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REVIEW

From Csaba Horváth to Quality by Design: Visualizing Design Space in Selectivity Exploration of HPLC Separations

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Abstract The present paper starts by taking a look back at some of the pioneering work in high pressure liquid chromatography (HPLC) that went on in Csaba Horváth's laboratory in 1970s, through the eyes of I. Molnár. It then goes on to describe a very modern approach to HPLC method development within the Quality by Design framework: the multifactorial optimization of three critical HPLC method parameters, i.e. gradient time (t_G), temperature (T), and ternary composition (B_1:B_2) based on 12 experiments. The effect of these experimental variables on critical resolution and selectivity was carried out in such a way as to systematically vary all three factors simultaneously.

Keywords Csaba Horváth · Solvophobic theory · Quality by Design · Design space · 3D computer modeling software

Introduction

This paper is the precipitation of a presentation given at the ISSS 2010 in Rome, where a special session was devoted to the memory of Prof. Csaba Horváth, the inventor of modern high pressure liquid chromatography (HPLC), who passed away in 2004 leaving a great emptiness in our hearts behind. The meeting was organized by the chairman Danilo Corradini, himself a student and a frequent visitor in Csaba's lab. I am trying to collect some of the major points of the work Csaba, Wayne Melander and I did together and show the development, which I took since leaving Yale to the present activities I am conducting today and which I am trying to continue in the spirit of Csaba (Fig. 1).

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Horváth's Goals in Summer 1975

As I arrived at Yale, Csaba explained the great need medical professionals in research were having. The separation of 100 organic acids according to the method of Scott was still taking 48 h. He thought that it must be possible to reduce this amount of time to 1 h. He envisioned a 10-μm pellicular stationary phase, which he modified to a C-18 material, packed in a capillary tubing of 1 m length and 1 mm ID to be able to achieve this goal.

Another project of Csaba was the separation of proteins, responsible for many health disorders. My colleague Wayne Melander, who arrived at Yale a few months earlier, was already working on this project. His job was to study the solubility of a great number of proteins in methanol, ethanol, propanol and other organic eluents. He used a Beckman UV-spectrophotometer and was quite frustrated to find that the solubility of proteins in organic solvents was quite low.

One day we had a separation problem of two organic acids vanillmandelic and homovanillic acids from a colleague from the department of Bertino. He tried to use anion-exchangers but with limited success. My protein separations went also slow: I was generating one Alpine landscape after the other, broad peaks; obviously kinetic problems slowed the equilibrium process [1] (Fig. 2).

Csaba had new C18 columns from Whatman. These columns were based on irregular silica particles and had

Fig. 1 This is a group picture, taken at Csaba Horváth's 70th birthday (Csaba in the *middle* with a *red* tie) with his students in Woolsey Hall at Yale in January 2000. The group is named the Csabaites, students, friends and frequent visitors

10-µm particle diameter. I said, why don't we try the two acids on this column? After we obtained one peak, and long time nothing else, we thought, this was it. I almost shut down the instrument and was ready to go home, as suddenly a second large peak appeared. What a separation on a C18 hydrophobic column! The reason was an OH-group. And we had a mobile phase with no organic component! We had plain water as eluent. This was for us almost a shock, as at that time reversed phase retention was explained to be based on partition chromatography. But in pure water there was no second liquid organic phase layer present, nothing for the sample molecules to partition between.

We started to read as much as we could about the water structure; Wayne Melander brought bands of photocopies from the library every day. We measured the retention of acidic, basic, zwitterionic and neutral compounds and calculated, how much retention we would have in water versus in that methanol: The difference between the capacity factors (at that time they were called *k*-prime or k'), was at least three orders of magnitude.

We continued our successful research on the HPLC-analytic of the catecholamines and published several excellent papers on their retention behavior [2].

Another on-going project was the separation of the urinary acids. We made an excellent separation with another new column, which gave us the separation shown in Fig. 3, 100 peaks in <30 min [3].

The work with proteins was stopped as we had good results with the smaller molecules, so we started to analyze amino acids and small peptides. I asked Csaba to provide the money for around 50 peptides and amino acids, which he did. My wife Petra and I were working hand in hand, each of us having a group of compounds and we built two different mixtures adding small amounts to the injection solution. Both of us made a sample solution with 14–15 peaks, which were clearly identified. Finally before 8 pm, our usual time to end the day, we injected both of our samples in equal amounts to obtain a wonderful separation. The resulting chromatogram is shown in Fig. 4, which opened a new era for analyzing amino acids and peptides as a new tool for life science [4].

Our separation spread quickly around the building, the Mason Laboratory in the Hillhouse Avenue in New Haven. Prof. Dr. John Fenn, one floor underneath of us in Mason was visiting our lab as he heard the news and was excited about the separation in Fig. 4 and decided to apply the technique in his Mass Spectroscopy Laboratory for protein-mass-spectrometry, leading to the discovery of the electro-spray-ionization (ESI) mass spectrometry technique, for which he received the Nobel prize in 2002.

This successful separation enabled us to elaborate the basics of reversed phase chromatography, the "Solvophobic Theory" [5–7], which delivered the first approach to Quality by Design (QbD) in HPLC in 1976, as we were

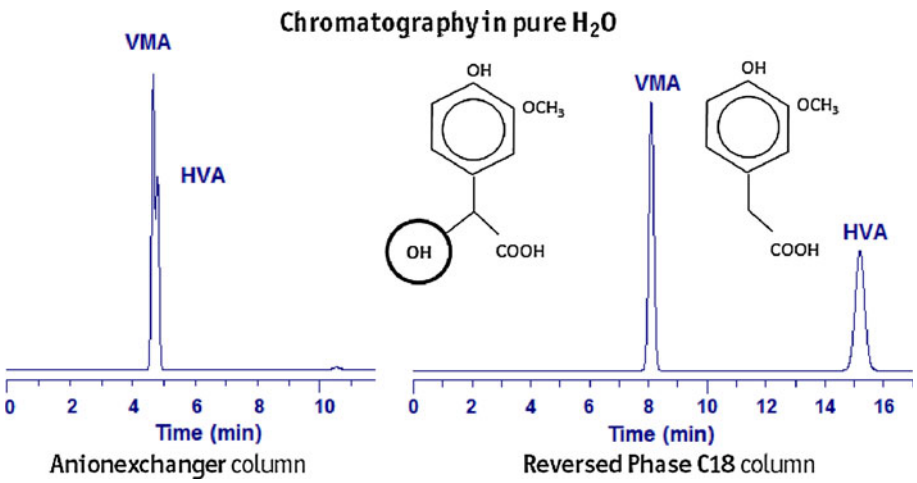


Fig. 2 The separation, which started the Solvophobic Theory: two similar acidic compounds with only an OH-group difference had no separation on an ion exchanger column (*left*) but an excellent separation on a C18-column in plan aqueous eluent (*right*)

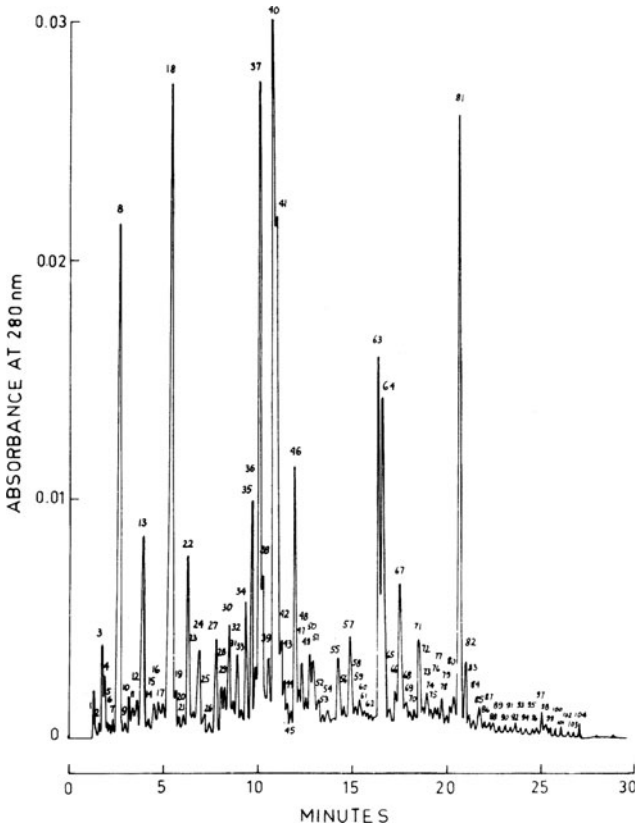
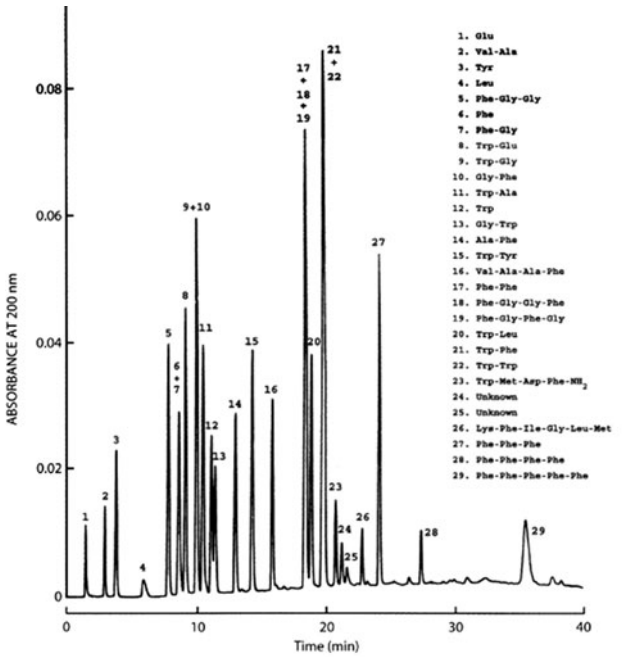


Fig. 3 Fast separation of approx. 100 organic acids for medical research on tumor metabolism studies opened new choices for R&D in Life Science in 1977—Analysis time was instead of 48 h <30 min—an improvement in speed of almost 100 times (reprinted [3])



Separation of nonpolar amino acids and small peptides in RPC Column: LiChrosorb RP-18, 5 μm, gradient elution from 0.5 M $HClO_4$ (to protonate the carboxyl-groups uniformically) pH 0.2, with acetonitrile as the gradient former. Temperature: 70 °C, flow rate: 2.0 mL min^{-1}, ΔP: 150 atm. Sample 10 μL containing ca. 1 μg of each component [12]

Fig. 4 First peptide separation on a LiChrosorb RP-C18-column (Merck, Darmstadt, Germany) in 1976, showing the high performance of RPC for peptides and later for proteins

investigating the influence of all important factors governing retention and forming a scientific fundament for a better understanding the retention process in RPC.

Solvophobic Theory

The work on the Solvophobic Theory started with the recognition that RP-HPLC works well in neat aqueous eluents. However, the question was what the role of the water is. Which energetic contributions are the strongest in the retention process? How can we explain to have in water a $k = 4{,}000$ and in MeOH or AN $k = 1$? (Fig. 5)

I suggested to Csaba to look at the different energetic contributions to this equilibrium process and hoped we could maybe find out, which term regulates the retention in the strongest way. After several long discussions, we came to the conclusion that the only physical property that fits the large retention difference between water and the organic mobile phase is the cavity reduction term, containing the surface tension of the water. In a long process of measuring, calculating and discussing these phenomena, we finally came up with the equation as follows:

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

The dominant term in this equation is the "cavity reduction term": $\mathrm{C}\Delta\mathrm{A} = \mathrm{N}\Upsilon\Delta\mathrm{A}/\mathrm{RT}$ (T temperature), which is approx. 10 times larger than the van der Waals term. This means that adsorption is not the main effect, but the eluent property "surface tension" Υ is. Υ is proportional to the percentage of water in eluent A, therefore, the most dominant influence in RPC is the composition of the eluent either as %B or as the gradient time (or slope) called t_{G}.

The quintessence of the Solvophobic Theory is the following: retention forces in RPC are based primarily on the "lipophobicity of the water" (LoW) (also called eluent "A" in RPC). The LoW can be reduced by dilution with the "strong" eluent, for instance MeOH, ACN, THF. This is done in gradient elution with excellent precision, leading to a reduction of retention values of any kind of substances in life science and offering an easy and simple way to generate information about processes of life by simply measuring the concentration of all related compounds involved. Additionally, the LoW against a molecule with partial non-polarity can be reduced by shifting the pH to form charged substituent such as $-NH_3^+$, or

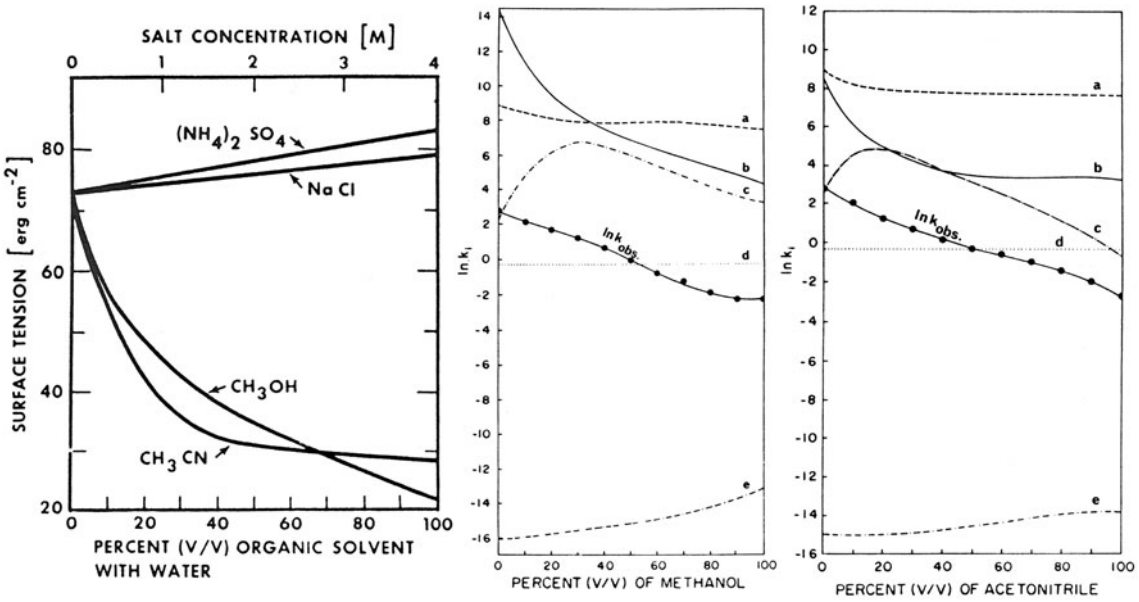


Fig. 5 Energetic contributions to retention in RPC: surface tension (on the *left*) and H_2O–MeOH system (in the *middle*) and H_2O–AN system on the *right* (reprinted [5])

–COO^--groups—offering improvements of separations with pH-changes (Fig. 6).

Where We are Today: Implementing QbD Principles into Method Development with Computer Modeling Software

For a number of years HPLC method development has been greatly eased with the aid of computer supported technologies [8] including one- (one-factor-at-a-time OFAT) and two-dimensional modeling. Retention mapping coupled with experimental design strategies have been continually used as a powerful method development and optimization technique. Indeed, HPLC method development software such as DryLab® [9–12] as well as LC-Simulator from Advanced Chemistry Development (http://www.acd.com) and Merck's ChromSword (http://www.chromsword.com), though tackling the method development task in different ways; still today use resolution maps to clearly identify optimal conditions. They are continually looking for ways to easily and practically optimize many factors simultaneously and change critical resolution seamlessly.

The shortcomings of an OFAT approach or of a 2D model are presented in Fig. 7 where an edge of the cube represents an OFAT method and a plane inside is presenting a 2D model. In Fig. 7, we can see three planes, each of them corresponds to a 2D resolution map. The total space as such, however, is experimentally not fully explored showing large volumes of unknown space. To experimentally fill out this space, an approach first

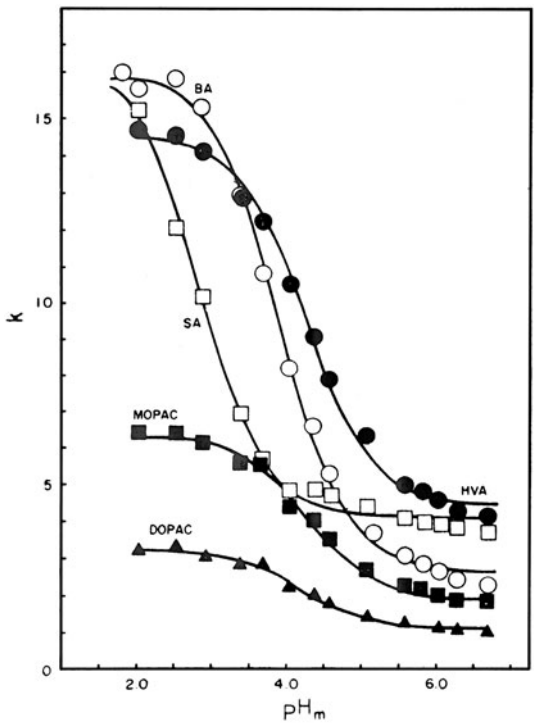


Fig. 6 pH influence on the retention of acidic compounds (reprinted [6])

described by Molnár et al. [10] was used. In difference to the mentioned work, here the number of critical factors considered is less therefore the optimization process is simplified to the generation of one cube rather than multiple cubes.

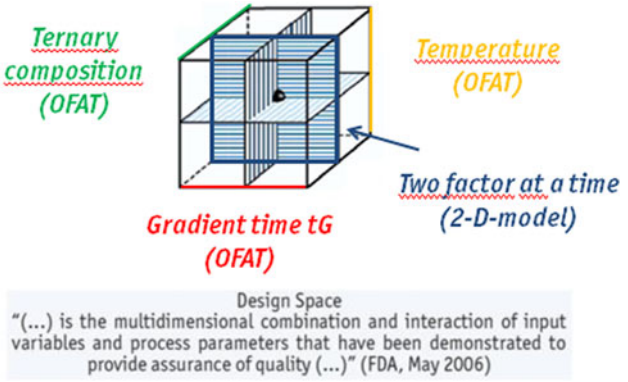


Fig. 7 Presentation of the Design space for three factors described by 1- and 2D planes. The working point is in the middle of the cube and represents a result of the best critical resolution. Three different resolution maps are shown here as individual parts of the whole Design space

High performance liquid chromatography method robustness and QbD [13] are playing an important role in global economy, where pharmaceutical and chemical products are distributed worldwide and the method transfer process has to be running smoothly for the same product in different countries and in different laboratories. Regulatory authorities (FDA, ICH, etc.) nowadays are promoting and requesting the application of QbD principles to ease the exchange of complex information about chromatographic selectivities and critical resolution values to support better method control, including method transfer. The result is a better understanding of how a method should be communicated and adjusted to ensure a given requested performance. Furthermore, the ICH Q8 (R2) [14] made a clear movement toward building in more flexibility to support the development of new products in industrial environments. The ever-increasing appearance of terms such as QbD and Design space (DS) within HPLC method development is an indication of this growing trend [15–18].

The present paper combines a more advanced resolution and selectivity mapping technology of the three above-mentioned factors with changes in column dimensions, gradient shape and flow rate, with the new mind set of the regulatory authorities to build in more scientific and multifactorial approach to HPLC method development and reduce costs caused by trial and error.

Experimental

Eluents

Methanol and acetonitrile (gradient grade), HPLC-water and all chemicals were purchased from Merck (Darmstadt, Germany). Eluent A was prepared by mixing 25% A_1 and 75% A_2 (*v*/*v*) where A_1 was a solution 25 mM phosphoric acid and A_2 was a solution 25 mM monobasic sodium monophosphate for a pH 2.6.

Eluent B was varied between acetonitrile (B_1) and methanol (B_2). Gradient elution between 0 and 100% B was used at a flow rate of 0.8 mL/min^{-1} unless indicated otherwise.

Sample

Model substance and reference materials used were phthalic acid (1), vanillic acid (2), isovanillic acid (3), anthranilic acid (4), vanillin (5), syringaldehyde (6), ferulic acid (7), ortho vanillin (8), benzoic acid (9) and were purchased from Merck. For systematic studies with three-dimensional resolution models, a stable sample mix which would be available over a longer period of time was needed. More practical examples of our work are published in the book of Ermer and Miller [19] and recent publication by Euerby et al. [20].

Equipment

High performance liquid chromatography separations were run on a Shimadzu LC-2010C with integrated 4-liquid gradient system, high-speed and cooled autosampler temperature controlled column compartment and Shimadzu UV–VIS detector (Shimadzu Europe, Duisburg, Germany). UV detection was performed at 240 and 254 nm. Double wavelength detection aided the identification of peaks in critical positions. The dwell volume was 1.06 mL and extracolumn volume was 0.016 mL. A HALO Phenyl-Hexyl (100 mm × 4.6 mm, 2.7 µm) column provided by MacMod Inc. (Chadds Ford, PA 19317, USA) was used.

Software

High performance liquid chromatography separations were generated using the automation option of DryLab®2010, which includes PeakMatch® v. 3.6.3 and DryLab® v. 3.9 (Molnár-Institute, Berlin, Germany) coupled with Shimadzu's LCsolution integration software. Peaks were identified and aligned based on peak areas using user-friendly tools, such as peak turnover and peak splitting functions of the software, reducing the usual problems of common misalignments between peaks. Modeling was performed in DryLab®2010 and predictions were compared with the original experiments to control the validity of the modeling process. Generation of 3D resolution models was carried out with a new proprietary algorithm in DryLab®2010.

Experiments for Modeling

Initial input data were acquired under the following conditions: gradient times (t_G) of 20 and 60 min (as recently reconfirmed by LoBrutto and his group at Novartis, USA

[21], producing an accuracy of 99% or better in retention time prediction), temperatures of 30 and 60 °C, largely in compliance with recommendations from Snyder et al. [22], eluent A was 25 mM phosphate buffer and had a pH value of 2.6.

Ternary eluent compositions were of (a) 100% AN, (b) AN:MeOH (50:50) (*v/v*), and (c) 100% MeOH and were combined to produce 12 experiments as shown in Fig. 8. Input data, 4 × 3 experimental runs, were performed overnight automatically with a Shimadzu LC-2010C controlled from PeakMatch®. After the runs were finished, they were exported automatically to PeakMatch for the peak tracking process. Finally, the data were transferred to DryLab®2010. The plate number was adjusted in various computer simulations of separation to the real column performance.

Results and Discussion

Work Scheme

The work flow followed in this project can be divided into six steps, which are described below in detail.

Design of Experiments

This initial step consisted of the selection of critical parameters, which were to be optimized and the establishment of the range over which they were to be varied.

Description of the Sample The selection of the critical parameters was done independently of the nature of the sample. Therefore, it is believed that all so-called "regular" samples could be treated in the same manner as described here. Special samples, such as organic ions, isomers, enantiomers, biological samples, peptides and proteins and other macromolecules such as oligonucleotides are also thought to be successfully separated following these steps, though slight adjustments are likely to be necessary.

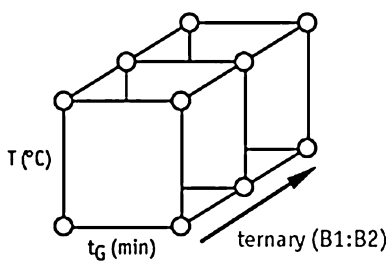


Fig. 8 Multifactorial optimization strategy of three measured critical HPLC method parameters: gradient time (t_G), temperature (T) and ternary composition (B_1:B_2), based on 12 experiments

Definition of Goals Defining separation goals before method development commences both prevents unnecessary experimentation and enables building in the required flexibility from the outset. Depending on the particular requirements of a separation, these goals can vary to have more or less constraints, though typically maximum separation of all or selected peaks and minimum run times are key. As previously stated, the goal of this optimization was the maximum separation of all compounds with a minimum run time, all within a known robust working region.

Definition of Critical Parameters The three parameters gradient time, temperature, and ternary eluent composition were chosen to be optimized first due to their known strong influential effect on selectivity. Flow rate, gradient slope, gradient shape, column parameters, dwell- and extra-column volume can be varied within the software to investigate selectivity changes, which is a well-known feature of DryLab®, but they are not treated here. In order to keep the number of experiments within a practical range pH of eluent A, buffer type and concentration (ionic strength) were kept constant, and an assessment of further additives was not carried out.

Column switching is a good way to change chromatographic selectivity. However, even after choosing the "best" column, selectivity is changing in gradient elution strongly with temperature, gradient form and shape, pH, ternary eluent, flow rate, column length and diameter, just to mention the most important influences. We intended to study primarily the above-mentioned factors on an excellent column to be able to control the method better according to the principles of QbD. All experiments took place therefore on the same column, selected on the basis of good peak shape and maximum number of obtained peaks, determined by preliminary basic scouting runs.

Two gradient times, two temperatures and three ternary eluent compositions were necessary in order to reliably create retention models of these factors with DryLab®. Gradient times should differ in a factor 3, temperatures in 30 °C, and ternary eluent composition was varied between MeOH and AN. These ranges are large enough to induce significant band movement to discover hidden peaks and small enough to enable relatively easy peak tracking.

Replacement of Acetonitrile by Methanol Due to the actual shortage and high price of acetonitrile, it was intended to find eluent compositions to reduce the amount of acetonitrile in the mobile phase. The ternary eluent was chosen so as to encompass the whole range from 100% acetonitrile to 100% methanol. This way the replacement of acetonitrile by methanol could be assessed to a large degree.

Automated Data Generation

Once the experiments were designed, chromatograms were acquired by means of automated data generation. This was executed within the software PeakMatch® coupled with Shimadzu's LCSolution software.

Chromatographic conditions, equipment, column and eluent data are first keyed into the input data interface. Then, upon "starting runs" the relevant experimental data are loaded into a batch file and read by the chromatographic system. Once the batch has been completed, the chromatograms are automatically integrated and imported ready for peak tracking.

Peak Tracking

Upon changing eluent properties and the temperature, chromatographic selectivity (the separation) also changes. Therefore, prior to importing data into DryLab® for modeling, peak tracking had to be carried out. Peak tracking refers to the matching of bands for the same compound between runs where conditions have been changed. In a t_G–T-model four experiments have to be aligned in a way that all data (retention times and peak areas) of one substance are in one horizontal line. The peak identification procedure was carried out on the basis of peak areas, which are fairly constant in the four runs of a t_G–T-model. The prerequisite for this process is the injection of the same sample mix and of identical sample volume. In this case, peak areas represent sample mass (concentration × volume) and are at constant flow rate constant in each individual run, fairly independent of other experimental conditions. In the case of overlapping peaks, the areas are additive.

As mentioned above, peak tracking was accomplished in a semi-automated fashion within the program PeakMatch®. First general complexity was reduced by dividing large peak area numbers to more manageable ones, relevant chromatogram areas were zoomed into and irrelevant bands below 0.1% were deleted. Then a semi-automated operation function was used to match peaks followed by manual adjustments. The result of this step is a fully matched peak table.

Automatic Generation of the Retention Model

Once a matched peak table was obtained, all data were automatically transferred to create the resolution map. Optimal conditions could be ascertained by a special comparison of the original experiments with their modeled versions to control the quality of the peak tracking process.

Exploration of the Design Space for t_G, T and Ternary Composition

Two-dimensional resolution maps were used to create a three-dimensional resolution space, in which the combined influence of three parameters could be simultaneously assessed, visualized and optimized. This was carried out for t_G–T–ternary eluent compositions.

Visualization and Numerical Definition of Robust Regions

By resetting the resolution options shown in the resolution maps, robust regions ($R_{s,crit} > 2$) could be isolated and studied and it was found that these robust regions were continuous voluminous shapes. With this, optimal and robust working conditions could be ascertained visually within the described Design space.

Experiments to Explore Parts of the HPLC Design Space

The 12 input runs underwent 3 peak tracking processes and resulted in 3 2D resolution maps, which were used for the calculation of additional t_G–T planes, resulting in a 3D-resolution space. The corresponding cube is shown in Fig. 9a for three values of the ternary eluent composition.

Robustness and Experimental Verification

3D resolution spaces represent the simultaneous influence of three parameters on selectivity and critical resolution ($R_{s,crit}$). The region for method robustness can be visualized as a geometrical body within the resolution space, in which critical resolution does not fall below a given $R_{s,crit}$, for instance, $R_{s,crit} > 2$ or $R_{s,crit} > 1.5$ (base-line separation). Figure 9b shows a t_G–T–ternary resolution space in which robustness could be defined as: t_G 56 ± 3 min, T 28 ± 2°C, ternary eluent composition: 52 ± 5 (% MeOH in AN). The robustness space can be moved also to other points within the same cube by changing the coordinates of the middle point of the robust sphere. However, the size of the tolerances may change to smaller values.

An experimental chromatogram was run for the predicted conditions of highest critical resolution and the comparison between prediction and experiment is shown in Fig. 10. Verification experiments were conducted with a newly mixed sample. Correlation was found to be excellent.

The average difference between predicted and experimental retention times is 0.06 min (4 s) and the largest difference is 0.15 min (9 s) as can be seen from Table 1.

As mentioned in Sect. 1, further optimization of flow rate and gradient shape was carried out within the software.

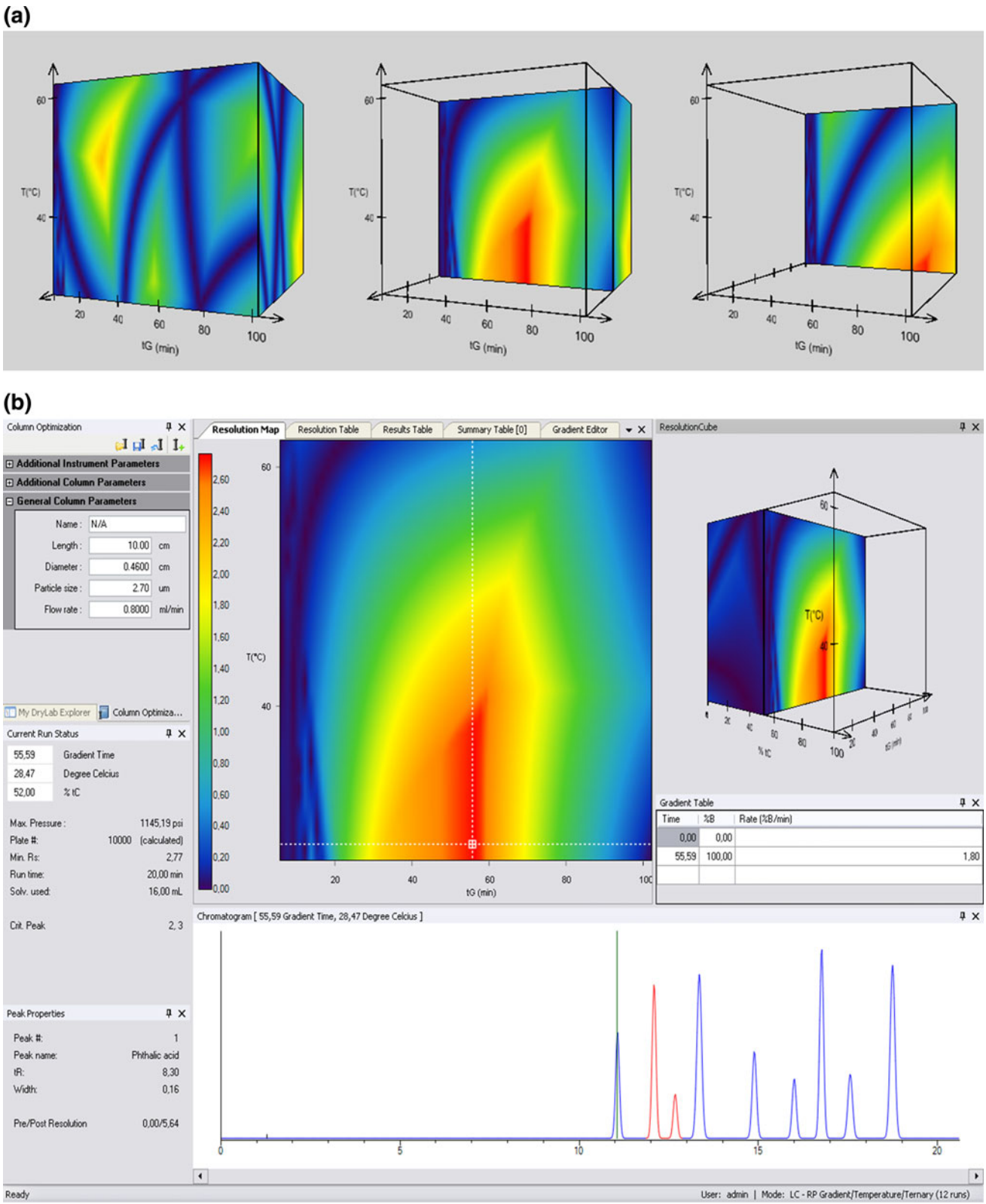


Fig. 9 **a** 3D resolution space modeling gradient-time, temperature and ternary eluent composition simultaneously in which the point of highest critical resolution within the robust region is depicted. **b** t_G–T–ternary cubes of 100% MeOH (*left*), AN:MeOH (50:50) (*v*/*v*) (*center*) and 100% AN (*right*)

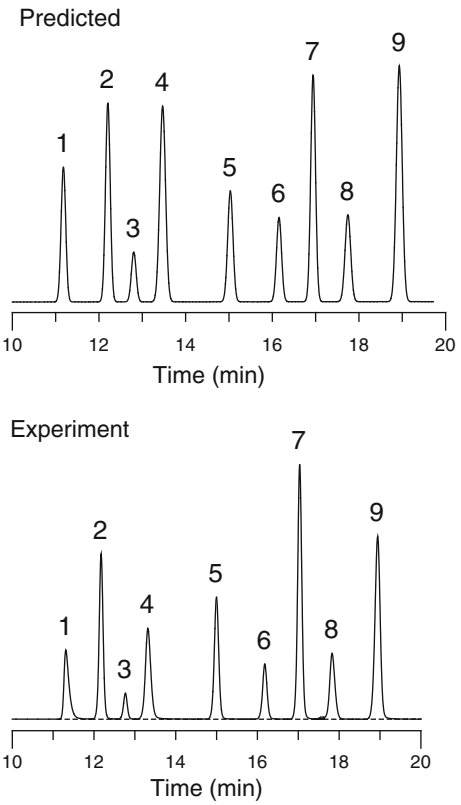


Fig. 10 Predicted and experimental chromatogram comparison for conditions of highest critical resolution: $t_G = 56$ min, $T = 28$ °C, ternary eluent composition = 52% MeOH, 48% AN, with flow rate 0.8 mL/min^{-1}

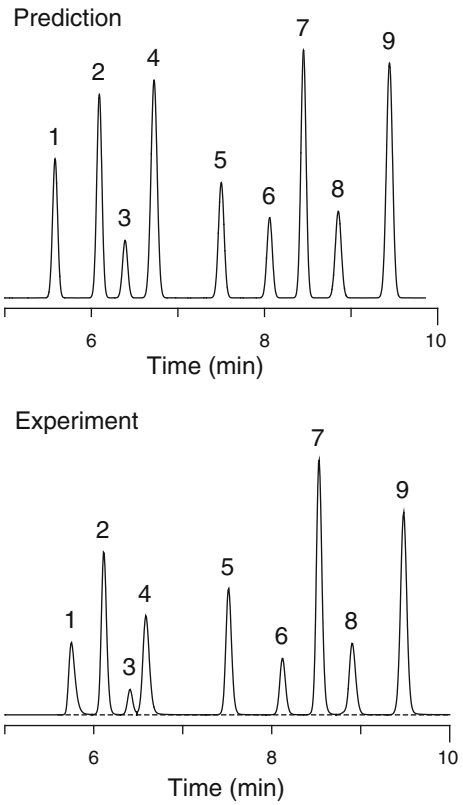


Fig. 11 Predicted and experimental chromatogram comparison for reduced run time conditions: $t_G = 28$ min, $T = 28$ °C, ternary eluent composition = 52% MeOH, 48% AN with flow rate 1.6 mL/min^{-1}

Table 1 Retention time comparison between experimental and predicted chromatograms at point of highest critical resolution

Peak no.	Retention time (min)			
	Experimental	Predicted	Difference[a]	% error[b]
1	11.31	11.18	0.13	1.14
2	12.17	12.20	−0.04	−0.30
3	12.76	12.80	−0.04	−0.30
4	13.14	13.47	−0.15	−1.14
5	14.99	15.03	−0.04	−0.25
6	16.18	16.15	0.02	0.13
7	17.03	16.94	0.09	0.51
8	17.82	17.74	0.08	0.45
9	18.94	18.93	0.01	0.04

[a] Difference = experimental − predicted retention time

[b] % error = [(experimental − predicted)/predicted] × 100

Table 2 Retention time comparison between experimental and predicted chromatograms under conditions of highest critical resolution and with run time reduced by half

Peak no.	Retention time (min)			
	Experimental	Predicted	Difference[a]	% error[b]
1	5.74	5.58	0.16	2.94
2	6.11	6.02	0.09	1.48
3	6.40	6.39	0.01	0.20
4	6.58	6.72	−0.14	−2.11
5	7.51	7.50	0.01	0.13
6	8.12	8.06	0.06	0.71
7	8.53	8.45	0.08	0.90
8	8.90	8.85	0.05	0.54
9	9.48	9.44	0.04	0.40

[a] Difference = experimental − predicted retention time

[b] % error = [(experimental − predicted)/predicted] × 100

It is possible to transfer the chromatogram at the best set of conditions to the gradient editor and make further improvements such as changing gradient shape, changing starting or/and final %B, column length, ID, dwell volume or flow rate. To test this, we increased the flow rate by a factor 2 and decreased the gradient time by the same factor. At the same time, gradient run time was reduced by half with preservation of the separation selectivity. The results were predicted by the software and, as seen in Fig. 11 and Table 2, correlate very well with experimental data.

Summary

Csaba Horváth revolutionized Life Science with a better understanding reversed phase chromatography. As a result of the better understanding of our RPC-technology, and the insight provided by HPLC modeling, method development can be carried out in a faster and scientifically more reliable way, in accordance with QbD principles.

A strategy for simultaneous multifactorial HPLC method optimization is presented in which automatic data generation, semi-automatic peak tracking, multidimensional retention modeling coupled with Design space visualization and organized method documentation can be performed under the DryLab®2010 platform. The resulting method gave a good separation of all nine peaks of a sample of nine substances with a critical resolution well above base-line separation and with a run time under 10 min. The method was proven to be robust with respect to three critical parameters. The methodology presented in this work is in accordance with QbD principles and results in the definition and visualization of the so-called Design space and posterior application to finding multiple robust working conditions of HPLC methods, giving the chromatographer a great degree of flexibility and the choice of developing orthogonal methods to control the mixture of the sample better.

High performance liquid chromatography computer simulation software has been shown to increase efficiency and to save time in routine method development, validation processes and method transfer time and time again. Here, it has been seen that other important factors such as building in flexibility and complying with regulatory demands can also be accomplished with the aid of an appropriate work flow and software. In this paper, a method and software are presented that locate the global optima for highly influential experimental parameters with respect to separation, analysis time and robustness. It has been shown that the graphical presentation of the critical parameters with an optimization Design space helps considerably in judging the robustness of the separation system. Moreover, inter-laboratory method transfer and documentation should be facilitated by this procedure.

Other potential applications of this methodology have also been touched upon. As well as its application within method development, transfer from one ternary eluent to another could also be assessed. This could be particularly relevant at a time of inflated acetonitrile prices and an ever-increasing tendency toward greener solvents.

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