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Fundamental properties of isoelectric buffers for capillary zone electrophoresis

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Abstract

Different isoelectric buffers are analysed theoretically, taking into account a fundamental parameter, i.e., the ratio between intrinsic buffering power and conductivity ($R=\beta/\lambda$). For a model ampholyte, the above parameter is analysed both as a function of the pI and the pK_b–pK_a values. For natural pH gradients, the variation of R, connected with approaching the isoelectric point, is evaluated. A case of oligo-protic ampholytes is also considered. © 1997 Elsevier Science B.V.

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1. Introduction

The technique of isoelectric focusing (IEF), as originally envisaged by Svensson [1–3] in a series of now classical articles, requires a special set of background electrolytes, called ''carrier ampholytes'' (CAs). This term defines two intrinsic properties of such buffers, i.e., they have to be amphoteric and be a ''carrier'' of buffering power and conductivity at the pH of the isoelectric point (pI), a special requirement that very few amphoteric compounds possess [4]. Consider amino acids: The vast majority (in fact, all the mono-amino, mono-carboxylic compounds) are totally useless as buffers in IEF, since at pH=pI (which is ca. 6.0 for all of them) cannot buffer or conduct any current, because their titration curve is flat over four pH units centred on the pI (i.e. from pH ca. 4 to 8). So, the hallmark of a ''carrier ampholyte'' is the absolute value of $pI-pK_{prox}$ (or $\frac{1}{2}\Delta pK$); the smaller this value is, the higher the conductivity and buffering capacity (at pH=pI) of the amphotere. A $\Delta pK=\log 4$ (i.e. $pI-pK=0.3$) would provide an incredible relative buffering power (β) at the pI, i.e. 2.0. A $\Delta pK=\log 16$ (i.e. $pI-pK=0.6$) offers a β value of 1.35 at pH=pI (note, however, that in the classical Svensson–Rilbe theory, the β power and conductivity of bulk water are neglected).

Such CA buffers possess another highly desirable property: At steady-state, they provide a medium of quite low conductivity (if compared with standard buffer media for zone electrophoresis), thus, they are compatible with very high voltage gradients along the separation axis. However, the use of such isoelectric buffers has not been explored much in the electrophoretic literature outside their natural use, i.e. for isoelectric focusing [5]. Nevertheless, recently, there has been an increasing number of articles on

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their use for zone electrophoresis. Perhaps one of the earliest reports has been the one of Mandecki and Hayden [6], who reported slab gel electrophoresis of nucleic acids in isoelectric His. Thus, in this experimental set-up, not a multitude of isoelectric buffers (as typical of IEF), but just a single amphoteric substance, dissolved in water, is adopted, this compound being the sole component of the background electrolyte and, therefore, being in a state very close to its isoelectric point. This concept was not noticed in the general literature of electrophoresis until, in 1995, Hjertén et al. [7] proposed it for capillary zone electrophoresis (CZE). In this paper, several ways of selecting buffers with low electric conductivity and ''yet satisfactory buffering capacity'' were proposed. In addition, a number of parameters were advanced for designing suitable buffers. Among them: (a) buffer constituents with relatively high M_rs and few charged groups (thus with low mobility); (b) ampholytes with relatively small pK values, this latter property in accordance with the Svensson–Rilbe requirements [1–4]. Their results with protein separations have been modelled also by Blanco et al. [8], who obtained simulated profiles in close agreement with the experimental ones of Hjertén et al. [7]. The use of such isoelectric buffers was proposed again in a capillary format by Gelfi et al. [9] in the analysis of oligonucleotides. In 75 mM His, these authors could obtain excellent separations of eighteen-mer antisense oligonucleotides and of failed and -truncated sequences thereof, in only 4–5 min of electrophoresis in 10% liquid linear polyacrylamide at voltages as high as 800 V/cm (as opposed to 25–30 min in standard TBE buffers). More recently, Righetti and Nembri [10] generated peptide maps in isoelectric aspartic acid and have shown that such maps could be developed in only 8–10 min, as opposed to 70–80 min in the standard phosphate buffer, pH 2.0, with much superior resolution.

In view of an expanding role of such buffers in CZE, here we are modelling the fundamental properties of these compounds. We have found that the intrinsic properties of ampholyte solutions are not adequately described with the help of the ΔpK value only and we propose here a new parameter, R, representing the ratio between buffering power and conductivity ($R=\beta/\lambda$). The calculations of this ratio have to take into account the contribution of water ions. In this article, we analyse this parameter as a function of the ampholyte's isoelectric point along the pH scale and as a function of ΔpK.

2. Theory

2.1. Notations

K_a, K_b	dissociation constants of the acidic and basic ionogenic groups of an ampholyte, respectively
[H]	hydrogen ion concentration
K_w	ion water product
C	analytical concentration of ampholyte (mol/l)
β	buffer power of the ampholyte solution in water
λ	conductivity of the solution in relative units
$R_{\beta/\lambda}$	buffering power to conductivity ratio

For any system that contains an arbitrary number of dissolved protolytic species, with known dissociation constants, by solving the system containing the electroneutrality equation and the dissociation equations for all of the acidic and basic groups, we obtain the equation that determines the hydrogen ion concentration, with the total degree depending on the total number of ionogenic groups:

$$[H]-\sum_i \frac{K_{ai}C}{[H]+K_{ai}}+\sum_i \frac{[H]C}{[H]+K_{bi}}-\frac{K_w}{[H]}=0 \qquad (1)$$

In the most common cases, we have an equation of the $N+2$ degree relative to [H], where N is the total number of groups capable of dissociation. The latter groups may be arbitrarily distributed within the number of substances present in solution. In the case of only two ionogenic groups (e.g. the simplest ampholyte, or a couple composed of a weak base and a weak acid), the degree of the resulting equation is four.

The expression for the buffering power (β) may be obtained by directly applying its definition in the form:

$$\beta = \frac{dc}{dpH}$$

where c is the concentration of strong basic titrant (or is the negative value of the concentration in the case of strong acid). By applying this procedure to Eq. (1), rewritten in the presence of titrant, we will have:

$$\beta = 2.303 \left([H] + [H]C \left(\sum_i \frac{K_{ai}}{[H] + K_{ai}} + \sum_i \frac{K_{bi}}{[H] + K_{bi}} \right) + \frac{K_w}{[H]} \right). \quad (2)$$

The conductivity is calculated according to the following definition:

$$\lambda = \sum_j C_j M_j Q_j \quad (3)$$

The mobility of each ion M_j is supposed to be proportional to its electric charge, Q_j and the latter, for an ampholyte molecule, can be written as:

$$Q = \sum_i \frac{[H]}{[H] + K_{bi}} - \sum_i \frac{K_{ai}}{[H] + K_{ai}} \quad (4)$$

The relative ampholyte mobility was taken as being 0.05 (in hydrogen mobility units) and the H/-OH mobility ratio was taken as being 1.7, as suggested in [12].

3. Results of simulations

3.1. Dependence of R (β power to conductivity ratio) from the pI value of a model ampholyte

Whereas pure water has a rather uniform R-ratio throughout the pH range (were it not for the mobility difference between the hydrogen and hydroxyl ions, it would in fact be an absolute constant), this ratio, when calculated for an arbitrary ampholyte without a water contribution, must have a strong (infinite) maximum at the isoelectric point. Note that, whereas both the buffering power of water and the water conductivity contribution have a tendency increase to infinity at pH extremes (see, e.g., Figures 4, 6 and 7 in Ref. [13]), these two values for an ampholyte are finite (here we consider β and λ separately, not their ratio!). Taking into consideration the fact that the ampholyte concentration is always negligible in comparison with that of water, we intuitively may expect that an acceptable property, with the point of view of a high R-value, is satisfied only in pH regions not so very far from neutrality.

Fig. 1 illustrates the behaviour of R, as a function of the relative position of its pI value along the pH scale, for a model ampholyte with only two ionogenic groups, possessing a very good buffering power ($\Delta pK = 1$) (the point of real interest is the R-value that corresponds to the real pH of the ampholyte solution; as a first approximation, we will assume that the real pH of this solution will coincide with the pI of the ampholyte; later on, this question will be discussed in detail). We can see substantial decrements of the R-ratio as the pI of the hypothetical ampholyte progressively decreases from p$I = 5.0$ to 4.0. For ampholytes with more ionogenic groups, we may have the very same tendency. However, the presence of an additional ionogenic group may result

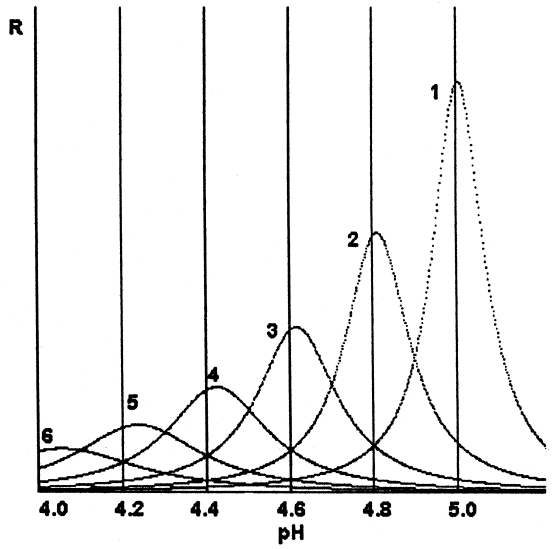

Fig. 1. Behaviour of the R (β/λ) parameter as a function of pH at different values of pI for a model ampholyte with $\Delta pK = 1$. Calculations were performed at ampholyte concentrations of 50 mM. The curves are numbered in order of decreasing pI values, at decrements of 0.2 pH units. Note the drastic decrease of the R parameter mainly due to increments in the conductivity of the bulk solvent, simply in going from pI 5.0 to 4.0.

in higher levels of buffering power and, also, in sharper conductivity decrements in the vicinity of the isoelectric point, which would bring about higher *R* maxima. This effect obviously depends on the total number of additional groups and on their p*K* values. However, if we consider an ampholyte with three ionogenic groups, where the new additional group is removed from the ampholyte's p*I* more than the other two (or where all of the p*K* values are rather far from each other), its influence will be rather negligible. For example, numerical calculations show very small differences between the *R* values for the following two series of ampholytes: ($pK_{a1}=5$, $pK_{b1}=7$; and $pK_{a1}=7$, $pK_{b1}=9$; $pK_{a2}=5$); ($pK_{a1}=4.5$, $pK_{b1}=6.5$; and $pK_{a1}=4.5$, $pK_{b1}=8.5$; $pK_{a2}=6.5$); ($pK_{a1}=4$, $pK_{b1}=6$; and $pK_{a1}=4$, $pK_{b1}=8$; $pK_{a2}=6$)... etc. (these ampholyte pairs exhibit essentially no differences in their respective p*I* values and Δp*K* parameters).

3.2. Dependence of R value on the pI and ΔpK of a model ampholyte

As a useful tool for selecting an adequate isoelectric buffer, let us consider a three-dimensional surface, which represents the *R*-ratio both as a function of p*I* and Δp*K* (Fig. 2). We can see that, in the vicinity of neutrality, even ampholytes that are usually treated as ''poor'' or even ''very poor'' (i.e. with large Δp*K* values), are able to provide an acceptable level of buffering power to conductivity ratio. On the contrary, at pH extremes, no ''good'' value of Δp*K* is able to compensate for *R* deterioration, due to the main contribution of bulk water. In addition, *R* falls off more rapidly towards pH 3 than towards pH 11, due to the higher mobility (thus conductivity) of protons compared with -OH ions. Note that this graph is, to a certain extent, an approximation, since it gives the *R* value precisely at the ampholyte's isoelectric point, rather than at the pH value of the real solution, but, for the purpose of selecting the buffer, we may neglect this difference.

Fig. 3 represents the deviation of pH from p*I* as a function of both changes in the ampholyte's p*I* and Δp*K*. We explore, in the abscissa, a series of p*I* values going from p*I* 7.0 to 1.0. The ordinate (pH − p*I*) gives the expected deviation of the solution's pH from the real p*I* of the ampholyte (in all case, the ampholyte's concentration in solution is kept constant at $C=50$ m*M*). The four curves show these deviations for four model ampholytes, as follows: I = Δp*K* of 0; II = Δp*K* of 1; III = Δp*K* of 2 and IV = Δp*K* of 3. It is seen that, as long as the ampholyte's p*I* is located at any pH value from ca. 4 up to pH 7, the four curves coincide and are located on the abscissa, i.e. there is no (or negligible)

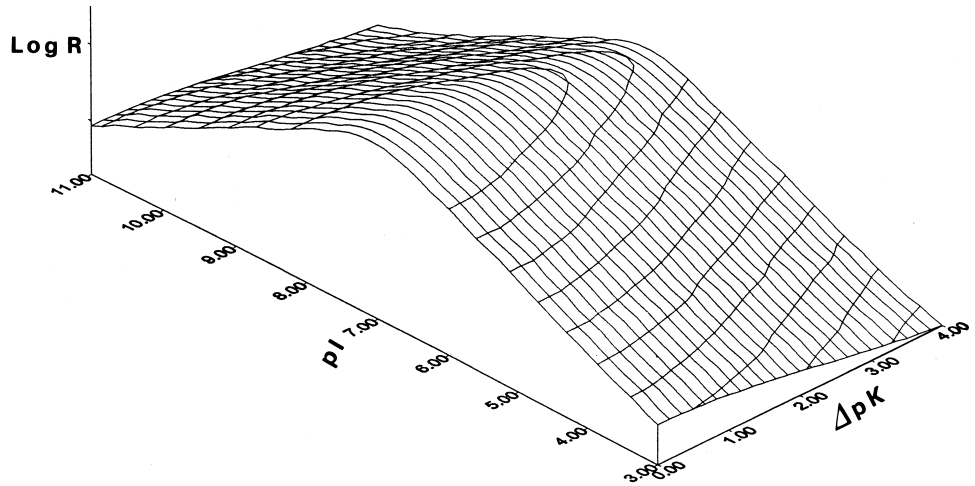

Fig. 2. Three-dimensional representation of the variation in the buffering power to conductivity ratio (*R*), calculated at the isoelectric point, as a function of p*I* and Δp*K*, for a constant concentration ($C=50$ m*M*) of the ampholyte. Note how *R* decreases more sharply towards pH 3 compared to alkaline pH values, due to the higher mobility of protons.

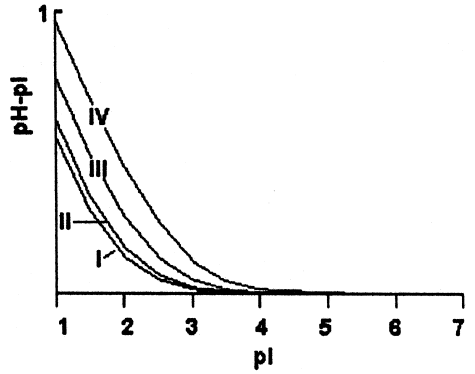

Fig. 3. Deviation of pH from the isoelectric point for a solution with a fixed ampholyte concentration ($C=50$ mM), as a function of the ampholyte's pI value (in the pH range of 1 to 7) at different values of ΔpK. The four curves are for $\Delta pK=0$ (I), $\Delta pK=1$ (II), $\Delta pK=2$ (III) and $\Delta pK=3$ (IV).

deviation from the expected pI value, no matter how ''good'' (ΔpK values of 0 and 1) or ''poor'' (ΔpK values of 2 and 3) such ampholytes are. However, as soon as the ampholyte's pI reaches a pH value of 4.0 or below, the four curves diverge substantially and the deviation becomes more and more pronounced as the hypothetical pI values reach pH 1.0. For example, an ampholyte with a $pI=1.0$ and a $\Delta pK=3$, if dissolved at a 50 mM concentration, will not give a pH of 1.0 in solution (as, in principle, it is expected to) but of 2.0 (see curve IV). However, under such adverse pH conditions, even an ''extremely good'' ampholyte ($\Delta pK=0$, something that could not exist in a real chemical compound!) does not fare much better; the pH of a 50 mM solution will not be 1.0 but 1.5 (see curve I).

3.3. R changes during the formation of a natural pH gradient

3.3.1. R increases with ampholyte concentration

As mentioned above, at infinite ampholyte dilution, R, must approach the appropriate value of pure water. With an increase in concentration, we should expect a progressive R growth, which, at considerable ampholyte concentrations, will tend to become linear, since the conductivity of the solution has a tendency to stabilise (see Eqs. (1) and (2)).

Let us consider the graph representing the set of R-curves with progressively increasing concentrations (at regular increments of 5 mM) in a narrow pH range (from pH 3.75 to pH 4.25; see Fig. 4) for a hypothetical ampholyte having a pI value of 4.0 and $\Delta pK=1$. The appropriate values of R, which correspond to the real pH of the solution for each ampholyte concentration, are visualised by black circles. It is thus apparent that the pH of the solution keeps deviating from the theoretical pI of the amphotere as a function of its concentration. At progressive dilutions, this deviation is quite appreciable; thus, when C=5 mM, the pH is 4.05.

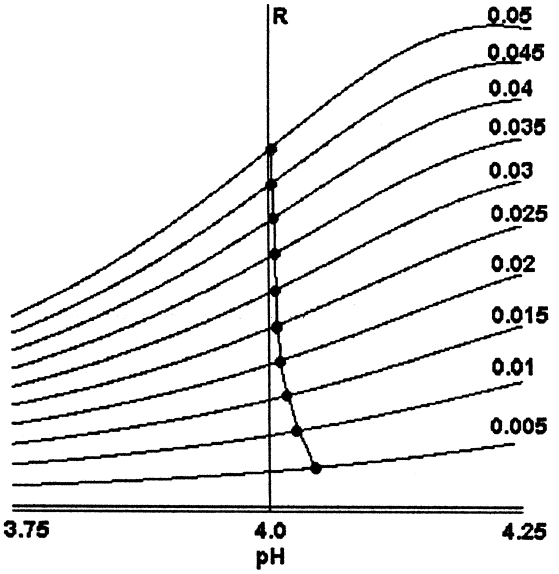

Fig. 4. Behaviour of the R ratio as a function of ampholyte concentration. The set of curves shows the variation of R as a function of pH at different values of ampholyte concentration, ranging from 5 up to 50 mM in increments of 5 mM. Simulations performed for an ideal ampholyte of $pI=4$ and $\Delta pK=1$. The black circles represent the deviation of the solution's pH from the theoretical pI at progressive ampholyte dilutions.

Fig. 5 shows the dependence of R on the concentration (in a log scale) of the same ampholyte with a pI value of 4.0 and $\Delta pK=1$. The line parallel to the abscissa corresponds to the R value of pure water and, in this example, this value is practically obtained when the ampholyte concentration in solution falls below the 1 mM value.

3.3.2. ''Ideal'' value of R for each ampholyte and R ''deterioration'' when approaching the pI

When considering the relationship of any physical

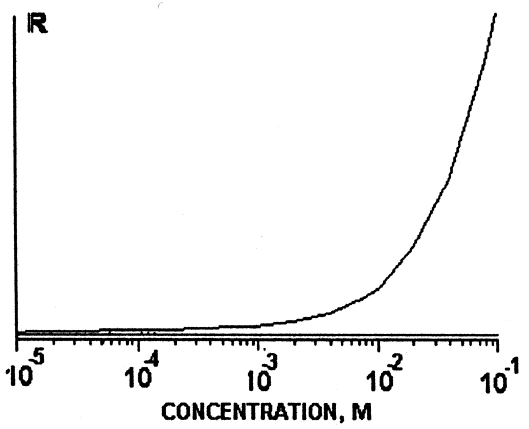

Fig. 5. Behaviour of the R ratio as a function of ampholyte concentration for a model ampholyte with pI=4 and ΔpK=1. Note that, even in the case of a good carrier ampholyte, the value of R is already extremely low for $C<10$ mM.

parameter, for a chemical system with a given composition, with pH, we have to remember that only the value of the real pH of the system will have a real physical sense. Nevertheless, since both the buffering power and the conductivity are additive values, the analysis of such properties for a single component in a wide pH interval is a useful tool in predicting the final properties of the system. If we consider now the set of curves representing R vs. pH for one ampholyte (Fig. 6a), we will see that the R maximum approaches the theoretical pI value, as the concentration increases. The simulation here refers to a model ampholyte with pI=4.0 and ΔpK=1, having concentrations ranging from 5 mM (lowest curve) up to 50 mM (uppermost curve) at 5 mM increments. At any ampholyte level, the point of the real pH of the ampholyte solution is situated between the pH that corresponds to R_{max} and the theoretical pI value of the species. This results in, at a first glance, a somewhat paradoxical situation, namely, when approaching the isoelectric point, the R value, calculated for one ampholyte, deteriorates to some extent. This effect is illustrated in Fig. 6b, which represents the ratio of two R values, one calculated at the real pH of the solution (ideal R value, R_{id}) and the other assessed at pH=pI (R^{pI}). We can see a visible deterioration at progressive ampholyte dilutions. Moreover, no ampholyte is able to achieve its pI value in pure water without some amount of

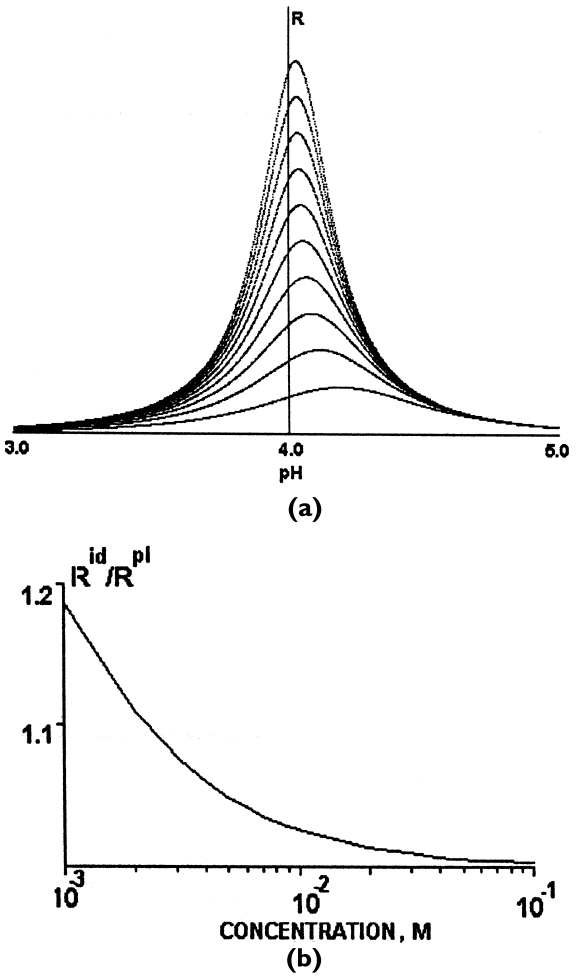

Fig. 6. Deterioration of the R parameter for a single ampholyte connected with the approximation to its pI in comparison with the ''ideal value''. (a) R maximum shift and pH shift with concentration for an ampholyte with pI=4 and ΔpK=1, in the 5 mM (lowest curve) to 50 mM (uppermost curve) concentration range, at 5 mM increments. (b) Ratio of the R value at the pH that corresponds to the pH of the ampholyte dissolved in pure water (R_{id}) to the R value at pH=pI (R^{pI}) (the calculations are performed under the same conditions as in Fig. 5).

titrant. In a natural pH gradient (i.e., under isoelectric focusing conditions), this role is played by neighbouring ampholytes, which are not precisely in their isoelectric state (due to the law of pH monotony, the Gaussian profiles of ampholytes at steady state have to overlap to some extent). This mechanism, in addition, must deteriorate the local value of R to some extent, since, not being in the isoelectric

state, such ampholytes provide an additional contribution to the local value of conductivity.

3.3.3. Mixture of several ampholytes

We may write the expression for R (in the case of a mixture of two components) as:

$$R = (C_1\beta_1 + C_2\beta_2)/(C_1\lambda_1 + C_2\lambda_2) \quad (5)$$

where C_1 and C_2 are the analytical concentrations. By introducing the term $k_\beta = \beta_2/\beta_1$, Eq. (6) can be rewritten in the form:

$$R/R_0 = (1 + sk\beta)/(1 + sk\beta/t) \quad (6)$$

where the symbol s denotes the C_2/C_1 ratio, and t stands for R_2/R_1. We can thus see that, by adding a small amount of ampholyte with a good R parameter, it is possible to improve the final R value for the mixture, provided that the second substance has a comparable (or better) buffering power. In the opposite case (negligible β, despite a high R value), essentially no improvement is obtained. When performing the qualitative evaluation of the properties of a mixture in this way, one should remember that, for each new added component, the buffering power and conductivity contributions should be taken into account, while the water contribution should be neglected (since it was already done with the first ampholyte). Also, the R values are to be taken at the resulting pH of the ampholyte solutions.

4. Discussion

The R-parameter of the buffer in which the electrophoretic analysis is to be performed is a matter of great importance, if one is interested in substantially reducing the analysis time. Each electrophoretic system has at least two important characteristics that restrict the opportunities of the experimenter, i.e. the maximum voltage applied V_{max} and, for a fixed capillary diameter, the maximum electric power that could be dissipated without any negative effects, P_{lim}. The R value gives us an opportunity to evaluate the suitability of any buffer substance starting from the minimal buffering power, β_{min}, required in a real experiment (this value depends on the substance to be separated, its concentration and, also, on some peculiarities of the separation process). We can thus write the following inequality:

$$R > \beta_{min}(V^2_{max}/2P_{lim}) \quad (7)$$

which links a desirable R value to the required β_{min} for the separation and to the ratio of V_{max} to P_{lim}. The utilization of CA buffers has some characteristic features, as we may see from the above considerations. Since, when approaching the isoelectric point value, we have some deterioration (see Section 3.3.2) in the local value of R, a system composed of a series of pure substances will possess some advantages, but this configuration is unfortunately unstable. The initial mixture of CAs is, as mentioned above, a rather bad buffer from the point of view of R, and a dramatic improvement can only be achieved with strong concentration increments of each ampholyte in its appropriate pI region, which will depend on the local electric field strength and on how steep the slope of the ensuing pH gradient is. Also, after formation of the pH gradient, we may obtain an essentially non-constant R profile. Aside from the above effects, some improvement in resolution may be achieved due to the stacking effect that takes place when the substances to be analyzed are migrating by zone electrophoresis against a stationary pH gradient as the background electrolyte.

Another interesting observation follows from our simulations: It is not true that, by adopting a single isoelectric buffer, one has to necessarily work at a fixed pH value (which would greatly limit the usefulness of the technique). One basic rule should be remembered: The pH produced in solution by an ampholyte has two limits; on the one hand (the upper limits for an acidic, the lower limits for an alkaline amphotere) is located the pI of water (i.e., pH 7.0), on the other hand, the true pI of the ampholyte. These two limits can be reached by modulating the concentration of the amphotere in solution [11]. At extreme dilutions (practically useless, of course, since the β power would approach that of water!), the pI of the ampholyte will be that of water, i.e. pH 7.0. At the correct concentration (which is not a universal value, as we have seen, since it depends on ΔpK, i.e. on how ‘‘good’’ or ‘‘bad’’ the carrier ampholyte is and on where the pI is located along the pH scale!), the pH in solution will approach the

p*I* of the amphotere. One could exploit this subtle rule for implementing separations that would not otherwise occur at a fixed pH value. A case in point has been shown by Righetti and Nembri [10]: By lowering the concentration of Asp, used as the buffering ion in their peptide maps, from 50 m*M* (pH 2.77) to 30 m*M* (pH 2.97), one can in fact move the pH of the background electrolyte by as much as 0.2 pH units. With this simple modification, one could move along the pH/mobility curves of the different peptides and find a pH window were no nodal (or cross-over) points exist among all of the curves, thus ensuring separation of otherwise inseparable peptides.

4.1. Note added

One referee questioned the validity of introducing the novel parameter $R_{(\beta/\lambda)}$ for assessing the performance of isoelectric buffers. The data presented in Ref. [13] should dispel such doubts. When comparing Lys and His as isoelectric buffers for oligonucleotide separations, one would be tempted to prefer Lys (if using only the β power criterion as the selection parameter) since, on an equimolar basis, Lys has a five-times higher β value. However, when re-assessed according to our novel $R_{(\beta/\lambda)}$ parameter, His would appear to be decidedly superior. Experimental electropherograms reported in Ref. [13] indeed were of better quality in His than in Lys buffer. Interestingly, His has also found a unique application in the high-speed separation of very large DNA fragments: In 178 m*M* His buffer, containing 0.7% hydroxypropyl cellulose, 10 min separations could be obtained by pulsed-field CZE of DNAs in the 72 to 166 000 base pairs (bp) range in the absence of aggregation, a most vexing phenomenon typically occurring with such large DNAs [14].

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