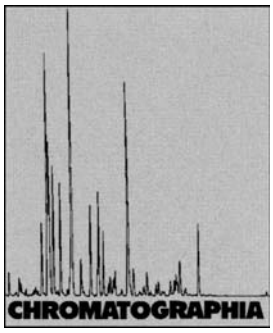
Searching for Robust HPLC Methods – Csaba Horváth and the Solvophobic Theory



I. Molnár

Institut für angewandte Chromatographie, Schneeglöckchenstr. 47, 10407, Berlin Germany; E-Mail: info@molnar-institut.com

In Memoriam of Professor Csaba Horváth (1930–2004)

Received: 23 June 2005 / Revised: 22 July 2005 / Accepted: 25 August 2005
Online publication: 10 October 2005

Abstract

This paper is written in remembrance of the work of Csaba Horváth on Reversed Phase Chromatography, (RPC) and the fundamental theory of the mechanism of retention on non-polar stationary phases, the ''Solvophobic Theory''. The paper discusses some steps in the development of this important theory and examines its consequences in developing robust methods for routine RPC. Reliable product quality requires the understanding of *selectivity changes*, which in RPC govern the development of robust and reliable methods involving *continuous changes of liquid chromatographic parameters in aqueous eluents*. The application of RPC is still growing in scientific research and in pharmaceutical and chemical production. The impact of the Solvophobic Theory in life sciences has been enormous but it was only a part of Horváth's scientific work. RPC is today one of the most popular, most widely used tools in analytical chemistry and will remain so for many years due to its stability and to its robustness.

Keywords

Csaba Horváth
Column liquid chromatography
Reversed phase chromatography
Solvophobic theory retention modeling
Continuous changes of selectivity

Introduction

After leaving Hungary in 1956, Csaba studied for his PhD in Frankfurt under the supervision of another famous Hungarian émigré, István Halász who became a full professor at the University of Saarbrücken in 1970. After my PhD Halász helped me to find a position with Csaba at Yale University as a postdoc for 2 years from 1975–1977.

Our common Hungarian background sustained our enthusiasm, and determination to succeed. We published 11 papers in two years, three of them on the Solvophobic Theory have been cited together more than 1800 times [1–3].

Csaba Horváth was a true pioneer. He developed pellicular stationary phases, first described by Kirkland, which were designed to reduce diffusion distances – a major step in making efficient separations in liquid chromatography. His supports were based on glass beads coated with a thin outer layer of silica. Csaba designed the first HPLC instrument for life science applications in the sixties. He told me, that one day, as his column pressure exceeded 1000 psi the first time, this couldn't be considered ''normal'' pressure chromatography anymore and consequently he coined the expression ''High Pressure Liquid Chromatography'', abbreviated to HPLC. This abbreviation became well known at the Pittsburgh Conference in 1969 and in Italy one year later [4].

When I arrived at Yale University in June 1975, Csaba wished to analyze proteins and enzymes on his pellicular C18 phases, which he packed into columns 1 m long and 1 mm in internal diameter. The packing material had 10 μm particle size and was surface coated with a porous layer of silica, which was derivatized with octadecylsilane. To be able to separate proteins on such a column, they had to be dissolved preferably in an organic solvent. Wayne Melander, another postdoc, was already working on the investigation of the solubility of proteins in different organic solvents. Our first experiments with proteins resulted in chromatograms with broad peaks like the Swiss Alps and it was not until nine years later that the seminal work

Part of this paper was presented at the meeting: ''In Memoriam of Csaba Horváth'', Hungarian Academy of Sciences, Budapest, Hungary, October 11, 2004.

Original
DOI: 10.1365/s10337-005-0645-1

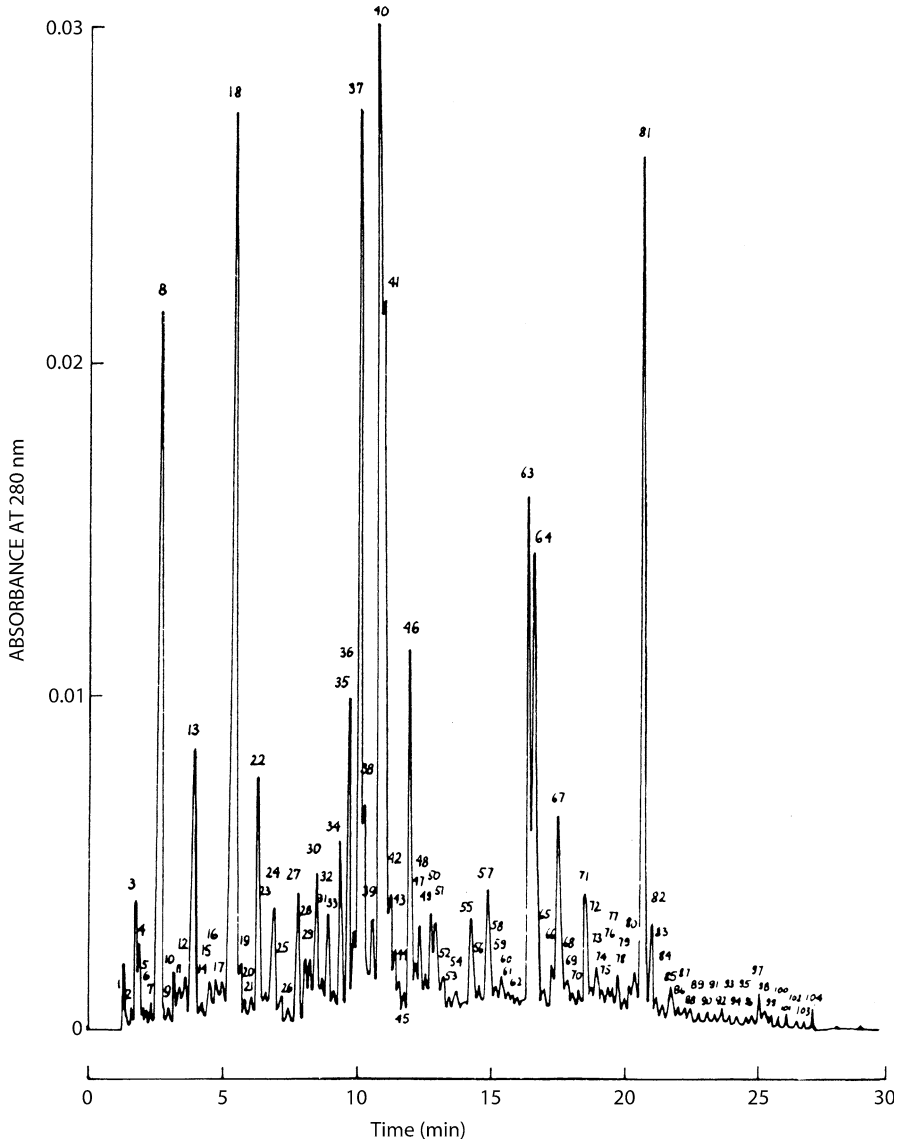


Fig. 1. Chromatogram of an extract of 100 µL acidified urine. Column: 5 µm octadecyl-silica, 25 × 0.46 cm, temperature 70 °C; flow rate: 2.0 mL min^{-1}. Gradient elution from 0.1 Mol phosphate buffer, pH 2.1, with acetonitrile to about 40% acetonitrile in 30 min [7]

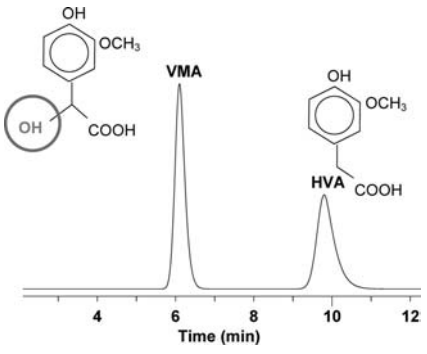


Fig. 2. The start of the Solvophobic Theory: Excellent separation of two very similar compounds VMA (left) and HVA (right), having only a hydrophilic OH-group as difference on a hydrophobic C18 phase in neat water, without any organic solvent added. Column: Partisil ODS, 10 µm, 25 × 0.46 cm; eluent: 0.05 M NaH_2PO_4, pH 4.3; flow rate: 0.66 mL min^{-1}, inlet pressure 450 psig [10]

of Barry Karger and Kalman Benedek, (another Hungarian), was published on the denaturation of proteins on RP materials, which explained how protein folding influenced their RP-retention and peak shape. Their paper was selected at "HPLC 1984" in New York, where Horváth was the chairman, as the best poster [5].

The biological function of enzymes was explained in those days with the "hydrophobic" effect. Some researchers, such as Sinanoglu, Professor of Theoretical Chemistry at Yale however used the word "solvophobic", as a more general expression as investigations on protein dissolution were often carried out in mixed aqueous-organic solvents.

In 1975 Horváth engaged in clinical applications of RPC, which was, according to his belief, well suited to solve problems in life sciences. In 1970 the separation of ca. 100 organic acids took over 40 hours on an ion exchange column using salt gradients [6]. Csaba postulated at the beginning of our project, that with RPC this separation might be done in less than one hour. He was right, as shown in Fig. 1, where we achieved the separation of more than 100 urinary acids in only 30 min with a special RP gradient [7].

In addition, a few years later he recognized the common basic relationships between RPC and Hydrophobic Interaction Chromatography (HIC), an important technique for the successful chromatography of proteins. Csaba also investigated displacement chromatography as a preparative tool for producing concentrated fractions of peptides from tryptic digests, using analytical columns in the quality control of recombinant proteins. He was greatly interested in microanalytical techniques also, especially in capillary electrophoresis (CE) and capillary electrochromatography (CEC) and was always deeply involved in the newest research areas.

The Start of the Solvophobic Theory of RPC

In 1975, R.W. Stout from Yale Medical School brought a problem to our lab – the separation of VMA and HVA, two very similar organic acids, metabolites of catecholamines, differing only in an alcoholic OH group. They are marker compounds for the development of pheochromocytoma and neuroblastoma and had to be separated from several basic catecholamines and quantitated for clinical screening. Stout could not separate these two acidic metabolites well on either an anion exchange or a cation exchange column and came to us to ask for advice.

There were no ways of performing a better separation on other ion exchange columns so I finally suggested that we try the separation "just for fun" on a reversed phase C18 material. After waiting ca. 6 min, a single peak arrived and after a further 4 min suddenly a second peak appeared. Injection of single reference compounds showed that the C18 phase was able to separate VMA as the first and HVA as the second peak with excellent resolution using a neat aqueous eluent. The retention time difference was

amazing and also fascinating. How could a hydrophilic OH group make such a difference in retention on the surface of a "hydrophobic" support? That day in September 1975, on which we achieved the highly efficient separation of VMA and HVA (Fig. 2) represented the birth of the "Solvophobic Theory of RPC", which will be discussed in more detail below.

It was not only the strong influence of a hydrophilic substituent leading to unexpected selectivity changes in RPC which was fascinating, but also the observation of other excellent separations of compounds with very slight differences in molecular structure, which are shown in Figs. 3 and 4. So two very similar basic compounds MET, (metanephrine, N-methyl-3-methoxy-phenylethanolamine) and 3MT, (3-methoxy-tyramine) could also be separated and radioactively labeled metabolites used to help to explain the metabolism of catecholamines [8].

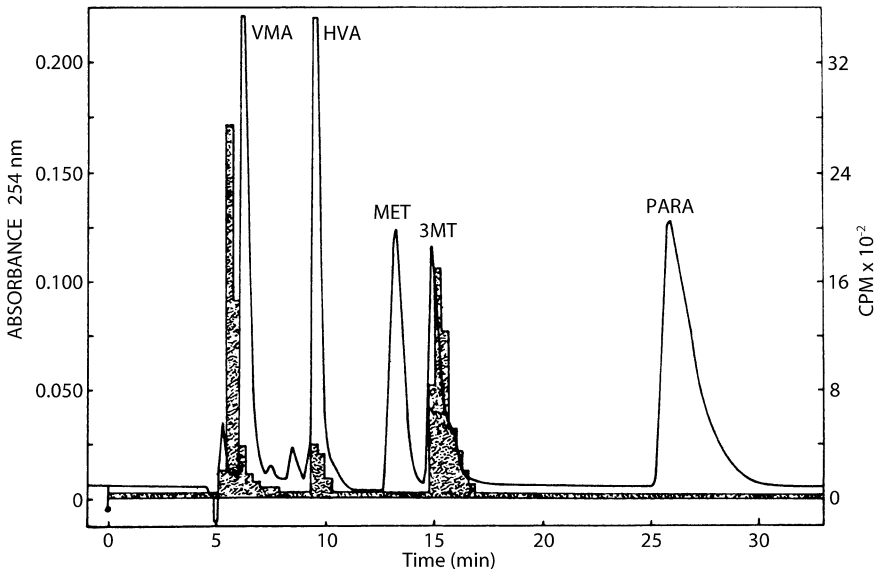


Fig. 3. Chromatogram of catecholamine derivatives obtained with a microparticulate Partisil ODS-column, 10 µm, 25 × 4.6 cm; eluent: 50 mMol Na2HPO4, pH 4.3; flow rate: 0.66 mL min^{-1}. In case of neuroblastoma cells ß-hydroxylated metabolites such as NMET, VMA are completely absent in the cell extract. This could be used to detect neuroblastoma in newborn babies [8]

RPC in Neat Aqueous Eluents

Based on such observations we started to look for a more general treatment of the retention process of RPC with samples of biological origin, such as catecholamines and their metabolites (Fig. 4a–d). The metabolism of the parent compound L-phenylalanine leads to dopamine and norepinephrine and after 3-O-methylation to other products like VMA and HVA, which could all be well separated in neat aqueous eluents without the presence of any organic modifier [9–12]. After returning to Berlin, I helped to establish this technique in the clinical laboratory of the Childrens Hospital Charlottenburg with Prof. Dr. E. Mönch to help to screen newborn children routinely for brain disorders.

Before this time it was believed, that in RPC there are two liquids in the column – an immobilized layer of organic solvent and the aqueous mobile phase with retention based on partition of the solute between both liquids. The fact, that the aqueous eluent did not contain any organic component, such as methanol or acetonitrile, was interesting for several reasons:

- There could not be any partition of the sample between two liquids. This led to an important conclusion: RPC must work by a different retention mechanism from "partition chromatography", but this was at that time not clear in detail.
- Work with aqueous eluents enhanced the further development and use of highly sensitive electrochemical detectors;
- We could investigate the influence of the eluent pH on the retention of acidic and basic compounds with less problems in neat aqueous eluents.

We grouped the metabolites into acidic and basic components and found that on changing the pH their retention behaved opposite to each other, as shown in Figs. 5a and 5b. The retention of acidic compounds decreased and the retention of basic compounds increased with increasing pH [11]. This finding had important consequences:

- The retention of acidic or basic compounds – but also of zwitterions, such as peptides and proteins – could be *continuously* changed by changing the pH of the eluent. This helped to optimize selectivity to achieve the best possible separation using the *eluent*.
- Method robustness: In industrial quality control, HPLC methods could be adjusted by changing separation selectivity, using the pH much easier than in the past.

Later the separation performance was considerably improved with newly developed C18 stationary phases, which had a better surface coverage and more retention power. This is shown in the gradient elution of physiological samples (Fig. 6 and Table 1), where VMA and HVA have much greater separation in the gradient mode, than in the previous isocratic mode on Partisil ODS [11].

Structure-Retention-Relationships in RPC

Many scientists tried to predict retention in liquid chromatography (LC) based on the structural properties of the analytes. We were also interested to find out which molecular descriptors governed retention in RPC.

We measured the retention factors of 32 closely related catecholamine compounds and their precursors and metabolites and estimated the effect of various substituents on the retention under similar chromatographic conditions. All experiments were carried out at 70 °C with neat aqueous eluents consisting of 0.1 M phosphate buffer at pH 2.1. The ionic strength was high enough to suppress silanophilic contributions to retention [10].

Later Jacobson, El Rassi and Horváth tried to predict retention for

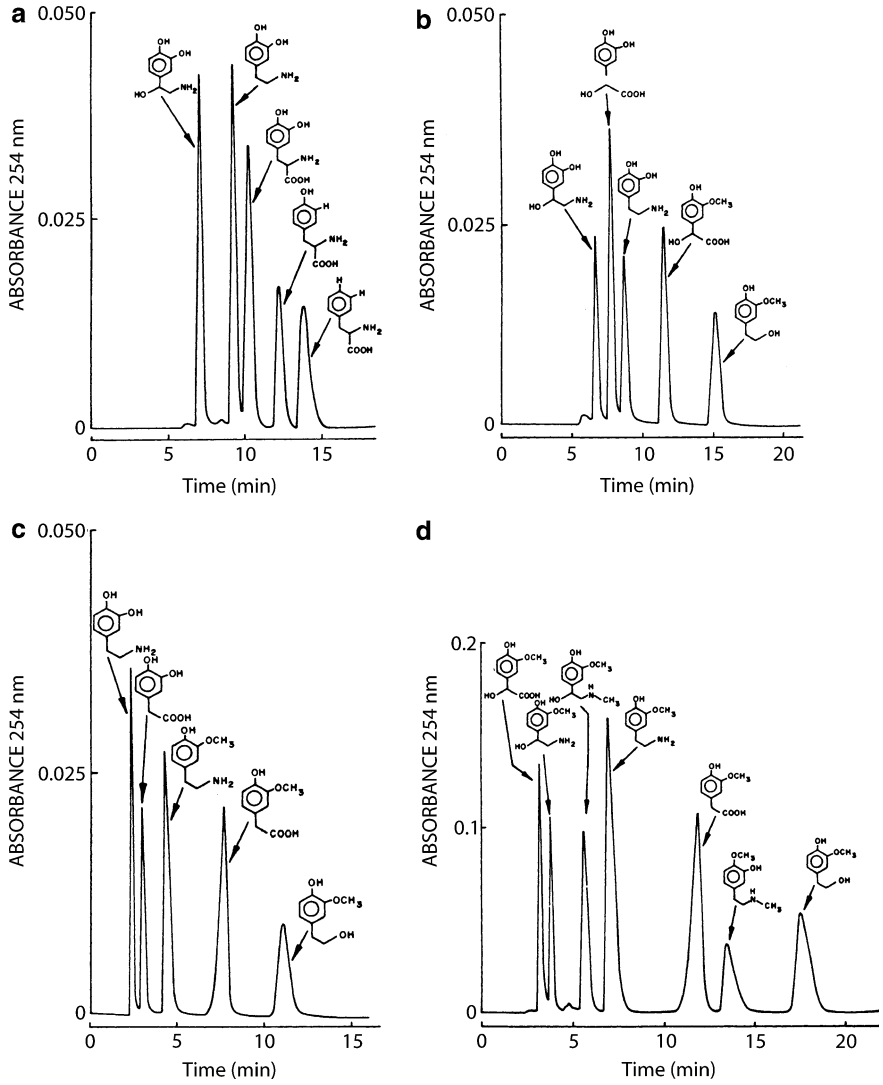


Fig. 4a. Chromatogram of intermediates of L-phenylalanine metabolism to norepinephrine. Column: Partisil 1025 ODS, 10 μm, 25 × 0.46 cm; eluent: 0.2 Mol L^{-1} H3PO4:NaH2PO4, pH 1.9; flow rate: 0.5 mL min^{-1}, inlet pressure 50 atm; temperature: 25 °C [10] **Fig. 4b.** Chromatogram of intermediates of dopamine metabolism to vanilmandelic acid Column: Partisil 1025 ODS, 10 μm, 25 × 0.46 cm; eluent: 0.05 Mol L^{-1} H3PO4:NaH2PO4, pH 2.0; flow rate: 0.5 mL min^{-1}, inlet pressure 50 atm; temperature: 23 °C [10]. **Fig. 4c.** Chromatogram of intermediates of intermediates of dopamine metabolism to homovanillic acid; Column: Partisil 1025 ODS, 10 μm, 25 × 0.46 cm; eluent: 50 mMol L^{-1} KH2PO4, pH 4.6; flow rate: 2.0 mL min^{-1}, inlet pressure 200 atm; temperature: 22 °C [10]. **Fig. 4d.** Chromatogram of intermediates of 3-O-methyl metabolites of dopamine Column: Partisil 1025 ODS, 10 μm, 25 × 0.46 cm; eluent: 50 mMol L^{-1} KH2PO4, pH 4.6; flow rate: 0.66 mL min^{-1}, inlet pressure 35 atm; temperature: 25 °C [10]

oligonucleotides using a simple linear model, based on the free energy contribution to retention by the component parts of the oligonucleotides. They introduced the τ-value for RPC, which was a log k increment for certain substituents. The meaning of the τ-value was similar to that of the p values introduced by Hantsch for use in quantitative structure-activity relationships (QSAR) in medicinal chemistry and related areas. They found a good correlation between predicted and experimental retention values under a given set of conditions [14]. The sample population was, however, not large enough to establish a statistically acceptable quantitative structure-retention relation. The results were only applicable if the pH was kept rigorously constant. If the experiments were carried out at another pH, the ionization of the basic and acidic groups could change.

The much more important consequence of these studies was that in RPC selectivity can be changed *continuously* using eluent parameters such as pH or ternary eluent composition. This is one of the reason, why RPC has become a more and more general analytical technique world wide.

The Separation of Amino Acids, Peptides and Proteins

From his earliest days at Harvard, Csaba's main interest was to improve separation techniques for the life sciences. After a period of trying to separate proteins on C18 phases, I investigated the separation of the smaller building units, amino acids and peptides. After several months of experimentation, the separation of different digests of the so called S-peptide opened an entirely new way into the analysis of peptides and allowed study of processes in life science using RPC (Fig. 7). It was shown for the first time on several peptide mixtures, that this group of compounds could be readily analyzed with high efficiency by RPC, demonstrating impressively, how to deal with these important compounds [12].

Although this is not the place here to go into details, I would like to mention the fascinating wealth of information, which came out of this research. Ordering a larger number of different peptides and investigating their retention behavior under our experimental conditions, namely under gradient elution and at high temperature, it was found, that the peptide backbone, $[Gly]_n$ did not contribute to RP retention, but was unimportant in terms of hydrophobic properties as shown in Fig. 7. Positive retention contributions came only from hydrophobic amino acids, such as Trp, Phe, Tyr, etc. On the other hand, negative retention contributions were obtained from polar amino acid side chains, such as Asp, Glu, etc. It was interesting, that there was a simple, but not quantitative additivity of retention times of several peptides in gradient elution, such as shown in Fig. 7:

$$t_R(\text{Tyr})(3)+t_R(\text{Trp})(12)\approx t_R(\text{Trp-Tyr})(15);$$

$$t_R(\text{Leu})(4)+t_R(\text{Trp})(12)\approx t_R(\text{Trp-Leu})(20);$$

$$t_R(\text{Val-Ala})(2)+t_R(\text{Ala-Phe})(14)\approx t_R(\text{Val-Ala-Ala-Phe})(16);$$

$$t_R(\text{Trp})(12)+t_R(\text{Trp})(12)\approx t_R(\text{Trp-Trp})(22);$$

(peak numbers in Fig. 7 are in parenthesis) using C18 stationary phases. The

neutral role of Gly can be seen in the coelution of Phe (6) with Phe-Gly (7) and in the coelution of Phe-Phe (17) with Phe-Gly-Phe-Gly (18) and with Phe-Gly-Gly-Phe (19). Phe-Gly-Gly (5) came even earlier than Phe (6) itself, probably due to a size exclusion effect. The distances between Phe, $(Phe)_2$, $(Phe)_3$, $(Phe)_4$ and $(Phe)_5$ were not equidistant as here a tertiary structure can be formed internally within the longer members.

The research on retention-structure relationship for peptides revealed the same dominance of hydrophobic contributions as we had found for catecholamines and metabolites, although here the variance due to the 21 different amino acid side chains was much larger, than in the catecholamine family. Retention prediction was here even more complicated because of the many parameters involved. We recognized that precise predictions of retention had to be based on actual measured data. These and other reasons later led to the development of the chromatographic software tools starting in 1986 where only direct measurements of retention times were used to model molecular retention behaviour in RPC with the help of the computer. Retention predictions, which were based on molecular structure were at that time not precise and reliable enough for the needs of the pharmaceutical and the chemical industries. The reason for this was the difficulty in generating precise data about molecular properties. However RPC can measure such molecular data simply by measuring retention in gradient elution. There are modeling softwares available which use measured retention values as input data. Their routine use worldwide in the R&D and quality assurance environment by companies such as Novartis, Pfizer, Astra Zeneca, Hoffmann la Roche, Aventis, Boehringer, etc., ensures efficient method development practices [13].

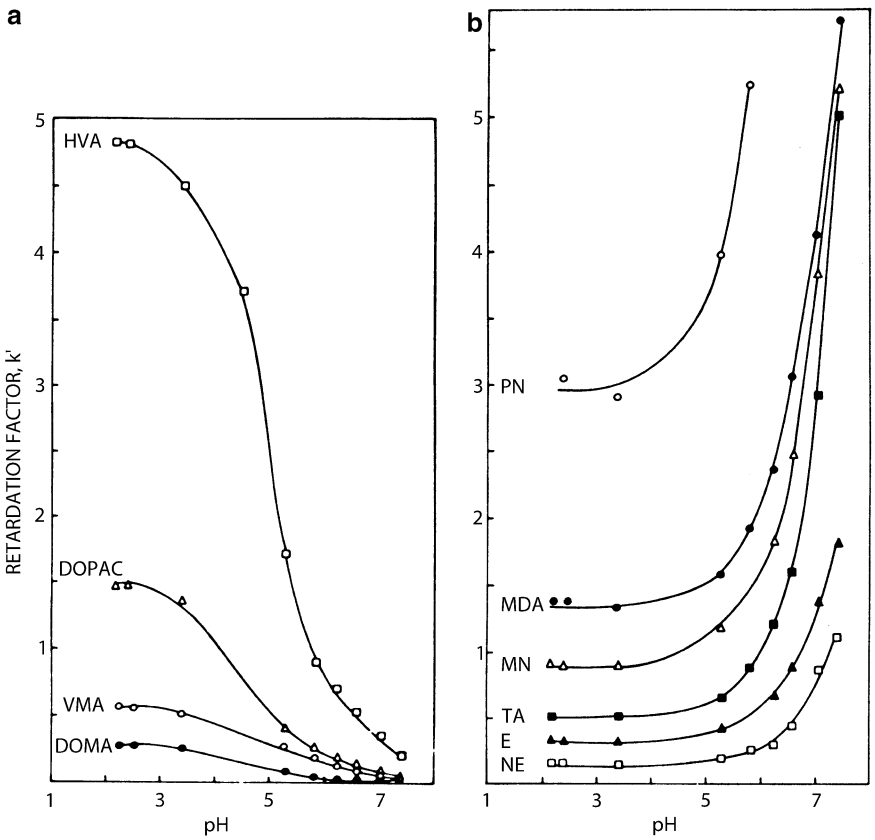


Fig. 5a. Effect of pH on the retention of acidic compounds. Stong selectivity changes can occure with pH changes. Column: Partisil 1025 ODS, 10 μm, 25 × 0.46 cm; eluent: 0.1 Mol L^{-1} phosphate buffers; flow rate: 1.0 mL min^{-1}, inlet pressure 100 atm; temperature: 25 °C, DOPAC: 3,4-dihydroxy-phenylacetic acid; DOMA: 3,4-dihydroxy-mandelic acid; HVA: 3-methoxy-4-hydroxy-phenylacetic acid (homovanillic acid); VMA: 3-methoxy-4-hydroxy-mandelic acid (vanilmandelic acid) [10]. **Fig. 5b.** Effect of pH on the retention of basic compounds. Conditions as in Fig. 4a. Biogenic amins: E: epinephrine; MDA: 3-O-methyl-dopamine; MN: metanephrine; NE: norepinephrine; PN: paranephrine; TA: tyramine [10]

Basic Rules of RPC Retention Equilibria

At the same time as studying structure-retention relations, we were trying to understand the reversed phase retention process itself in much more detail. We had to break down the energetic contributions, which were governing the retention process, into individual terms [1–3]. Csaba used the idea of "solvophobic" interactions developed by Sinanoglu to understand molecular associations of biological materials in aqueous-organic solvents. We used a simple mass balance equation (Fig. 8), which led to the simplified expression of the different free energy contributions to the overall retention process:

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

$$C = N\Delta A/RT \quad (2)$$

(γ is proportional to the amount of water in the eluent, % H_2O)

ln k is the logarithm of the retention factor $k = (t_R/t_o) - 1$, t_R and t_o are the elution time of sample and eluent molecule, respectively. With the exception of the constant A all other terms of Eq. (1) are eluent dependent. The logarithmic retention factor, ln k, is the sum of 6 energetic contributions:

A depends on column parameters and entails the free energy of the association process in the gas phase,

B, C, D and E and the last term are approximately constant,

E represents van der Waals forces between solute and ligand. These forces are stronger in organic solvents than in water.

The so called "cavity-term" Cγ, where

$$C = N\Delta A/RT, \quad (3)$$

with N: Avogadro number, γ: surface tension of the mobile phase (H_2O: 72 erg/cm^2, AN, MeOH ca. 25 erg/cm^2), R: universal gas constant, T: absolute temperature, ΔA is the accessible hydrophobic contact surface area between solute S and ligand L, and it is the difference between the hydrophobic surface areas of the solute, (A_S), the ligand, (A_L), minus that of the associated complex SL (A_{SL}) resulting in

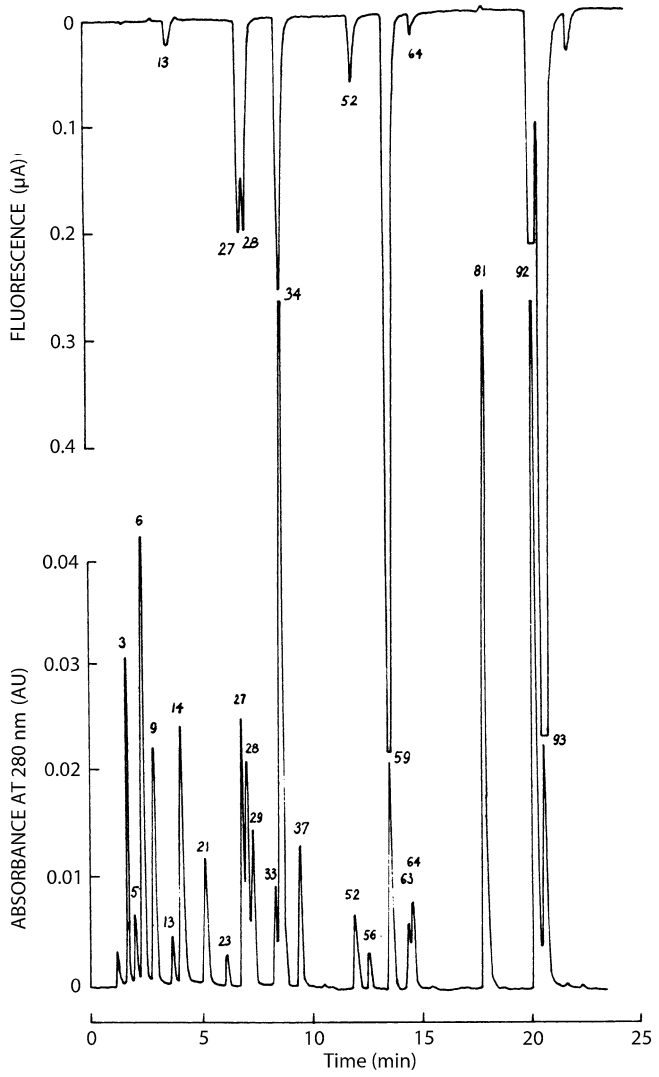


Fig. 6. Chromatogram of aromatic acids. The numbering of the peaks corresponds to that shown in Table 1. Column: LiChrosorb RP-18, 5 μm, flow rate: 2.0 mL min^{-1}; temperature: 70 °C; eluent A: 0.1 Mol phosphate buffer pH 2.1, eluent B: acetonitrile; gradient elution 0- > 35%B in 30 min; initial inlet pressure: 160 bar. Fluorescence detector: excitation 238 nm, emission 340 nm [7]

$$A = A_S + A_L - A_{SL} \qquad (4)$$

κ: correction term to γ to correct the surface tension for curved cavities vs. planar surfaces,

$\ln(RT/P_oV)$ is the entropy-of-condensation term [1].

After studying retention phenomena in RPC, we became aware of the fact, that the water structure had an overwhelmingly important role in the retention process. We can see the magnitude of the forces in water-rich eluents with the picture in Fig. 9. The structural forces of H-bond interlinked water molecules, which are mainly dipole-dipole interactions, represent an energetically low state of the water structure. This means that in the neighborhood of hydrocarbonaceous molecular surfaces (like the C18 chain) the water is in an energetically "excited" state as it has no contact with neighboring water molecules. Therefore the water tries to reduce such surfaces by squeezing species without ionic or dipolar properties out of its structure. One way to return to an energetically low state is an enforced association among the hydrocarbonaceous species themselves; another way is to combine ligand C18 and solute to an associated complex. The latter we called the Reversed Phase Retention Process. The disappearing contact surface area (ΔA) multiplied by the surface tension (γ) represents the energy which is released upon association of the ligand with the analyte molecule, which means, the retention is an exothermic process. In a linear RP gradient, the surface tension is continuously decreasing and the function R_s = f(%B) can be conveniently adjusted to continuously optimize peak distances.

We found by careful calculations, that the dominant term in this equation was the cavity term, $C\gamma = N\gamma\Delta A / RT$ (Figs. 10 a and b), which expresses the large energy needed to separate neighboring water molecules in order to form a cavity in the aqueous eluent around a hydrocarbonaceous molecule. This energy is proportional to the

- accessible hydrocarbonaceous contact surface area of the molecule A_S and the available hydrocarbonaceous surface of the stationary phase A_L, which depends on
- the ligand surface density and and the specific surface area of the silica
- ligand hydrocarbon chain length of the packing material and furthermore on the
- surface tension of the eluent (water, 72 erg/cm^2), which is about 3 times larger, than the surface tension of methanol or acetonitrile.

The high surface tension of water is the result of its highly ordered structure, in which each oxygen is in the center of a tetrahedron with 4 protons. This ordered structure is responsible for the forces of water on hydrocarbonaceous molecules. The addition of organic eluents, such as methanol or acetonitrile, reduces the surface tension of water, i.e., weakening the electrostatic "order" or strength in the water structure. This in turn means the reduction of retention forces.

The expression "hydrophobicity" has to be treated in view of the Solvophobic Theory in a new way. In terms of the magnitude of forces, "hydrophobic interactions" are not the result of the phobicity of fatty molecules to water. The opposite is true: Water is phobic to all molecules with atomic bonds, such as hydrocarbon molecules – or molecular regions – and it tries to exclude them out of its structure with strong forces. The more appropriate expression for "hydrophobic interaction" would be "Hydrocarbon-phobicity of water" or "fatphobicity" for short. Attraction forces in "hydrophobic interactions" between hydrocarbonaceous regions of associated pairs on molecules (such as the complex of a C18 chain with a solute molecule) are the *weakest forces* in water rich eluents (s.Fig. 10a and b, curve "e"). The C18 ligands and hydrocarbonaceous

solutes are kept together not because of "attraction", but by the overwhelmingly strong, external force from the aqueous environment. The saying "The enemy of my enemy is my friend" is well applicable to this process. Added organic eluents such as acetonitrile or methanol reduce the expelling force by reducing the surface tension of water. This effect is what we use successfully in gradient elution to shorten retention time.

The dominance of the cavity term in Reversed Phase Chromatography is also clearly visible in several column databases, such as in "Column Match®", (Rheodyne, USA), showing the clear and dominant effect of the lipophobic contributions of water (called "Hydrophobic Interaction Term"), which is 80–95% responsible for determining the magnitude of retention, depending on solute structural properties. Other contributions, such as shape selectivity, hydrogen-bond acidity, and basicity or ion exchange properties of the stationary phase are of much smaller magnitude. They might be useful, however, to find stationary phases of different selectivities in the optimization process.

The Primary Retention Equilibrium

Csaba called the equation in Fig. 8 "primary retention equilibrium" in which retention (the right side of the equation) is mainly proportional to the amount of water in the eluent and to the hydrophobic contact surface area between the ligand (C18) and the solute (or analyte). The great success of gradient elution in RPC is based on the fact, that the total retention is proportional to the amount of water pumped through the column, which is in modern gradient equipment highly reproducible and is mainly responsible for the exact reproducibility of solute retention times, often down to $\pm$ 5 s.

Secondary Equilibria

Horváth purchased a PDP-11 computer in 1977 to calculate the effects of pH on retention in RPC. He was interested in equations, which could help to predict the retention of compounds having one or more polar groups (-COO^- or NH_3^+) [2].

Table 1. Identification of the peaks on the UV-chromatogram at 280 nm shown in Figs. 1 and 2

Peak No.	Substance	Amount in sample (ng)
3	3, 4-dihydroxymandelic acid	489
5	4-hydroxymandelic acid (POMA)	500
6	3, 4, 5 trihydroxybenzoic acid	500
9	3-methoxy-4-hydrxymandelic acid (VMA)	850
13	3, 5-dihydroxybenzoic acid	517
14	2, 3, 4-trihydroxybenzoic acid	540
21	3, 4-dihydroxyphenylacetic acid	522
23	2, 6-dihydroxybenzoic acid	500
27	2, 4-dihydroxybenzoic acid	493
28	5-hydroxyindolacetic acid	500
29	4-hydroxyphenylacetic acid	519
33	3-methoxymandelic acid	500
34	3, 4-dihydrxycinnamic acid and	513
	3-methoxy-4-hydroxybenzoic acid	515
37	3-metoxy-4-hydroxyphonylacetic acid (HVA)	491
52	2-hydroxybenzoic acid	488
56	Benzoic acid	300
59	Indolacetic acid	500
63	3-methoxyphenylacetic acid	500
64	4-methoxyphenylacetic acid	500
81	Cinnamic acid	254
92	3-hydroxy-β-naphthoic acid	482
93	α-naphthoic acid	486

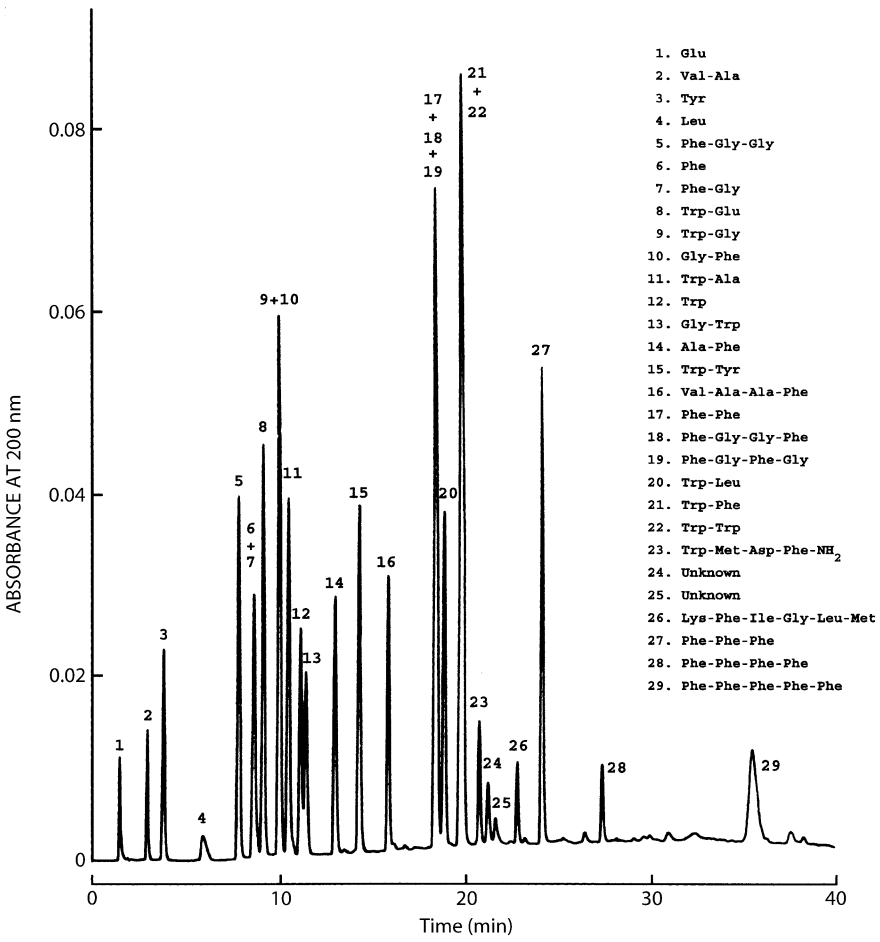


Fig. 7. Separation of nonpolar amino acids and small peptides in RPC Column: LiChrosorb RP-18, 5 μm, gradient elution from 0.5 M $HClO_4$ (to protonate the carboxyl-groups uniformically) pH 0.2, with acetonitrile as the gradient former. Temperature: 70 °C, flow rate: 2.0 mL min^{-1}, ΔP: 150 atm. Sample 10 μL containing ca. 1 μg of each component [12]

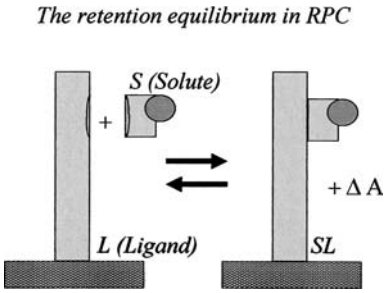


Fig. 8. The retention equilibrium in RPC. L(Ligand) represents a C18 or a C8 hydrocarbon chain, S (Solute) represents some compound of interest. The equation is shifted to the right (=retention) with increasing ΔA ($=A_S+A_L-A_{SL}$) [1]

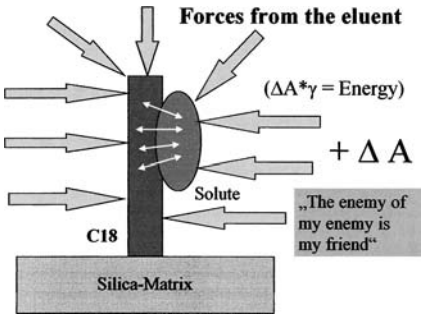


Fig. 9. Magnitude of the retention forces in RPC: The structural forces of water, mainly dipol-dipol interactions represent an energetically low state, which means, that on the surface to hydrophobic molecular surface areas the water is in an energetically "loaded" state and it tries to reduce such surfaces by minimizing them through enforced association of hydrocarbonaceous molecules and other species with nonpolar bonds as well. The disappearing contact surface area (ΔA) multiplied with the surface tension (γ) is the energy, which is released upon association of ligand and analyte. The larger ΔA and the larger γ, the larger is the retention time in RPC [1]

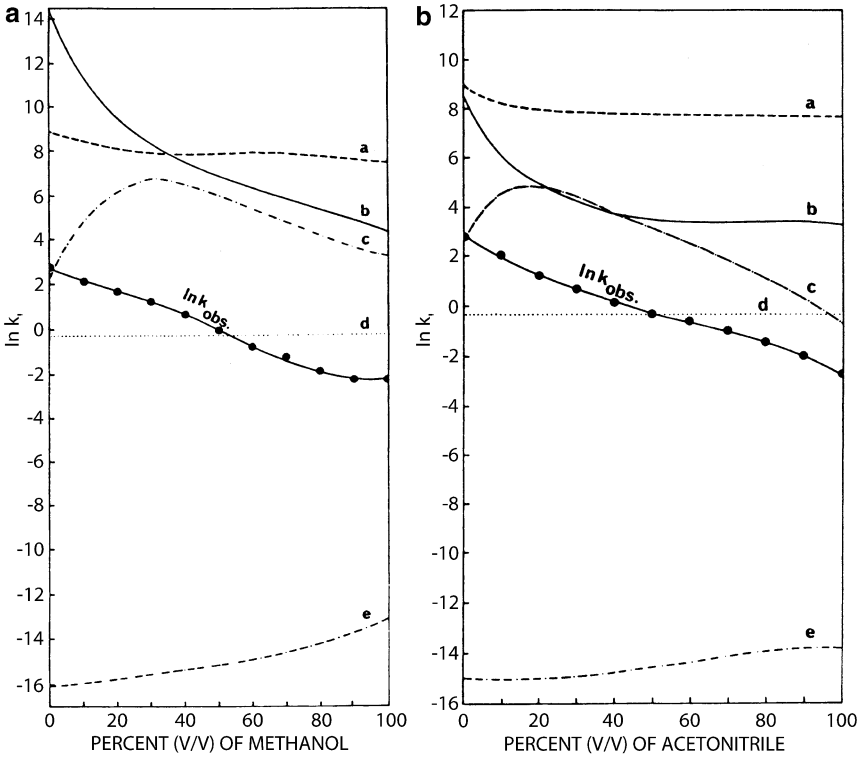


Fig. 10a, b. Effect of the solvophobic strength of the eluent on the capacity factor. The ordinate compares the individual energetic contributions ln k_i to the observed overall retention ln k_{obs} measured for o-toluic acid on C18-phase in a. methanol and b. acetonitrile at 25 °C. The ln k_i values were calculated with Eq. (1), the curves represent the individual terms as follows: (a) $\ln(RT/P_oV)$, (b) $C\gamma$, (c) $D(\kappa^e-1)V^{2/3}\gamma$, (d) B_D and (e) E. The value for A is in methanol (Fig. 10a) −7.00, in acetonitrile (Fig. 10b) −6.60 [1]

Secondary equilibria are based on pH influences on acid-base equilibria and ion-pair effects due to ionic additives to the mobile phase. Both equilibria can be shifted in RPC in a *continuous mode,* giving additional degrees of freedom to change retention and selectivity in a desired direction, as shown in Figs. 5 a and b, to improve the separation.

Secondary equilibria govern the retention of polar solutes by:

- *protic equilibria*: acidic and basic molecules and other polar compounds, where the retention and the selectivity is changing depending on the *pH of the aqueous eluent continuously* [2];
- *ion-pair equilibria*: Horváth coined the name for the ion-pair-former "ionic companion", from the Greek, "hetaera-on" [$\varepsilon\tau\alpha\iota\rho\alpha-ov$], to augment RPC retention of charged eluites with polar "hetaeric" additives to the eluent, to *continuously* alter selectivity [3];
- retention shifts due to *continuous* changes in *ionic strength* of the mobile phase, which play an important role in "Ion-Exchange" [IEC] and in "Hydrophobic Interaction Chromatography" [HIC] [3];
- *stationary phase influences*: interactions of polar eluites with surface silanols, with metal ions in the silica and with polar chemically-bonded ligands offer a further choice to alter the selectivity of a chromatographic system (column to column changes).

The magnitude of the change in retention by changing pH depends on the position of the –COOH or $-NH_2$-group in the molecule, which will be becoming charged upon dissociation into COO^- or $-NH_3^+$. Such ionic centers are well integrated into the dipolar water structure and increase the solubility of the charged analyte and reduce their retention on a C18 phase, sometimes up to 500% or more [2, 3, 20–22].

Retention also changes due to differences in molecular structure, such as added or missing substituents. For example, the closer a polar OH group is located to a hydrocarbonaceous region of the solute molecule, the larger the retention changes, i.e., selectivity changes, will be. So it is in VMA that the accessibility of hydrophobic molecular surface area is greatly reduced by the solvated OH group in the alkyl side chain and therefore the retention is much weaker, compared to HVA (Fig. 5a).

Compared to acids, basic compounds move into the opposite direction and their retention increases with increasing pH (Fig. 5b, Fig. 11a). The opposing peak movements of acids in mixtures with basic compounds and zwitterions are the reason for many of the unexpected separation problems in routine HPLC work.

Continuous peak position shifts can be seen in a mixture of acidic and basic compounds, where peak overlaps at pH 3.7, 4.0, 4.9 or 5.3 can simply be resolved by changing the pH slightly to pH 6.0–6.5 (Fig. 12). Zwitterionic compounds such

as amino acids, peptides and proteins behave according to their pK_A and pK_B-values in a mostly unpredictable manner, so that measuring the effects of the pH for this class of compounds is absolutely necessary. This is an important consideration in the development and in the security of quality control for safe biotech products.

Continuous Changes of Selectivity in Reversed Phase Chromatography

The changes of selectivity in RPC in aqueous media, which are dominant in life science, are *continuously variable over changes in pH, gradient slope, %B, ionic strength or ternary eluent composition*. This is another reason for the great success of RPC, as optimizing separations is technically much easier than in other chromatographic techniques such as GC, due to continuous variability of the selectivity with the above parameters. In RPC, there are many other factors in addition, which can be used to profoundly change peak distances to a desired separation, like ion-pairing agents, or the use of different columns. But we have to remember that column to column changes bring selectivity changes in a *discontinuous* way.

Additionally in RPC there are a number of *unexpected choices* to change selectivity to a better separation in a *continuous way:* Better separations can be achieved often at a higher temperature and better separation is often found with a steeper rather than with a more flat gradient [19, 23, 24].

In the pharmaceutical industry the control of drug product quality is extremely rigorous, therefore the systematic investigation of the influence of pH and other parameters is absolutely necessary. In industrial production control the method quality is just as important as product quality, as product quality is verified by HPLC methods. Small pH changes can sometimes cause great confusion in the interpretation of a chromatogram as the proof for the quality of a drug product.

In the past once a method was validated, changes were not desired as it was very expensive to change the method and the corresponding renewal of the applications to regulating authorities such as the FDA were time consuming. No changes could be done once the method had been validated. However most methods are depending on a number of parameters, such as pH, column batch, temperature, eluent type, buffer concentration, etc. In a global economy, where the product is traveling between countries, the results often do not fulfill the expectations even if a validated method is used. The problem is that the validation process is mainly based on statistical tools and there are no requested checks about the quality of the chromatographic method. Therefore, today we need HPLC methods, which are adjustable from location to location to perform the analytical goal of correct quantitation of the product components. This is only possible, if the method is developed in such a way as to allow necessary adjustments between certain clearly defined limits. The best tools to achieve this goal are the so called "Maps of critical resolution" [13], which express the quality of a working point in a simple and understandable way.

Csaba also studied solvophobic effects with a focus on the separation of macromolecules with many of his coworkers. Günther Bonn, John Frenz, Wayne Melander, Avi Nahum, Danilo Corradini, Ziad El Rassi and other "Csabaites". He

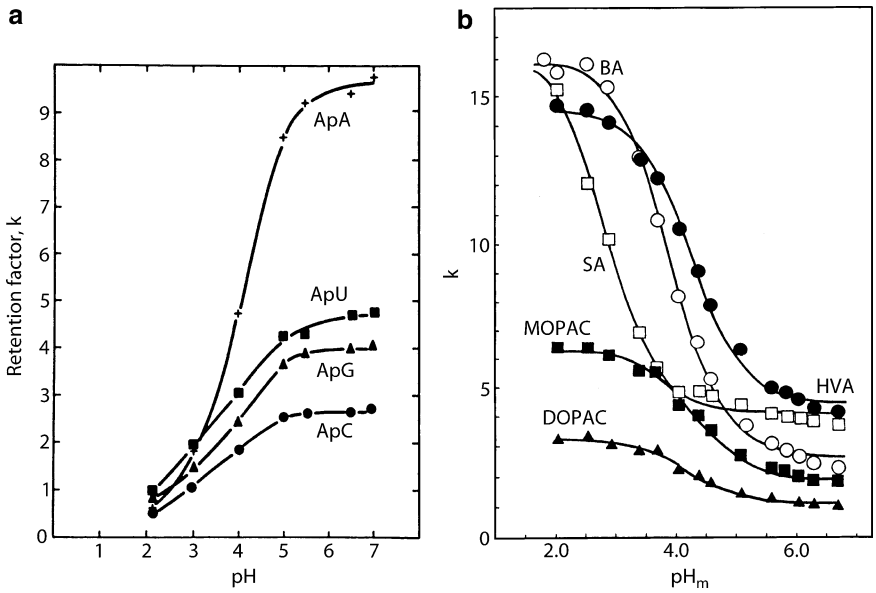


Fig. 11a. Plots of the retention factor of dinucleotides against the pH of the mobile phase. Eluent: 0.1 M phosphate or acetate buffer, containing 5% v/v acetonitrile [15]. **Fig. 11b.** Plots of the retention factor of monoprotic acids vs. the pH of the eluent. Column: Partisil 1025 ODS; eluent: 1.0 M Na2SO4 in 0.05 M phosphate buffers; temperature: 25 °C. The acronyms are: BA, benzoic acid; DOPAC, 3,4-dihydroxyphenylacetic acid, HVA, homovanillic acid; MOPAC, parahydroxyphenylacetic acid; SA, salicylic acid [2]

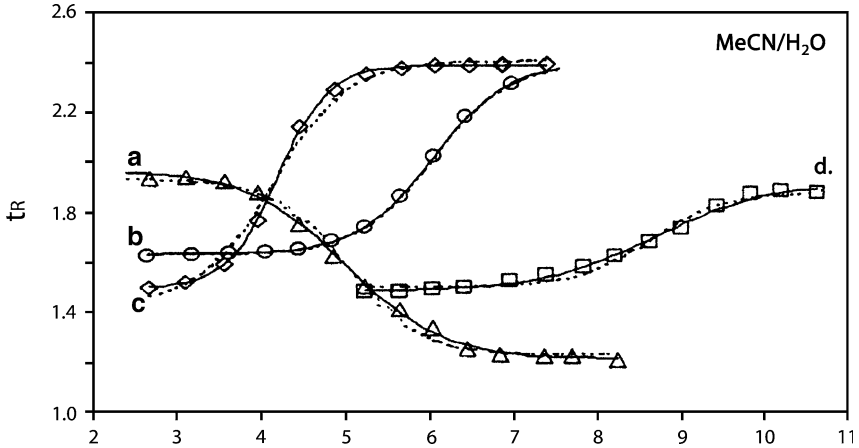


Fig. 12. Retention of acidic and basic compounds in RPC. Compounds: (a) benzoic acid (b) lidocaine (c) 4-tert-butylpyridine (d) ephedrine [17]

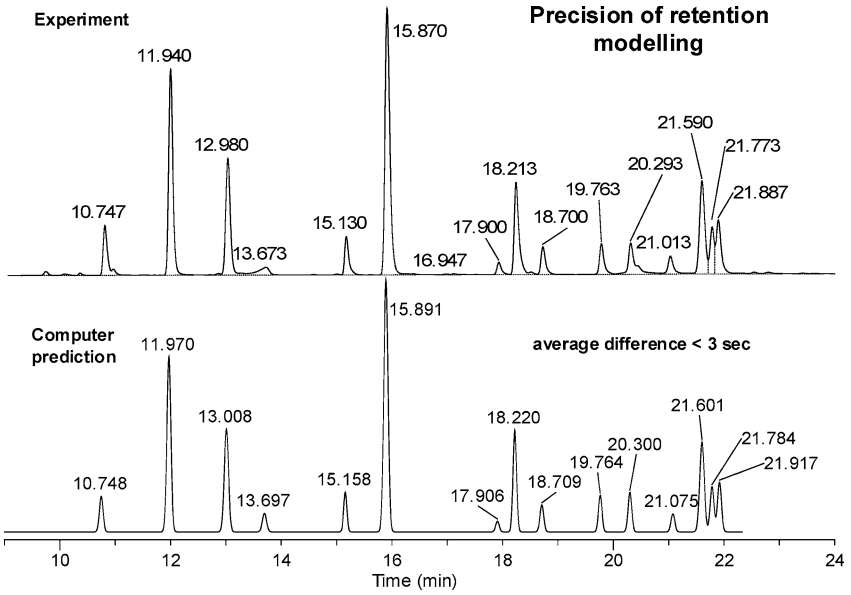


Fig. 13. Precision of predictions in RPC after correct peak assignment with PeakMatch and retention modeling with DryLab based on 4 experiment: 2 gradients with 30 and 90 min runtime from 5 to 95% acetonitrile, at two temperature: 40 and 70 °C, eluent A: 5 mMol phosphate buffer pH 2.40. Column: Water Xterra C18, 150 × 4.6 mm, 3 µm. Instrument: Agilent 1100, dwell volume: 1.06 mL. Sample from mixture of unknown components after stressing a drug in a drug stability study. Average difference between prediction and experiment is ca. 3 seconds [13]

published numerous papers on hydrophobic interaction chromatography (HIC), with totally aqueous mobile phases and mild forces between proteins and the short chain RP material, to avoid denaturation of the biomacromolecules. To improve column performance, Csaba developed a new type of 2 μm stationary phases with Krishna Kalghathgi in 1987 and with this material they could separate peptides and proteins much faster than with conventional columns.

With Jan Stahlberg Csaba developed a theoretical framework for the separation of proteins on ion exchangers. With Firoz Antia he encouraged the use of higher temperatures and tried to mitigate the "thermophobia" of biochemists.

Csaba and Anant Vailaya revisited the solvophobic theory after a period of 20 years and found that several other possible theories could be well explained within the frame of the solvophobic theory, which was capable of describing the energetics of processes involving hydrophobic interactions. It was stated, that there are difficulties in distinguishing between partition and adsorption mechanisms in RPC by using partition models based on the lattice approach. They concluded further, that

- the ligand length has a much smaller influence on the selectivity, than the mobile phase;
- the eluent has the dominant role in determining retention in RPC [16].

In summary, the publications of Csaba Horváth and his coworkers helped a great deal to develop more robust and more reliable HPLC methods.

Systematic Studies in the Development of Robust HPLC Methods

Several attempts were made in the 1970s to develop HPLC methods using eluent properties. Lloyd Snyder classified solvents in 1978 and introduced the well known solvent triangle principle [25], Glajch and Kirkland modeled solvent strength in multicomponent gradient elution [26]. Nyiredy and Sticher created the PRISMA model in 1989 for TLC and for HPLC and developed software tools for the prediction of retention from measured data [27]. Although the focus was primarily on normal phase systems, more and more chromatographers also used the solvent triangle and the PRISMA modeling in reversed phase chromatography with excellent success.

Horváth always did systematic evaluation of eluent influences. His curiosity to look at new findings in depth taught us that systematic work is always worth doing even if at the beginning it seems to be a time-consuming chore. He found, it is better to make a small number of experiments and to learn a great deal about the strengths and weaknesses of the method, rather than do a large number of runs by trial and error without knowing, whether the method is at a true optimum or not.

Today the quality of an RPC method can be easily expressed with software tools. The software adapts the so-called maps of the critical resolution, introduced by Berridge [28], and demonstrates the correctness of the established working conditions in a highly complex HPLC method in a transparent way and in only a few seconds [13].

Robust regions of the method can be elaborated with one- or two-dimensional maps after a correct peak match.

The most promising start is to carry out four runs in gradient mode at two different temperatures. The gradient range is typically 0 to 100% acetonitrile or methanol. Ideal temperatures are 40 and 70 °C, due to better salt solubility and lower mobile phase viscosity, so it is easier to apply small particle size columns.

In the next step it is preferable to use mixtures of polar compounds, such as acids, and bases or zwitterions to further optimize the pH in the aqueous eluent A, which should have a pH between 3-4, to find the largest peak movements. The pH difference between three basic runs should be not more than 0.5–0.6 pH units. After the discovery of all hidden bands we can look for more robust pH regions and evaluate different column selectivities to complete the process.

We can use ternary eluents to change the selectivity in RPC for mixtures of non-polar compounds, like steroids and other molecules without charged groups.

Further systematic changes in buffer and/or ion-pairing agent concentration can reveal additional possibilities to achieve peak movements towards a desired separation. We usually try to maximize peak distances at peak pairs of small resolution and try to reduce the distance between peaks, which are far apart. The final chromatogram should have peaks with "equal band spacing", from where we can increase the speed of analysis using shorter columns, smaller particles and higher flow rate.

Precision of Virtual RPC Models

The use of the *continuous* modeling of resolution with appropriate parameters such as gradient time, temperature, eluent pH, ternary eluent composition and ionic strength, as established in the Solvophobic Theory has been continued in the past 25 years resulting in the development of the tool DryLab and recently an addition, called PeakMatch (Molnar, Berlin). The accuracy of predictions is excellent, as shown in (Fig. 13). The deviations between predictions and experiments are in the range of a few seconds (13).

Outlook

The basic message of the Solvophobic Theory is that in aqueous chromatographic systems in HPLC, CE or CEC the selectivity can be *continuously* modified using eluent properties and the temperature. *Continuous* modification of the chromatographic selectivity is important to understand the peak tracking process. Here peak movements can be extremely large, and a resolution maps can help to find overlaps and regions of best separation (red), where optimum working conditions can be provided and avoid conditions where peaks overlap.

The still difficult step of matching peaks in unknown mixtures using systematically generated multiple runs for the calculation of a retention model to reveal resolution optima at the shortest possible run time is presently a major challenge in several research groups. The author is continuing his research in this direction.

In the future new instruments will run systematic experiments to show how the separation selectivity might be changed by eluent influence. Establishing the best and fastest separation in automated overnight operation will be routinely used. Method robustness and transferability will be easily developed and clearly documented for easier product transfer in a global economy. Measurable HPLC method quality will support excellent product quality.

In this way the Solvophobic Theory of Csaba Horváth and his co-workers will help us to define more robust methods in the future and to ensure a better quality of research and production in *life sciences* on a high scientific level. The Solvophobic Theory will also influence the development of robust methods in electrochromatography in aqueous-organic media, and will contribute to a large degree to the industrial success of CE and CEC for the safe characterization of new products in the pharmaceutical and chemical industry as in genetic engineering as well.

Csaba Horváth left his research projects unexpectedly and very suddenly at the relatively early age of 74. The gap left by his departure is tremendous and we all miss him every day. But he still lives with us through his scientific work, through his many achievements, through his spirit and his friendly personality. We have the memories of him with us, and so he is still present. He will be with us for many years and many generations to come. His scientific contributions helped a great deal to improve our health and our future life. Thank you Csaba for your untiring hard work, for your love to science and for your kindness to all of us.

References

1. Horváth Cs, Melander W, Molnár I (1976) J Chromatogr 125:129–156
2. Horváth Cs, Melander W, Molnár I (1977) Analyt Chem 49:142–154
3. Horváth Cs, Melander W, Molnár I, Molnár P (1977) Analyt Chem 49:2295-2305
4. Csaba Horváth, private communication Aug. 1975
5. Benedek K, Dong S, Karger BL (1984) J Chromatogr 317:227–243
6. Scott CD (1968) Clin Chem 14:521–528
7. Molnár I, Horváth Cs (1977) J Chromatogr 143:391–400
8. Stout RW, Michelot RJ, Molnár I, Horváth Cs, Coward JK (1976) Analyt Biochem 76:330–341
9. Molnár I, Horváth C (1978) J Chromatogr 145:371–381
10. Molnár I, Horváth Cs (1976) Clin Chem 22:1497–1502
11. Molnár I, Horváth Cs, Jatlow P (1978) Chromatographia 11:260–265
12. Molnár I, Horváth Cs (1977) J Chromatogr 442:623–640
13. Molnár I (2002) J Chromatogr A 965:175–194
14. Jacobson J, El Rassi Z, Horváth Cs (1983) In: Molnár I (ed) Practical Aspects of Modern HPLC. Verlag Walter de Gruyter & Co., Berlin, New York, pp. 15–23
15. El Rassi Z, Horváth Cs (1983) In: Molnár I (ed) Practical Aspects of Modern HPLC. Verlag Walter de Gruyter & Co., Berlin, New York, pp. 1–14
16. Vailaya A, Horváth Cs (1998) J Chromatogr A 829:1–27
17. Canals I, Valkó K, Bosch E, Hill AP, Rosés N (2001) Anal Chem 73:4937–4945
18. Jupille TH, Dolan JW, Snyder LR, Molnár I (2002) J Chromatogr 948:35–41
19. Chloupek RC, Hancock WS, Snyder LR (1992) J Chromatogr 594:65–70
20. Lewis JA, Lommen DC, Raddatz WD, Dolan JW, Snyder LR, Molnár I (1992) J Chromatogr 592:183–195
21. Lewis JA, Lommen DC, Raddatz WD, Dolan JW, Snyder LR, Molnár I (1992) J Chromatogr 592:197–208
22. Bilke HW, Molnár I, Gernet C (1996) J Chromatog A 729:189–195
23. Schmidt AH, Molnár I (2002) J Chromatogr 948, 51–59
24. Rieger HJ, Molnár I (2002) J Chromatogr 948:43–49
25. Snyder LR (1978) J Chromatogr Sci 16:223–230
26. Glajch JL, Kirland JJ, Snyder LR (1982) J Chromatogr 238:269–277
27. Nyiredy Sz, Dallenbach-Toelke K, Sticher O (1989) J Liq Chromatogr 12:95–116
28. Berridge JC (1985) Techniques for the Automated Optimization of HPLC Separations. John Wiley & Sons