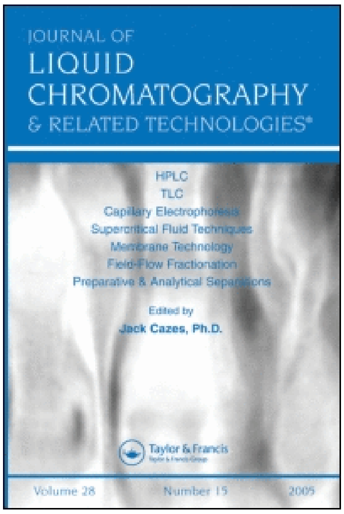

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Abstract: The formation of decreasing internal pH gradients in carboxylic cation-exchange columns, with a single buffering component in the eluent, was investigated. Two types of carboxylic cation exchangers (hypercrosslinked polystyrene and polymethylmetacrylate based) were used. To develop a pH gradient, the column was pre-equilibrated at pH 7.5 with Tris buffer, followed by washing with different organic acid solutions, including acetic, oxalic, tartaric, citric, and glutamic acids. The smoother quasi-linear gradients in the pH range from 7.5 to 3.5 were obtained with 0.2–1 mM citric acid as eluent. The noticeable role of ionic strength of the starting buffer and of the eluent on the profile of pH gradient was demonstrated. The optimal pH gradient profiles were obtained at equal ionic strengths of both mobile phases.

Keywords: Chromatofocusing, Decreasing pH gradient, Weak cation-exchange column, Carboxylated sorbents, Single-component eluent

INTRODUCTION

Chromatofocusing is a well-established ion-exchange separation technique, allowing preconcentration and separation of biological macromolecules, such as proteins, peptides, and amino acids, on a single ion-exchange

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column in accordance with their isoelectric points.[1,2] Usually, the efficient separation of polyampholyte molecules is possible, due to linear a pH gradient formed by passing eluent with a lower pH value through the weak anion-exchange column which has been pre-equilibrated at a higher pH value. The main advantages of chromatofocusing include high column loadability, high resolving power, allowing separation of two proteins differing in less than 0.02 pI units, and high efficiency, due to both gradient elution mode and the special focusing effect of polyampholytes.[2]

Recently, the new technique — chromatofocusing of transition metals combining chelation ion chromatography with decreasing pH gradients on a weak anion-exchange column — was proposed by Nesterenko.[3] The separation of metals with this technique is based on the ability of primary or secondary amino groups of anion-exchangers to bind metal ions at the high pH as complexes, followed by subsequent destruction of these complexes, under a decreasing pH gradient, in accordance with their conditional stability. The separation of 4–5 transition metals was achieved with oligoethyleneamine functionalised Sepharose or silica.[3,4] However, the kinetics of dissociation of complexes of metal ions with oligoethyleneamines is rather slow and affects peak shape and restricts, significantly, possibilities of the method.

Weak cation-exchange columns can be also used in chromatofocusing. In this case, the increasing pH gradients are mainly produced.[2,4–6] The use of cation-exchangers could be a possible solution of the problem in chromatofocusing of metal ions, as the kinetics of dissociation of the complexes are much faster in this case. For this reason, the interest in generating pH gradients in carboxylic cation-exchange columns has surfaced. In our previous communication, the potential of decreasing pH gradient formation within a cation-exchange column packed with carboxymethylcellulose Whatman CM-52 was demonstrated.[7] A linear, smooth, decreasing pH gradient between pH 7.5 and pH 3.0 was obtained in a cation-exchange column pre-equilibrated with Tris-HCl as the starting buffer and polyampholyte Polybuffer-96 (Pharmacia) as the eluent.[7] A similar system producing an increasing pH gradient was recently described by Frey.[6]

Polybuffer-96, consisting of synthetic polyampholyte macromolecules, is strongly adsorbed by cation-exchangers; this causes some problems in regeneration of the cation-exchange columns. The use of multicomponent buffers, consisting of low molecular weight components, offers a chance to avoid these problems. The few attempts using a 3–5 component buffer eluent for generation of pH gradients in anion-exchange column are published.[5,8,9] Recently, a single-component eluent was used for production of a linear or quasi-linear pH gradient in an anion-exchange column and applied to separation of model mixture of amino acids.[10,11] The term "quasi-linear gradient." proposed by Bates and Frey,[8] means nearly linear gradient with sufficiently smooth steps or including several linear segments with different slopes. This approach could be useful for generation of pH gradients in cation-exchange columns; this represents the aim of this work.

EXPERIMENTAL

Equipment

The high-pressure pump, Beckman 114 M, (Berkeley, CA, USA) was used for eluent delivery. The flow rates of mobile phases were maintained at 0,9 mL/min, if otherwise not stated. The digital pH-meter, HM-20S, (TOA Electronics, Tokyo, Japan) equipped with flow cell of volume 0.5 mL and combined glass electrode GST 5211C was used for pH-measurements. The UV-photometer, Uvicord-2238, (LKB-Bromma, Sweden) at 254 nm was used for detection of metal ions separation. The signal was recorded by PC with Ecochrom (Institute of Organic Chemistry, Russia) DOS software.

Columns and Ion-Exchangers

Two cation-exchange columns (PEEK, 50 × 4.6 mm) packed with carboxylated hypercrosslinked polystyrene MN (Purolite, Pontyclun, UK) and with Macro Prep 50 CM (Bio-Rad, Richmond, CA, USA) were used. The properties of ion-exchangers are listed in Table 1.

Reagents

10 mM Tris (Merck, Darmstadt, Germany) with pH 7.5 adjusted by 2 M HCl (Germed, Dresden, Germany) was used as a starting buffer for equilibration of ion-exchange columns. 0.5–5 mM aqueous solutions of acetic, oxalic, tartaric, citric, (Reakhim, Moscow, Russia) and glutamic acid (Reanal, Budapest,

Table 1. Properties of used sorbents

Sorbent	MN	Macro-Prep 50 CM
Manufacturer	Purolite (UK)	Biorad (USA)
Matrix	hypercrosslinked polystyrene	polymethylmetacrylate
Groups	$-COOH$	$-CH_2COOH$
Particle size, μm	5	50
pH-range of hydrolytical stability	1–14	3–13
Ion-exchange capacity, mmole/ml	no data	0.17–0.25

Hungary), at their native pH values, were used as the eluents. The ionic strengths in the starting buffer and in the eluents were created by addition of 2 M NaCl (Germed, Dresden, Germany). Since carboxylated hypercrosslinked polystyrene revealed some hydrophobic properties, the additives of 5 vol.% of acetonitrile in the starting buffer and eluent were used. A model mixture of metal ions (Mn^{2+}, Zn^{2+}, and Co^{2+}) was prepared from their 20 mM chloride solutions. All chemicals were of analytical grade.

RESULTS AND DISCUSSION

In accordance with the results of our previous work,[4,10] aqueous solutions of carboxylic and amino acids having a reasonable buffer capacity can be used as single-component eluents for generation of linear pH gradients in weak acid cation-exchange columns. As a first step, the effects of the number of carboxylic groups in a molecule and their pK values on the pH gradient profile were studied. At the second stage, the optimisation of the obtained pH gradients by varying the ionic strength of the eluent was undertaken.

Effect of the Type of Carboxylic Acids on pH Gradient Shape

Aqueous solutions of organic acids with different numbers of carboxyls having pK_a values within the required pH range were tested as eluents. The set of acids included acetic (pK_a 4.75), oxalic (pK_{a1} 1.25, pK_{a2} 4.27), tartaric (pK_{a1} 2.98, pK_{a2} 4.34), and citric (pK_{a1} 3.10, pK_{a2} 4.76 and pK_{a3} 6.40) acids. The concentrations of the acids were varied from 0.05 mM to 5 mM. A set of pH gradients was obtained for each acid at these concentrations.

In general, three separate segments can be outlined for any pH gradient obtained with organic acid as eluent and a carboxylic cation-exchange column (Figs. 1–3):

- the initial segment with constant pH value equal to the pH of the starting buffer used for cation-exchange column equilibration;
- the "anomalous" segment corresponding to pH increase to a value higher than the pH of the starting buffer;
- the segment with linear decrease of pH until the pH of the eluent.

With varied concentrations of citric acid within the 0.05–5.0 mM range, the length of the initial segment at constant pH is approximately unchanged (Fig. 1). The reason for this is displacement of starting buffer from the volume of column bed.

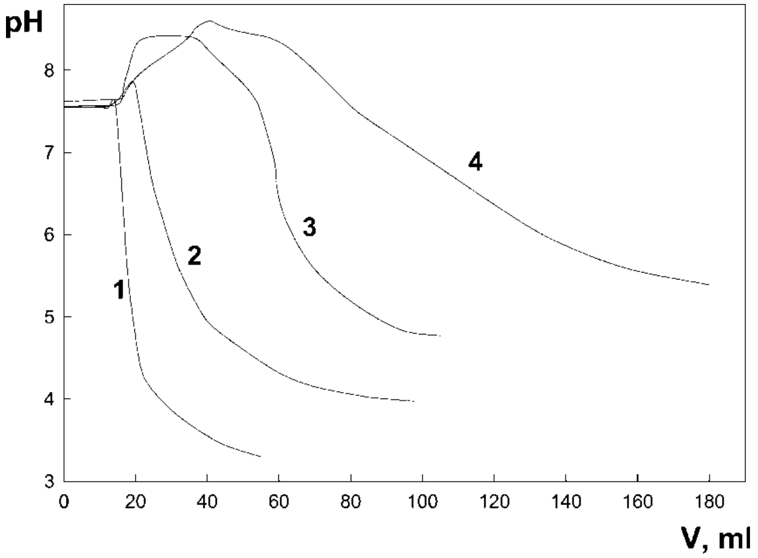

Figure 1. The profiles of pH gradients obtained in cation-exchange column using citric acid. Column: 50 × 4.6 mm, carboxylated MN, 5 μm. Start buffer: 10 mM Tris-HCl—5% CH_3CN (pH 7.5). Eluent-citric acid (*1*—5 mM, *2*—1 mM, *3*—0.2 mM, *4*—0.05 mM)—5% CH_3CN.

At the same time, the pH increase at the second "anomalous" segment becomes larger with decrease of citric acid concentration in the eluent (Table 2). Probably, this is connected to the appearance of citrate anions in the effluent. Before switching from the starting buffer to the eluent, the weak cation-exchange column with fully dissociated carboxylic groups is saturated with protonated Tris molecules. Depending upon the concentration of citric acid in the eluent, the buffer and the eluent may have different ionic strengths. After switching buffer to the eluent with an ionic strength lower than that in starting buffer, the protonation of sorbent carboxylic groups takes place, and some amount of non-retained citrate anions Cit^{3-} is released from the column. As a result, a temporary pH increase in the effluent occurs. The larger the difference in ionic strength between eluent and buffer, the higher will be the protonation degree of carboxyls in cation-exchanger and, hence, the concentration of citrate in the effluent is higher too. Under low concentration of citric acid in the eluent, the process takes a longer time; this explains further extension of this segment (Fig. 1).

The third segment is caused by the upcoming continuous displacement of protonated Tris molecules by acid from the column, with consequent protonation of functional groups. The duration of this process depends upon the concentration of the acid. Smoother pH gradient profiles were observed for diluted citric acid solutions (Fig. 1, curves 3, 4), but the time of the pH gradient was

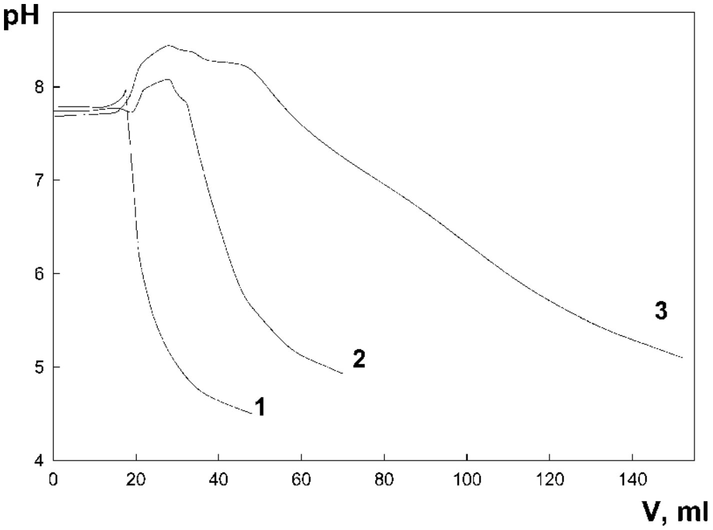

Figure 2. The profiles of pH gradients obtained in cation-exchange column using glutamic acid. Column: as in Fig. 1. Start buffer: 10 mM Tris-HCl—5% CH_3CN (pH 7.5). Eluent—citric acid (*1*—5 mM, *2*—1 mM, *3* - 0.2 mM)—5% CH_3CN.

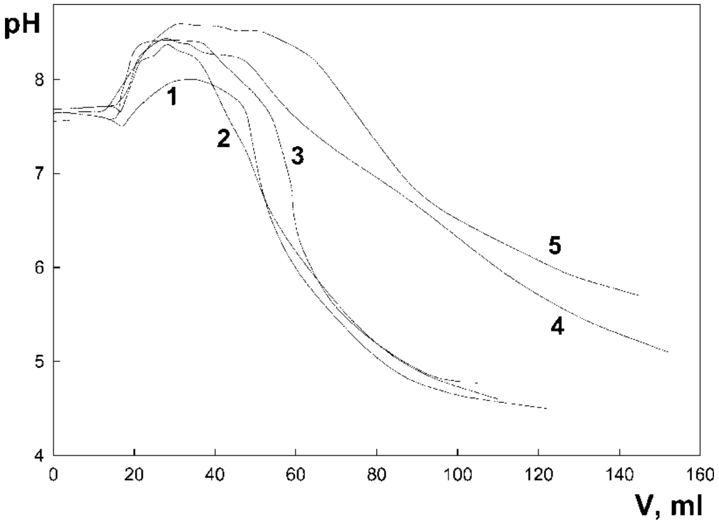

Figure 3. The effect of the nature of organic acid on pH gradient profile. Column: as in Fig. 1. Start buffer: 10 mM Tris-HCl—5% CH_3CN (pH 7.5). Eluent—0.2 mM acid (*1*—oxalic, *2*—tartaric, *3*—citric, *4*—glutamic, *5*—acetic)—5% CH_3CN.

Table 2. The influence of eluent composition on characteristics segments of pH gradient profile

Eluent				"anomalous" pH increase, pH units	Extension of "anomalous" segment, mL
Acid	Concentration, mM	pH	Ionic strength[a]		
MN					
acetic	0.2	4.30	$\sim$0	0.85	60
oxalic	0.2	3.63	$<$0.001	0.40	27
tartaric	0.2	3.70	$\sim$0	0.70	25
citric	5	2.80	0.002	0.10	2
	1	3.23	$<$0.001	0.30	9
	0.2	3.75	$\sim$0	0.80	38
	0.2	3.92	0.01 (NaCl) (a)	0.05	13
	0.2	3.97	0.1 (NaCl) (b)	0.02	7
	0.05	4.25	$\sim$0	1.00	65
glutamic	5	3.42	0.005	0.10	5
	1	3.70	0.001	0.35	13
	0.2	4.10	$<$0.001	0.70	43
MacroPrep 50 CM					
citric	1	3.25	0.01 (NaCl) (a)	0.03	10
	0.5	3.47	0.1 (NaCl) (b)	0	—

[a] calculated by "Acid-Base Calculator".[12]

Ionic strength in start buffer: a—0,01 (NaCl) and b—0,1 (NaCl).

longer. Similar gradient profiles were observed for all studied carboxylic acids.

Glutamic Acid

Glutamic acid solutions can also be used as eluents for generation of internal linear pH gradients. The pK_a values of glutamic acid, i.e., ($pK_{a1(COOH)}$), 2.19, $pK_{a2(COOH)}$ 4.25, $pKa_{(NH2)}$ 9.67; pI 3.22)[13] provides a buffering capacity for its solutions over a relatively broad pH range. In fact, linear gradients of 2.5–3 pH units have been obtained with glutamic acid solutions (Fig. 2). In the case of glutamic acid, the same regularities as for carboxylic acids were observed and an optimal pH gradient was obtained with 0.2 mM glutamic acid.

Effect of the Nature of the Eluent on the Gradient Profile

It is interesting to compare the pH gradients obtained for different acids, at equal concentrations, in the eluents (Table 2). The differences in pH gradient profiles can be clearly noted for 0.2 mM solutions (Fig. 3). The length of the first segment of the gradient profile before pH increase was the same for all studied eluents, but absolute values and length of pH increase at the second segment depends upon the nature of acid used. This pH increase was observed in the series from oxalic to acetic acid that is in accordance with the decrease in pK_a values for corresponding carboxylic acids.

Ionic strengths of equimolar solutions of carboxylic acids depend upon pK_a values of carboxyls in their molecules; so, the difference between ionic strengths of the starting buffer and the eluent of the same concentration varies. For example, the difference in ionic strength values between 0.2 mM acetic acid used as the eluent and start buffer is maximum. It leads to the highest degree of additional protonation of carboxyl groups in the cation-exchanger. Also, further extension of the second segment takes place. The most extended second segment with a pH increase of more than one pH unit in the effluent was observed for 0.2 mM acetic acid as the eluent (Fig. 3, curve *5*). For stronger carboxylic acids, like oxalic, the segment is shorter with an effluent pH increase in 0.4–0.5 units. (Fig. 3, curve 1; Table 2).

The pH decrease after the second segment is practically important to make the pH gradient smooth. The acid strength and pH range of its buffering capacity affects the slope of third segment of the pH gradient. The smooth segments of pH gradients were observed for glutamic and acetic acids (Figure 3, curves 4, 5), but in the case of acetic acid, the linear pH gradient was obtained over a relatively narrow pH range because of too big a difference between the pH of the acetate buffer in the eluent and the starting buffer.

How Can We Eliminate the pH Increase of the pH Gradient?

The "anomalous" segment with pH increase is undesirable for any linear pH gradient. As proposed,[6] it can be eliminated by adjustment of ionic strength in both the starting buffer and in the eluent. The best effect should be achieved under equal ionic strengths of these mobile phases.

This approach was checked with citric acid as an eluent (Figs. 4 and 5). The pH gradients obtained on a hypercrosslinked polystyrene carboxyl column have no pH increase at the second segment when the ionic strength of the starting buffer and eluent was equal to 0.01 (Fig. 4, curve *2*). At higher ionic strengths (0.1) pH gradient profile becomes more linear and smoother over a pH range broader than 3 pH-units (Fig. 4, curve 3). Similar results were also obtained for the column packed with MacroPrep 50 CM (Fig. 5). The gradient, shown in curve 2, can be considered as quasi-linear. Some distinctions in pH gradient profiles can be due to residual anion-exchange ability of hypercrosslinked polystyrene matrix.[14] Obviously, the increase in ionic strength of the eluent and starting buffer caused a partial dissociation of carboxyl functional groups of the cation-exchanger. So, in accordance with above proposed mechanism, no pH increase in the first portions of the effluent was observed (Fig. 5, Table 2).

The quasi-linear pH gradient formed with citric acid at high ionic strength (0.2) was applied for preparative separation of metal ions on MacroPrep

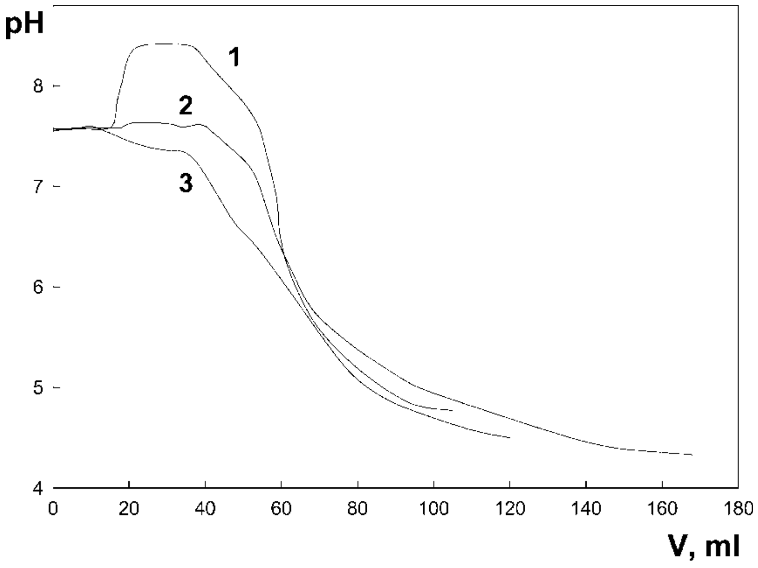

Figure 4. How to eliminate pH raise on gradient profile. Column: as in Fig. 1. Start buffer: 10 mM Tris-HCl - 5% CH_3CN (pH 7.5). Eluent—0.2 mM citric acid—5% CH_3CN. Ionic strength (NaCl) in both mobile phases: *1*—0, *2*—0.01, *3*—0.1.

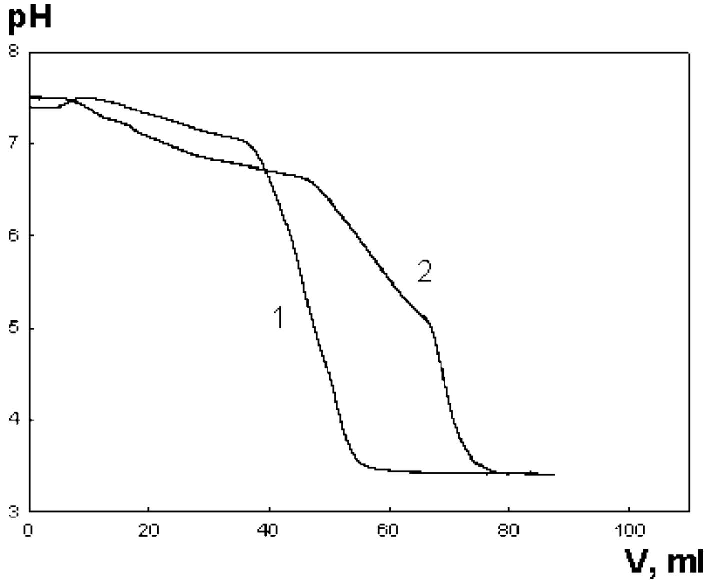

Figure 5. The verification of ionic strength effect on pH gradient profile. Column: 50 × 4.6 mm, MacroPrep 50 CM, 50 μm. Start buffer: 10 mM Tris-HCl (pH 7.5). Eluent—citric acid (*1*—1 mM, *2*—0.5 mM). Ionic strength created by NaCl in both mobile phases: *1*—0.01, *2*—0.1.

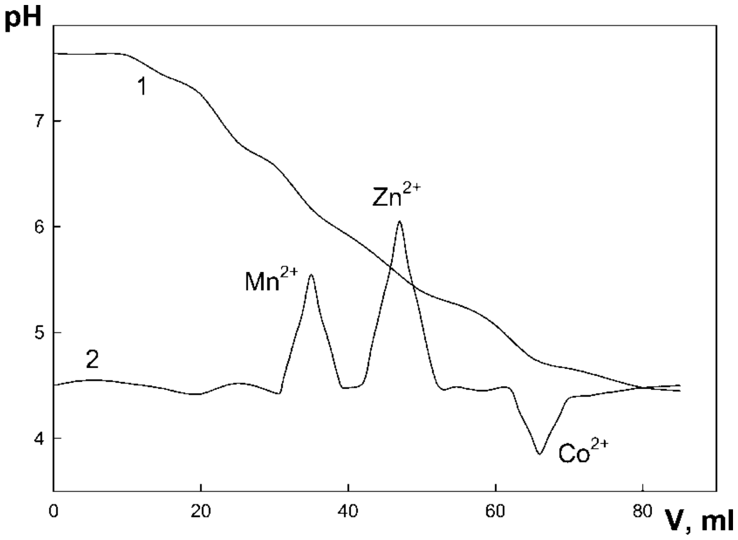

Figure 6. The separation of metal ions mixture with pH gradient elution. Column: 50 × 4.6 mm, MacroPrep 50 CM, 50 μm. Start buffer: 10 mM Tris-HCl (pH 7.5). Eluent—0.5 mM citric acid. Ionic strength (NaCl) in both mobile phases—0.2. *1*—pH gradient, *2*—optical density at 254 nm.

50 CM (Fig. 6). As was shown earlier under static conditions, MacroPrep is more selective towards metal ions than MN.[15] The retention of each metal ion (Mn^{2+}, Zn^{2+}, and Co^{2+}) with pH gradient elution was previously determined at 254 nm for individual solutions. The chromatographic peaks of the metal ions were symmetrical due to a focusing effect. Probably, the quite large particle size (50 μm) resulted in peak broadening.

CONCLUSIONS

The formation of a decreasing pH gradient within a carboxylic cation-exchange column was studied. The role of the eluent nature, its concentration, and ionic strength created by addition of strong electrolytes to the buffer was investigated and the crucial effect of ionic strength on the gradient profile was determined. The optimum linear or quasi-linear decreasing pH gradient in a cation-exchange column can be obtained with 0.2–1 mM citric acid, while the ionic strength of both starting and eluent buffers should be between 0.01–0.1 M. This quasi-linear pH gradient was applied for separation of the metal ions model mixture.

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