Control of Column Influence on the Wide Range pH Gradient in Ion-Exchange Chromatography

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Summary
A method for optimization of the mobile phase composition in ion exchange chromatography with a wide range external pH gradient (approx. over 5 pH units) has been formulated. It is shown that programming of various wide range pH profiles which are not impaired by buffer interactions of the mobile phase with sorbent is possible. Utilization of the wide range external pH gradient in ion-exchange chromatography of bovine haemoglobin is also demonstrated.

Introduction
Ion-exchange chromatography (IEC) is a method in which interactions between charged functional groups and ionic solute are the basis for adsorption. The strength of electrostatic interactions is mainly mediated by either the ionic strength or the pH of the mobile phase. IEC is known to be an efficient technique for the separation of charged macromolecules, e.g. proteins [1, 2].

When biological samples arc to be separated by IEC, gradient elution comes most often into consideration. The reason is that samples of biological origin are often quite complex, and different sample components may vary widely in their retention. Most applications of gradient IEC are realized by increasing ionic strength at fixed pH, see Refs. [1-4] and references therein. Since the proteins have the smallest retention at the eluent pH close to the protein isoelectric point, pl, [2], one would expect a wide use of the pH gradient for their separation. One reason for the relatively little acceptance of the wide range pH gradient in IEC can be that "... it is difficult to make a continuous pH-gradient using a simple apparatus owing to the buffer action of the elution buffer and of the ion exchange." [5]. However, the choice of the conditions for IEC of proteins with a pH gradient is still only empirical, e.g., IEC of haemoglobin in the gradient range over one to two pH units was realized on either weak or strong, anionic or cationic ion exchangers using either cationic or anionic, or both, buffers in the mobile phase, see e.g. reviews [6, 7]. Nevertheless, IEC of proteins with the wide range pH gradient was accomplished under conditions of internal pH gradient (chromatofocusing). In this mode of IEC, the interactions of the mobile phase buffer with the ion exchanger are advantageously used for the pH gradient generation. The mobile phase can be either a solution of a mixture of synthetic polyampholytes [8-10] or that of low-molecular compounds [11, 12]. The rules for the proper choice of the ion exchanger and mobile phase buffers were formulated [13] and a method for design of the internal pH gradient was suggested [14].

Theory
The method is described for a cation-exchanger, the similar treatment can be shown for an anion exchanger. A decrease of the migration velocity of some pH values along the column relative to the velocity of the mobile phase is caused by the buffer action between the elution buffer and the ion exchanger in the column. It holds for the migration velocity of a particular pH value along the column, \( u_{pH} \) (c.f. also Refs. [8, 10, 12, 13]):

\[
\frac{u_{pH}}{u_0} = \frac{a_m + a_s}{a_m} \quad (1)
\]

where \( u_0 \) is the average linear velocity of the mobile phase along the column, and \( a_m \) and \( a_s \) are the buffer capacities of mobile and stationary phases per unit column length, respectively. Values of \( a_m \) and \( a_s \) are those at the pH considered for the migration. For the ease of introduction of a pH step at the top of the column the retention volume of the particular pH value, \( V_{pH} \), is [14]:

\[
V_{pH} = V_0 \cdot \frac{u_0}{u_{pH}} \quad (2)
\]

where \( V_0 \) is the column dead volume.

Further, it holds:

\[
a_s/a_m = q \cdot b_s/b_m \quad (3)
\]
Here, \( b_m \) and \( b_s \) are the buffering capacities of the mobile and stationary phase, respectively and \( q \) is the phase ratio defined by the relationship:

\[
q = s/V_o
\]  

(4)

where \( s \) is the amount of the stationary phase within the column.

By insertion of Eqs. (2) and (3) into Eq. (1), we obtain after rearrangement:

\[
V_{pH} = V_o \cdot \left(1 + q \cdot \frac{b_s}{b_m}\right) \tag{5}
\]

The volume, \( V_{pH2} \), corresponding to the delivery of some \( pH \) value by the pump, is function of the programmed external \( pH \) gradient, generally, it is:

\[
V_{pH2} = f(pH) \tag{6}
\]

In practice, the gradient profile is programmed as a sequence of linear steps. The steepness, \( B \), of the linear \( pH \) gradient slope is:

\[
B = \frac{\Delta pH}{\Delta V} \tag{7}
\]

Further, there is always some delay between the programmed profile and the profile at the pump outlet. This is generally due to the volume, \( V_m \), between the gradient mixer and the pump outlet. Thus, the volume needed for the delivery of some \( pH \) value is:

\[
V_{pH} = (pH - pH_o)/B + V_p \tag{8}
\]

where \( pH_o \) is the \( pH \) value at the gradient start. The actual \( pH \) at the column outlet, \( V_{pH} \), can be obtained as a sum of \( V_{pH1} \) and \( V_{pH2} \):

\[
V_{pH} = V_o \left(1 + q \cdot \frac{b_s}{b_m}\right) + (pH - pH_o)/B + V_p \tag{9}
\]

The first term on the RHS of Eq. (9) represents the gradient delay caused by the column which is equal to \( V_{pH1} \). Minimizing the influence of the column buffer capacity on the overall gradient \( pH \) profile can be quantified by minimizing the second term in the parentheses in RHS of Eq. (9). The decrease of this term can be achieved by enhancement of the buffer concentration in the mobile phase (larger \( b_m \)) and/or by decrease in \( b_s \). It means that the buffer capacity of an ion exchanger should be the lowest possible. It helps to use the diluted solution of buffers in the mobile phase which favours the focussing of the ampholytic analyte in the \( pH \) gradient [8]. On the other hand, a high solute retention requires a large exchange capacity of the sorbent. In order to fulfil both requirements simultaneously, the strong ion exchanger should be preferred. The actual buffer capacity of the sorbent in the \( pH \) range of interest can be obtained from derivation of the sorbent titration curve.

Buffer components in the mobile phase should not be retained on the column packing in the \( pH \) range used. It means that the buffer components should possess either no charge or a charge with the same sign as the column packing. Components with a high solubility in the mobile phase should be used. The buffer capacity of the mobile phase should be high enough to maintain the deviation of the actual \( pH \) profile, at the column end, from the programmed profile acceptably small. The actual buffer capacity of the mobile phase within the \( pH \) range considered can be calculated from equation:

\[
b_m = 2.3 \sum C_i \cdot \left[10^{\text{pK}_a - \text{pK}_a} / [10^{\text{pK}_a} + 1] \right]^2 \tag{10}
\]

where \( C_i \) and \( pK_{ai} \) are buffer component concentration at its \( pK_a \).

It is advantageous to choose such compositions of mixed buffer solutions so that the linear correlation between the programmed volumetric composition and the \( pH \) profile leaving the pump is maintained. It can be achieved by maintaining the \( b_m \) of solution A (with starting \( pH \) constant in the \( pH \) range considered, while the solution B is concentrated enough not to contribute to the \( pH \) change by dilution. As follows from Eq. (10), the simplest way is to choose for the solution A equal concentrations of buffers with their \( pK_a \)'s differing by a constant value.

**Experimental**

**Instrument**

A liquid chromatograph PU 4100M (Philips, Cambridge, UK), equipped with a Model 7125 injection valve (Rheodyne, Cotati, CA, USA) and multichannel detector PU 4021 (Philips, Cambridge, UK), were used. The data collection and post run evaluation were controlled by PU 6003 diode array detector system (Philips, Cambridge, UK). An analytical 8 \( \mu L \) and/or microbore 1 \( \mu L \) flowcell was used and chromatograms at the wavelength of 412 nm were selected from the corresponding Chromascan data files.

The UV flowcell output was connected with a \( pH \) capillary flow-through electrode (Model OP-0745P, Radelkis, Budapest, Hungary) by means of a 20 cm long PTFE tubing (0.25 mm i.d., 1.6 mm o.d.). The effluent was collected in a 20 mL reservoir with an immersed capillary reference electrode (Model OP-0830P, Radelkis, Budapest, Hungary). A Model OP-208/1 digital \( pH \) meter (Radelkis, Budapest, Hungary) and a line recorder TZ 4200 (Laboratorní přístroje, Prague, Czechoslovakia) were used for monitoring of the \( pH \) signal.

**Column**

The 150 x 2 mm Tescs Separim HEMA-BIO 1000 SB ion exchanger column (Tescs Ltd., Prague, Czechoslovakia) was used as received. The column packing is spherical, 10 \( \mu m \) particle diameter, 75-150 nm pore diameter Separim HEMA copolymer which is modified by strongly acidic sulphotutyl groups [15]. Its ion-exchange capacity is in the range of 1.2 to 1.6 meq g\(^{-1}\). The column dead volume is 0.37 ml and the sorbent amount in the column is 140 mg. It gives the phase ratio \( q = 0.40 \) g ml\(^{-1}\).