ISSN 0027-1314, Moscow University Chemistry Bulletin, 2007, Vol. 62, No. 4, pp. 204–209. © Allerton Press, Inc., 2007.
Original Russian Text © A.V. Ivanov, D.V. Kurek, M.S. Vakstein, A.P. Bayunov, 2007, published in Vestnik Moskovskogo Universiteta. Khimiya, 2007, No. 4, pp. 250–256.

Ionic-Strength Gradient during Chromatofocusing in Carboxylic Columns

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Received October 10, 2006

Abstract—The presence of a strong electrolyte in mobile phases essentially influences the pH gradient profile generated by chromatofocusing. The variation in the strong electrolyte concentration in the chromatofocusing system is studied. The ionic strength gradient ends far more early than the pH gradient; anomalous segments of the pH gradient profile match the general trend of the ionic strength in the system. The pH gradients obtained under identical conditions on carboxylic sorbents MacroPrep 50 CM and MN are compared. The influence of the KNO3 concentration in the mobile phases on the gradient profiles is studied. Optimum systems are chosen for generating the smoothestlinear pH gradients.

DOI: 10.3103/S0027131407040074

Ion-exchange chromatography knows two methods for creating pH gradients. The precolumn method is in most use. In this method, the gradient is generated upstream the column by mixing two or several buffer solutions, having different pHs, in a mixer system or a programmed gradient pump, thus controlling the velocities of mobile phases [1]. The pH gradient created in this way can be called an external gradient. The linear ranges of external gradients are seldom longer than 2.0–2.5 pH units [2]. In the second (in-column) method, pH gradients are created inside a chromatographic column due to the buffer properties of the eluent and the buffering ability of the ionic groups of the sorbent preequilibrated with the starting solution at another pH [2, 3]. This method creates smooth, virtually linear gradients over wide pH ranges (up to 4–5 units). In-column (or internal) pH gradients are used in chromatofocusing, a method for separating biologically important bipolar compounds according to their isoelectric points [3]. Despite the simplicity of instrumental implementation and the accumulation of a large body of experimental data, the physicochemical modeling of the creation of internal pH gradients by chromatofocusing remained until recently immature [4, 5]. Now, there are several models of the generation of pH gradients inside anion-exchange columns with account of ion exchange and protolytic equilibria in a heterogeneous dynamic system [4]. However, the understanding of gradient generation in a cation-exchange system is still in its infancy, remaining at the level achieved in 1981 [6].

Recent studies demonstrated the utility of pH gradients in cation-exchange systems: descending gradients can be used for the preconcentration and determination of metal ions [7, 8], and ascending gradients, for the separation of proteins and peptides [9–11]. The physicochemical modeling of gradient formation in such systems is made topical by the use of cation-exchange sorbents in chromatofocusing. These models would make it possible for researchers to calculate the pH gradient from the set parameters, thus saving time.

The parameters of the system in physicochemical modeling should be categorized as follows: (1) known from the literature, (2) previously unknown but subject to experimental determination (this category includes the ion-exchange capacity, protonation constant, and other sorbent parameters), and (3) unknown and indeterminable directly from an experiment [4, 12, 13].

The last category should include approximate parameters, i.e., empirical parameters and the parameters that change in a complex manner during the experiment [12–14]. For anion-exchange systems, for example, it was shown that the ionic strength of the starting solution and eluent has a complex influence on pH-gradient formation: virtually linear gradients over wide pH ranges can be generated by varying the concentration of the strong electrolyte (such as NaCl) in both mobile phases [4, 13]. The complex influence of the ionic strength of the mobile phases on the pH gradient profile was also observed in cation-exchange systems [7]. In the context of modeling, it is interesting how the strong-electrolyte concentration changes when an internal pH gradient is formed. A combination of a pH gradient with an ionic-strength gradient evidently pertains to the separation of proteins and other bipolar biomolecules [11]. This work concerns the variation in the concentration of a strong electrolyte during the creation of a pH gradient inside low-acidity cation-exchange columns.

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Table 1. Characteristics of the sorbents used

Sorbent	MacroPrep 50 CM	MN
Manufacturer	Biorad (USA)	Purolite (Great Britain)
Matrix	Poly(methyl methacrylate) (PMMA)	Hypercrosslinked polystyrene (HCPS)
Functional groups	$–CH_2COOH$	–COOH
Particle size, μm	50	5
pH range of hydrolytic stability	3–13	1–14
Ion-exchange capacity (as certified by the manufacturer)	0.21 ± 0.04 mmol/(mL gel)	Less than 0.10 mmol/g*

* Estimated from acid–base titration [7, 8].

EXPERIMENTAL

Instruments. The chromatographic system used comprised Milton-Roy A-802 or Shimadzu LC-10AT VP high-pressure pumps; a Rheodyne 7125 injector with loop volumes of 100 μL to 1 mL; a cation-exchange chromatographic column; and a TOA Electronics HM 20-S digital pH meter including a GST-5211C combination glass electrode or a pH-121 millivoltmeter/pH meter equipped with a EM-NO_3-01 (for NO_3^- ion) or EM-K-01 (for K^+ ion) ion-selective electrodes and a silver/silver chloride reference electrode, all electrodes being dipped into a home-designed flow-through cell. The signals from the pH meter or ion meter were recorded on an LKB-Bromma LKB-2210 two-channel recorder and, then, processed.

Chromatographic columns (50 × 4.6 mm) made of PEEK polymer were packed with sorbents MacroPrep 50 CM or MN. The characteristics of these sorbents are displayed in Table 1.

Reagents and solutions. In generating pH gradients by means of chromatofocusing, 10 mM Tris-HCl with pH 7.3–7.6 was used as the starting solution and 1 : 20 Polybuffer-96 (from Pharmacia) with pH 3.0 was used as the eluent. The required solution acidity was adjusted by conc. HCl. The ionic strength of the mobile phases was adjusted by addition of calculated amounts of a 2 M KNO_3 solution, which was prepared from a dry reagent grade chemical (from Reakhim).

Procedures. A cation-exchange column was equilibrated with a starting solution until it reached the pH of the starting solution (~7.5), which corresponded to the onset point of the gradient. Then, a buffer eluent with pH 3.0 was admitted to the column, and the effluent pH at the column outlet was continuously controlled in a flow-through cell. The pH-meter signal was recorded, and the time during which the final point of the gradient was reached was written. After this, the column was again equilibrated with the starting solution, a millivoltmeter/pH meter equipped with an ion-selective electrode was connected, and the anion (NO_3^-) or cation (K^+) potential was continuously recorded in a flow-through cell at the column outlet during the same period of time as the pH gradient developed. Ion concentrations were derived from calibration curves through the potential. The volume rate of the mobile phases was 1 mL/min; in some cases, 0.6 mL/min. All gradients were normalized to the volume of the mobile phase that passed the column.

RESULTS AND DISCUSSION

Choice of the chromatographic system. Sorbent MacroPrep 50 CM, having a sufficiently high ion-exchange capacity (Table 1), was chosen to be the model for studying the main tendencies of the pH gradient profile as a function of the ionic strength of the mobile phases. In addition, it is well known how to create descending and ascending pH gradients in this sorbent using polyampholite and one- or two-component eluents [7, 11]. To ease the understanding of the processes that occur in the chromatographic system, we chose Polybuffer-96 (a well known polyampholite) as the eluent, while the starting solution was Tris-HCl, which is routinely used in protein chromatofocusing on anion- and cation-exchange sorbents [3]. Although the influence of a strong electrolyte for anion-exchange systems was noticed for sodium chloride [13], in this work we chose potassium nitrate to be the model electrolyte in order to avoid a possible influence of chloride ions on the potential of the silver/silver chloride electrode and, through this, on the measured signal.

The following notations will be used hereafter: I_{ss}, the ionic strength of the starting solution, crated by KNO_3 addition; I_e, the eluent ionic strength; descending gradient, a decrease in the ionic strength or ion concentration; and ascending gradient, an increase in these parameters.

Descending ionic strength gradient. pH and nitrate-ion concentration gradients were obtained for the chromatographic system where the KNO_3 concentration in the starting solution was 0.1 mol/L and a strong electrolyte was not added to the eluent (I_e ~ 0) (Fig. 1). A so-called anomalous segment was detected on the pH gradient curve: in this segment, pH was 0.5 unit higher than in the starting solution; its length was ~20 min (Table 2). Similar segments with rising pH are well known from previous experiments for anion- and

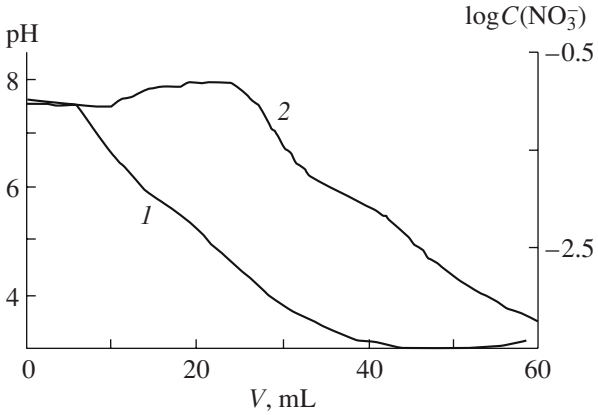

Fig. 1. pH gradient for the descending ionic strength gradient in the mobile phases: (*1*) $\log C(NO_3^-)$ and (*2*) pH gradient. Sorbent: MacroPrep 50 CM (50 × 4.6 mm). Starting solution: 10 mM Tris-HCl, I_{ss} = 0.1. Eluent: 1 : 20 Polybuffer-96 (I_e ~ 0).

cation-exchange systems; they are explained by a change in the acidity/basicity constants of the grafted groups of the sorbent in response to changing ionic strengths of the mobile phases [4, 7, 13]: the result is an additional protonation of the ion exchanger and, thus, an increase in the concentration of bases in the effluent.

At the same time, the nitrate-ion concentration decreases monotonically, without anomalies (Fig. 1, curve *1*). During the first 7–8 min, the nitrate-ion concentration does not change; then, the ionic strength in the system starts to decrease almost linearly: in 30 min, the NO_3^- concentration decreases to 10^{-3} mol/L and below. Similar plots were obtained during the potentiometric control of the potassium-ion concentration; however, because an EM-K-01 ion-selective electrode operates within a limited pH range, all subsequent measurements were carried out for nitrate ions. Thus, potassium nitrate is gradually eluted from the pores and interstitial space of the sorbent by an eluent free of a strong electrolyte. The anomalous rise in the pH gradient profile coincides with the decrease in the ionic strength in the system by one and a half to two orders of magnitude. This confirms that the anomalous segment appears due to a change in the conditional ionization constants of the functional groups of the sorbent in response to changing ionic strength in the mobile phases. A plateau or a rise in the concentration gradient can be expected in the presence of two-charged cations in the system and secondary anion sorption on previously sorbed cations [4, 13].

Creating an ionic strength of at least 0.025 in the eluent slightly decreases the height and length of the rise in pH along the initial segment (Table 2); however, such a system approaches the case where a strong electrolyte is absent in the eluent. When the ionic strengths of the starting solution and eluent are not high and when they differ as little as by a factor of about two (e.g., I_{ss} = 0.1 and I_e = 0.05 or I_{ss} = 0.05 and I_e = 0.025), the rise in pH lasts no longer than 8–9 min, and the pH gradient develops in a shorter time. Descending ionic strength gradients are hardly pertinent to chromatofocusing.

Ascending ionic strength gradient. In cases where the starting electrolyte does not contain a strong electrolyte and where I_e = 0.1, the nitrate-ion concentration gradient develops in a far shorter time than the pH gradient (Fig. 2). Thus, neither pH nor the ionic strength changes during the first 7 min. Then, the ionic strength starts to change, while pH still remains constant (for about 3–4 min), which is followed by the opposite vari-

Table 2. pH-gradient parameters as a function of the ionic strengths of mobile phases

Sorbent	Starting solution (pH, ionic strength)	Eluent (pH, ionic strength)	Rise, pH units	Rise length, mL	Gradient slope, ΔpH/Δ*V*
MacroPrep 50 CM	10 mM Tris-HCl (pH 7.5; *I* 0.1)	1 : 20 Polybuffer (pH 3.0, *I* ~0)	0.45	18–20	0.13
	The same (*I* 0.1)	The same (*I* ~0.025)	0.32	17–18	0.19
	The same (*I* ~0)	The same (*I* ~0.1)	0.30*	10–11	0.10 (0.45)**
	The same (*I* 0.1)	The same (*I* ~0.1)	0	–	0.12
	The same (pH 7.1; *I* 0.05)	The same (*I* ~0)	0.30	9–10	0.18
	The same (*I* 0.05)	The same (*I* ~0.025)	0.20	8–9	0.17
	The same (*I* 0.05)	The same (*I* ~0.05)	0	–	0.21
MN	10 mM Tris-HCl (pH 7.2; *I* 0.05)	1 : 20 Polybuffer (pH 3.0, *I* ~0)	0.40	4–5	0.58
	The same (*I* 0.05)	The same (*I* ~0.025)	0.30	4	0.42
	The same (*I* 0.05)	The same (*I* ~0.05)	0.15	2–2.5	0.48

Notes: * In the pH range of 6.7–6.4.
** In the pH range of 7.6–6.4.

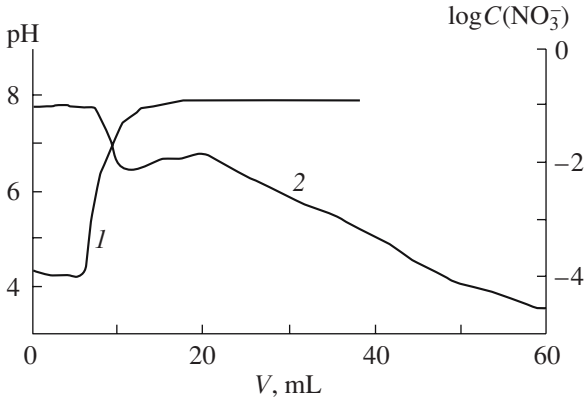

Fig. 2. pH gradient and the ascending ionic strength gradient in the mobile phases: (*1*) $\log C(NO_3^-)$ and (*2*) pH gradient. Starting solution: $I_{ss} \sim 0$. Eluent: $I_e = 0.1$. The other parameters as in the legend to Fig. 1.

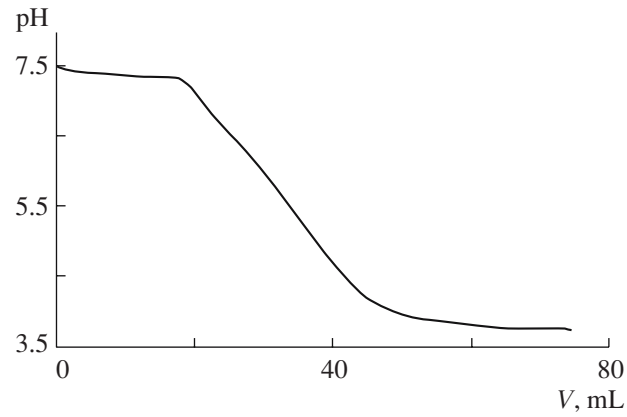

Fig. 3. pH gradient without an anomalous segment for equal ionic strengths of the mobile phases ($I_{ss} = I_e = 0.1$). The other parameters as in the legend to Fig. 1.

ations in the nitrate-ion concentration and pH. The most significant increase in the ionic strength (up to 0.05) occurs during 8–9 min (the linear segment on curve *1*); the subsequent saturation to 0.1 and development to the plateau take another 6–7 min. Along these segments, potassium cations from the eluent are exchanged for protons in the sorbent phase until equilibrium is reached at the given pH:

$$-\overline{COO^-H^+} + K^+ + NO_3^- = -\overline{COO^-K^+} + H^+ + NO_3^-.$$

The interstitial volume in the column is also filled-in by the mobile phase with the ionic strength equal to 0.1. A anomalous segment with an insignificant rise in pH, 11–12 min long, appears on the gradient curve (Table 2). The new gradient segment, with a smooth decrease in pH, approximately matches the development of the nitrate-ion concentration to a plateau (Fig. 2). The conditional dissociation constants in the system change; the sorbent starts to manifest its polyelectrolyte nature [4, 13], which induces a smoother variation in pH inside the column. The slope of the gradient in this segment is far smaller than in the initial segment in a pH range of 7.6–6.4 (Table 2).

Notice that ascending ionic strength gradients are usually used for elution with a constant pH. In chromatofocusing, ionic strength gradients have not yet been created. The combination of an ascending ionic strength gradient with a pH gradient can be of particular interest for the chromatofocusing separation of macromolecules.

Constant ionic strength. For systems in which the ionic strength is sufficiently high and has the same value in both mobile phases ($I = 0.1$), the smoothest pH gradient was obtained with a wide linear range and without anomalous rise or steps (Fig. 3). The potential measured for the nitrate ion remained constant over the whole gradient length, verifying the absence of secondary anion sorption. Provided that the sorbent is equilibrated, protons are exchanged for the cations of the strong electrolyte during eluent passage, but at a higher degree of equilibrium:

$$-\overline{COO^-K^+} + K^+ + NO_3^- + H_nPoly$$

$$\longrightarrow -\overline{COO^-H^+} + K^+ + NO_3^- + KH_{n-2}Poly^- + H^+.$$

Here, H_nPoly denotes a polybasic acid in Polybuffer. Because the sorbent is saturated with the cations of the strong electrolyte, the carboxy groups of the sorbent are protonated in time, with some protons leaving the column on account of partial dissociation of Polybuffer and, thus, decreasing the effluent pH. An analogous gradient was observed when an ionic strength of 0.05 was created in both mobile phases (Table 2).

Thus, the smoothest, virtually linear pH gradients in low-acidity cation-exchange columns can be generated by adjusting equal ionic strengths (0.05–0.1) in both mobile phases.

Comparison of sorbents. Sorbent MN, based on hypercrosslinked polystyrene with grafted carboxy groups, more tightly fills-in the chromatographic column due to its small particle size, decreasing the interstitial volume. According to our estimates, the ion-exchange capacity of MN is three to four time lower than that of MacroPrep 50 CM. Thus, the effects caused by the presence of a strong electrolyte in the mobile phases should be enhanced. In this set of experiments, the ionic strengths in the mobile phases were not higher than 0.05. For comparison, pH gradients were also created on sorbent MacroPrep 50 CM.

In the former case, where the starting solution contained a strong electrolyte (KNO_3) in a concentration of 0.05 mol/L, a peaked segment was observed in the gradient for sorbent MN, with a rise in pH of 0.4 units, lasting no longer than 5 min (Fig. 4a, Table 2). The gradient length was 19–20 min, while for MacroPrep 50 CM, the

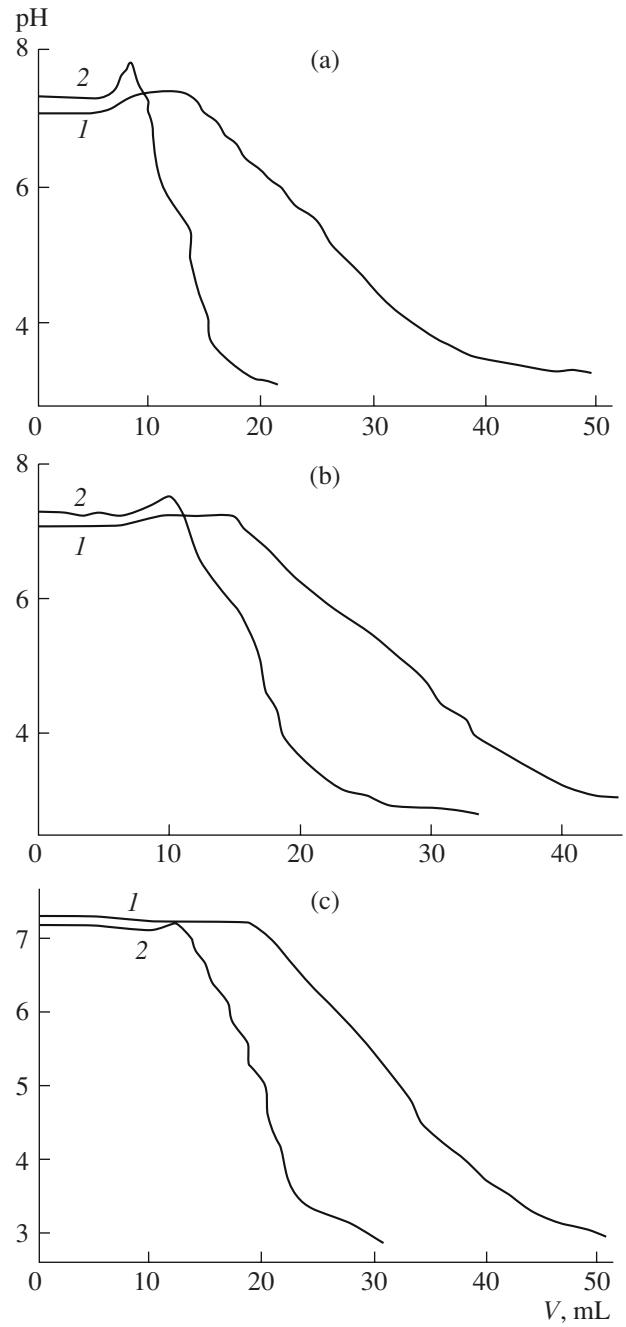

Fig. 4. Comparison of the pH gradients obtained in 5 × 4.6 mm columns packed with sorbents (*1*) MacroPrep 50 CM and (*2*) MN. Starting solution: 10 mM Tris-HCl with I_{ss} = 0.05. Eluent: Polybuffer-96 (1 : 20) with an ionic strength of (a) ~0, (b) 0.025, and (c) 0.05.

final gradient point is reached in less than 50 min, which circumstantially verifies the different ion-exchange capacities of the two sorbents. In addition, the gradient for sorbent MN is less smooth; it consists of several linear segments, which can also be due to the lower ion-exchange capacity of sorbent MN. The nitrate-ion concentration gradient for sorbent MN is a analogous to that shown in Fig. 1, but it develops in a far shorter time. The slope of the ΔpH versus Δ*V* curve is an essential parameter of the pH gradient [15]. The optimum ΔpH/Δ*V* value lies in the range 0.17–0.25 (the gradient has a reasonable development time but still remains sufficiently smooth). Table 2 lists ΔpH/Δ*V* for the test systems. One can see that the slope of the gradient obtained for I_{ss} = 0.05 on sorbent MacroPrep 50 CM precisely falls into the optimum range, while for sorbent MN, ΔpH/Δ*V* has a too high value (0.58), which is also due to its low ion-exchange capacity.

For both sorbents, pH gradients were obtained with a minimum difference between the ionic strengths of the mobile phases: I_{ss} = 0.05 and I_e = 0.025 (Fig. 4b). In this case, a rise in pH was also observed at the initial segment of the gradient, being higher and steeper on sorbent MN (Table 2). The gradient development time for this ratio between the ionic strengths of the mobile phases slightly increases, especially for sorbent MN. The pH gradient profile is smoothed, which is most evident for sorbent MN: the transitions between the linear gradient segments are less abrupt than for the gradient shown by curve *2* in Fig. 4a; ΔpH/Δ*V* decreases to 0.42. For sorbent MacroPrep 50 CM, the slope of the gradient remains almost unchanged (Table 2).

With the ionic strengths in the starting solution and eluent being equal (I_{ss} = I_e = 0.05), the gradient obtained for sorbent MN still contains a segment with an insignificant rise (0.15 units) in pH, no longer than 2.5 min (Fig. 4c). The slope of the gradient increases only insignificantly (Table 2), but smoothed steps appear on the gradient profile. The gradient obtained for MacroPrep 50 CM does not show an anomalous segment with rising pH. The slope of the gradient here also increases to 0.21, yet remaining within the optimum ΔpH/Δ*V* range. The linear decrease in pH at I_{ss} = 0.05 on sorbent MacroPrep 50 CM starts 18–20 min after the onset of the gradient; on sorbent MN, it starts 10–12 min after the onset of the gradient and is virtually independent of the eluent ionic strength (Figs. 4a–4c). With the ionic strength in the starting solution equal to 0.1, for sorbent MacroPrep 50 CM the onset of decreasing pH was strongly affected by the KNO_3 concentration in the eluent.

In summary, the following systems look the best from the standpoint of anomalous pH rises and slopes of the gradients:

for **sorbent MacroPrep 50 CM**,

I_{ss} = 0.05; I_e = 0.05 (0.025) or I_{ss} = I_e = 0.1;

for **sorbent MN**,

I_{ss} = 0.05, I_e = 0.025.

Although anomalous steps appear on the pH gradient profile, when the eluent ionic strength is sufficiently high (0.1) and when there is no strong electrolyte in the starting solution, the system can yet be suitable for the separation of bipolar biomolecules: in this case, an ascending ionic strength gradient is generated inside

the column in addition to the pH gradient. Systems where a strong electrolyte is contained in both the starting solution and the eluent but where $I_{ss} < I_e$, can be of particular interest. Such systems will be studied later.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 05-03-33069).

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