrocarbons in both acetonitrile–water and methanol–water systems. Predicted and actual retentions typically differed by only 1% [70].

In other pharmaceutical applications, Dappen from Ciba and Molnar were looking at the reliability of HPLC methods, developed by computer supported means. They found a high precision between prediction and experiment, using a special test. The average difference between prediction and experiment for 10 compounds was less than 10 s. They showed further that in the peak tracking process, area ratioing is a helpful tool to track peak movements between different experiments. Depending on the accuracy of the integration in both runs, errors were found to be less than 10% in area ratios [71].

Noel Mellish from American Cyanamid investigated five impurities in an anxiolytic drug. Using two different gradient slopes, he discovered a coelution of two peaks on the basis of peak areas, which are additive in overlapping peaks. In a second steroidal sample, 15 impurities were found. In a β-lactamase inhibitor, the separation of eight potential precursor impurities was optimized. The results have shown that computer simulation is a useful tool in routine analytical work [72].

Coenen et al. optimized the separation of the Rp and Sp diastereomers of phosphate-methylated DNA and RNA dinucleotides on reversed-phase with respect to pH, organic modifier type and concentration, and RP-packing material. Computer simulation was used to deduce the optimum conditions. On the basis of the work conducted, the gradient operation with volatile buffers could be improved. Elution order changes were found and pH values for maximum resolution of 14 diastereomeric pairs could be well established [73].

15. Precision

Further applications were shown in the method development for atenolol by Hofmann and Molnar. The desire to obtain information about synthesis byproducts led to an optimization using gradient elution and different temperatures. The method development period of ca. 1 week was rather short [74] and enabled to differentiate between rough products of various origin on the basis of the impurity pattern.

Bonfichi developed methods for glycopeptide antibiotics, characterized by highly modified sugar containing heptapeptidic structures. DryLab G/plus has proved to be very practical and useful for shortening the time required to develop analytical methods including analysis of complicated mixtures. The usefulness of the computerized approach was even more evident as it did not refer to any definite class of chemical substances. After determining the dwell volume, method transfer was easy. With the help of the resolution map not just the critical peak pair, but also the separation of other bands could be optimized [75].

Fritsch and colleagues optimized the separation of arachidonic acid metabolites, one of the major lipid constituents in lipid membranes, which can be converted into a variety of extremely potent mediators exhibiting important physiological roles. The prediction of the retention times of the 20:4 metabolites with a four-step gradient was very accurate with a maximum deviation of 1.2% in retention times [76].

Optimization of separations in plant protection research and control of the environment among other issues were demonstrated by Molnar in 1993 [77].

Wrisley studied drug compounds and pharmaceutical intermediates. He used computer simulation in the development of isocratic and gradient HPLC methods as well [78].

16. Accuracy in pH-dependent prediction of retention times

This subject was discussed by Lewis et al. [79]. Although in earlier studies, mathematical equations for the description of retention for acidic, basic and zwitterionic compounds were derived [48], significant deviations can occur, if the pH is far away from the pK_a of the compound concerned. It was therefore clear that the mathematical prediction will be less accurate than the direct measurement of the pH in the pH area of interest. As there is a general scientific interest to find the maximum number of peaks in a mixture, the distance between three experiments in pH-value of eluent A was established to be a maximum of 0.5–0.6 pH units. The pH range for weak acids, in which measurements should be carried out, have to be logically close to the expected pK_a, as here the strongest peak position changes are to be expected. Later on the optimization at lower or higher pH-values could be continued.

Another aspect of the computerized treatment of retention is to set the range of retention factor values at $1<k<20$ for the first and the last peak. This could also be assured using the new tool DryLab I/mp (''isocratic multiparameter version''), the update after DryLab I. The paper, which was published together with the introduction of DryLab I/mp established accuracy limits in pH-optimisation and helped to estimate pK_a-values of compounds of unknown identity [79].

Chemometric approaches in method development are using a reduced number of experiments and change working parameters between certain limits. However the tracking of peaks for complex mixtures with more than five components is very difficult.

Therefore, the dependency of the critical resolution was first studied in DryLab by changing only one parameter at a time. Interdependencies of the parameter under each other could not be observed. As the technology proceeded and computers also became more powerful, the development of two-dimensional retention models and corresponding resolution maps became possible. A comparison of practicability in changes in the optimization parameters indicated that the simultaneous variation in pH and %B as a means of maximizing sample resolution is very useful [80].

One of the most significant series of papers for the development of new products in biotechnology was published on the development of HPLC methods for the quality control of recombinant human growth hormone at Genentech. Chloupek et al. have shown how peaks are moving in a peptide digest with changing temperature and changing gradient time t_G [81,82] (Fig. 6a,b). Their observations were so novel that the development of a new version, called DryLab 2.00, became a reality, which opened up new possibilities of separating complex peptide mixtures. The paper also discussed the importance of peak matching. Agreement between predicted and actual retention was 0.3%.

The power of unified forces on selectivity by changing two parameters at the same time brought a more efficient way to adjust selectivity to the best

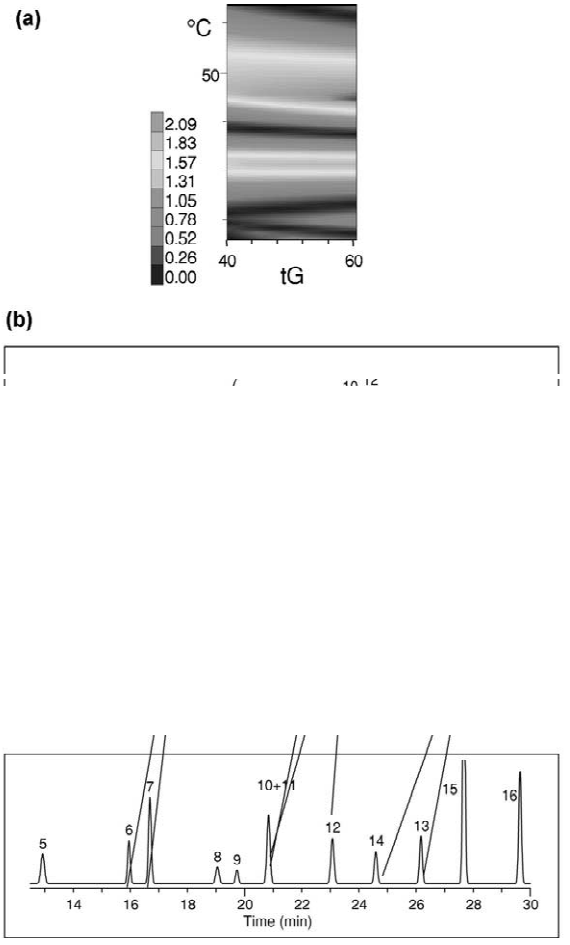


Fig. 6. (a) Resolution map for the peptide digest sample in the range of $40 < t_G < 60$ min and of $18 < T < 63$ °C. It contains four zones of potential peak coelutions and selectivity changes, shown by the dark lines. (b) Strong peak movements from 63 °C (bottom) over 38 °C (middle) to 18 °C (top) show the strong dependence of peptide retention values on column temperature.

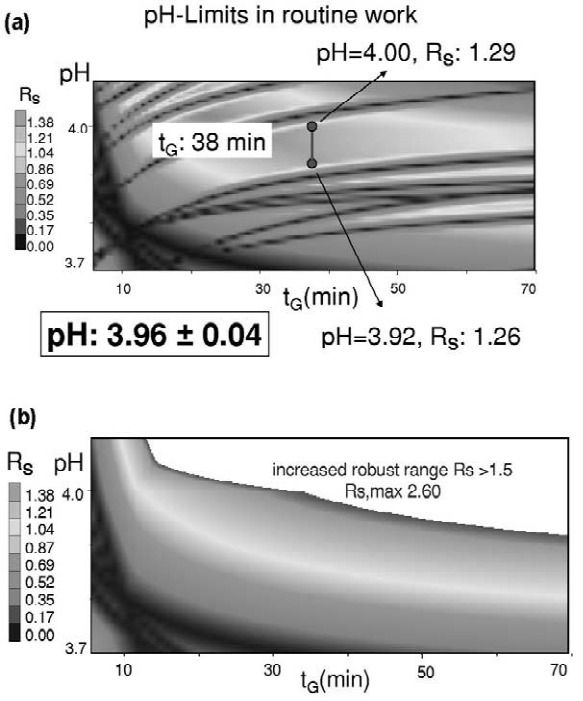


Fig. 7. (a) Two-dimensional resolution map t_G versus pH (enlarged) for a mixture of 12 acidic and basic compounds. Basic experiments: t_G 20 and 60 min, pH 3.0, 3.5 and 4.0. There are extremely tight limits for robust work, shown by the vertical bar at t_G: 38 min between pH 3.92 and 4.00 = 3.96 ± 0.04 (!) with a given maximum R_s of 1.26–1.29. Here, the precision of the pH meter has to be far better than the usual ±0.1 pH-units—which is the standard value in adjusting the pH in routine work for eluent A—to be able to maintain robust working conditions. Dark lines represent peak overlaps for different critical band pairs. (b) Resolution map, after sample pretreatment and consequent removal of four basic compounds. For the remaining eight acidic compounds, the robust region becomes much larger and the critical resolution increases up to 2.60 at pH 4.00.

available resolution and thus helped to obtain the fastest and most reliable analysis, which is required with routine processes in an industrial setting [83–85].

The two-dimensional simulation opened up new possibilities to study complex mixtures by Zhu et al. Especially important was the fact that the trend towards gradient elution could be combined with access to other variables, to the temperature in the first place, then to the pH (Fig. 7a,b) and to ternary composition of eluent B (acetonitrile, methanol, THF and propanol, etc.). This could be followed by individual modeling of the steps in the gradient and changing flow-rate, the latter of which often changes separation selectivity (see also Table 1). Investigations on neutral and charged compounds were studied separately. An experimental demonstration of the relative effectiveness of temperature or gradient time to change selectivity for nine different neutral samples showed an average change in α of 23% for temperature variation and 23% for change in gradient steepness [86].

Validated methods are often different in various

Table 1
General strategy in computer-supported method development for a mixture with ca. 20 components, starting with gradient elution

Step	Action
1	Carry out two gradients 0→100%B (acetonitrile) with eluent A: 0.05 *M* phosphate, (or other volatile buffer for LC–MS) pH 2.1 at 40 °C in 40 and 120 min
2	Repeat the same two gradients at 70 °C, transfer data into DryLab
3	Optimize separation with DryLab: Look for best temperature and best gradient run time. Shape gradient form. This is the ''best gradient no.1'' at the best temperature
4	A. Keeping the gradient form and the temperature constant, change now the pH of eluent A to pH 2.7
5	B. repeat 4A but with an eluent A of pH 3.3
6	Optimize the pH with DryLab: Look for the highest critical resolution between $1.8<pH<3.6$. Run experiments at the pH of the highest critical resolution. This your ''best gradient no.2''. Now you have three parameters at their optimum: gradient form, temperature and pH
7	Keeping these conditions, run a further set of experiments: Change eluent B from acetonitrile to methanol, and to a mixture of (50:50) (AN/MeOH) (v/v)
8	Make a resolution map for the ratio of MeOH/AN, look at the best value and fix the new method at the ''best'' conditions This is your ''best gradient no.3''
9	In case you have still unresolved peaks, change eluent B to isopropanol
10	Try out other columns
11	Finally, the column length, I.D., particle size and the flow-rate can be optimized, considering the allowed and the actual column pressure
12	Test the possibility for isocratic elution using DryLab. If the *k*-values of the sample components are $1<k<10$, isocratic work can be recommended

laboratories as one would expect. Smooth running robust HPLC methods without using computer-assisted method development is often a burden in QC, if the separation is not done properly. Molnar discussed the problems of method robustness and illustrated how to use robust resolution maps as provided by DryLab to improve method quality and enable easier electronic method transfer to other laboratories [87,88].

Computer supported studies of pH influence in HPLC were carried out in most cases in isocratic mode. However, the increasing number of components down to the >0.1% w/w level forces the chromatographers to use gradient elution. The combination of gradient runs with pH optimization was tried first by Bilke and colleagues [50]. A pharmaceutical stability study of impurities and degradation products using varying gradient times and pH resulted in the adequate separation of a 15-component sample, but the method was very sensitive to changes in pH. There were groups of peaks, which moved closer, other peak moved apart with changing pH. As DryLab only offered an isocratic pH modelling, this shortcoming was circumvented by subdividing the chromatogram into three regions and each region was modelled with different plate numbers. In the later stages of the development, Snyder found a solution to use measured peak widths to model the peak widths correctly. Experimental results were in good agreement with DryLab predictions. The average deviation between predicted and experimental retention times of 16 bands was less than 8 s [89].

The relative advantage of using different variables to optimize selectivity and resolution was compared in a number of paper in the following years. Since most samples could be separated using any variables for this purpose, it was important to consider other consequences of this choice: convenience, costs, method robustness, etc. It is concluded that the use of either: (1) temperature with either gradient time or isocratic %B, or (2) changes in the (acetonitrile/methanol)-ratio in eluent B with either gradient time or isocratic %B, are generally superior in this respect for compounds, like peptides, furanocoumarins, etc. [90–93].

17. New fields of applications of computer assisted design of separations

Another field for difficult separations is the class

of *enantiomeric mixtures*, where method development mainly concentrates on the use of different columns. Here computerized eluent optimization might be a great possibility. This was shown by Lindner et al., who separated for the first time 14 derivatized chiral DPN-amino acids on a quinine carbamate type chiral stationary phase. The application of DryLab to the separation of chiral isomers reduced the analysis time from 230 to 65 min and considerably improved the resolution at the same time [94].

Another rather new application of computerized method development is in gradient ion chromatography. Molnar could show that the rules of gradient elution are valid also in this type of chromatography, showing an excellent correlation between predictions and results [95].

18. Transferring results from gradient elution to isocratic mode

Snyder and colleagues found that a single reversed-phase gradient run could be used to accurately predict the best %B value for a corresponding isocratic separation with an accuracy of about 1%. A synthetic mixture of 11 substituted benzenes was used to evaluate a new approach to method development. A single gradient run is carried out initially and used to select conditions for separation as a function of various ternary solvent mixtures in isocratic reversed-phase HPLC [96,97].

The question which variable should be tried first to change selectivity in RPC was addressed by Snyder et al. The relative effectiveness of different ways to change selectivity was compared. Mixing two organic solvents such as methanol and tetrahydrofuran was best, changing solvent strength (%B) or column type next, and finally temperature provided the smallest change in values of α. However, all of these changes in conditions can be effective for a given sample [98].

The development of new algorithms allowed easier calculation of other two-dimensional optima in the selectivity control in HPLC method development. It revealed that of the gradient time and temperature or gradient time and pH, both were needed in trying to find the maximum number of components. Problems with complex samples and method transfer could be solved by means of computer simulation [99,100].

In a summary, Snyder and Dolan looked at the present technology of method optimization with emphasis on two different approaches for optimizing: (a) the %-acetonitrile and %-methanol in the mobile phase, or (b) temperature and either gradient time or isocratic %B [101].

19. Difficult samples

Difficult samples in RPC are typically mixtures of toxicology samples, plant extracts, etc. Hill et al. used DryLab to optimize the separation of 14 different mixtures having 9–40 components, by means of changes in temperature and gradient time. Most of these samples could be separated with $R_s > 1.00$. Predicted separations agreed closely with experimental results [102]. Various means were explored in order to further improve separation after optimizing temperature T and gradient time t_G: (a) optimizing the initial %B in the gradient, (b) using segmented gradients, (c) changing some other variable (pH, solvent, column), followed by reoptimizing T and t_G. Option (a) resulted in a 0–20% further increase in R_s; option (b) resulted in a $<10\%$ increase in R_s; option (c) resulted in an 0.1- to 3-fold increase in R_s. However, option (c) required further experiments, whereas options (a) and (b) did not. A review of the best current model for reversed-phase gradient elution showing how it can be used to predict separation as a function of gradient conditions, was published in a review paper [103].

The use of a newly introduced version of DryLab was described by Dolan and Snyder. They showed how to optimize reversed-phase isocratic separations by varying temperature and %B in a two-dimensional simultaneous optimization in isocratic RPC. They used four initial experiments at two different temperatures, starting with either isocratic elution or (better) gradient elution. If isocratic experiments are chosen for computer simulation, it is necessary to select appropriate values of %B for these initial runs. Literature data for solute retention as a function of T were reviewed as a basis for estimating values of %B at the two values of T selected [104].

Maintaining fixed band spacing when changing column dimensions in gradient elution is a common desire of chromatographers, if they go to LC–MS. The usual rule for maintaining the same gradient separation is to keep (gradient time)×(flow-rate)/(column volume) constant. However, this also requires maintaining the equipment dwell volume constant as well. Some examples showing how large these changes in separation can turn out to be when the dwell volume is ignored, were given by Snyder and Dolan [105].

Wollcott et al. studied various temperature-related problems that can result in a failure of method transfer for non-ambient RPC methods, were examined. Means for correcting for such effects, and thereby ensuring method transferability, were described. When using temperature to optimize HPLC separations, care must be taken to ensure that the column is at the correct temperature. An experimental study was described that leads to simple rules for ensuring good method transfer for methods run at temperatures higher than ambient [106,107].

The separation of samples that contain more than 15–20 analytes is typically difficult and usually requires gradient elution (Table 1). Dolan et al. have examined the reversed-phase separation of 24 samples with 8–48 components each as a function of temperature T and gradient time t_G. The required peak capacity was determined for each sample after selecting T and t_G for optimum selectivity and maximum sample resolution. It was concluded that samples with $>$15–20 components would be difficult to separate with $R_s > 1.0$. Other means of optimizing resolution using mixed organic solvents appear to be no better in this respect [108]. An alternative approach is to carry out two separations with different conditions (T, t_G) in each run. The combination of results from these two runs would then allow the total analysis of the sample, providing that every sample component is adequately resolved in one run or the other. Examples of this approach, carried out by means of computer simulation, were shown for several samples of varying complexity [109].

Using RPC for separation of complex samples by optimizing temperature and gradient time, Dolan et al. made a detailed examination about the accuracy of predictions, when either gradient time, %B or temperature was varied. It was concluded that these predictions should be generally adequate, except in the case of using gradient data for isocratic predictions. The latter are less reliable, with an average error equivalent to 0.5–1.0 R_s units [110].

20. Recent developments: visualization of band movements

In the industrial world, validated methods have to be used. The emphasis is on statistical tests of the quantitative results. Such data are important if clear decisions about product quality are needed. Insufficient optimization of the chromatographic system, based on short available time, leads to methods where according to my experience, 20–30% of the peaks are double or triple bands. These peaks might look gaussian, the symmetrical form falsely suggesting purity. Therefore a systematic ‘‘shaking’’ of the peaks is needed by purposely changing working conditions in a rather drastic manner.

Very early in 1994, experiments were carried out by Molnar and colleagues to see the scientific effect of changes in conditions by animating peak movements. The real progress however became first possible after the introduction of the 32-bit DryLab version in 1999. Due to fast calculation capabilities, changes in chromatograms could be shown in ‘‘slow motion’’. Band movements resulting from changes in temperature- or pH-values relative to neighboring peaks and relative to the time scale were clearly demonstrated. Changing the values with the mouse, one could now better understand the reasons for problems in routine work. Undiscovered coelutions could be found early, avoiding poor results in quantitative analysis.

Another big step forward occurred in gradient elution, using the same principles, where similarly to the cursor in resolution maps, gradient points could be moved to new positions with the mouse. The user could see at the same time, which peak was moving to another location and when a coelution would happen. In this way, scientifically optimized gradients could be developed in even shorter time than was possible with previous versions.

21. Column performance comparisons

One of the hot topics to use computer modeling in chromatography is for testing and comparing column performance using two-dimensional t_G versus *T*- or t_G versus pH-models. There are several approaches to the problem of column variability, which assume that small changes in conditions can restore the original separation with the preceding column. This convenient procedure for selecting altered conditions for this purpose is also promising for the routine QC laboratory.

22. Separation of isomers

Snyder collected experimental data for 137 isomer-pairs, presented as a function of temperature and gradient times. For 90% of these compounds, reasonable changes in temperature and gradient time resulted in their separation with a resolution $R_s > 1$. This is another example of the unique ability of these conditions to control separation in reversed-phase HPLC. The use of DryLab in this connection allows such separations to be optimized by means of only four experiments.

Introduced in 2000, the new 32-bit DryLab 2000 version revolutionizes the use of computer simulation by extending its application to any chromatographic system: HPLC in reversed-phase, normal-phase, ion-exchange, ion-pairing or other mode and any other chromatographic procedure, such as GC, CE, CEC, etc. Applications are already shown: (a) in the optimization of a RPC by simultaneous changes in pH and gradient time and (b) the optimization of a CE separation by simultaneous changes in pH and buffer concentration [111–117].

23. Outlook

HPLC method development of complex pharmaceutical assays such as those used for stability testing or related compound analysis is a complex and time-consuming process. Trial and error is still a common approach, but many researchers prefer more efficient approaches, utilizing chromatographic modeling software that relies upon theory to decrease the time and resources required (Table 1). However, while automated HPLC systems exist to run the methods, there is a gap between the chromatographic and modeling software, resulting in a manual process requiring operator intervention for interpretation and implementation.

For ionizable compounds such as organic acids, best results were obtained recently by Jupille et al. with simultaneous optimization of %B and pH, regardless of ionic strength or temperature. Changes in the pH of eluent A, adjusted to bracket the p*K*-values of acids (works also for bases and zwitterions), help to understand changes in critical resolution values due to shifts in peak positions [118].

A new strategy for neutral compounds, contained in many phytopharmaceuticals, was presented at HPLC 2001 in Maastricht by Molnar and Schmidt. The systematic work with kava pyrones and three different organic modifiers, methanol, acetonitrile and 2-propanol, by simultanously changing gradient slope versus temperature or gradient slope versus pH reveals the true composition of such mixtures [119].

The analytical chemist is interested to learn more about the influence of the experimental parameters on the resolution, but can often only rely on experiments, he was able to carry out in a given time in a project. There are however often as many chances to improve resolution in the ‘‘unexpected’’ direction as by varying them in the ‘‘expected’’ way. The tools for understanding the method and discover all chances for improved selectivity are the different resolution maps [120].

Waters and LC Resources are working on a new approach to HPLC method development, which will automate the entire process from method requirements and definition to method implementation. This automated approach encompasses both software and hardware, which are operated by an iterative decision engine, driven by a graphical user interface. After the input of basic separation requirements, starting conditions are proposed to the user and experiments are carried out, evaluated, optimized, and automatically implemented by the chromatographic system. The process is repeated, until the separation goals are achieved. The result is an automated method development system capable of unattended operation

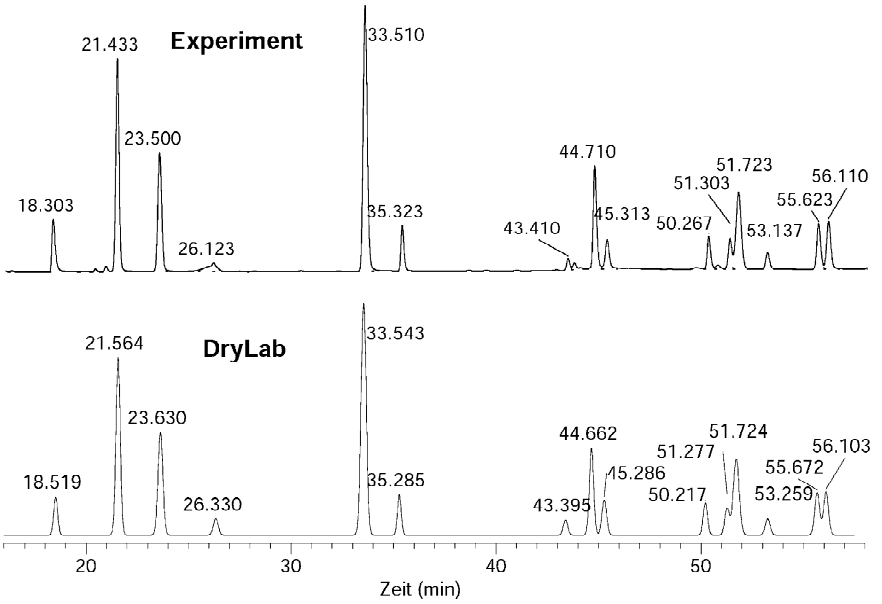


Fig. 8. Precision of predicted chromatograms from a t_G–temperature DryLab model of a drug research project at t_G: 90 min, temperature: 55 °C. Retention times are typically predictable for linear gradients with an average difference of ±0.2 min or less. For multisegmented gradients, the average t_R-difference is typically ±0.5 min or less, due to rounding of gradient steps, caused by band spreading of the eluent B-front. The instrument used here was an Agilent 1100 (Molnar, unpublished results).

increasing throughput and efficiency. The application of this system is planned for the development of complex pharmaceutical assays that take advantage of the selectivity afforded by high pH mobile phases and columns designed specifically for this systematic approach [121].

Waters also explores with ACD the challenges and benefits of integrating chemical structures into HPLC analysis. They focus beyond the basics of including chemical structures on chromatographic results to integrate structural information with chromatographic information for advanced searching capabilities. A database of successful separations is linked to chemical structures as a valuable tool to interpret HPLC data.

Computer supported optimization is certainly still new in many areas, especially in the chemical industry. Here the chromatographer still does the experiments. Many of the newcomers to the field, but also some experts can hardly believe what the software can simulate: ''Is this all really true?'' and the answer is first a doubtful ''No!''. However, if they have a chance to learn to work with DryLab and see the unexpected choices which the computer can show to them, the hesitation normally turns into enthusiasm [122]. There are so many variants of a solution, which, if modeled properly, can be the alternative to time consuming trial and error experiments and struggle. And virtual experiments bring the expected results (Fig. 8).

They are also great fun to do.

References

[1] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, Wiley, New York, 1997.
[2] L.R. Snyder, in: Cs. Horváth (Ed.), High Performance Liquid Chromatography. Advances and Perspectives, Vol. 1, Academic Press, New York, 1980, Chapter 4.
[3] W. Melander, Cs. Horváth, in: Cs. Horváth (Ed.), High Performance Liquid Chromatography. Advances and Perspectives, Vol. 2, Academic Press, New York, 1980, Chapter 3.
[4] J. Berridge, Techniques for the Automated Optimization of HPLC Separations, Wiley, New York, 1986.
[5] P.J. Schoenmakers, Optimization of Chromatographic Selectivity, Elsevier, Amsterdam, 1986.
[6] R.W. Stout, J.J. DeStefano, L.R. Snyder, J. Chromatogr. 282 (1983) 263.

[7] L.R. Snyder, M.A. Stadalius, M.A. Quarry, Anal. Chem. 55 (1983) 412A.
[8] M.A. Stadalius, H.S. Gold, L.R. Snyder, J. Chromatogr. 296 (1984) 31.
[9] M.A. Quarry, R.L. Grob, L.R. Snyder, J. Chromatogr. 285 (1984) 19.
[10] M.A. Quarry, R.L. Grob, L.R. Snyder, J. Chromatogr. 285 (1984) 1.
[11] L.R. Snyder, P.E. Antle, LC Mag. 3 (1985) 98.
[12] M.A. Stadalius, H.S. Gold, L.R. Snyder, J. Chromatogr. 327 (1985) 93.
[13] M.A. Stadalius, H.S. Gold, L.R. Snyder, J. Chromatogr. 327 (1985) 27.
[14] L.R. Snyder, J.W. Dolan, Am. Lab. 18 (8) (1986) 37.
[15] L.R. Snyder, J.W. Dolan, M.P. Rigney, LC·GC 4 (1986) 921.
[16] L.R. Snyder, M.A. Stadalius, in: Cs. Horvath (Ed.), High Performance Liquid Chromatography. Advances and Perspectives, Vol. 4, Academic Press, New York, 1986, p. 195.
[17] M.A. Quarry, R.L. Grob, L.R. Snyder, Anal. Chem. 58 (1986) 907.
[18] J.L. Glajch, M.A. Quarry, J.F. Vasta, L.R. Snyder, Anal. Chem. 58 (1986) 280.
[19] J.W. Dolan, L.R. Snyder, Chromatography 2 (1987) 49.
[20] M.A. Stadalius, B.F.D. Ghrist, L.R. Snyder, J. Chromatogr. 387 (1987) 21.
[21] J.W. Dolan, L.R. Snyder, M.A. Quarry, Am. Lab. 19 (8) (1987) 43.
[22] M.A. Quarry, R.L. Grob, L.R. Snyder, J.W. Dolan, M. Rigney, J. Chromatogr. 384 (1987) 163.
[23] L.R. Snyder, M.A. Quarry, J. Liq. Chromatogr. 10 (1987) 1789.
[24] B.F.D. Ghrist, M.A. Stadalius, L.R. Snyder, J. Chromatogr. 387 (1987) 1.
[25] B.F.D. Ghrist, L.R. Snyder, J. Chromatogr. 459 (1988) 25.
[26] B.F.D. Ghrist, B.S. Cooperman, L.R. Snyder, J. Chromatogr. 459 (1988) 1.
[27] T.H. Jupille, J.W. Dolan, L.R. Snyder, Am. Lab. 20 (12) (1988) 20.
[28] J.W. Dolan, L.R. Snyder, M.A. Quarry, Chromatographia 24 (1988) 261.
[29] J.W. Dolan, L.R. Snyder, LC·GC 5 (1988) 970.
[30] B.F.D. Ghrist, L.R. Snyder, J. Chromatogr. 459 (1988) 43.
[31] I. Molnar, R.I. Boysen, V.A. Erdmann, Chromatographia 28 (1989) 39.
[32] L.R. Snyder, M.A. Quarry, J.L. Glajch, Chromatographia 24 (1988) 33.
[33] P.E. Shaw, C.W. Wilson, J. Chromatogr. Sci. 26 (1988) 478.
[34] J. Fulper, J. Chromatogr. 485 (1989) 579.
[35] J.D. Stuart, D.D. Lisi, L.R. Snyder, J. Chromatogr. 485 (1989) 657.
[36] D.J. Thompson, W.D. Ellenson, J. Chromatogr. 485 (1989) 607.
[37] R.G. Lehman, J.R. Miller, J. Chromatogr. 485 (1989) 581.
[38] I. Molnar, R. Boysen, P. Jekow, J. Chromatogr. 485 (1989) 569.
[39] J.L. Glajch, L.R. Snyder (Eds.), Computer-Assisted Method Development for High Performance Liquid Chromatography, J. Chromatogr. 485 (1990).
[40] T. Sasegawa, Y. Sakamoto, T. Hirose, T. Yoshida, Y. Kobayashi, Y. Sato, J. Chromatogr. 485 (1989) 533.
[41] E.P. Lankmayr, W. Wegscheider, J.C. Gfeller, N.M. Djordjevic, B. Schreiber, J. Chromatogr. 485 (1989) 183.
[42] L.R. Snyder, J.W. Dolan, D.C. Lommen, J. Chromatogr. 485 (1989) 63.
[43] J.W. Dolan, D.C. Lommen, L.R. Snyder, J. Chromatogr. 485 (1989) 91.
[44] J.W. Dolan, D.C. Lommen, L.R. Snyder, J. Chromatogr. 535 (1990) 55.
[45] L.R. Snyder, J.W. Dolan, D.C. Lommen, J. Chromatogr. 535 (1990) 75.
[46] I. Molnar, Cs. Horvath, Clin. Chem. 22 (1976) 1497.
[47] Cs. Horvath, W. Melander, I. Molnar, J. Chromatogr. 125 (1976) 129.
[48] Cs. Horvath, W. Melander, I. Molnar, Anal. Chem. 49 (1977) 142.
[49] F. Erni, J. Chromatogr. 509 (1990) 141.
[50] H.W. Bilke, I. Molnar, Ch. Gernet, poster presented at ''HPLC 93'', Hamburg, June 1993.
[51] L.R. Snyder, J.W. Dolan, LC·GC 8 (1990) 524.
[52] W. Markowski, T.H. Dzido, E. Soczewinski, J. Chromatogr. 523 (1990) 81.
[53] D.E. Bautz, J.W. Dolan, W.D. Raddatz, L.R. Snyder, Anal. Chem. 62 (1990) 1560.
[54] G.N. Abbay, E.F. Barry, S. Leepipatpiboon, T. Ramstad, M.C. Roman, R.W. Siergiej, L.R. Snyder, W.L. Winniford, LC·GC 9 (2) (1991) 1001.
[55] D.E. Bautz, J.W. Dolan, L.R. Snyder, J. Chromatogr. 541 (1991) 1.
[56] J.W. Dolan, L.R. Snyder, D.E. Bautz, J. Chromatogr. 541 (1991) 21.
[57] L.R. Snyder, D.E. Bautz, J.W. Dolan, J. Chromatogr. 541 (1991) 34.
[58] A. Jayatilaka, C.F. Poole, J. Chromatogr. 617 (1993) 19.
[59] R.L. Grob, E.F. Barry, S. Leepipatpiboon, J.M. Ombaba, L.A. Colon, J. Chromatogr. Sci. 30 (1992) 177.
[60] J.W. Dolan, L.R. Snyder, J. Chromatogr. Sci. 28 (1990) 379.
[61] A. Drouen, J.W. Dolan, L.R. Snyder, A. Poile, P. Schoenmakers, LC·GC 9 (1991) 714.
[62] K. Valko, G. Szabo, J. Röhricht, K. Jemnitz, J. Chromatogr. 485 (1990) 349.
[63] S.V. Galushko, A.A. Kamenchuk, LC–GC Int. 8 (1995) 581.
[64] T. Baczek, R. Kaliszan, H.A. Claessens, M.A. van Straten, LC–GC Eur. 14 (2001) 304.
[65] C.T. Mant, T.W.L. Burke, N.E. Zhou, J.M.R. Parker, R.S. Hodges, J. Chromatogr. 485 (1990) 365.
[66] I. Molnar, K.H. Gober, B. Christ, J. Chromatogr. 550 (1991) 39.
[67] P.A. Ryan, B.A. Ewels, J.L. Glajch, J. Chromatogr. 550 (1991) 549.
[68] T.H. Dzido, E. Soczewinski, J. Gudej, J. Chromatogr. 550 (1991) 71.
[69] J.D. Stuart, D.D. Lisi, J. Chromatogr. 550 (1991) 77.
[70] T-Y. Liu, A. Robbat Jr., J. Chromatogr. 539 (1991) 1.
[71] R. Dappen, I. Molnar, J. Chromatogr. 592 (1992) 133.
[72] N.G. Mellish, LC·GC 9 (1991) 845.

[73] A.J.J.M. Coenen, L.H.G. Henckens, Y. Mengerink, S. van der Wal, P.J.L.M. Quaedifleig, L.H. Koole, E.M. Meijer, J. Chromatogr. 596 (1992) 59.
[74] H. Hofmann, I. Molnar, Pharm. Ztg. Wiss. 1 (5) (1992) 137.
[75] R. Bonfichi, J. Chromatogr. A 678 (1994) 213.
[76] H. Fritsch, I. Molnar, M. Wurl, J. Chromatogr. A 684 (1994) 65.
[77] I. Molnar, LaborPraxis 10 (1993) 40, in German.
[78] L. Wrisely, J. Chromatogr. 628 (1993) 191.
[79] J.A. Lewis, D.C. Lommen, W.D. Raddatz, J.W. Dolan, L.R. Snyder, I. Molnar, J. Chromatogr. 592 (1992) 183.
[80] J.A. Lewis, J.W. Dolan, L.R. Snyder, I. Molnar, J. Chromatogr. 592 (1992) 197.
[81] (a) R.C. Chloupek, W.S. Hancock, L.R. Snyder, J. Chromatogr. 594 (1992) 65;
(b) W. Hancock, R.C. Chloupek, J.J. Kirkland, L.R. Snyder, J. Chromatogr. A 686 (1994) 31.
[82] R.C. Chloupek, W. Hancock, B.A. Marchylo, J.J. Kirkland, B. Boyes, L.R. Snyder, J. Chromatogr. A 686 (1994) 45.
[83] P.L. Zhu, L.R. Snyder, J.W. Dolan, N.M. Djordjevic, D.W. Hill, L.C. Sander, T.J. Waeghe, J. Chromatogr. A 756 (1996) 21.
[84] P.L. Zhu, J.W. Dolan, L.R. Snyder, J. Chromatogr. A 756 (1996) 41.
[85] P.L. Zhu, J.W. Dolan, L.R. Snyder, D.W. Hill, L. Van Heukelem, T.J. Waeghe, J. Chromatogr. A 756 (1996) 51.
[86] P.L. Zhu, J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. HIll, J.-T. Lin, L.C. Sander, L. Van Heukelem, J. Chromatogr. A 756 (1996) 63.
[87] I. Molnar, LC·GC Int. 9 (12) (1996) 800.
[88] I. Molnar, LC·GC Int. 10 (1) (1997) 32.
[89] H.W. Bilke, I. Molnar, Ch. Gernet, J. Chromatogr. A 729 (1996) 189.
[90] L.R. Snyder, in: W. Hancock (Ed.), New Methods in Peptide Mapping for the Characterization of Proteins, CRC Press, Boca Raton, FL, 1996, p. 31.
[91] T.H. Dzido, A. Sory, Chem. Anal. (Warsaw) 41 (1996) 113.
[92] L.R. Snyder, Today's Chemist Work 5 (1) (1996) 29.
[93] W. Markowski, K.L. Czapinska, Chem. Anal. (Warsaw) 42 (1997) 353.
[94] M. Lammerhofer, P. Di Eugenio, I. Molnar, W. Lindner, J. Chromatogr. B 689 (1997) 123.
[95] I. Molnar, Ionenanalyse mit Chromatographie und Kapillarelektrophorese, Proceedings of the Ion Chromatography-Symposium, Dionex, Sept. 1999, Idstein, Germany, ISBN 3-00-007001-X, p. 277.
[96] L.R. Snyder, J.W. Dolan, J. Chromatogr. A 721 (1996) 1.
[97] J.L. Lewis, L.R. Snyder, J.W. Dolan, J. Chromatogr. A 721 (1996) 15.
[98] L.R. Snyder, J. Chromatogr. B 689 (1997) 105.
[99] L.R. Snyder, J.W. Dolan, I. Molnar, N.M. Djordjevic, LC·GC 15 (1997) 136.
[100] I. Molnar, L.R. Snyder, J.W. Dolan, LC·GC Int. 11 (1998) 374.
[101] L.R. Snyder, J.W. Dolan, Chem. Anal. (Warsaw) 43 (1998) 495.
[102] J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. Hill, D.L. Saunders, L. Van Heukelem, T.J. Waeghe, J. Chromatogr. A 803 (1998) 1.
[103] J.W. Dolan, L.R. Snyder, D.L. Saunders, L. Van Heukelem, J. Chromatogr. A 80 (1998) 33.
[104] L.R. Snyder, J.W. Dolan, Adv. Chromatogr. 38 (1998) 157.
[105] J.W. Dolan, L.R. Snyder, J. Chromatogr. 799 (1998) 21.
[106] R.G. Wolcott, J.W. Dolan, L.R. Snyder, J. Chromatogr. A 869 (2000) 3.
[107] R.G. Wolcott, J.W. Dolan, L.R. Snyder, S.R. Bakalyar, M.A. Arnold, J.A. Nichols, J. Chromatogr. A 869 (2000) 211.
[108] J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. Hill, L. Van Heukelem, T.J. Waeghe, J. Chromatogr. A 857 (1999) 1.
[109] J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. Hill, L. Van Heukelem, T.J. Waeghe, J. Chromatogr. A 857 (1999) 21.
[110] J.W. Dolan, L.R. Snyder, L.C. Sander, P. Haber, T. Baczek, R. Kaliszan, J. Chromatogr. A 857 (1999) 41.
[111] Essential guides to method development in HPLC, in: J.W. Dolan, L.R. Snyder (Eds.), Encyclopedia of Separation Science, Academic Press, London, in press.
[112] J.W. Dolan, L.R. Snyder, LC·GC 17 (1999) S17.
[113] R.G. Wolcott, J.W. Dolan, L.R. Snyder, J. Chromatogr. A 869 (2000) 3.
[114] R.G. Wolcott, J.W. Dolan, L.R. Snyder, S.R. Bakalyar, M.A. Arnold, J.A. Nichols, J. Chromatogr. A 869 (2000) 211.
[115] J.W. Dolan, L.R. Snyder, T. Blanc, L. Van Heukelem, J. Chromatogr. A 897 (2000) 37.
[116] L.R. Snyder, J.W. Dolan, J. Chromatogr. A 892 (2000) 107.
[117] P. Haber, T. Baczek, R. Kaliszan, L.R. Snyder, J.W. Dolan, C.T. Wehr, J. Chromatogr. Sci. 38 (2000) 386.
[118] T.H. Jupille, J.W. Dolan, L.R. Snyder, I. Molnar, J. Chromatogr. A 948 (2002) 35.
[119] A.H. Schmidt, I. Molnar, J. Chromatogr. A 948 (2002) 51.
[120] H.J. Rieger, I. Molnar, J. Chromatogr. A 948 (2002) 43.
[121] M. Swartz, L.R. Snyder, J. Dolan, R. Lee-Berman, T. Jupille, Paper no. 634, presented at the Pittsburgh Conference, 2002, New Orleans, LA.
[122] I. Molnar, LC–GC Int. 17 (2001) 231.