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Short communication

# Characterization of unstable ion-exchange chromatographic separation of proteins

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**Abstract**

Very fine separation of proteins by stepwise elution ion-exchange chromatography is very often a unstable process. To characterize the unstability of such processes the elution volume variations were examined by the model equation which contained the ion-exchange capacity and the number of adsorption sites. The data needed for the model calculation were obtained from gradient elution experiments. As a model separation system stepwise elution of a model protein (β-lactoglobulin) near the isoelectric point on a weak cation-exchange chromatography column was chosen. The elution volume varied significantly with a small change in the ion-exchange capacity. It was found that the ionic strength of the elution buffer must be adjusted in order to compensate a change in the elution volume due to the ion-exchange capacity variations. The ionic strength and the pH of the elution buffer were also found to be important variables affecting the elution volume. In this model separation system, it was indicated that the pH should be within ±0.1 unit and the ionic strength within ±0.002 mol/l in order to meet the criteria (±5% elution volume variation). It is recommended that gradient elution data be obtained for predicting elution volume variations in stepwise elution. By using the gradient elution data the process diagnosis can be performed, and the important information on the process stability can be obtained. 

*Keywords:* Retention models; Mobile phase composition; Proteins

## 1. Introduction

Ion-exchange chromatography (IEC) is a very versatile protein separation method [1–6], and widely used as large production scale protein purification processes [2–4]. When IEC is used for high resolution separation of proteins, retention and peak broadening are not always reproducible. This is especially true for stepwise elution where the separation is very sensitive to small changes in typical operating factors such as pH, ionic strength and packing media (gel) ion-exchange capacity. As characterization of such unstable IEC processes is quite important, the process diagnosis by mathematical models must be performed so that possible fluctuations (variations) of elution volume and peak width with small changes in the above mentioned operating variables can be predicted or estimated. This is also useful for trouble shooting in the operation of the actual production processes as some variations of pH and/or ionic strength of the buffer solution from run to run, and lot to lot variations of ion-exchange capacity are unavoidable.

In this paper the effects of the ion-exchange capacity, the pH and the salt concentration on the elution volume in stepwise elution IEC were ex-

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amined on the basis of our retention model [4]. The data needed for the model calculation were obtained from gradient elution experiments [4]. From the model which was based on the ion-exchange equilibrium, the distribution coefficient was related to the effective ionic capacity and the number of adsorption sites. By using a model separation system (β-lactoglobulin near the isoelectric point on a weak cation-exchange gel column), the variations of the elution volume with the ion-exchange capacity, the ionic strength and the pH of the elution buffer were investigated. Factors affecting the variation of the ionic strength in preparing acetate buffer solutions were discussed and a method for preparing constant-ionic strength acetate buffer solutions was proposed.

## 2. Results and discussion

We chose a model separation system of β-lactoglobulin on CM-Sepharose at pH 5.2 [7]. Although the isoelectric point of this protein is 5.1–5.2, it is retained on both anion- and cation-exchange chromatography columns at pH around 5.2 [8–10]. From the ion-exchange equilibrium model [4,8,10–13] the following equation was derived [7].

$$K = K_e \Lambda^B I^{-B} \quad (1)$$

where $K$ = protein distribution coefficient, $K_e$ = equilibrium association constant, $\Lambda$ = effective total ion-exchange capacity, $I$ = ionic strength (NaCl concentration), $B$ = the number of sites (charges) involved in protein adsorption, which is basically the same as the '$Z$' number in the literature [2,8,12]. The values of $B$ (=2) and $K_e \Lambda^B$ (=0.1179) were obtained from the linear gradient elution experiment data [4,7,10,14]. In the actual purification processes, the column size, the flow-rate and the sample loading are usually fixed. So the important operating variables are the ion-exchange capacity due to a lot-to-lot variation, the salt concentration and the pH of the elution buffer which may vary from batch to batch (or run to run).

The stepwise elution is assumed to be performed with an elution buffer of $I_E = 0.1$ $M$, where $K = 11.8$ from Eq. (1). According to the manufacturer, the ion-exchange capacity $\Lambda$ of CM-Sepharose FF ranges from 90 to 130 μmol/ml-gel. As such, a lot-to-lot variation is often encountered in the actual production process, the influence of the variation of $\Lambda$ was investigated. Fig. 1 shows the relative elution volume $V_R/V_t$ calculated by Eq. (2) as a function of $\Lambda$.

$$V_R/V_t = \epsilon + (1 - \epsilon) K \quad (2)$$

Here $V_R$ = elution volume and $V_t$ = column bed volume. The void fraction $\epsilon$ was assumed to be 0.4. $V_R/V_t$ at $\Lambda = 110$ was set to be a reference value where the protein is eluted at 7.47 (column volume). The +5% and −5% values were also shown in the figures (Figs. 1–3). Even a 5% lot-to-lot variation of $\Lambda$ (105 or 116 μmol/ml gel) affects the elution volume significantly.

In order to elute the protein within ±5% of the reference elution volume value, it is needed to adjust the NaCl concentration of the elution buffer $I_E$. Fig. 2 shows the relationships between the relative elution volume and $I_E$. When the reference value curve **r** ($\Lambda = 110$) is compared with the low $\Lambda$ value curve (1: $\Lambda = 90$) and with the high $\Lambda$ value curve (2: $\Lambda = 130$), it is seen that the NaCl concentration must be adjusted ±0.015 mol/l so that the relative elution volume falls in the ±5% reference elution volume. The preparation of large volumes of the buffer solution at production processes sometimes results in a certain variation of the salt concentration and/or pH. Fig. 2 also indicates that the specification of the buffer salt concentration must be ±0.002 mol/l in order to meet the criteria.

The pH of the buffer solution is also an important variable affecting the elution volume. The ion-exchange capacity of CM-Sepharose (weak cation-exchange gel) decreases with pH below 6 [5]. The relative elution volume change with pH due to the change of the ion-exchange capacity $\Lambda$ is shown in Fig. 3. Although variations of the relative elution volume is smaller compared with those shown in Figs. 1 and 2, it is still important that the buffer pH should be prepared within ±0.1 pH unit. However, the effect of pH is much more complicated than the salt concentration or the ion-exchange capacity. The interaction between protein and ion-exchange ligands changes with pH especially near the isoelectric point. Namely, the values of $B$ and $K_e \Lambda^B$ as well as $\Lambda$ vary with pH. In addition, the method for preparing buffer solutions affects both pH and the salt concentration.

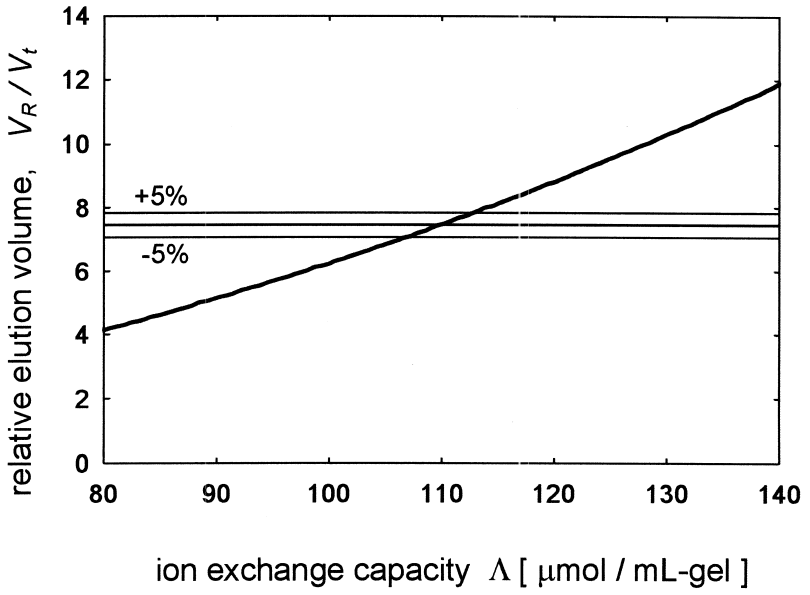


Fig. 1. Relative elution volume vs. ion-exchange capacity. The reference value = $V_R/V_t$ = 7.5, +5% value = 7.9, −5% value = 7.1. The same three curves are drawn in Figs. 2 and 3.

Often a large scale buffer solution is prepared by titrating acetic acid with sodium hydroxide. Consequently, a slight overtitration with caustic also increases the ionic strength. Unfortunately, both factors (pH and conductivity) influence the relative elution volume as mentioned above. This ionic strength shift is further amplified by chemical equilibrium effect in the buffer system [15–17]. The

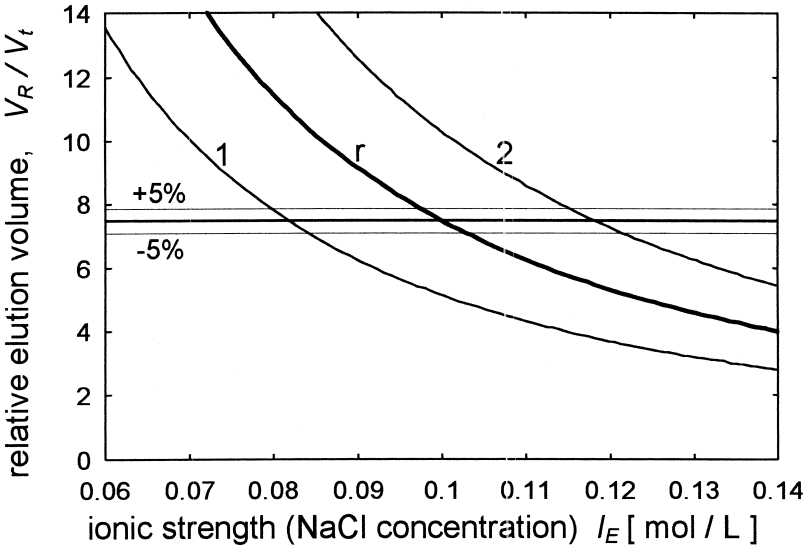


Fig. 2. Relative elution volume vs. ionic strength. Curve **r** = reference value $\Lambda$ = 110, curve 1: $\Lambda$ = 90, curve 2: $\Lambda$ = 130.

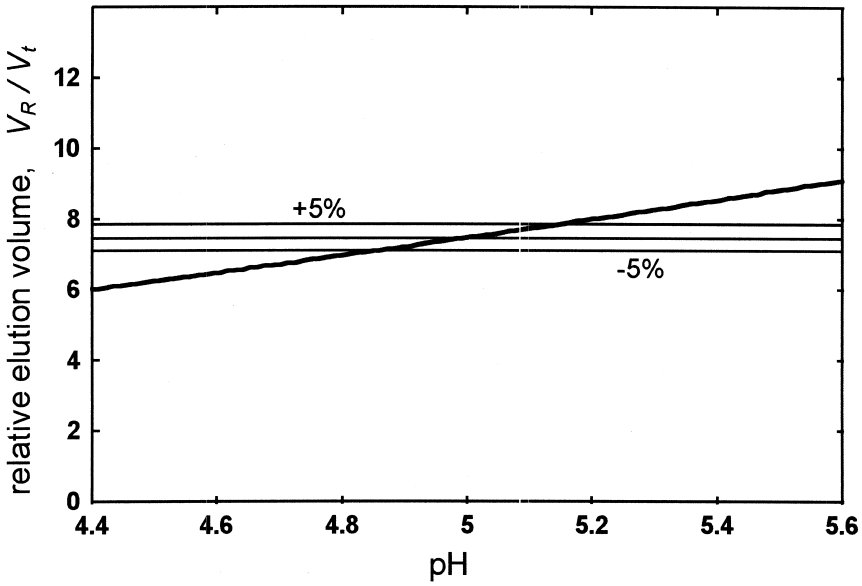


Fig. 3. Relative elution volume vs. pH reference value = pH 5.

following equilibrium is written for acetate buffer solutions

$$K_a = [H^+][A^-]/[HA] \quad (3)$$

where $K_a$ = dissociation constant (4.76 at 25°C), HA = non-dissociated acetic acid, and $A^-$ = acetate ion. For any pH value the ratio between dissociated and non-dissociated acetic acid is estimated. For increasing pH values, i.e. decreasing concentration of $H^+$ ions, the equilibrium is shifted to the side of the dissociated ions. As only the dissociated acetate ions contribute to the conductivity of the buffer, the titration with NaOH increases the ionic strength by the addition of $Na^+$ and the release of acetate ions. At pH 5.4 the buffer contains 84.5% ions and 15.7% non-dissociated acetic acid. Within pH specification of ±0.1 units the dissociation can change ±3%.

It is also a matter of propagation of error to expect higher variability for a procedure titrating with caustic than titrating with acetic acid. For the amount of acetic acid the titration starts with, a specification limit of ±1% is set. For the 20 m*M* acetic acid approximately 17 m*M* NaOH are needed for titration. ΔpH/Δ[NaOH] is approximately 0.17/m*M*. Consequently, for a specification range of ±0.1 pH units, the amount of NaOH will vary by approximately ±0.58 m*M*. For 17 m*M* NaOH, this is a range of ±3.4%. This is a notably extended range compared to being able to set it to ±1%. For a procedure starting with NaOH, the variability for NaOH can be reduced whereas the range for acetic acid increases. Fortunately, that does not change the ionic strength significantly (Fig. 4).

The buffering capacity $\beta$ can be calculated by the Henderson–Hasselbach equation [15–17]. The amount of strong acid or base [B] needed to cause an incremental change in pH is defined by

$$\beta = d[B]/dpH$$
$$= 2.303\{K_a c [H^+]/(K_a + [H^+])^2 + [H^+] + K_w/[H^+]\} \quad (4)$$

where $c$ = sum of concentrations of acetic acid and acetate ions and $K_w$ = ionic product of water. The second and third term are only significant below pH 3 and above pH 11. Fig. 4 shows $\beta$ as a function of pH (Fig. 4a) and the calculated ionic strengths of buffers prepared by the conventional and the proposed method (Fig. 4b).

Although gradient elution is much more robust compared with stepwise elution, the stepwise elution is preferred in production processes because of the simplicity of the operation and the equipment. The

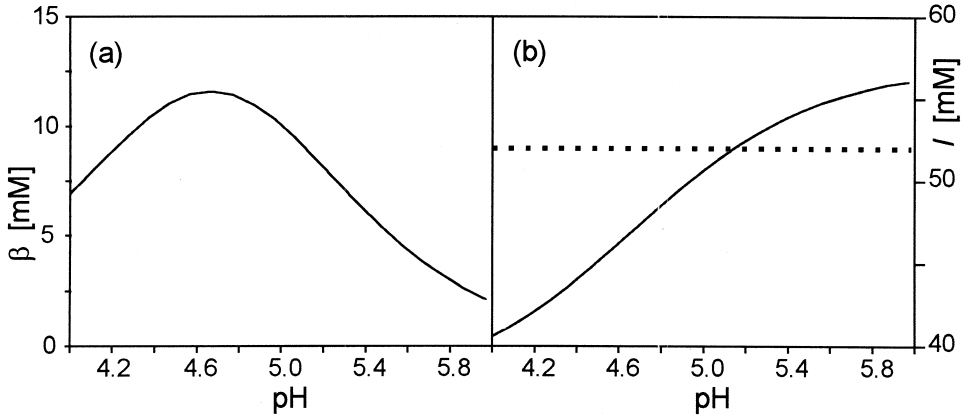


Fig. 4. (a) Buffering capacity $\beta$ of acetate buffer for different pH. (b) Calculated ionic strength of buffers [17] prepared by the conventional method (solid curve) and the proposed method (dotted curve).

stepwise elution conditions are very often sensitive to pH, ionic strength and ion-exchange capacity especially when very fine separation of the product protein from similar contaminants is performed as pointed out in this paper. Care must be taken to buffer preparation procedures as well as lot-to-lot variations of the ion-exchange capacity. Sometimes the salt concentration of the elution buffer must be adjusted in order to control the retention volume. This is usually done by trial and error. However, as shown in this paper, the distribution coefficient as a function of ionic strength can be obtained from gradient elution experiment data. Once this information is obtained, elution volume can be predicted. It will serve as a convenient tool for tuning and troubleshooting very sensitive and unstable stepwise elution ion-exchange chromatography processes.

## Acknowledgements

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