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Properties of buffer systems with charges immobilized on a gel matrix and their potential use in capillary electrophoresis

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Abstract

Some physicochemical properties, such as conductivity and buffering capacity, are evaluated and compared for two types of buffer systems: soluble, amphoteric, isoelectric species and immobilized weak buffering ions and counter-ions grafted onto a polyacrylamide gel (Immobiline technology). Theoretical calculations are performed for a model system comprising two Immobilines in a wide range of concentrations and compared with the properties of model ampholyte solutions. The results obtained show that the conductivity of any system composed of ampholytes dissolved in pure water is mostly connected with the contributions of the hydrogen and hydroxyl ions, and that the intrinsic ampholyte contribution is negligible at any value of concentration, although it increases when the ampholyte isoelectric point (pI) is removed from neutrality. The buffering capacity behaviour is also analysed in the range of small ampholyte (Immobilines) concentrations as a function of ΔpK values (i.e., how far the pK values are removed from the pI of the ampholyte). Due to the high flexibility in the preparation of isoelectric, Immobiline buffers (which can cover essentially any pH value), their potential use as background electrolytes for separations in capillary electrophoresis is evaluated. © 1998 Elsevier Science B.V.

Keywords: Buffer composition; Isoelectric buffers; Conductivity; Buffer modelling

1. Introduction

In recent times, isoelectric buffers possessing low conductivity have been exploited more and more frequently as background electrolytes in capillary zone electrophoresis (CZE) for the separation of different bioorganic substances [1–5]. This technique was reported initially in a flat bed configuration [6] and, at present, is gaining acceptance in capillary electrophoresis [7,8]. Since we are dealing with such a highly popular subject, there comes a time to give

a precise definition of ‘isoelectric buffers’. In a more general definition, we can state the following: “a buffer composed of one or more amphoteric substances, each of them being at or rather close to the isoelectric point and thus possessing very low electric mobility”. Thus the main characteristic feature of such buffers is the absence of relatively highly charged mobile particles, contributing to the conductivity (provided that such buffers do not contain any titrants, salt ions and non-isoelectric ampholytes as well).

As initially proposed, a buffer of this type represented a water solution of a single ampholyte. The extension to the case of a mixture of several am-

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photic substances (e.g. two or three different carrier ampholytes (CAs), or to a multitude of CA species, is possible, provided we have achieved a stationary, or quasi-stationary state during the electrolysis of our solution). As a consequence, the system will reach a stationary state characterized by a rather low conductivity level, thus compatible with high voltage gradients without Joule overheating, with the consequence of much reduced analysis times, which in turn favour high resolution. The general theory and fundamental properties of such isoelectric buffers have been given by us recently [9,10]. We have introduced a new parameter $R_{\beta/\lambda} = \beta/\lambda$, i.e. the buffering power to conductivity ratio, which provides an opportunity of evaluating the potential application of different isoelectric buffers in CZE.

Returning to the definition given above, it should be noted that it applies to soluble amphoteric buffers which, when used as single species, represent a particular case in the general theory of isoelectric focusing (IEF) [11], as they permit to work at a single value of the pH scale, rather than along a pH gradient, as is customary with IEF. However, in 1982, immobilized pH gradients (IPGs) were introduced [12], in which non-amphoteric buffers, comprising weak acrylamido acids and bases, are grafted onto a neutral backbone composed of acrylamide and cross-linker (*N,N*-methylenebisacrylamide). The IPG methodology represents a major advance in the field of focusing techniques, since it overcomes all the intrinsic problems of conventional IEF (pH gradient instability, irregular buffering capacity profiles, inability to produce very shallow pH gradients). Formally, the ‘immobilized system’ does not satisfy the above definition precisely; nevertheless, such systems do not contradict the main idea of ‘isoelectric buffers’, since they contain neither mobile non-isoelectric species nor mobile molecules (except for H^+ and $-OH^-$). Thus, generalising the above definition, we may write a new definition for isoelectric buffers as follows: “these systems may contain as mobile ions nothing but (quasi-)isoelectric ampholytes (if any), in addition to water ions”.

In the present report, we consider some properties of ‘immobilized isoelectric buffers’ in comparison with conventional, soluble amphoteric species. Although such immobilized buffers have not been used

in CZE as yet, they certainly represent a novel background electrolyte worth exploring. Additionally, they have been recently proposed as coating materials for the inner capillary wall, since they can provide ‘tuneable’ positive and negative charges which can be modulated by the pH of the buffer bathing the wall and can offer reproducible charge densities and thus reproducible electroosmotic flow inside the capillary lumen [13].

2. Model description

The general approach to ampholyte dissociation was formulated in Ref. [14]. In that report, we concluded that the ampholyte dissociation scheme should be treated within the framework of highly relaxing models. That means that, despite the many possible different protolytic states A_i (with electric charge q_i , and relative concentration c_i), which an ampholyte can exhibit in solution, the conductivity must be determined with the following equation:

$$\lambda_a \approx \mu_a C^T \left[\sum (c_i q_i) \right]^2 \quad (1)$$

where μ_a is the effective mobility of the single ampholyte molecule, which may depend on the total charge, and C^T its analytical concentration, rather than with the expression:

$$\lambda_a \approx C^T \sum \mu_{a_i} c_i (q_i)^2 \quad (2)$$

where μ_{a_i} are the relative mobilities of each proteolytic form.

The latter expression contradicts the fact that we observe a phenomenon of ampholyte focusing, and it gives overstated values for the ampholyte conductivity contribution.

The concentrations of each protolytic state are determined by the dissociation scheme. In the case of a biprotic ampholyte, the scheme of parallel dissociation is free from any contradiction although, in some particular cases, the practical application of the scheme with stepwise dissociation does not generate considerable errors [15]. In this work we used the variant of an independent dissociation scheme [14]. Calculations were performed by taking into account

the contribution of water ions and the ratio of hydrogen/hydroxyl ion mobility was assumed to be 1.85. The relative ampholyte mobility was taken as 0.05 in hydrogen mobility units.

3. Results of simulations

3.1. Conductivity of conventional 'isoelectric buffers' and 'immobilized' buffer systems

From the point of view of calculating the buffering capacity (β), there is no difference whether the ionogenic groups are immobilised on the gel matrix, located in the same molecule, or distributed within different ones, since in all cases they are subjected to the additive summing of the above value. For the simple case of only two ionogenic groups, the only difference is the 'rigid concentration ratio' between them (1:1) for soluble, individual ampholyte vs. complete freedom of manipulation in any Immobiline system (in IPGs, a buffering ion is typically titrated with a counter-ion Immobiline within ± 0.5 pH units centred on the pK value; this allows the generation of a vast number of 'polymeric ampholytes' with widely different buffer/titrant ratios).

With regard to the conductivity, however, the situation is quite different, since an ampholyte molecule is capable of maintaining an electric current whereas, in any Immobiline system, the conductivity is due to contribution of water ions (H^+ and $-OH^-$) only. The shape of the ampholyte conductivity curve versus pH is determined by the ΔpK values (Fig. 1). The simulation is performed for a series of ampholytes all having isoelectric point (pI) = 4.0 and progressively wider ΔpK values (from 0 to 4). Note that, paradoxically, at $pH = pI$, there is a conductivity minimum since the molecule should bear zero net charge and thus, in principle, should be immobile in the electric field. This minimum rises steeply for small pH variations about the pI value in the case of 'good carrier ampholytes' (i.e., $\Delta pK = 0$), whereas such a minimum tends to be distributed over a wider region of the pH axis for 'poor carrier ampholytes' ($\Delta pK = 4$). When the pH is sufficiently removed from the pI (3 or more pH units, depending on the ΔpK value) the conductivity value tends to a plateau (a maximum).

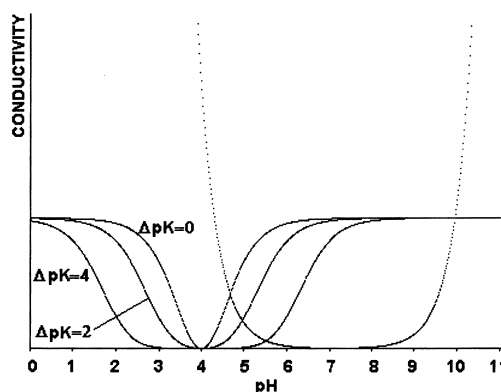


Fig. 1. Ampholyte conductivity contribution as a function of pH, for a $pI = 4.0$ ideal ampholyte (100 mM concentration) possessing three different ΔpK values: 0, 2 and 4. The U-shaped profile to the right of these curves represents the conductivity of pure water.

Let us now consider Fig. 2a: at vanishingly small concentrations of ampholyte the conductivity tends to zero. At progressively increasing concentrations, the conductivity also increases until it reaches a maximum and then steadily declines to values again close to zero, at very high concentrations. This is because, at high concentrations, the pH of the solution coincides with the pI value, at which value the net electric charge of the ampholyte becomes zero (cf. Fig. 1). On the contrary, the water conductivity contribution increases progressively and stabilises at the level corresponding to the appropriate value of the final pH of the ampholyte solution (Fig. 2b). This latter curve can also be interpreted as the conductivity of an Immobiline system with equal concentrations of each component.

3.2. Behaviour of the conductivity of an ampholyte solution as a function of concentration

As we should expect, the conductivity of an ampholyte solution will approach that of pure water at the value $pH = pI$, with an infinite ampholyte concentration increase (see Fig. 2), but let us consider the range of intermediate concentrations in greater detail.

In Fig. 3 we have plotted the ratio of the intrinsic ampholyte conductivity to the contribution of the water ion. The relative value of the ampholyte contribution depends on the ampholyte pI (the more

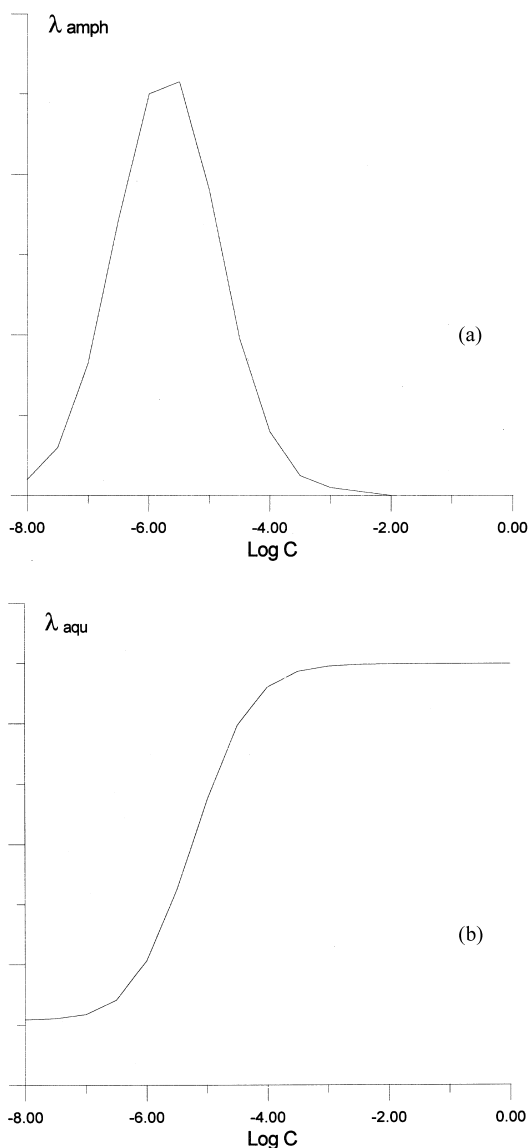


Fig. 2. Separate conductivity contributions of an ampholyte (a) and bulk water (b) as a function of ampholyte concentration, in the range 10^{-8} to 1 M.

removed the pI from neutrality, the higher the relative contribution), and the maximum of this ratio also varies with pI , but this maximum is always located in the region of very small concentrations (of the order of micromolar) and, moreover, the value of the above ratio never exceeds a few percent even in the case of very acidic (basic) ampholytes (for $pI=2$,

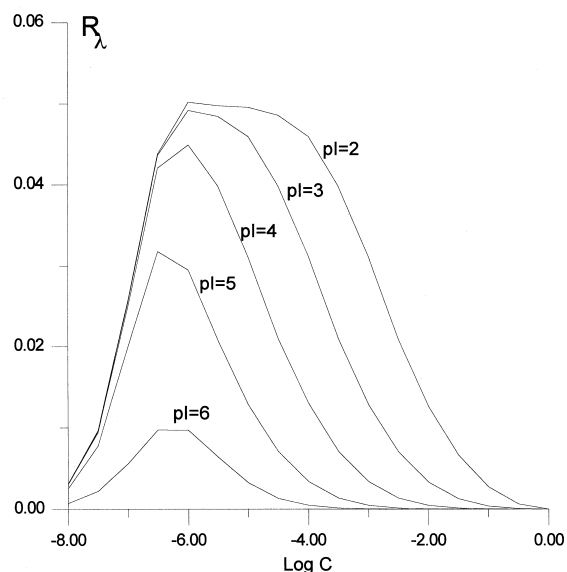


Fig. 3. Ratio of ampholyte conductivity contribution to water conductivity at different ampholyte pI values (ranging from $pI=6-2$) in a wide region of ampholyte concentration (ranging from 10^{-8} to 1 M). Each curve represents an ideal ampholyte with ΔpK of 2.

$R \sim 0.01$ and $R \sim 0.04$ at concentrations of 10 and 0.1 mM, respectively). This is a rather paradoxical result, since it shows clearly that the current level maintained with ampholyte molecules by themselves is negligible, and almost all the conductivity increase with ampholyte concentration is due to the fact that the solution pH is removed from neutrality.

3.3. Buffer system comprising two immobilized components

When working with a buffer composed of one ampholyte only, dissolved in pure water, we are able, to a certain extent, to vary the pH of the solution by means of changing the ampholyte concentration [5,9,16]. But, if we are dealing with a buffer/titrant couple grafted on to a gel matrix (Immobiline technology), we may operate in a very wide range of immobilised concentrations, the only restriction imposed being the acidic to basic component ratio. The appropriate expression linking the molarity of the basic component (C_b) relative to that of the acidic counter-ion (C_a) for a given pH value (which is here assumed to be constant, $H^+ = \text{const} = h^*$) is easily

derived from the electroneutrality equation and is given by:

$$C_b = C_a(1 + b)/(1 + 1/a) + (K_w/H - H)(1 + b) \quad (3)$$

where $a = K_a/h^*$ and $b = K_b/h^*$ are the respective dissociation constants of the acidic and basic components and K_w is the ionic product of water.

Let us now consider the ‘symmetrical case’ in which the pH of the solution, defined by two Immobilines, is equidistant from the pK values of the acidic and basic Immobilines. If we thus define $\Delta_1 = (\text{pH} - \text{p}K_a)$ and $\Delta_2 = (\text{p}K_b - \text{pH})$, the ‘symmetrical case’ will be characterized by $\Delta_1 = \Delta_2$. In this case, Eq. (3) rewrites as:

$$C_b = C_a + (K_w/H - H)(1 + b) \quad (4)$$

which, in turn, in cases sufficiently away from neutrality (acidic ranges), can be simplified into:

$$C_b = C_a - H(1 + b) \quad (5)$$

Fig. 4 shows how C_b varies as a function of C_a [$C_b = C_b(C_a)$] for the three cases $\Delta_1 = \Delta_2$ (central curve tending to unity), $\Delta_1 = 1/2\Delta_2$ (lower curve), and $\Delta_1 = 2\Delta_2$ (upper curve).

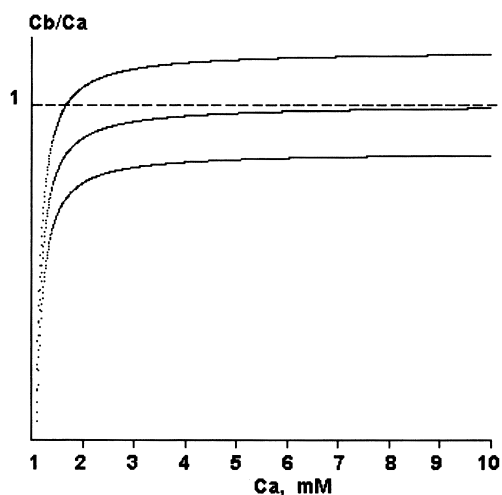


Fig. 4. C_b/C_a ratio for a constant pH value (maintained at pH=4) of a two-Immobiline system as a function of an acidic Immobiline concentration. The central curve represents the case $\Delta_1 = \Delta_2$; the lower curve is for $\Delta_1 = 1/2\Delta_2$ and the upper curve depicts the case $\Delta_1 = 2\Delta_2$.

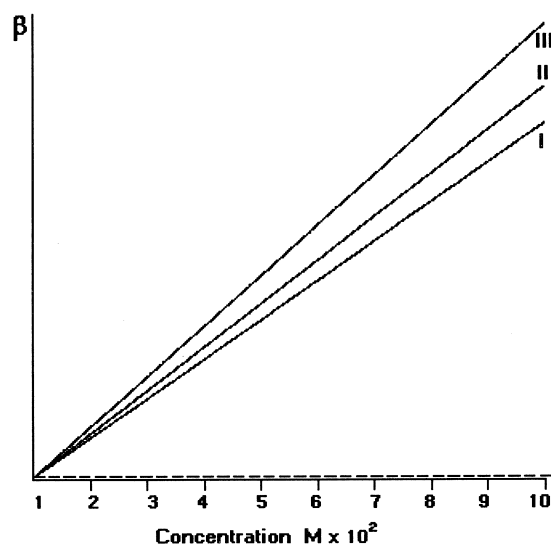


Fig. 5. Buffer capacity for two-Immobiline model systems with different pK distributions about the pI value. In each case the pI is maintained at pH=4 (by means of an appropriate C_b/C_a ratio; see Eq. (3)). Line I corresponds to $\text{pH} - \text{p}K_a = \Delta_1 = \Delta_2 = \text{p}K_b - \text{pH}$, ‘symmetrical case’; line II corresponds to $\Delta_1 = 1/2\Delta_2$; and line III corresponds to $\Delta_1 = 2\Delta_2$. ($\Delta\text{p}K = \text{p}K_b - \text{p}K_a = 2$).

If we now calculate the β power for these three systems we obtain an almost linear increase with C_a (Fig. 5). The slope of these curves will be a little bit different, it is connected with the fact that, for non-symmetrical cases, one group is sufficiently more close to the final pH (also in case III, i.e. $\Delta_1 = 2\Delta_2$, the total Immobiline concentration is considerably higher) than the other. Since there is no principal difference in behaviour between symmetrical and non-symmetrical systems, further on we will restrict our considerations only to symmetrical cases, since this permits us to treat the properties of both ampholyte and Immobiline systems within the framework of the same approach.

3.4. Buffer capacity of ampholyte buffers in a wide region of ampholyte concentrations

The expression for the buffer capacity gives us a relationship with respect to concentration in first degree, but we should remember that the pH is also a function of ampholyte concentration. Neglecting the pH changes with concentration (which correspond to rather high levels of ampholyte concentration), we

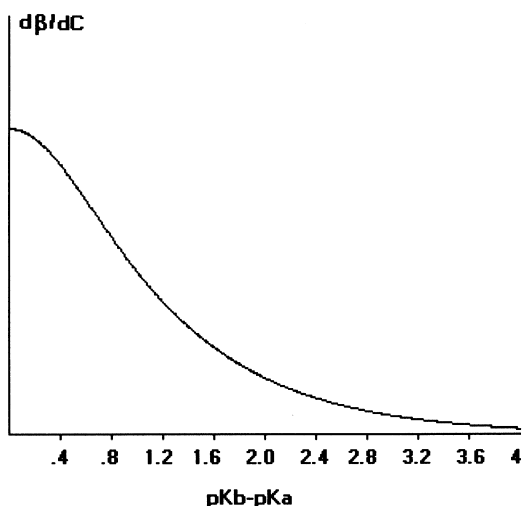


Fig. 6. The derivative of buffering power (β) to concentration ($d\beta/dc$) as a function of ΔpK . It is seen that, at very small ΔpK values, such a derivative reaches a constant, maximum value.

obtain a linear buffer power increase with a slope determined by the pK difference (ΔpK ; see Fig. 6). If we calculate the value of buffering power, taking into account the water contribution in the region of practical use, we will see approximately equal slopes for lines corresponding to the same ΔpK , but the absolute value of β power will be different (the latter increases as the ampholyte pI is removed from neutrality) (Fig. 7).

In order to understand this phenomenon in a better way, let us consider the buffer power behaviour in wide range of concentrations. Let us place our attention on the relative ampholyte contribution (Fig. 8). At extremely low ampholyte concentrations (less than micromolar), the specific ampholyte contribution is major in the case of neutral pI values (see the $pI=6$ curve to the left in Fig. 8). But the situation changes with an ampholyte pI removed from neutrality. For example, for the ampholyte with $pI=2$ at a concentration of 10 mM we have an approximate parity between the ampholyte and water contributions. With an infinite concentration growth this ratio, under any conditions, must become unity, but not all the amphoteric substances possess such a good solubility.

The high relative level of the contribution of water ions in β power gives us an explanation of the

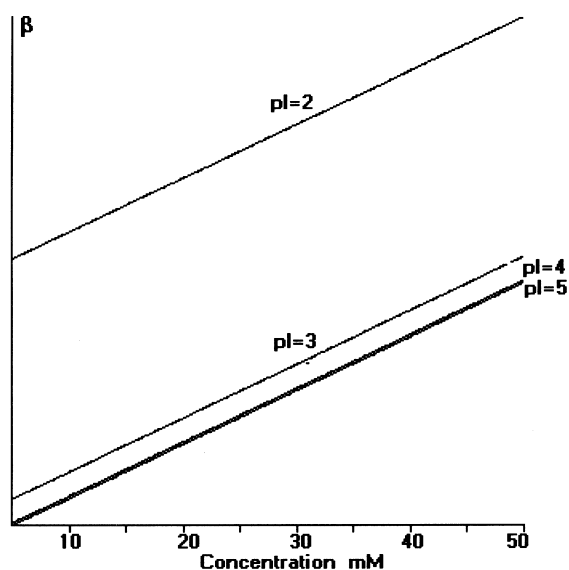


Fig. 7. Buffer capacity (β) as a function of ampholyte concentration (in the 10–50 mM range) at different ampholyte pI values ($pI=2-5$).

quicker increase of such β power in the range of very small or intermediate ampholyte concentrations (Fig. 9). Here too the increments are more pronounced for lower pI amphoterics.

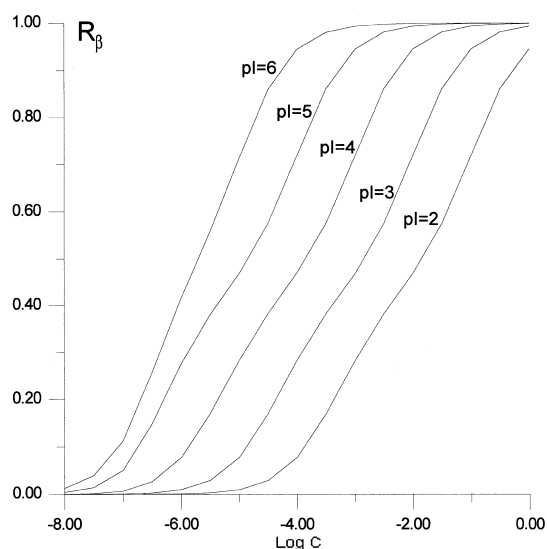


Fig. 8. Relative ampholyte buffer power contribution as a function of concentration, for a series of ampholytes with pI values from 2.0 to 6.0.

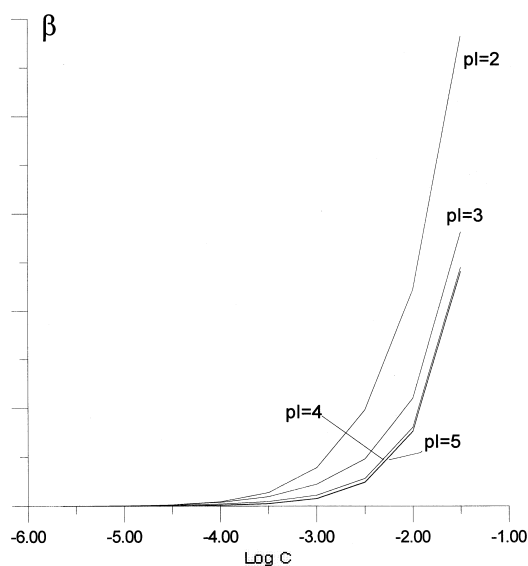


Fig. 9. Buffer capacity in a wide region of ampholyte concentration at different ampholyte pI values ($pI=2-5$).

4. Discussion

Whereas in IPG technology any recipe utilizes weak acidic and/or basic ionogenic groups, which are immobilized onto a gel matrix, and thus the conductivity of such a system is determined by hydrogen and hydroxyl ions only, ampholyte molecules are able to provide some contribution to the overall conductivity. However, as we have seen in the case of single ampholytes dissolved in pure water, the latter value is rather small. So, the conductivity increments with ampholyte concentration are explained, mostly, by the fact that the pH of our solution approaches ampholyte pI . Thus, in practice, we have no great difference in conductivity between an ampholyte solution and any 'immobilized' system with the same pH.

A great advantage of the Immobiline system is an opportunity of building an almost endless number of amphoteres covering any desired pH value, and simultaneously the possibility of regulating the buffering capacity value in a very wide range. These features render the IPG technology very attractive for a potential use outside of the standard framework of isoelectric focusing (at present available only in the polyacrylamide gel format, due to the fact that Immobilines can only be grafted onto such a poly-

mer). It should be realized, however, that, for use as soluble, isoelectric buffers as background electrolytes in CZE, such Immobiline buffers can only be made polymeric, since, in the process of polymerizing the weak acids and bases, admixed with the neutral monomer (acrylamide, but in the absence of cross-linker), an amphoteric, isoelectric polymer will be generated. Such a polymer might have substantial viscosity, and thus the advantage of using an isoelectric buffer (high-speed separations engendered by very high voltages) might be offset by the frictional resistance to migration due to the higher viscosity. Experiments are in progress to verify the suitability of such polymeric, isoelectric, soluble buffers for CZE separations. It must be emphasized, however, that the use of such high- M_r buffers has also been advocated by Hjertén et al. [1]. These authors observe that a buffer made of polyoxyethylene bis(3-amino-2-hydroxypropyl)/polyoxyethylene bis(acetic acid) would give an operative pH of 8.6 and exhibit a rather low conductivity, although not truly being an amphoteric molecule in the proper sense, since the acidic and basic groups are located on two different polymers, mixed in adequate ratios so as to obtain the desired pH value. These authors also describe a potential polymeric buffer, truly amphoteric, which could be made by grafting, onto a polymeric backbone (e.g., polyoxyethylene), an amino derivative at one end and a boric acid derivative at the other end. Since boric acid has a pK of 9.2 and, e.g. 2-amino-2-methyl-1,3-propanediol has a pK of 8.8, at the theoretical pI (9.0) this amphoteric polymer would exhibit an extraordinary buffering power, since the ΔpK would be only 0.4 pH units (in this case both the acidic and basic groups would act simultaneously as buffering and titrant ions!).

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