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Review

Csaba Horváth and preparative liquid chromatography

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Abstract

Few chromatographers have been interested in furthering preparative liquid chromatography. The pioneers, Tswett, Kuhn and Lederer, A.J.P. Martin, Tiselius, isolated fractions but as an intermediate step in the analysis of their samples. The progress in electronics and sensors, and in their miniaturization has lead to the paradoxical situation that the analysts never see the transient pure fractions that their detector quantitates. Yet, over the last 25 years, preparative liquid chromatography has become an important industrial process for the separation, the extraction, and/or the purification of many pharmaceuticals or pharmaceutical intermediates, including pure enantiomers, purified peptides and proteins, compounds that are manufactured at the relatively large industrial scale of a few kilograms to several hundred tons per year. This development that has strongly affected the modern pharmaceutical industry is mainly due to the pioneering work of Csaba Horváth. His work in preparative HPLC was critical at both the practical and the theoretical levels. He was the first scientist in modern times to pay serious attention to the relationships between the curvature of the equilibrium isotherms, the competitive nature of nonlinear isotherms, and the chromatographic band profiles of complex mixtures. The thermodynamics of multi-component phase equilibria and mass transfer kinetics in chromatography attracted his interest and were the focus of ground-breaking contributions. He investigated displacement chromatography, an old method invented by Tiselius that Csaba was first to implement in HPLC. This choice was explained by the essential characteristic of displacement chromatography, in that it delivers fractions that can be far more concentrated than the feed. Remarkably, once the basics of nonlinear chromatography had been mastered in his group, most of the applications that were studied by his coworkers dealt with peptides of various sizes and with proteins. Thus, all the applications of preparative HPLC in the biotechnologies derive directly from Csaba's work. Although displacement did not pan out as a general method, the reasons are related more to practical constraints of the production of pharmaceuticals and to the long period of cheap energy that might be ending now. This report reviews Csaba's work in nonlinear chromatography.

Keywords: HPLC; Competitive isotherms; Displacement chromatography; Frontal analysis; Gradient elution; Isotherm determination; Nonlinear chromatography; Stoichiometric displacement model

Contents

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1. Introduction

Csaba Horváth had a 50-year scientific career and for each decade of his work he made remarkable contributions to differing areas of research. First, in the 1950s, while preparing his Ph.D. dissertation, Csaba synthesized new adsorbents for gas chromatography (GC). These were inorganic materials, mostly silica-based. He used them first to coat the wall of GC open tubular columns [1], producing the porous-layer open tubular columns that provide high saturation capacity and new selectivities, then to coat the surface of glass beads [2]. He never lost interest in the synthesis of packing materials and his skills in synthesis were often critical for the success of new experiments (e.g., the rapid analysis of proteins using sub-micrometer particles [3]).

Second, in the early 1960s Csaba imagined, developed, and implemented the concept of an integrated instrument for analytical liquid chromatography somewhat similar to the gas chromatographs that were becoming hugely popular then. At about the same time, several other scientists thought of this transposition, proceeded to pack metal columns, and sought means to control the flow rate through the chromatographic column. However, it was Csaba who described the yet-to-come liquid chromatographs as complete systems that would incorporate a dedicated solvent delivery system, a sample injection device, a highly efficient column, an on-line detector, and a chromatogram recorder [4]. Furthermore, he envisioned the main field of applications as being biochemical analyses [5]. Admittedly, at this time, biochemistry was more interested in analyzing fats, sugars, steroids, and amino acids than in unraveling the complexities of the proteome, still most early work in HPLC was dedicated mostly to the products of the petro-chemical industry, many of which were easily analyzed by GC which gave a strangely unrealistic character to much of the early work. Csaba never lost track of his original goal. At the same time, in what may be his most consequential endeavor, Csaba pioneered the development of immobilized enzyme reactors [6] that have become at the heart of many modern biochemical diagnostic tools.

Third, to enhance the versatility of HPLC and further the development of its biochemical applications, Csaba conceived and developed reversed phase liquid chromatography (RPLC) that allowed separation scientists to free themselves from the drastic limitations of silica- or alumina-based adsorbents that in the 1970s were not as homogeneous as they have now become. Of greater import is the fact that the separation of the often highly polar compounds contained in most samples of biochemical origin would not be possible without the chemically bonded phases that provide weak interactions and a great flexibility. Csaba proposed a general conceptual framework to explain retention mechanisms in RPLC [7,8]. His initial work in this area sparked a worldwide interest in the analytical community that is not yet extinguished.

The fourth achievement of Csaba's scientific career was preparative liquid chromatography. He realized very early that the life sciences and particularly the pharmaceutical industry would need this most powerful separation method in order to produce the highly pure intermediates required for modern pharmaceuticals. In contrast to analytical applications that call for sensitive detectors, success in preparative applications requires successful operations at high concentrations. In the presence of high concentrations, equilibrium isotherms are no longer linear, band profiles depend on the composition of the feed and the size of the sample injected, and the purity, the concentration, and the yield of the purified fractions are the critical requirements of successful separations. In the early 1980s, Csaba undertook investigations of nonlinear chromatography at a time when the field had been practically abandoned by fundamental chemical engineers and not yet found by bioengineers. He rediscovered displacement chromatography and made a thorough investigation of this process.

Finally, in the 1990s, Csaba began working in electrochromatography, a chromatographic process in which the flux of mobile phase percolating through the chromatographic column is driven by a high electric field instead of the mechanical means of pumps. He was a prime mover in this field until the end of his life.

This review is dedicated to the work done by Csaba in preparative chromatography, a field that progressed due to his remarkable research. We discuss successively his contributions to the study of equilibrium isotherms, to the theory

of displacement chromatography and to the applications of that process.

2. Investigations in nonlinear chromatography

Csaba Horváth began working on displacement in 1980. The following year, he published a landmark paper that marked the second birth of displacement chromatography, this time combined with the HPLC technique that he had pioneered earlier [9]. Displacement was an old mode of chromatography, invented by Tiselius [10] and Claesson [11], later used by the separation group of the Manhattan project [12] to purify cations of the rare earth and the uranide series of elements and by the separation group of the API project to separate and identify pure hydrocarbon compounds from crude oil samples [13]. Using conventional, low-efficiency columns, the last authors needed a 16 m long column to achieve the formation of a constant pattern or isotachic trains (see later). This impractical requirement prevented wide spread applications before the use of HPLC columns.

The need for a profound understanding of the displacement process lead Csaba to investigate the procedures for the acquisition of equilibrium isotherm data, the modeling of isotherm data, and the theory of nonlinear chromatography. In all these fields he made important progress, published major papers, and contributed considerably to their progress. In so doing, he reconnected the theory of nonlinear chromatography, which had been improved by the works of Helfferich [14] and of Rhee et al. [15], with the study of actual chromatographic separations. The contributions of Csaba's group to the acquisition and modeling of isotherm data, to the advancement of the theory of nonlinear chromatography, and to the development of original separations by displacement chromatography will be discussed.

2.1. Isotherm determinations

Economical considerations require that chromatography-based procedures of preparative separations be carried out in the displacement or in the over-loaded elution modes, that is with feed concentrations that are much higher than those used in linear chromatography. Under such conditions, the Henry law coefficient that controls the migration of peaks in linear chromatography is no longer constant. It depends on the concentration and this dependence has a profound influence on the shape of elution or displacement bands. Interpretation of the results and computer-assisted optimization of the parameters of preparative separations by chromatography require detailed knowledge of the relationship between the equilibrium concentrations in the two phases of the compounds of interest in the feed over a wide range. This relationship is the isotherm. Its experimental determination has become important with the advent of preparative chromatography.

Single-component isotherms are relatively easy to measure. The important isotherms in chromatography, however, are competitive binary isotherms because chromatography is a separation method and a feed component always competes for retention with the components eluted just before and/or after it. Because methods are available that predict, often with a reasonable accuracy, competitive isotherms for pairs of compounds based on the single-component isotherms of the mixture components, the experimental determination of single-component isotherms remains an important topic of investigation.

2.1.1. Single-component isotherms

Jacobson et al. presented a comprehensive and cogent review of the procedures developed for the determination of experimental isotherms [16]. Their analysis of the advantages and drawbacks of these different methods remains valid today and frontal analysis is still the preferred method because of its accuracy and precision. They showed that chromatographic methods are at least as accurate and much faster than the static method (so-called 'shake-flask method'). Among chromatographic methods, frontal analysis (FA) is a titration method with results essentially independent of the column efficiency. It is more accurate than methods analyzing the shape of the diffuse boundary of large peaks, known as ECP and FACP methods. These authors developed an instrument for the acquisition of equilibrium isotherm data for pure compounds (single-component isotherms) and for binary mixtures (competitive isotherms) [16]. This instrument acquires frontal analysis data, using HPLC columns. Several versions were built, using standard (4.6 mm I.D.) or narrow (1.18 mm I.D.) bore columns (see Fig. 1). The version of the instrument designed to measure competitive isotherms incorporates two HPLC instruments and a valve allowing the transfer of selected fractions of the eluent of a first column to analyze them on the second column, as explained later, a feature essential for the determination of competitive isotherms using the staircase variant of frontal analysis [16,17]. The single-component isotherms of phenol, alkylphenols and a few other aromatic compounds were determined.

Using the same instrument and method, Huang and Horváth [18,19] determined the equilibrium isotherms of a large number of compounds of biochemical interest in RPLC systems with a C_{18} bonded column in a buffered aqueous mobile phase [18] and those of proteins on cation exchangers [19]. In the first system, they measured the equilibrium isotherms of some aminoacids, di- and tri-peptides, of the nucleic bases and of some nucleotides [18]. They investigated the influence of the pH, the ionic strength and the presence of different additives on these isotherms. Most isotherms were convex upward and the experimental data fitted well to the Langmuir or Jovanovic models. The adsorption of L-phenylalanine was found to be maximum at its isoelectric point. The adsorption isotherm of adenosine monophosphate had an S-shape under certain conditions, '*a behavior that was attributed to solvent-mediated conformational changes*

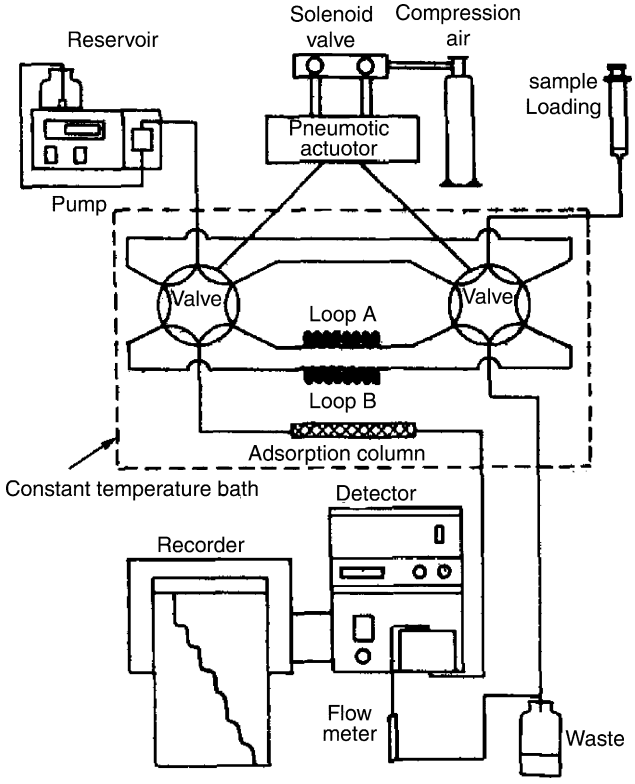

Fig. 1. Flow sheet of the dual chromatographic unit employed for the measurement of multicomponent isotherms. The effluent from detector A of the frontal chromatograph is continually sampled by the flow-through microsample injector and solute concentrations are measured with the analytical chromatograph. Reprinted from Ref. [22] with permission. Copyright 1987, American Chemical Society (Fig. 2).

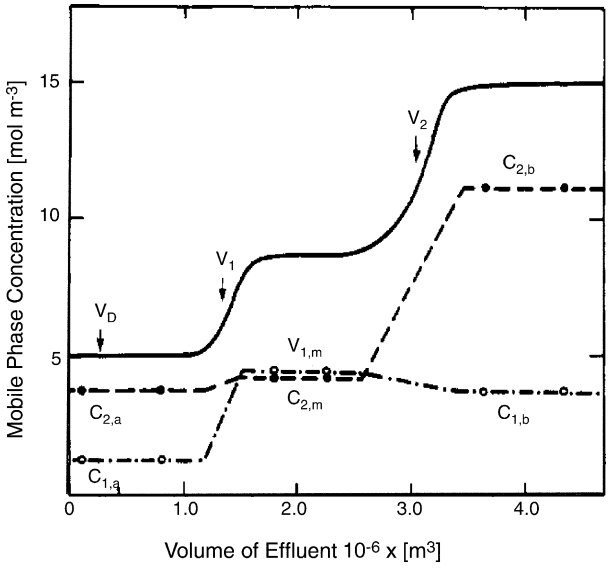

Fig. 2. Diagram of the concentration profiles of the effluent after a stepwise change in the concentration of a binary solution has been made at the inlet of the column. The solid line was recorded by the optical detector at the outlet of the adsorption column. Two fronts emerge at retention volumes V_1 and V_2. V_D is the hold-up volume of the system. Reprinted from Ref. [22] with permission. Copyright 1987, American Chemical Society (Fig. 1).

and molecular stacking'. The experimental isotherm data of ribonuclease A, α-chymotrypsinogen A, cytochrome C, and lysozyme were measured on several cation exchangers [19]. Some serious difficulties arose from adsorption of the proteins on the stainless steel parts of the system, which was limited by coating their surface with a layer of a hydrophilic polymer. In most cases, the experimental data were found to fit well to the Langmuir model and reasonably well to the Jovanovic model. These data were in excellent agreement with those afforded by the static method. The isotherms of a protein on the different ion-exchange resins used were similar. The procedures for the determination of isotherm data are discussed in great detail in refs. [20,21] where numerous applications can also be found.

2.1.2. Competitive isotherms

Jacobson et al. made a comprehensive investigation of the procedures of experimental determination of competitive isotherms [22]. They selected two methods, one general, giving the competitive equilibrium data for any composition of the binary mixture, whatever the nature of the isotherm behavior but requiring a considerable amount of work, hence long and tedious, the other, far simpler and faster, working only when the system follows competitive Langmuir isotherm behavior. Again, their analysis was sound and remains valid today. The former method is competitive FA in the staircase mode. In frontal analysis, a stream of a solution of the feed in the mobile phase is pumped into the column. The concentration of the feed is changed by step. Upon such a step change, the composition of the eluent adjusts and becomes equal to that of the input solution. This takes place as two successive waves, separated by an intermediate plateau (Fig. 2). The composition of the first plateau in the figure is that of the solution pumped into the column during the previous step; that of the third plateau is that of the solution pumped during the current step. The composition of the intermediate plateau is related to the parameters of the isotherm. If this composition is determined directly, it allows the calculation of the amounts of each compound adsorbed at equilibrium at the end of step *n*, hence a data point, q_1, C_1, q_2, C_2, of the binary isotherm. For the sake of convenience, the data points in a series are determined at constant relative composition (i.e., $C_1/C_2 = \text{const}$, see Fig. 3). We have successfully used this method many times and it is still implemented in our laboratory (see ref. [23], chapter 4). The second method is based on the use of the theory of interferences in chromatography [14]. It calculates the parameters of the Langmuir model by regression of the velocities of composition changes in the column (i.e., V_i in Fig. 2) that are measured experimentally. The method, which uses the *h*-transform, assumes that the system follows competitive Langmuir isotherm behavior and affords the best estimates of these coefficients. Unfortunately, we have now come to realize that Langmuir isotherm behavior is far from universal, that relatively few compounds follow it closely and

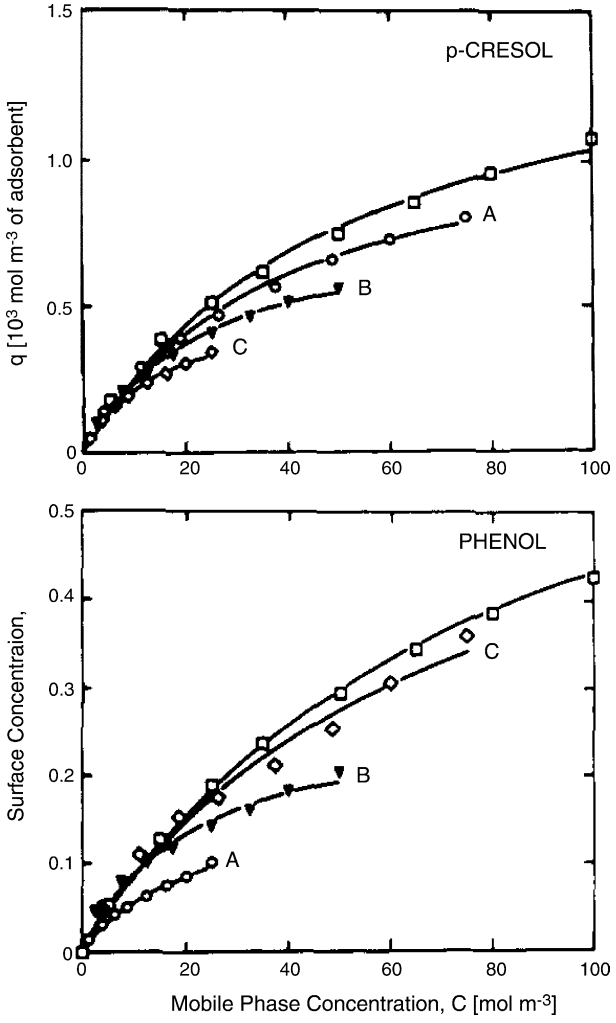

Fig. 3. Compositions of the binary mixtures (phenol and *p*-cresol in the case in point) for which isotherm data are measured. The lines A, B, and C show the composition of binary mixtures of fixed mole ratio, 3:1, 1:1, and 1:3 employed in the study. Reprinted from Ref. [22] with permission. Copyright 1987, American Chemical Society (Fig. 3).

that it is rare to have this model followed in multi-component systems. Fig. 4 shows the competitive isotherms obtained for phenol and *p*-cresol on porous silica [24].

2.1.3. Competitive isotherms for ionized molecules

Velayudhan and Horváth investigated in detail the particular problem of the determination of the competitive isotherm of a pure compound using a solution of an adsorbing mobile phase modulator [25]. This type of measurement is necessary for the acquisition of the data required to account for band behavior in gradient elution. They also showed that, under linear conditions, the logarithm of the retention factor of proteins in ion-exchange chromatography increases linearly with increasing logarithm of the salt concentration [25]. The slope of the straight line is the ratio of the apparent charge of the protein to the charge of the competing ion of the salt.

The chromatographic separation of proteins by ion-exchange chromatography is an important process. However,

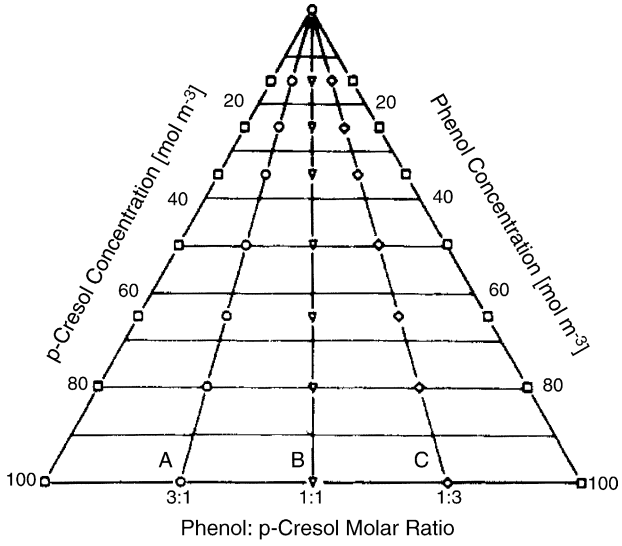

Fig. 4. Isotherms of phenol and *p*-cresol on octadecylsilica from aqueous solutions having compositions given in Fig. 3. The top isotherm in each figure were measured with solutions containing each singe solute whereas the isotherms denoted by A, B, and C were obtained with solutions containing phenol and *p*-cresol at mole ratios of 1:3, 1:1, and 3:1, respectively. Reprinted from Ref. [22] with permission. Copyright 1987, American Chemical Society (Fig. 4).

the phenomenon can be complex. At low salt concentrations, proteins are often irreversibly bound to ion-exchange resins at the time-scale of the chromatographic analyses, which may cause considerable difficulties. The composition of the mobile phase has a considerable influence on their retention. The stoichiometric displacement model originally suggested by Glueckauf and Coates [26] is useful to account for these effects. Velayudhan and Horváth [27] made a detailed mathematical analysis of the competitive isotherms of multivalent ion exchange for proteins and discussed the influence of the mobile phase composition.

The exchange reaction is written

$$aA_m + bS_s \rightleftharpoons aA_s + bS_m \quad (1)$$

where the symbols A_m, A_s, S_m, or S_s stand for the ions in the mobile (m) or the stationary (s) phase and a and b are the charges of the studied ions and the counter-ion. Ion-exchange equilibrium provides that:

$$K = \frac{[A_s]^a[S_m]^b}{[A_m]^a[S_a]^b} \quad (2)$$

The electroneutrality condition gives:

$$b[A_s] + a[S_s] = \Lambda \quad (3)$$

where Λ is the concentration of the binding sites on the ion-exchange resin surface.

The combination of Eqs. (2) and (3) permits the calculation of the concentrations of protein and counter-ion in the stationary phase when their mobile phase concentration and Λ are known. Of particular interest in this model is that the influence of the mobile phase composition on the binding

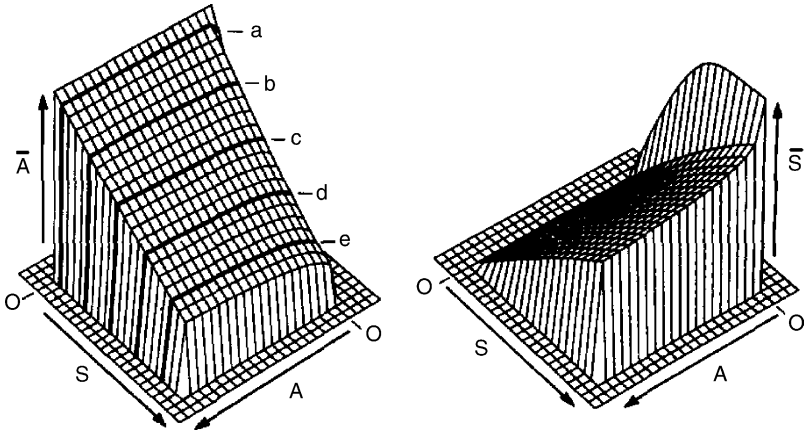

Fig. 5. Single-protein isotherm in the presence of a salt. Two-dimensional surface representations of the stationary phase concentrations of the protein A and the salt as a function of their mobile phase concentrations; the figure on the left is the protein isotherm surface while the salt surface is shown on the right. Reprinted from Ref. [27], Copyright 1988, with permission from Elsevier (Fig. 2).

isotherm of the protein is explicitly given in Eq. (2). An example of the results obtained, illustrating the progressive passage from a quasi-rectangular isotherm to a Langmuir isotherm, is shown in Fig. 5 [27]. However, the isotherm equation is quite different from that of a competitive Langmuir isotherm involving the protein and the salt. This last isotherm model would not explain how a small change in the ionic strength of the mobile phase may cause a protein that was nearly irreversibly bound to be eluted with a very small retention factor. The shape of the surface obtained by plotting the adsorbed concentration versus its mobile phase concentration and the salt concentration in a 3-D plot (Fig. 5) clearly illustrates the isotherm properties [27]. This led Velayudhan and Horváth [27] to distinguish three regions of binding, depending on the limit retention factor at 0 concentration, hence on the mobile phase composition.

(1) In the region of strong interactions, the retention is practically infinite at low concentrations ($k'_0 \equiv \infty$) and the isotherm is nearly rectangular. This region is useless in linear chromatography. However, because the saturation capacity and the separation factors are high, it can be most useful in preparative chromatography [27].
(2) In the intermediate region ($100 > k'_0 > 1$), the protein begin to behave like small organic ions. This is the region frequently used in preparative applications because the proteins behave under quasi-linear conditions and it is not necessary to use special measures to regenerate the column between successive separations.
(3) In the low binding region, the protein is barely retained ($k'_0 < 1$). This region is accessed relatively easily in step gradient mode.

The approach can be extended easily to multicomponent mixtures. The exchange reactions are written

$$nA_m + aS_s \rightleftharpoons nA_s + aS_m \quad (4a)$$

$$nB_m + bS_s \rightleftharpoons nB_s + bS_m \quad (4b)$$

where n, a and b are the charges of the counterion and of the two studied ions A and B. Ion-exchange equilibrium provides that:

$$K_a = \frac{[A_s]^n [S_m]^a}{[A_m]^n [S_s]^a} \quad (5a)$$

$$K_b = \frac{[B_s]^n [S_m]^b}{[B_m]^n [S_s]^b} \quad (5b)$$

The electroneutrality condition gives:

$$a[A_s] + b[B_s] + n[S_s] = \Lambda \quad (6)$$

In Fig. 6, a typical set of competitive isotherms for two proteins is shown for a given salt concentration [27]. Note that the competitive isotherms exhibit a behavior that is farther removed from a rectangular one than the single-component isotherms. This is because the separation factor, K_b/K_a, is much smaller than each of these equilibrium constants.

Later, Velayudhan and Horváth [28] investigated the relationship between adsorption and ion-exchange isotherms. There is a certain similarity between the interactions of a protein with the binding sites in ion-exchange resins and the hydrophobic patches on the surface of RPLC adsorbents. Helfferich had already shown the equivalence between the multicomponent Langmuir isotherm and the monovalent ion-exchange isotherm. The latter can be converted into the former by the addition of a dummy component [14]. The general case of adsorbates having different saturation capacities was shown to parallel that of the heterovalent ion-exchange equilibrium. The application of a general adsorptive formalism to RPLC yields a relationship between the retention factor and the modifier concentration that reduces to well known results in two different cases. For small molecules, it gives the classical linear or quadratic dependence of $\ln k'_0$ on the modifier concentration, C. For large molecules, it gives a linear relationship between $\ln k'_0$ and $\ln C$ that is conventional for proteins [28]. Details on the calculation of complex competitive isotherms and of chromatograms can be found in ref. [29].

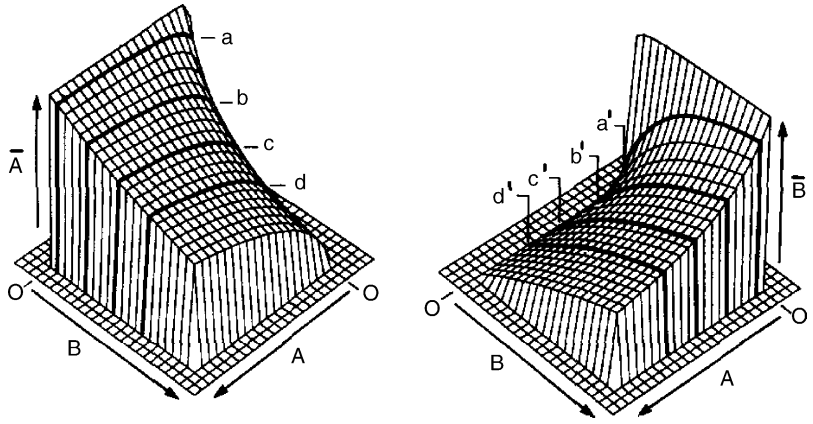

Fig. 6. Multicomponent protein isotherms. Two-dimensional surface representations of the stationary phase concentrations of two proteins A and B as a function of their mobile phase concentrations at fixed concentration of mobile phase additive. A has an apparent charge of 10, B one of 6. Reprinted from Ref. [27], Copyright 1988, with permission from Elsevier (Fig. 5).

2.2. Mass transfer kinetics

Horváth and Lin [30,31] derived a general plate height equation. This equation accounts for the effects of axial dispersion, the mass transfer resistances at the boundary and in the interior of the porous particles of the stationary phase, and for the kinetic resistances associated with the reversible binding of the eluite to the stationary phase. The authors assumed that axial dispersion takes place only in the stream of mobile phase outside the stagnant film surrounding each particle of the packing. The thickness of this film, δ, decreases with increasing fluid velocity. To estimate it, δ was assumed to be equal to the thickness of the Nernst diffusion layer, D_m/k_f, with D_m molecular diffusivity and k_f rate coefficient of mass transfer. Using experimental results from Pfeffer and Happel [32] who showed that the Sherwood number, $Sh = k_f d_p/D_m$, increases in proportion to the power 1/3 of the reduced velocity, they obtained

$$\delta = \frac{d_p}{\omega \nu^{1/3}} \tag{7}$$

This value of the stagnant film thickness allowed the derivation of the following plate height equation

$$h_a = \frac{2\gamma}{\nu} + \frac{2\lambda}{1 + \omega \nu^{-x}} + C\nu \tag{8}$$

with $x = 1/3$. An earlier study of the same problem by Giddings [33] had led this author to derive a similar equation but with $x = 1$. A complex investigation of the dependence of the experimental values of the column HETP on the different physical parameters included in the explicit expressions of λ and ω in Eq. (7) led Horváth and Lin [31] to results that were in good agreement with this equation. Later, Arnold et al. [34] showed that the relationship derived by Pfeffer and Happel [32] between the Sherwood number and the reduced velocity is valid only at high values of ν, above ca. 50. At low values of ν, they suggested that the Sherwood number would become constant. Taking this new value into account, they showed that the Horváth and Lin equation would reduce to the Van Deemter equation. However, Nelson and Galloway [35] have shown that, although the Sherwood number becomes constant at low velocities for a single particle, the situation is quite different and far more complex for a densely packed bed. In this case, the Sherwood number becomes proportional to the Reynolds number. If this dependence is substituted in the thickness of the Nernst layer (Eq. (7)), we obtain for the plate height equation Eq. (8) with $x = 1$, i.e., an equation which is identical to the Giddings equation. Obviously, however, the meaning of the numerical constants, γ, λ, and ω of Eq. (8) is entirely different in the two models. Independent measurements by Tallarek et al. [36] have shown that Eq. (8) with $x = 1$ accounts remarkably well for experimental data in a wide range of reduced velocities, from 0.2 to 140.

Frey et al. compared the plate height equations for chromatography under linear conditions with conventional and with perfusion columns [37]. In the latter case, convection takes place in large macropores inside the packing particles, in addition to conventional dispersive transport. This convection accelerates mass transfers across the particles, hence improves their efficiency. There is no convection inside the particles in columns packed with materials devoid of sufficiently large macropores. A model was developed to account for the various contributions to the mass transfer kinetics in such columns. It was demonstrated that packing materials exhibiting a bimodal porosity with the larger pores being large enough to be percolated by the mobile phase, present significant advantages for the achievement of fast separations that do not require very large efficiencies. The faster mass transfer kinetics allows operation of the columns at high flow rates which are compatible with the high permeability of columns packed with large particles. The results of the model were used to examine the performance of columns packed with materials thought to exhibit the perfusive effect. The experimental data collected were in agreement with the predictions of the model. The analysis shows that these materials have advantages over the conventional ones for

applications that do not require a high column efficiency. However, the rapid, high-resolution HPLC separation of biopolymers can be more effectively achieved through the use of micropellicular particles [37]. The use of these packing materials was studied in great detail elsewhere [38,39].

2.3. Theory of nonlinear chromatography

Early in his work, Csaba Horváth understood that preparative HPLC could not be carried out using the basic tools of analytical (i.e., linear) chromatography but that a profound grasp of nonlinear chromatography was necessary for the proper understanding of frontal analysis, displacement chromatography, and overloaded elution. Although most of his work in this area was devoted to displacement chromatography (see later), he undertook important and successful investigations in these other areas. His work with Frenz and Jacobson [20,22] on the frontal analysis of binary mixtures was reviewed earlier in this paper. In the next two subsections, we discuss the work done on gradient elution and system peaks before giving a deeper look at the work done on the displacement theory.

2.3.1. Regeneration policies in liquid chromatography

Based on the ideal model theory of Rhee, Aris, and Amundson (see Ref. [23], chapter 7) and assuming a Langmuir isotherm for the compounds involved, Frenz and Horváth analyzed various column regeneration schemes and developed optimum policies for efficient equilibration [40]. They showed that a train of different solvents in series is more effective than any single regenerant and that square wave inputs are better than gradient schemes. Experimental results confirmed the validity of the calculations. This approach is particularly useful for optimizing processes in gradient elution, frontal analysis or displacement chromatography which all need a regeneration step between successive runs.

2.3.2. Gradient elution

Antia and Horváth studied the theoretical aspects of the separation of a binary mixture by gradient elution chromatography in the orverloaded elution mode as a ternary competitive problem [41]. Using a ternary Langmuir competitive isotherm model with all the coefficients of the two feed components being functions of the modifier concentration, they calculated numerical solutions of the system of mass balance equations with an orthogonal collocation program. Although the isotherm model selected predicts a separation factor that is independent of the concentrations of the two feed components under isocratic conditions, it allows consideration of a separation factor that is a function of the organic modifier concentration, hence varies during the separation. The elution profiles of the single components (Fig. 7) and the separation of binary mixtures (Fig. 8) were compared with the results obtained in the isocratic mode, under comparable conditions. In part, the shape of the band profiles is due to the duration of the feed injection which, at high loading factors, may be significant compared to the retention volume and, consequently, causes the beginning of the separation to take place under the frontal analysis mode [41].

Under overloaded conditions, gradient elution was shown to be superior to isocratic separation elution in terms of production rate, recovery yield, and enrichment

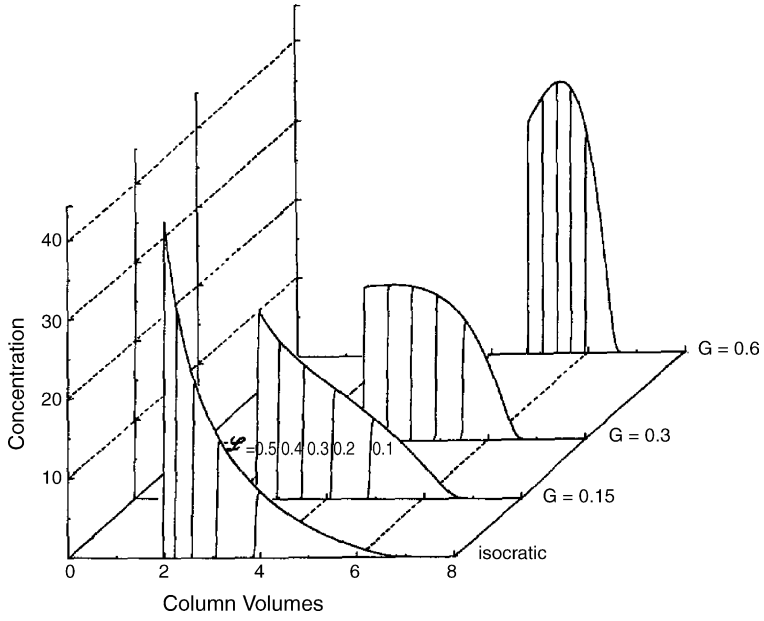

Fig. 7. Effect of the elution conditions and the degree of column overloading on the peak profiles of a single component in gradient elution. The peaks calculated for different loading factors under otherwise identical conditions are superposed in each case. Reprinted from Ref. [41], Copyright 1989, with permission from Elsevier (Fig. 2).

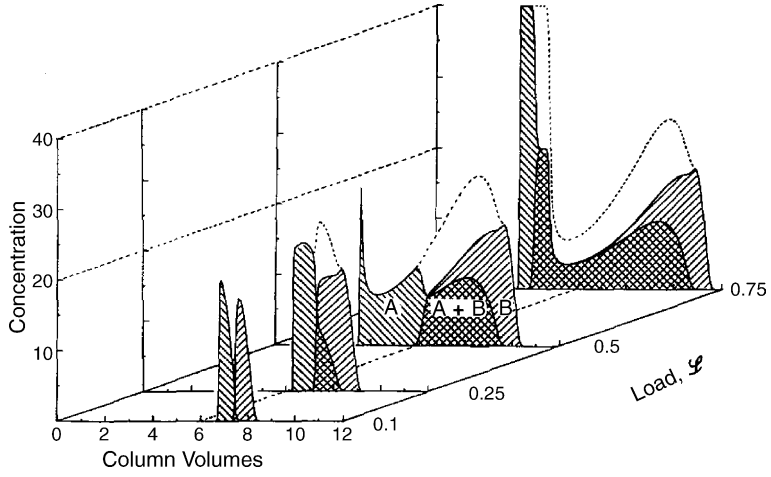

Fig. 8. Elution profiles of a 1:1 binary mixture in nonlinear gradient elution at different loads. The envelope (dotted line) represents the response of a linear, nonselective detector, i.e., the total concentration. The two hatched areas correspond to the two feed components. Reprinted from Ref. [41], Copyright 1989, with permission from Elsevier (Fig. 3).

factor when the separation factor remains independent of the modifier concentration [41]. The cases in which the separation factor increases or decreases with increasing modifier concentration were also studied. The plots of the logarithm of the retention factors of both feed components at infinite dilution versus the modifier concentration are assumed to be linear, as most often they are experimentally found to be. Obviously, the results of the separation depend greatly on the modifier concentration corresponding to the intersection of the two plots (separation factor equal to 0) compared to the range of modifier concentrations used in the gradient separation. Examples of possible results are shown in Fig. 9. More detailed discussions are found in ref. [42].

2.3.3. Eigen or system peaks

Within the framework of his investigations on the fundamentals of the retention mechanism in RPLC, Csaba Horváth investigated the formation of system peaks (which he preferred to call eigen-peaks) [43,44]. System peaks are formed upon perturbation of the equilibrium between the stationary phase and an eluent that is a solution of compounds retained in the stationary phase. These peaks migrate along the column at velocities that are proportional to the eigenvectors given by the characteristic solutions of the mass balance equations for the system [15]. The heights of these peaks are related to the composition of the mobile phase and to the chemical structure of the solutes. Melander et al. found that the heights of the system peaks are proportional to the mass of eluite injected over the range of sample sizes investigated. This was explained on the basis of the solvation model that takes into account the solvation effects that depend also on the retention of the eluite considered. The results obtained with binary and ternary eluents were quantitatively accounted for by the solvation model. It was observed that isomeric eluites that have the same retention factors exhibit significantly different system peak patterns [44].

2.3.4. Fundamentals of displacement chromatography

Displacement chromatography was developed by Tiselius [10] at a time when on-line detection was not available and the sensitivity of the analytical methods used off-line was many orders of magnitude less than it is today. A separation method delivering concentrated fractions was then invaluable. Glueckauf [45,46] gave the first theoretical analysis of this method, using the ideal model with which nearly all theoretical work has been done in this field until recently. Beyond this pioneering work, two contributions were critical, those of Helfferich and Klein [14] and of Rhee et al. [47].

The “theory of interference” [14] was originally developed for stoichiometric ion-exchange systems. The set of concentrations C_i of the components of a system (feed components and retained mobile phase modifiers) is replaced by a new set of variables, h_i, using the h-transform, which is based on the assumption that the system follows competitive Langmuir isotherm behavior [14]. Adsorption processes can be viewed as equivalent to ion-exchange processes by introducing a fictitious component to account for the exchanged ion and an n-component adsorption system becomes equivalent to an $(n+1)$-component ion-exchange system. The behaviors of the two systems can be handled similarly. The approach assumes the validity of both the ideal model and the Langmuir competitive isotherm. It has been widely used to account for experimental results obtained in displacement chromatography. For example, Frenz and Horváth used it for the calculation of the separation of phenol and p-cresol in reversed phase chromatography [48]. Very good agreement with experimental results was reported.

Like Helfferich and Klein [14], Rhee et al. [47] studied the separation of multicomponent mixtures by displacement chromatography using the same restrictive assumptions of the validity of the Langmuir isotherm model and the ideal model. They used a different approach, based on the method of characteristics, and studied the interactions between concentration shocks and centered simple waves [15]. This

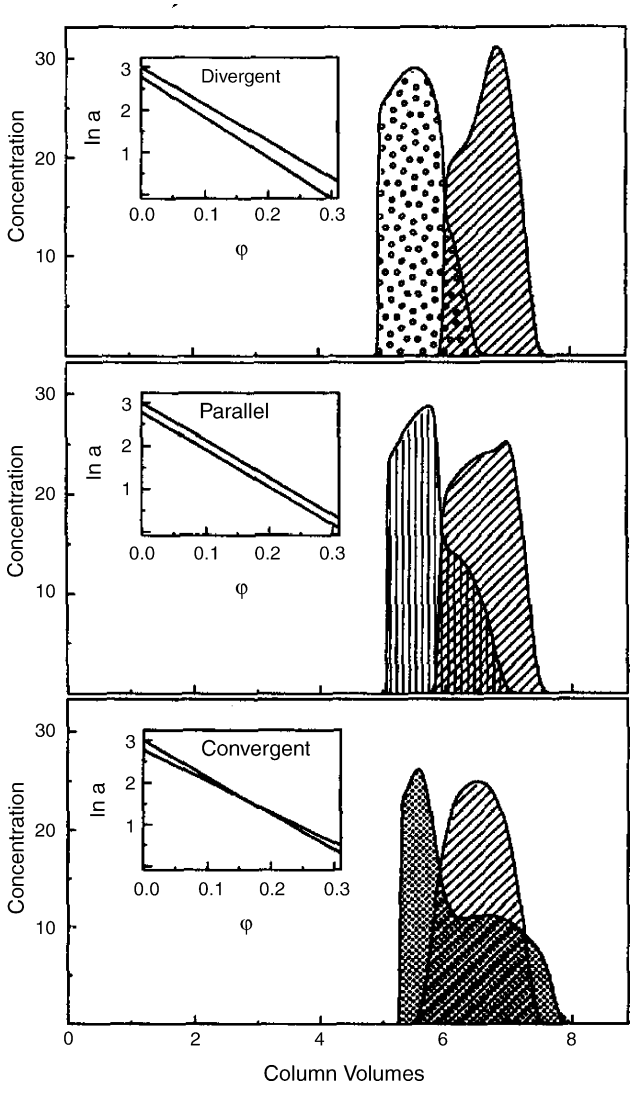

Fig. 9. Elution profiles of a 1:1 binary mixture with convergent, parallel and divergent retention plots. Initial separation factor, $\alpha_0 = 1.67$, loading factor 0.325. Reprinted from Ref. [41], Copyright 1989, with permission from Elsevier (Fig. 6).

approach is more directly suited to adsorption chromatography than the method of Helfferich and Klein, but the results of both methods are equivalent [49]. A comprehensive review of the history and development of displacement chromatography and of its applications was published by Frenz and Horváth [50]. Systematic investigations by Horváth and his co-workers [9,50–63] led to the reintroduction of displacement chromatography in the 1980s. Their experimental results on numerous systems verify the effect of the different operating parameters as determined by theoretical calculations. They identified displacers and the operating conditions leading to an isotachic train (see next section). These authors capitalized on various technological advances, such as the development of high-efficiency columns for ion-exchange or reversed phase liquid chromatography. Many results could be accounted for with the ideal model. Others are not and considerations of mass transfer kinetics must be introduced.

(1) *Description of displacement chromatography*. In displacement chromatography, a rather large amount of feed is injected into a column saturated by a solvent (the carrier) that is so weak that all the feed components are strongly retained. Almost immediately after the feed introduction is completed, a stream of a displacer solution is pumped into the column, pushing the feed sample ahead. The displacer is a compound selected for being more strongly retained than any feed component. The feed components are expelled (displaced) from the stationary phase by this solution and form a series of bands eluting ahead of the displacer breakthrough front. When the experimental conditions are properly chosen, a steady state is achieved before the sample leaves the column. A series of successive bands, separated from each other is formed and moves along the column at the velocity of the displacer front. This series of bands in synchronous motion is called the isotachic train. The formation of the isotachic train requires a certain time and is a complex phenomenon [14,15,47]. Following completion of the displacement separation, the column must be regenerated and reequilibrated with the pure carrier.

(2) *The operating line*. The general requirement for performing displacement chromatography is that the competitive isotherms of the mixture components and the displacer should be convex upward and should not intersect each other (Fig. 10). What happens otherwise was investigated by Frenz et al. [48,51], Antia and Horváth [42,62], and Velayudhan and Horváth [28,29]. Since the isotherms are convex, the band fronts are self-sharpening [23]. In the ideal model, the column efficiency being infinite, each front is a concentration shock. We now assume that an asymptotic solution (constant pattern) exists and that an isotachic train will form, as can be demonstrated [9,14,15]: all the band fronts move at the same velocity. A shock separates two successive bands of pure components. The concentration of the pure component is constant between the two shocks which limit a band. The height or concentration of each component zone must be such that its front shock travels at the same velocity as the displacer front. The set of plateau concentrations and the common migration velocity can be controlled by choosing the displacer concentration. The velocity of the displacer front is proportional to the flow rate and depends on the displacer concentration and on the displacer isotherm. The isotachic migration of all component fronts requires that the velocities of the concentration shocks at the front of their bands, $U_{s,1}$, be all equal and that we have:

$$U_{s,1} = U_{s,2} = \cdots = U_{s,i} = \cdots = U_{s,n} \quad (9)$$

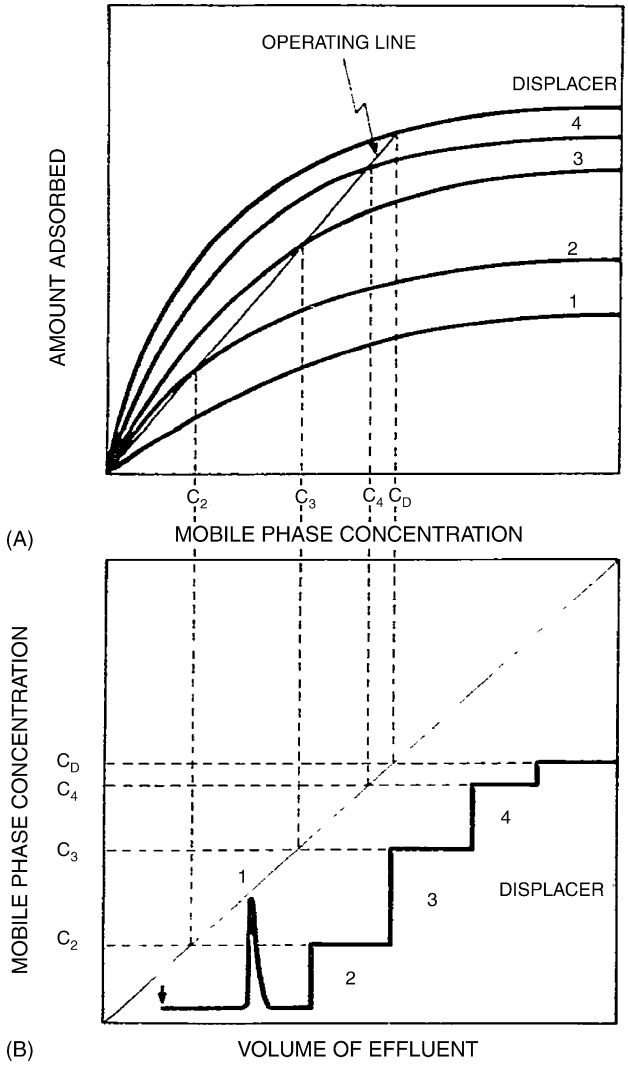

Fig. 10. Graphical representation of the isotherms of the feed components and of the displacer, operating line (A), and of the fully developed isotachic train (B). The concentrations of the component zones are determined by the intersection of the operating line and the adsorption isotherms of the components. Because the isotherm of the first component lies below the operating line at all concentrations, it elutes as a conventional peak. Reprinted from Ref. [9], Copyright 1981, with permission from Elsevier (Fig. 2).

The velocity of the displacer front is given by [9,23]

$$U_{s,n} = \frac{u}{1 + F(\Delta q_n/\Delta C_n)} = \frac{u}{1 + F(q_n/C_n)} \quad (10)$$

where u is the mobile phase velocity, F the phase ratio, Δq_n and ΔC_n the differences between the stationary and the mobile phase concentrations of the displacer just before and just after the shock, respectively. Since the former concentrations are 0, we have $\Delta q_n/\Delta C_n = q_n/C_n$. If we assume a Langmuir isotherm for the displacer, $q_n = a_n C_n/(1 + b_n C_n)$ (with a_n, b_n numerical coefficients), the retention time of its front is

$$t_n = t_0 \left(1 + \frac{Fa_n}{1 + b_n C_n}\right) = t_0 \left(1 + \frac{k'_{0,n}}{1 + b_n C_n}\right) \quad (11)$$

where t_0 is the hold-up time and $k'_{0,i}$ the retention factor of compound i at infinite dilution. The velocity of each band front is given by a similar equation, replacing q_n/C_n in the denominator by q_i/C_i for each component i. In almost all cases, the initial displacer concentration in the mobile phase, as well as the initial concentration of all the mixture components, is 0. Combination of Eqs. (9) and (10) gives

$$\frac{q_1}{C_1} = \frac{q_2}{C_2} = \cdots = \frac{q_i}{C_i} = \cdots = \frac{q_n}{C_n} \quad (12)$$

The term q_i/C_i in these equations is the slope of the chord of the isotherm of component i joining the origin to the point of coordinates q_i, C_i (Fig. 10). Eq. (11) shows that in order to form an isotachic train, in which all the bands move at the same velocity, the concentration of each band is given by the intersection of the corresponding isotherm and the chord of the displacer isotherm. This chord is called the operating line (Fig. 10).

(3) *The isotachic train*. The isotachic train forms as a series of bands whose heights, C_i^P, are given by the intersection of the single-component isotherms and the operating line. By changing the slope of the operating line, i.e., by changing the displacer concentration, it is possible to change the heights (or concentration) of all the bands but it is not possible to adjust the height of each band independently. For a given concentration of the displacer, the band height of each component at steady state is constant. Since the area of each band must remain equal to the area of the injected profile, the width of each band is proportional to the amount of the corresponding compound injected. As the amount of any feed component injected in the column increases, the width of its band in the isotachic train increases at constant height (Fig. 11).

Note that in the illustration of this effect, the isotachic train is given the realistic look of an actual train, not the idealistic view suggested by the ideal model that assumes the chromatographic columns to have an infinite efficiency. There are mixed regions between successive bands of the isotachic train where the concentrations of the two components involved vary continuously, at a rate that depends on the column efficiency and the separation factor of the two components. Csaba was never fooled by this model nor did he suggest that the method could do more than it does. This figure also predicts the effect that will be observed later by Frenz et al. [65] in the LC–MS analysis of peptides digests by displacement chromatography, the very sharp but high peaks of trace components, an attractive approach to trace analysis.

2.3.5. Optimization of displacement chromatography

Frenz et al. performed a profound investigation of the factors that influence the production rate in displacement

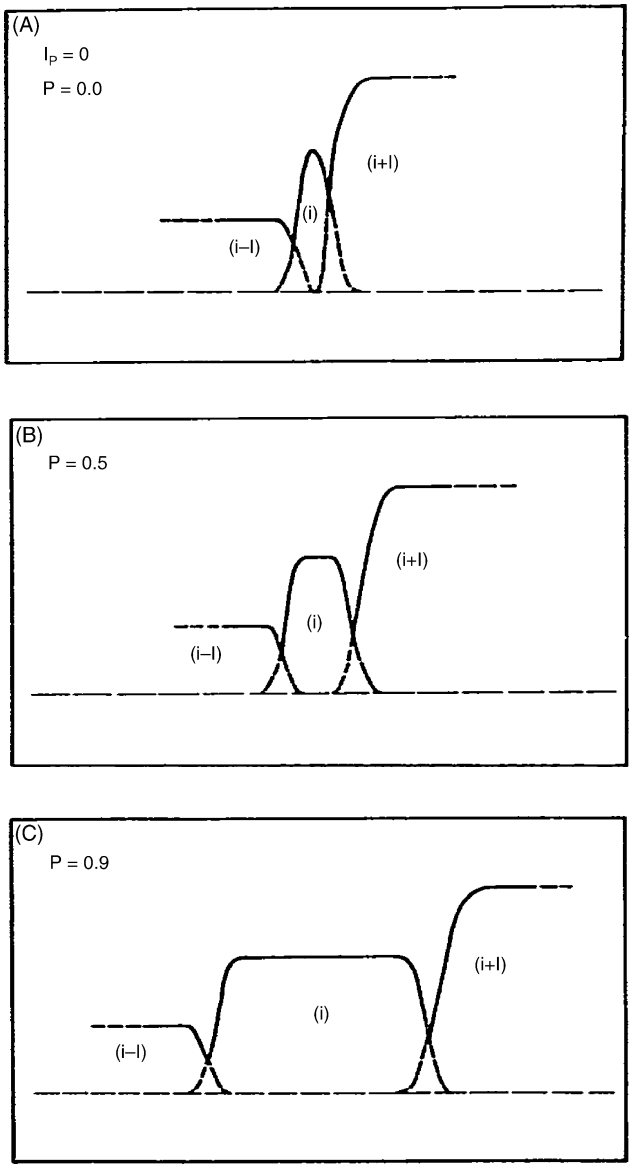

Fig. 11. Schematic illustration of the effect of increasing the amount of component *i* in the feed on the shape of its zone and the purity. Reprinted from Ref. [9], Copyright 1981, with permission from Elsevier (Fig. 4).

chromatography [51,57]. Three mixtures were studied, the hydrolysis products of methylfurylbutyrolactone, the diastereoisomers of benzoyl-L-phenylalanyl-L-alanyl-L-proline, and Carbowax 400 and the throughput was taken as the objective function. Columns of analytical size were used. They were packed with octadecylsilica but different brands gave different results and showed that the selection of the displacer is influenced by the brand of column used. The dependence of the throughput on the flow rate and the feed load indicated that non-equilibrium effects may be significant. Displacement is a powerful separation method but its optimization requires the understanding of its theory and the measurement of a considerable amount of data.

The requirements for the selection of the displacer are relatively severe. First, the displacer must have an isotherm that overlies the isotherms of all the feed components that must be displaced. It should not react with any of these compounds. It must have a relatively high solubility in the mobile phase and must give solutions of low viscosity. It must be safe and inexpensive. The difficulty of the choice of a proper displacer is clear in RPLC: the displacer must be strongly retained on C_{18}-bonded silica, yet soluble in water. Ethers or ether-alcohols, quaternary ammoniums provide acceptable solutions. Although it has to be more strongly adsorbed than all the feed components, the displacer should not be too strongly adsorbed on the stationary phase either. A suitable, reasonably fast scheme should be available for the removal of the displacer from the column and its regeneration. It should also be easily removed from the products of the separation [57]. Unfortunately, the accumulation of these requirements have combined to make the selection of the displacer a major obstacles to the diffusion of displacement chromatography.

The concentration of the displacer must be optimized for maximum throughput. At high concentration, the separation is fast but the bands are high and narrow, the mixed zones between successive bands of the isotachic train are important and the recovery yield is relatively low. It increases with decreasing displacer concentration. The emerging bands occupy larger volumes, allowing the recovery of a higher fraction of pure product. The experimental results are illustrated in Fig. 12(a). The influence of the amount of feed, of the flow rate of the mobile phase (that does not affect the thermodynamics of the separation but the thickness of the mixed zone between successive bands in the isotachic train, see Fig. 12(b)), of the nature of the carrier and the displacer, of the brand of stationary phase were discussed in detail. The experimental results obtained regarding the optimization of the throughput were later shown to agree well with the theoretical results of an analysis of the production rate based on the shock layer theory [23,66].

2.3.6. Displacement chromatography with complex isotherm models

Most fundamental investigations of displacement chromatography were based on the use of the competitive Langmuir isotherm model. However, there are numerous cases in which the feed components do not follow the adsorption behavior predicted by this model. Antia and Horváth [62] studied displacement chromatography in cases when the isotherms of the feed components intersect. They analyzed the stability of the isocratic pattern and the stability of the band boundaries under such conditions. The isotherm models that they used were those predicted by the ideal adsorbed solution when the single-component isotherms follow Langmuir behavior but the saturation capacities for several compounds are different [23]. In the case of the separation of a binary mixture exhibiting that kind of isotherm behavior, the results show that there are three different regions [42,62]. At low concentrations, the two components are separated and

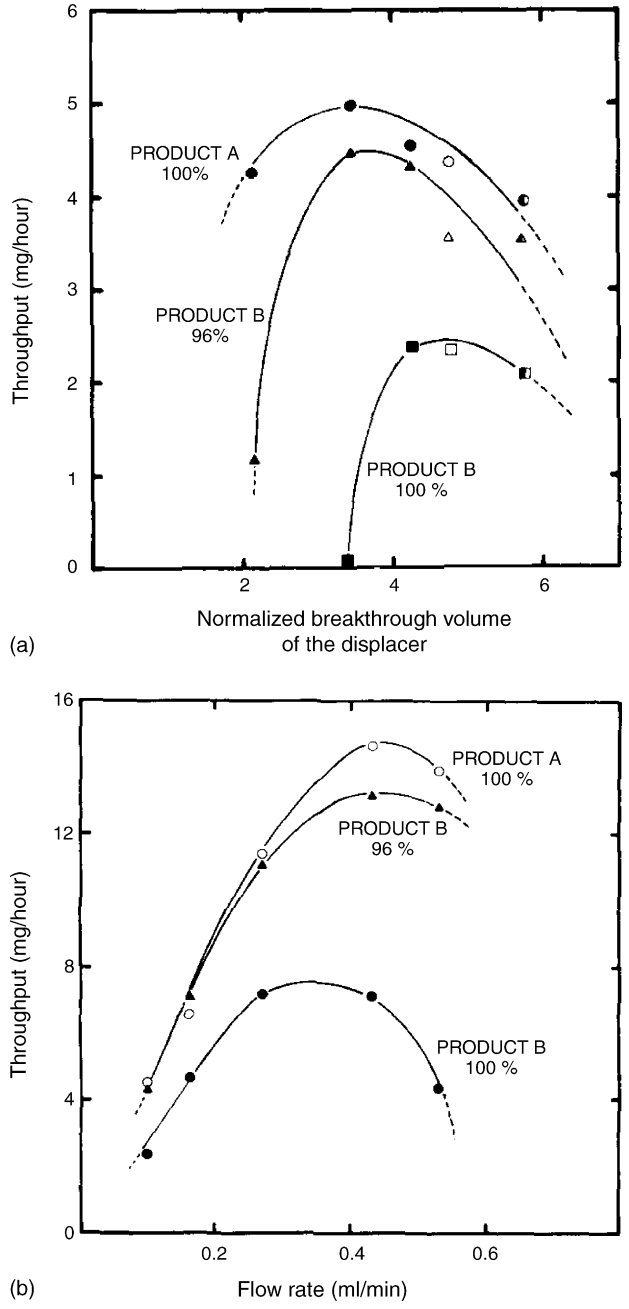

Fig. 12. (a) Plot of the throughput of the displacement separation of the degradation products of methylfulrylbutyrolactone versus the normalized breakthrough volume of the displacer. Column 25 cm × 0.46 cm, 5 μm Zorbax ODS; carrier: water, 0.1 ml/min; $T = 22\,^{\circ}C$; feed: 31 mg in 1 ml water; displacer, solid symbols, tripropylene glycol monomethyl ether at 40, 21, and 15 mg/ml; open symbols, dipropylene glycol monomethylether at 10 mg/ml; half-solid symbol, 2-(2-butoxyethoxy)ethanol at 11.8 mg/ml. From Ref. [57] (Fig. 6). (b) Plot of the throughput of the displacement separation of the degradation products of methylfulrylbutyrolactone versus the flow rate. Same conditions as for (a). Reprinted from Ref. [57], Copyright 1985, with permission from Elsevier (Fig. 8).

appear in the same order as they would if their isotherms did not intersect. At high concentrations, the bands are separated but are eluted in the reverse order. In the intermediate region, the separation is not possible, an isotachic train forms but it contains a mixed zone of thermodynamic, not kinetic origin. The stability analysis presented permits the prediction of the outcome of displacement without the need for arduous calculations. It might apply to systems following different competitive isotherm models.

2.4. Applications of displacement chromatography

In the first publication of the group on displacement chromatography, Horváth et al. described an instrument that was used in Csaba's laboratory for many years [9]. This instrument combines two liquid chromatographs. The first one is a fractionator, designed to perform displacement chromatography, with provisions to load the column with long plugs of feed, to switch abruptly from a stream of weak solvent or carrier to a stream of displacer and finally to a stream of regenerant, and a fraction collector. The second one is an analyzer, designed to determine the composition of the successive fractions eluted from the displacer column.

2.4.1. First results

The possibilities and advantages of displacement chromatography were first demonstrated on small molecules, 4-hydroxy-, 2-hydroxy-, and 3,4-dihydroxy-phenyl acetic acids, in a 0.1 M phosphate buffer, displaced by a 0.87 M aqueous solution of *n*-butanol on a C_{18} silica column (Fig. 13) [9]. Note that the first fraction is as concentrated as

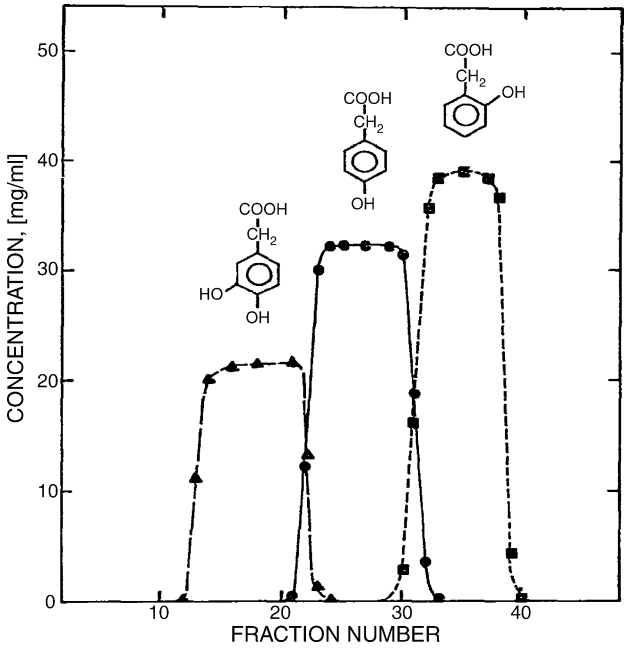

Fig. 13. Separation of hydroxyphenylacetic acids by displacement on a Partisil ODS-2 column (25 cm × 0.46 cm). Carrier: 0.1 M phosphate buffer; displacer: 0.87 M aqueous solution of *n*-butanol. Flow rate: 0.05 ml/min; temperature 25 °C; feed volume: 1.5 ml; sample weights: 30, 35 and 45 mg of 4-hydroxy-, 2-hydroxy-, and 3,4-dihydroxy-phenyl acetic acids, respectively. Fraction size 0.15 ml; fraction 40 had an elution volume of 12 ml (hence retention time, 4 h). Reprinted from Ref. [9], Copyright 1981, with permission from Elsevier (Fig. 7).

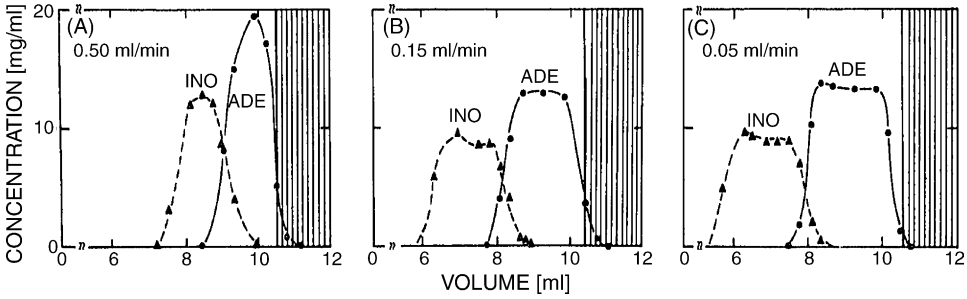

Fig. 14. Effect of the flow rate on the displacement separation of inosine and adenosine. Flow rates: A, 0.05; B, 0.15; C, 0.50 ml/min. Column: 25 cm × 0.46 cm, 10 μm Lichrosorb RP-18. Feed: 10 and 15 mg of inosine and adenosine, respectively. Displacer: 0.66 M *n*-butanol in 0.10 M phosphate buffer, pH = 3.5. Reprinted from Ref. [9], Copyright 1981, with permission from Elsevier (Fig. 11).

the feed and the third one is more concentrated. This is the major advantage of displacement chromatography. Similar results were obtained with resorcinol and catechol displaced by a 0.8 M aqueous solution of *n*-propanol. The separation of the oligomers ($n = 6$–15) of Carbowax 400, with complete resolution up to $n = 12$ and that of inosine and adenosine were also described [9]. The band heights in the isotachic train are close to the values calculated from the intersection of the operation line and the isotherms of the compounds concerned (Fig. 10).

2.4.2. Experimental parameters

The influence of the operational parameters was discussed in great detail with the goal of optimizing the performance of the method for purity of the fractions, recovery yield, and production rate. The need for the formation of the isotachic train to be just complete when this train begins to exit the column was recognized. Because the numerical integration of the system of mass balance equations of the chromatographic problem was not a practical possibility at the time, an empirical study of the effects of the feed concentration and volume, the column length, the mobile phase flow rate, the displacer concentration and its nature was undertaken. Fig. 14 illustrates the effect of the flow rate on the separation of adenosine and inosine. This effect is moderate. We know now that it arises merely from the influence of the flow rate on the column efficiency, the latter controling the thickness of the shock layer between the bands of the isotachic train (see Ref. [23], chapters 12 and 14). The interdependence of the influences of the feed amount and volume and of the column length were illustrated. Column overload (too much feed or too short a column) result in the elution of incompletely developed concentration zones, as illustrated in Fig. 15. Under the same experimental conditions, an isotachic train was obtained with a feed containing 15 and 37.5 mg of 3,4-dihydroxyphenyl acetic acid and 4-hydroxyphenylacetic acids, respectively (see also Fig. 13). This phenomenon comes from the mixed mode of chromatography used in preparative applications of displacement chromatography. Under the conditions of the separation, the feed in injected as in frontal analysis and a beginning of separation takes place. However, a large part of the feed component are still mixed in the rear of the feed

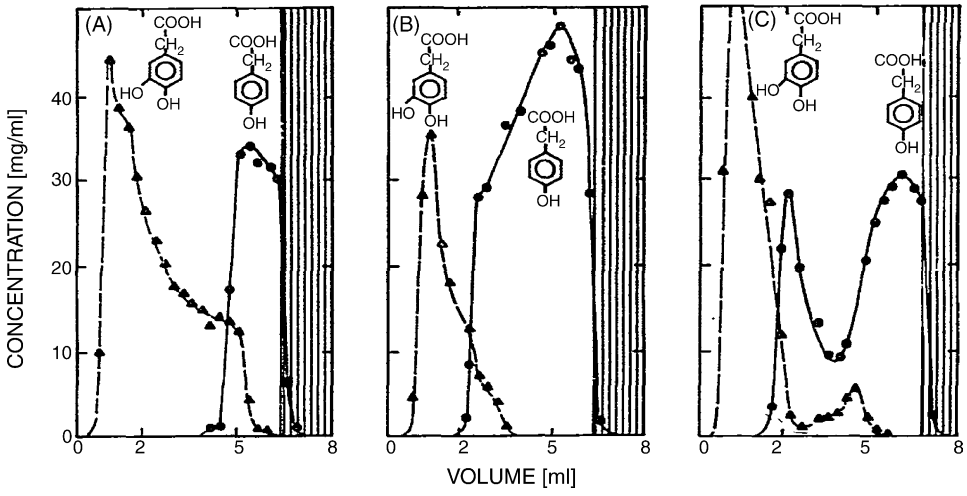

Fig. 15. Effect of the feed size on the separation of 4-hydroxy- and 3,4-dihydroxy-phenyl acetic acids by displacement chromatography. Displacer: 0.64 M phenol in 0.10 M phosphate buffer at pH 2.12 and 25 °C. Flow rate: 0.3 ml/min. Amounts of feed: A, 114 and 57 mg; B, 57 and 114 mg; C, 114 and 114 mg of 3,4-dihydroxy phenyl acetic acid and 4-hydroxyphenylacetic acids, respectively. Reprinted from Ref. [9], Copyright 1981, with permission from Elsevier (Fig. 12).

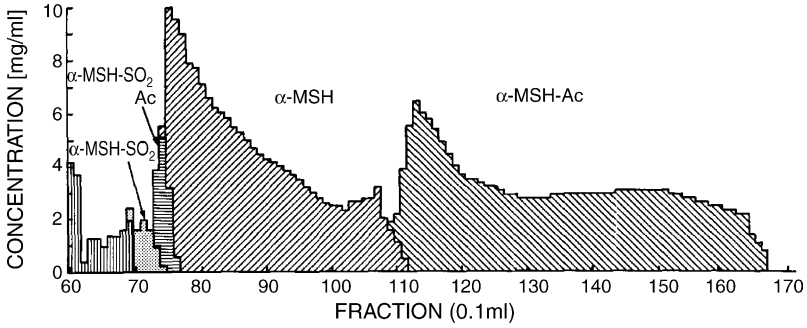

Fig. 16. Displacement chromatogram of crude α-MSH mixture. Column: 25 cm × 0.46 cm, packed with an octadecyl silica, 5 μm. Carrier: water with 0.25% formic acid, 0.5% triethylamine, and 19% acetonitrile. Displacer: 50 mM benzyldodecyldimethyl ammonium bromide in water with 0.25% formic acid, 0.5% triethylamine, and 21% acetonitrile. Flow rate: 0.1 ml/min. Feed: 0.1 ml with 35 mg crude mixture. Reprinted from Ref. [60], Copyright 1988, with permission from Elsevier (Fig. 3).

band. The forward migration of the displacer front reorganizes the distribution of the two components but this process takes time and a certain column length is necessary to reach constant pattern [9].

2.4.3. Biochemical applications

Numerous applications of displacement chromatography for the preparation of pure components have been described. The separation of the polymixins B_1 and B_2 antibodies and their purification from small amounts of their main impurities, colistin A and B, was the first one to be described [52]. The displacement of feed samples containing 150 mg of each polymixin by a solution of octyl dodecyl dimethyl ammonium chloride at 0.1 ml/min gave an isotachic train on a 25 cm long column, with elution of the displacer front in about 4 h. The use of more conventional displacers such as alkanols [9] did not allow the formation of the isotachic train. This was due to the insufficient affinity of these displacers for the stationary phase. Success with the long alkyl chain quaternary ammonium ion resulted from realizing that both hydrophobic and silanophilic interactions were involved in the bonding of the polymixins. This points to one of the critical problems encountered in method development when using displacement chromatography, the selection of the best displacer, discussed earlier [52].

Numerous peptides were separated by displacement chromatography [52,57,59,60]. For example, Viscomi et al. discussed the purification of two biologically active peptides, α and β-MSH (melanocyte stimulating hormones, a 13 and a 22 amino-acid-residue peptide, respectively) by displacement chromatography. The separation from crude mixtures containing various impurities was performed on a 25 cm × 0.46 cm column packed with an octadecyl silica and displaced with aqueous solutions of benzyldodecyldimethyl ammonium bromide. Significant amounts (30 mg) were prepared using an analytical instrument and an analytical size column [60]. The possibility to use such instruments and perform preparative applications at the laboratory level is an important advantage of displacement chromatography. Although the isotherm of α-MSH is concave upward while that of the displacer is langmuirian, displacement was possible after adjustment of the solvent composition to make sure that the isotherm of α-MSH underlie that of the displacer in the concentration range of operational interest. Although a true isotachic train was not generated under the experimental conditions selected for the production, an important production rate was achieved (Fig. 16).

Kalghatvi et al. purified melittin (a 26 amino acid peptide, the main component of bee venom) and separated it from its variant by displacement chromatography on columns (3 cm × 0.46 and 10.5 cm × 0.46 cm) packed with small (2 μm) beads of solid silica covered with a pellicular C_{18} hydrocarbonaceous layer, using benzyldimethylhexadecyl ammonium chloride as the displacer [3]. The melittin of a 10 mg sample of bee venom could be extracted in about 13 min, operating the column at 0.6 ml/min, at 40 °C. The advantages obtained in using pellicular particles and operating at temperature above ambient were a considerable reduction in the separation time and an improvement in the recovery yield.

A particular displacement separation is instructive (Fig. 17). It shows the separation of inosine, deoxyinosine, adenosine and deoxyadenosine. As shown in Fig. 10, the height of a band in the isotachic train is given by the intersection of the operating line and the isotherm of the corresponding compound. Thus, when two successive bands have nearly the same height, their isotherms are close. This is the case of inosine and deoxyinosine on the one hand, of adenosine and deoxyadenosine, on the other. By contrast, deoxyinosine and adenosine have different heights, hence their isotherms are remote. This means that the three successive separation factors are close to 1, much larger than 1, and close to 1. The width of the first and third mixed bands are large, that of the second is very narrow. This illustrates what happens in displacement when two components have a separation factor close to 1 and are difficult to separate in chromatography. In displacement, the width of the mixed zone between their two bands becomes large.

Numerous other separations of mixtures of biochemical interest and purifications of the components of these mixtures were discussed by Csaba Horváth and his coworkers.

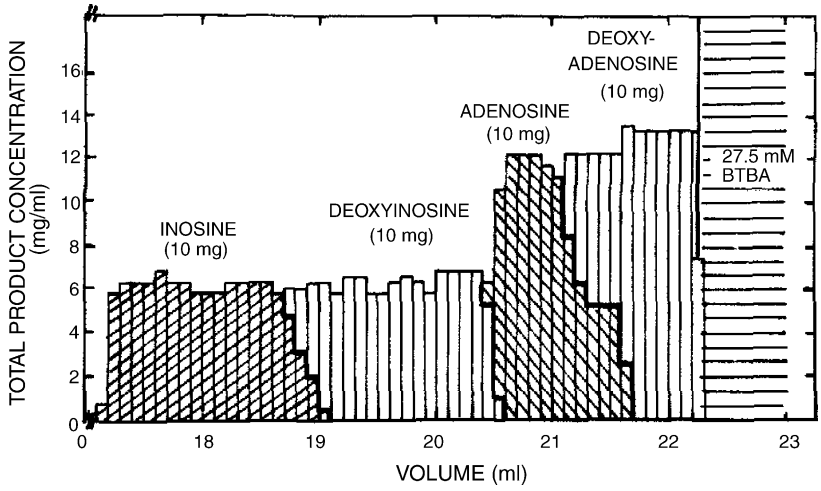

Fig. 17. Separation of inosine, deoxyinosine, adenosine and deoxyadenosine by displacement chromatography on Supelcosil LC-18 (5 μm). Column: 40 cm × 0.46 cm; carrier: 10 mM acetate buffer at pH 5.0; displacer: 27.5 mM benzyltributyl ammonium chloride in carrier; flow rate: 0.1 ml/min; feed: 10 mg of each component in 2 ml of carrier. Reprinted from Ref. [55], Copyright 1983, with permission from Elsevier (Fig. 3).

A brief sample is given here: 5′-AMP, 3′-AMP, 2′-AMP, and adenosine [55]; di- and polypeptides [56]; tripeptides [57]; α and β-lactoglobulins [58]; peptides obtained by enzymatic synthesis [59]. Numerous examples can be found in the Ph.D. dissertations of his students, notably in [39,42,51,64].

3. Conclusion

Among the many successful achievements of his rich career, we must list the profound influence that Csaba Horváth's work had on the origin and development of modern preparative liquid chromatography. He steered this development toward the applications of interest in the biotechnologies, particularly toward the separation and purification of peptides and proteins. Directly and indirectly, through his publications, presentations, personal discussions and through the influence of a score of brilliant students, he caused numerous separation scientists to investigate all the various aspects of the method. He lead chromatographers to revisit the theory of nonlinear chromatography that had long been neglected and was considered as exhausted by chemical engineers.

Under his leadership, separation scientists learned to combine the knowledge of chromatography acquired through the study of analytical applications, the flexibility offered by the immense variety of the retention mechanisms offered by HPLC and the separation power offered by HPLC, with the theoretical and practical tools of the engineers to solve difficult preparative separation problems. They were able to use with great success a method that is still considered by chemical engineers as a mere adsorption process. Csaba's work in the different aspects of chromatography at finite concentrations, its theory, its implementation and its applications made him a true pioneer of that field.

Finally, we should wonder what happened and will happen with displacement chromatography. Currently, this method is not used for many large scale applications. Overloaded elution and simulated moving bed are the only two processes that are used at the industrial scale, with possibly a few exceptions. Yet, at the laboratory scale, displacement presents some major advantages that seem to have been lost, possibly because some oversold the method. Displacement allows the production of a few hundred mg of pure compounds in a relatively short time, a few hours to a few days, with an analytical instrument and analytical-size columns. This might be most helpful for isolating the amount of material needed for the acquisition of spectro-chemical data, for characterization, to perform chemical reactions, or to study the biochemical properties of a compound. The price to pay for this important advantage is that displacement chromatography remains a complex method, that it is poorly understood by most analytical chemists, and that it requires the combination of a profound understanding of nonlinear chromatography and of the retention mechanisms in the system studied, together with much persistence in the quest for a displacer. That much is made abundantly clear to those who read the application papers published by Csaba's group. Success in developing a displacement chromatography method was not so easy even there.

At the industrial level, two significant drawbacks have played heavily against a more widespread use of displacement chromatography. Separation engineers in the pharmaceutical industry do not like to introduce another compound, the displacer, in the feed and, in exchange for the advantage of collecting concentrated fractions, to have another purification problem to solve. The extraction of silica from pharmaceuticals, which must be ashless, is already a sufficient source of headaches. Finally, energy is cheap now, still cheaper than it was 25 years ago when Csaba started working on displacement chromatography. The great advantage of this method over overloaded elution is that it delivers more concentrated fractions. Presently, the cost of concentration of the fractions

collected from overloaded elution is quite bearable. Besides, SMB affords streams that are about as concentrated as displacement. Maybe, in 10 years from now, if energy is far more expensive than it is today, displacement chromatography will return at the forefront of separation sciences.

Each one of Csaba's groundbreaking contributions to the separation sciences is truly remarkable. Taken as a whole they represent a treasure trove for the scientific community to draw from for decades to come.

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