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CHROMATOPHORESIS: A NEW APPROACH TO THE THEORY AND PRACTICE OF CHROMATOFOCUSING

I. GENERAL PRINCIPLES

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SUMMARY

Accurate mathematical analysis reveals that a smooth pH gradient in a chromatofocusing column cannot be produced by the buffering action of the ion exchanger and running buffer. The generation of the pH gradient is attributed to the mechanism of classical frontal analysis chromatography. Computer simulations predict a dependence of the pH gradient shape on the buffer composition.

Utilization of the displacement technique is expected to produce an improved ampholyte separation. The pattern distribution of steady-state stacked ampholytes has been computed, together with ionic strength and pH of the mobile phase. The term "chromatophoresis" is proposed for this method of pH gradient generation and the conditions for the efficient separation of ampholyte mixtures are discussed.

INTRODUCTION

One of the most modern separation techniques in biochemistry is chromatofocusing[1]. Because of the expensive commercial materials involved, we have searched for a substitute for Polybuffer[2], including a dilute (0.5% w/w) aqueous solution of tetraethylenepentamine adjusted to pH 8.0 with HCl. This polyamine has about the same buffer capacity as polybuffer over a wide pH range (*cf.*, Fig. 1 in ref. 3 and Fig. 8 in ref. 2). Hence, in accordance with chromatofocusing theory[1], a smooth pH gradient of the column effluent has to be produced. Insertion of buffer capacity data into eqns. 3 and 4 in ref. 1 result in an S-shaped pH gradient throughout the entire column, provided the column is ten-sectioned. However, instead of a smooth pH shift from 10.0 to 8.0, in actual experiments the pH first rose to 11.0 and then fell abruptly to 8.0.

There is another observation that cannot be explained in terms of the "buffering mechanism"[1]: in actual experiments with Polybuffer (*e.g.*, Figs. 13, 16 and 17 in ref. 2) a small pH increase precedes the descending pH gradient. However, the addition of more acidic to a less acidic buffer is never expected to increase the pH of the resulting mixture.

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These two observations constituted the starting point of our investigations. The initial ideas were conceived by Dr. Andrew Murel. Dr. Maret Pank and Svetlana Vilde carried out most of the experimental work and Dr. Igor Shevchuk manipulated the mathematical equations for the computer.

THEORETICAL

Buffering action for producing a pH gradient in chromatofocusing

Sluyterman and Elgersma[1] considered the addition of nineteen buffer aliquots to a ten-section column. Assumption that the buffer capacities of one aliquot and column section are equal yields

$$pH_{f,j} = \frac{pH_{f-1,j} + pH_{f,j-1}}{2} \quad (1)$$

where f is the aliquot number and j denotes the section number. In order to approach real conditions, however, the column should be divided into many more sections and smaller aliquots should be considered. Ideally, one section should correspond to the height of one theoretical plate.

Calculated pH values of the effluent from 10 and 100 sectional columns are shown in Fig. 1. A further increase in the theoretical plate number results in an abrupt pH jump that resembles a characteristic sigmoid curve of "breakthrough and total capacity" (Fig. 4.3 in ref. 4) rather than a pH gradient for focusing.

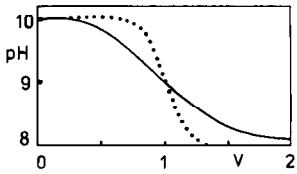


Fig. 1. Buffering mechanism of chromatofocusing: pH of the effluent from the column as a function of the relative elution volume. Solid line, 10-section column; dotted line, 100-section column.

The failed experiment with tetraethylenepentamine as a buffer can now be explained. However, the same data prevent the acquisition of smooth pH gradients with ampholyte mixtures. In attempting to solve this problem, we considered Sluyterman and Elgersma's remark (footnote on page 18, ref. 1) that in the actual mechanism of buffering action a pH increasse results from retention of the more acidic components from the buffer. Although it was stated[1] that the "actual mechanism" does not alter the final equation, we have derived a new equation to reflect the "retention amendment".

Let us assume that the initial buffer aliquot ($j = 0$) consists of equal concentrations of eleven ampholytes with pI values ranging from 3 to 13. The pH of this mixture is $(3+4+5+\ldots)/11 = 8.0$, the initial buffer capacity being $A^m = 1.0$. Suppose one section of column possesses the same capacity as the stationary phase, $A^s = 1.0$, displayed as the ability to retain two ampholyte molecules. Hence, every

column section adsorbs two more acidic components from the first aliquot, and allowance should be made for the loss of buffering material from the mobile phase. The assumption that the buffer capacity of the mobile phase, A^m, is proportional to the ampholyte concentration gives

$$A^m = \frac{(13 - pH_{1,j-1}) \cdot 2 + 1}{11} \tag{2}$$

and eqn. 1 has to be rewritten as

$$pH_{f,j} = \frac{pH_{f-1,j}A^s + pH_{f,j-1}A^m}{A^s + A^m} \tag{3}$$

As far as the first aliquot is concerned, $pH_{0,j}$ and A^s are constant (10.0 and 1.0, respectively):

$$pH_{1,j} = \frac{10 + pH_{1,j-1}A^m}{1 + A^m} \tag{4}$$

Calculated pH values of the first aliquot display an even steeper descent than that based on a "pure" buffering action. Moreover, the "retention amendment" does not elucidate the impact of adsorbed ampholytes on the equilibrium with successive aliquots.

As neither a smooth pH gradient nor a pH jump are explained in terms of the buffering mechanism, we turned to classical frontal development chromatography (for instance, see page 228 in ref. 4).

Frontal development for producing a pH gradient

For the sake of simplicity, let us consider a strong ion exchanger with no buffer capacity in the pH range concerned. The smallest section of the column that still retains the properties of an ion exchanger defines the volume of the buffer aliquot: one aliquot is the buffer volume consisting of a number of ampholyte species equal to the number of "sites of exchange" (page 200 in ref. 4) in one column section, Q (μequiv./ml).

Let $j = 1,2,...$ denote the number of successive column sections, $1j = 0$ being the buffer reservoir. Let the buffer contain L types of ampholyte species, each type i designated by the initial concentration $C_{i,0}$ and by the isoielectric point pI. For one aliquot one can write

$$\sum_{i=1}^{L} C_{i,0} = Q \tag{5}$$

Let us consider an anion exchanger in the OH^- form. The first buffer aliquot is entirely adsorbed in the first column section. The second portion of ampholytes will compete for the "sites of exchange" with the species from the first aliquot. The concentration of type i ampholyte that adheres to the j section, $C^s_{i,j}$, is

$$C^{s}_{i,j} = \frac{C_{i,j}k'_i}{\sum_i (C_{i,j}k'_i)} \cdot Q \tag{6}$$

where $C_{i,j} = C^{m}_{i,j} + C^{s}_{i,j}$ is the total concentration of type i ampholytes in section j and k'_i is a capacity factor reflecting the relative strength of the i–ampholyte–exchanger ion complex.

The ampholyte concentration in the mobile phase is calculated as

$$C^{m}_{i,j} = C_{i,j} - C^{s}_{i,j} \tag{7}$$

and pH is given by:

$$\mathrm{pH}_j = \frac{\sum_i (C^{m}_{i,j}\mathrm{p}I_i)}{\sum_i C^{m}_{i,j}} \tag{8}$$

It is assumed here that ampholyte electric charge Z_i is proportional to the slope of the titration curve near its isoelectric point and that all ampholytes have the same charge dependence:

$$Z_i = \mathrm{p}I_i - \mathrm{pH}_j \tag{9}$$

and the ionic strength of the mobile phase is

$$I = \sum_i (C^{m}_{i,j}Z^2) \tag{10}$$

Eqns. 6, 7, 8 and 10 permit the calculation of component composition, pH and ionic strength of aliquots as they pass successively through the column. Similarly, ascending pH gradients can be derived for cation exchangers in the acidic form*.

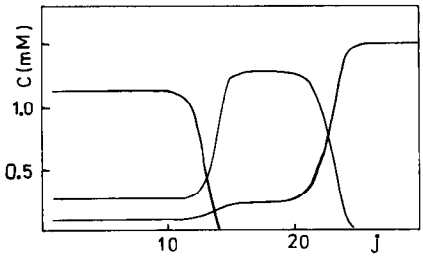
Fig. 2. shows the stationary phase composition addition of 30 infinitely dilute aliquots, the buffer being a three-ampholyte mixture ($k'_1/k'_2/k'_3$ = 4:2:1). Fig. 3 shows the pH of the mobile phase in the nineth section, or the effluent pH from a 9-ml column provided the sizes of the aliquot and the section volumes are 1 ml.

The effluent composition, ionic strength and pH data shown in Fig. 4 correspond to an ampholyte mixture with a lower separation selectivity $\alpha(k'_1/k'_2/k'_3$ = 2.25:1.5:1).

Displacement development for producing a pH gradient

Suppose that ten ampholyte buffer aliquots are applied to an anion-exchange column and the column is washed with dilute HCl. If the Cl^- ion has a higher affinity for the resin it will displace all other counter ions from exchanger. As calculations

* For computer programs in BASIC and FORTRAN, contact Dr. I. Shevchuk.



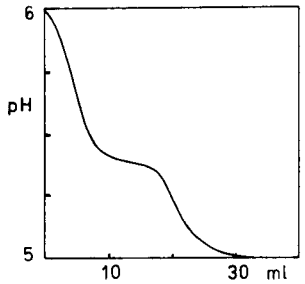


Fig. 2. Chromatofocusing: calculated component distribution inside a column at the moment when 30 infinitely dilute buffer aliquots are loaded. Initial buffer consists of a mixture of three ampholytes; k_i' ratio = 4:2:1; $C_{i,0}$ = 0.5 mM.

Fig. 3. Chromatofocusing: pH of the effluent from the column, V = 9 ml, Q = 1.5 μequiv./ml. Initial buffer consists of a mixture of three ampholytes, $k_1'/k_2'/k_3'$ = 4:2:1; $pI_1/pI_2/pI_3$ = 4:5:6; $C_{i,0}$ = 0.5 mM.

reveal, the displacement technique furnishes zones of pure components and zones of mixed fractions, the quality of the separation being dependent on the experimental conditions. A steady component distribution can be achieved by repetition of the displacement process.

Displacement development is reminiscent of isotachophoretic separation where the counter ions are substituted for the charged matrix of the exchanger and where the stack is driven by a solvent flow instead of electric force. To define this new means of producing a pH gradient via the displacement technique on an ion exchanger we propose the term "chromatophoresis" (CHPH).

Subsequently, a simple equation was conceived to reflect the link between the molarity of the mobile phase, C^m, and that of the displacing (or terminating) constituent, C_t:

$$C^m = \frac{Q_a}{Q_t} \cdot C_t \tag{11}$$

where Q_a and Q_t are the capacities of the exchanger for ampholytes and for the terminating constituent, respectively. This equation gives the effluent concentration provided that steady-state stacking is establishing, *i.e.*, the column is large enough for the given ampholyte load to mould a stable stack. Thus, one act of CHPH caused by the addition of the displacing constituent solution can be calculated mathematically as an addition of the first section stationary phase to the second:

$$C_1^s + C_2^s = C_2^{*s} + C_2^m \tag{12}$$

where the asterisk denotes a new ampholyte composition in the section. Then C_2^m is added to C_3^s to yield C_3^{*s} and C_3^m, etc.

This approach permits the simulation of the CHPH process for the mobile phase concentration range $0 < C^m \leqslant C^s$. For $C^m > C^s$ it is necessary to sum more than two sections. For instance, Fig. 10 depicts the sum of the first three sections:

$$C_1^s + C_2^s + C_3^s = C_3^{*s} + C_3^m \tag{13}$$

where C_3^{*s} is a new stationary phase of the third section, $C_3^m = 2C_3^{*s}$. The next step is $C_3^m + C_4^s = C_4^{*s} + C_4^m$, etc. The second displacement wave is initiated by addition of a new portion of the displacing constituent: $C_3^{*s} + C_4^{*s} + C_5^{*s} = C_5^{**s} + C_5^m$, etc.

Figs. 4–11 show the effluent composition, the top panel representing the pH and ionic strength. The arrows in each instance indicate the point that divides the first and third bands into fractions of equal purity, with the number giving the precentage purity of the first component with respect to the third. Buffer components display their concentration maxima in the column effluent in succession that depends inversely on k': lower affinity patterns are eluted first and the most acidic ampholyte is the last to be displaced from the anion exchanger.

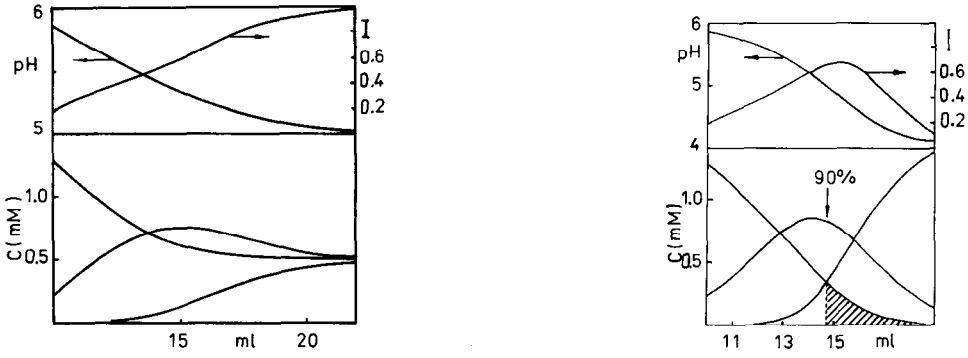


Fig. 4. Chromatofocusing: calculated composition of the effluent. Column: V = 9 ml; Q = 1.5 μequiv./ml. Initial buffer consists of a mixture of three ampholytes, $k'_1/k'_2/k'_3$ = 2.25:1.5:1; $pI_1/pI_2/pI_3$ = 4:5:6; $C_{i,0}$ = 0.5 mM. Top panel: pH and ionic strength of the effluent.

Fig. 5. Chromatophoresis: calculated composition of the effluent. Column: V = 9 ml; $Q_a = Q_t$ = 1.5 μequiv./ml. Initial buffer consists of a mixture of three ampholytes, k'_i ratio = 2.25:1.5:1; pI_i ratio = 4:5:6; $C_{i,0}$ = 0.5 mM. Ampholyte load is 10 aliquots, then the column is washed with 1.5 mM displacing solution. Top panel: pH and ionic strength of the effluent.

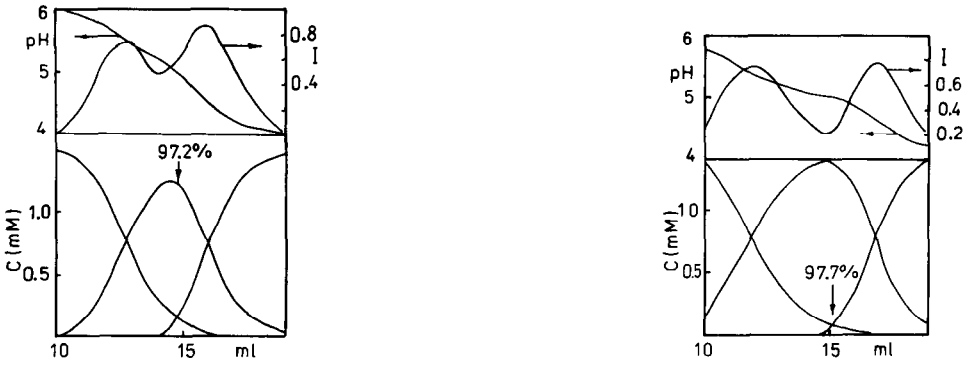
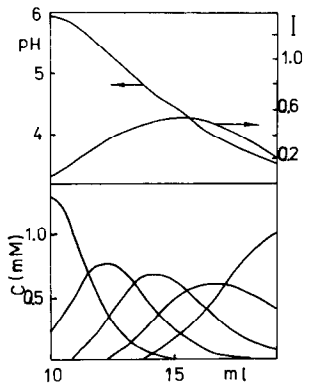


Fig. 6. Chromatophoresis: conditions as in Fig. 5 except for capacity factor ratio, $k'_1/k'_2/k'_3$ = 4:2:1.

Fig. 7. Chromatophoresis: conditions as in Fig. 5 except capacity factor ratio $k'_1/k'_2/k'_3$ and component concentrations in the initial buffer; $C_{i,0}$ ratio = 0.38:0.74:0.38.



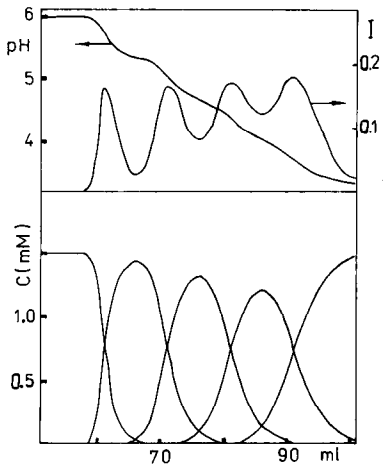
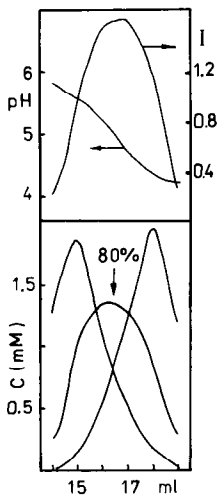


Fig. 8. Chromatophoresis: calculated composition of the effluent. V = 9 ml; $Q_a = Q_t$ = 1.5. μequiv./ml. Initial buffer consists of a mixture of five ampholytes; k_i' ratio = 5:4:3:2:1; pI_i ratio = 3.33:4.00:4.66:5.33:6.00; $C_{i,0}$ = 0.3 mM. Ampholyte load is 10 aliquots, then the column is washed with 1.5 mM displacing solution.

Fig. 9. Chromatophoresis: conditions as in Fig. 8 except column volume = 50 ml and ampholyte load is 50 aliquots.



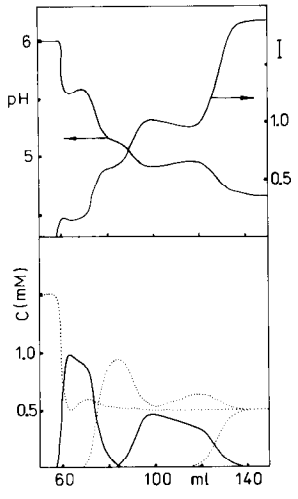


Fig. 10. Chromatophoresis: conditions as in Fig. 5 except column volume = 13 ml and displacing constituent concentration C_t = 3 mM.

Fig. 11. Chromatofocusing: conditions as in Fig. 9 except for the displacing step. Instead of displacement the frontal development is continued with a new buffer composition where the second and the fourth ampholytes are withdrawn. First sample ampholyte with pI = 5.33 is explicitly focused. Embowered in high ionic strength the second sample ampholyte is rather blurred. The divergence between observed and actual pI is *ca.* 1 pH unit for this pI = 4.00 ampholyte.

In order to plot the calculated pH, I and C values *versus* conventional eluent units, it is suggested that one aliquot (except for the data displayed in Fig. 10) consists of a 1-ml volume and one column section has $V = 1$ ml.

DISCUSSION

The frontal development mechanism permits the generation of pH gradients even on a strong ion exchanger with no need for a buffering action of the resin. The larger the column, the higher is the pH gradient volume. While the first ampholyte emerges in a nearly pure state, its successors are more and more contaminated. This contamination is reflected in Fig. 4 as a steady increase in ionic strength and its is supported by experimental data (Fig. 26 in ref. 2).

Note that the equilibrated part of the column (sections 1–10, Fig. 2) represents a component concentration ratio in the stationary phase other than the 1:2:4 that one would expect from the ampholyte affinity k' ratio. This is a good example of how carefully one should approach chromatographic techniques. An increase in the separation selectivity, $k'_{i+1}/k'_i = \alpha$ and in the column size can furnish a stepwise pH gradient (Figs. 3 and 4). Addition of species with intermediate pI and k' values to the initial buffer will have an opposite effect. Finally, the frontal development mechanism elucidated the pH jump in some actual experiments as an elution of less adhesive components at their isoelectric points, provided that the column was initially adjusted to a lower pH than the pI of the leading ampholyte.

Chromatophoretic separations are affected by several factors:

(1) Alteration of the separation selectivity, α; *cf.*, Figs. 5 and 6.

(2) Alteration of the relative "spacer" contents to hold apart components of interest; *cf.*, Figs. 6 and 7.

(3) Alteration of the sample load as reflected by comparison of two five-component mixture separations (Figs. 8 and 9). A reduction in the exchanger capacity, also improves CHPH.

(4) Alteration of the column length, leading to a different CHPH span. For instance, 9- and 22-ml columns furnish purities of 93.4% and 95%, respectively (Fig. 8). However, the effective increase in column length is limited as the component distribution attains the steady state, and the greater separation selectivity the more promptly the stack is trimmed.

(5) Alteration of the ampholyte concentration in the mobile phase with respect to the stationary phase by lowering the displacing constituent concentration (eqn. 11), as shown in Figs. 5 and 10.

The chromatophoretic technique has several advantages over chromatofocusing (CHF):

(1) Utilization of the entire pI range of the buffer ampholytes; *cf.*, Figs. 4 and 5. CHF takes advantage of only half of the available pI values unless the pH of the buffer solution is adjusted with acid to equal the pI of the most acidic ampholyte.

(2) A low ionic strength in CHF has been emphasized (1) as a decisive factor for separation quality. CHPH gives a lower ionic strength of the effluent; *cf.*, Figs. 9 and 11.

(3) The cost of the eluent constitutes the major factor in the experimental costs. While all buffer components are recovered on completion of the run in CHPH, in CHF a column volume of ampholytes is lost.

(4) The sample composition is important in CHF[2] and its should not contain large amounts of salts ($I < 0.05$). CHPH permits the removal of salts from ampholytes and from the sample, provided that the column is large enough to adsorb all counter ions applied.

The sample load in isotachophoresis (ITP) has recently been reported to consist of bare proteins with no need for carrier ampholytes or other spacers[5]. From the analogy between ITP and CHPH, there appears to be no theoretical objection to the possibility of a similar approach in CHPH.

The molecular weight distribution of ampholytes can be obtained by CHPH. The effluent concentration is proportional to the molecular weight of an ampholyte, provided that every "site of adsorption" is occupied by one counter ion, *i.e.*, when $Q_a = Q_t$ in eqn. 11. A pellicular or superficially porous strong ion exchanger with a low capacity is likely to comply with this requirement. Theoretically, displacement development permits the determination of the molecular weights of non-amphoteric electrolytes on ion exchangers, and the same is valid for the displacement technique with other types of adsorption.

Terminology

The term "ampholyte displacement chromatography" has been employed in several papers to define the process of elution of proteins from ion exchangers with ampholyte solutions[6–9]. Other authors[10] have observed the formation of a pH gradient, which was explained as a "retardation phenomenon in which ampholytes, depending on their charge in aqueous solution, are retarded by the ionic groups of the gel", and several column volumes of water are required for complete elution of ampholytes from the exchanger.

However, the "displacement development" denotes washing columns with a solution of the displacing constituent and the term "frontal development" implies passing the sample solution continuously through the column, both terms being conventional terms in liquid chromatography texts[4]. Thus, regarding carrier ampholytes only, "ampholyte displacement chromatography" and CHF should be considered as elution and frontal development techniques, respectively.

The founders of the CHF method[1] distinguish ampholyte and protein separation mechanism, the latter being described as elution with a pH gradient. In fact, unlike continuous ampholyte feeding during frontal development (or CHF), the protein sample is applied to the column in a finite volume. However, owing to the amphoteric nature of both buffer ampholytes and sample proteins, their behaviour should be attributed to a single mechanism (Fig. 11).

In Part II, the frontal development mechanism (for CHF) and the displacement development mechanism (for CHPH) are experimentally verified, and some applications of the new chromatophoretic procedure are presented.

SYMBOLS

$A^{s,m}$	Buffer capacity of the stationary and the mobile phase, respectively;
a	suffix indicating ampholyte constituent;
C	mean concentration of a constituent, mM;
$C^{m,s}$	concentration in the mobile and stationary phase, respectively;

f suffix indicating aliquot number;
I ionic strength;
i suffix indicating a certain pI type of ampholytes;
j suffix indicating column section number;
k' capacity factor reflecting the affinity of a constituent for an ion exchanger;
L total number of ampholyte types;
m suffix indicating mobile phase;
pI isoelectric point;
$Q_{a,t}$ capacity of exchanger for ampholytes and for the terminating constituent, respectively, equiv./ml;
s suffix indicating stationary phase;
t suffix indicating terminating (or washing or displacing) constituent;
V colume volume, ml;
Z electric charge of an ampholyte;
α separation factor, k'_{i+1}/k'_i.

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