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Carrier ampholytes as potential buffers in electrophoresis: physico-chemical study

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Abstract

Joule heating is a limiting factor when separating proteins in capillary zone electrophoresis (CZE). Low conductivity buffers, are required for high-speed separations. We investigated the use of carrier ampholytes (CA) as background electrolytes (BGE) in CZE. We prepared 25 "narrow pH cuts" of wide pH range (3–10) CA mixture in order to know if these fractions were suitable to be used as BGE in CZE. Each fraction was characterised by CZE analysis, giving an idea of its heterogeneity (number and relative abundance of molecular ampholytes). Conductivities and buffering capacities of each fraction have been also measured. Our conclusion is that "narrow pH cuts" of CA might be well suited buffers for electrophoretic separations.

Keywords: Carrier ampholytes; Background electrolyte; Conductivity; Buffering capacity

1. Introduction

The concept of low conductivity buffers based on amphoteric compounds for their use in capillary electrophoresis has been introduced by Hjerten et al. [1]. They show, by theoretical considerations and experiments, various advantages of this kind of compounds for their use in capillary zone electrophoresis (CZE). Later, Blanco et al. [2] obtained simulation results in close agreement with the protein separations performed [1]. The use of amphoteric buffers in gel electrophoresis was also reported [3]. Although Hjerten et al. proposed different kinds of compounds to be used as background electrolyte (BGE) in CZE, amino-acid-based isoelectric buffers were the most studied and have been proven to be useful for the accomplishment of CZE separations. By their low conductivity allowing the use of higher electric field strength than in conventional buffers, very fast separations were obtained. For example, the peptide map of bovine β-casein was performed in less than 10 min in an aspartic acid (Asp) based buffer whereas in an 80 mM phosphate (pH = 2) common buffer, it was done in 80 min [4]. Other amino-acids have been tested as isoelectric buffers, cysteic acid [5], histidine or the His–Gly dipeptide [6]. Amino-acids are an interesting alternative but only few of them present a sufficient buffering capacity to be used as BGE [7]. Carrier ampholytes (CA), also introduced by Hjerten et al. [1], originally synthesised for isoelectric focusing (IEF) are perhaps a better alternative because a mixture of CA permits to establish a pH gradient over a large pH range [2–11] allowing protein solubility and separation. Thus, considering CA for a hypothetical use in CZE, buffering capacities may be supposed not to be a limitation. In order to support this point, one can notice that in electro-titration curve (ETC) experiments, first introduced by Rosengren et al. [8], CA were already used as BGE in "zone electrophoresis" in the last step of ETC. Such technique has already been used for several purposes, for optimising pH conditions for zone electrophoresis separation [8], or for the determination of p*I* [9]. Results obtained by this technique are a good experimental proof that CA can be used as low conductivity buffers in CZE to separate proteins.

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As no single purified CA is available, we decided to fractionate a batch of CA (3–10 pH range) into 25 "narrow cuts" by preparative gel (Sephadex G-75) IEF. Then, the "narrow pH cut" heterogeneity (number and relative abundance of the CA) has been studied by CZE in conventional buffers in order to approximate the molecular population of each fraction. The use of "narrow pH cuts" of ampholytes as BGE in CZE rises however some questions. Can we expect the formation of a narrow pH gradient in each "narrow pH cut" solution? How this will affect the quality of the protein separation? Moreover, such a study is a valuable tool to control the synthesis reproducibility from batch to batch and to show differences existing between several CA synthesis methods. Each "narrow pH cut" fraction has also been characterised according other key-importance parameters when dealing with BGE in CZE: pH, conductivity and buffering capacity.

2. Materials and methods

2.1. Chemicals

Sephadex G-75 granulated gel, Pharmalytes (3–10) and Agarose-IEF were purchased by Amersham Biosciences (AB) (Orsay, France). Anolyte and Catholyte solutions were obtained from Serva (Coger, Paris, France). All chemicals used were of analytical reagent grade. Citric acid, boric acid, sodium hydroxide, Na_2HPO_4, NaH_2PO_4, benzyl alcohol and CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France). Buffer solutions compositions were computed by Phoebus software and were prepared with water produced by an alpha Q Millipore system (Molsheim, France) and filtered through 0.2 μm filter units before their use in capillary electrophoresis.

2.2. Ampholytes synthesis

Three to ten pH range carrier ampholytes were produced by Thierry Rabilloud using the following method: 0.1 mole of pentaethylene hexamine was dissolved in 40 mL of water, 0.2 mole of glycidol and 0.04 mole of 1,3-butadiene diepoxyde were added. The mixture obtained was placed for 4 h at 50 °C and then 0.15 mole of itaconic acid was added to it. The solution was heated at 95 °C during 48 h and contained approximately 40% (w/v) of CA's.

2.3. Preparative IEF and fractionation of the "narrow pH cuts"

For preparing the "narrow pH cuts", a preparative gel (Sephadex G-75) IEF at 50% (v/v) of CA in distilled water was performed. The IEF was performed at constant power (5 W) during 24 h. A Multiphor II electrophoresis system and an EPS 3500 XL power supply, both from AB, were used. The preparative gel presented the following dimensions: 12.5 cm long, 12.5 cm wide and 0.7 cm thick.

Then, 25 gel fractions have been collected with a spatula; each one was approximately 0.5 cm wide. Ampholytes were eluted from each fraction with distilled water. A peristaltic pump was used to facilitate the ampholyte elutions. Considering the different dilutions performed, each "narrow cut" contains 10% (w/v) of ampholytes.

2.4. "Narrow pH cuts" characterisation

To measure the pH and the buffering capacity of the "narrow pH cuts", a Radiometer Analytical combined pH electrode pHC3359 was used.

The conductivity measurements and the CZE experiments were carried out with an $HP^{3D}CE$ apparatus (Agilent, Waldbronn, Germany) equipped with a diode-array detector, an autosampler and a power supply able to deliver up to 30 kV. Data were handled by an HP Chemstation software. Bare fused silica capillaries (Phymep, Paris, France) of 35 cm length, 26.5 cm effective length and 50 μm internal diameter were used.

In order to calculate the conductivity of the "narrow pH cut", the following equation can be obtained from the Ohm's law.

$$K = \frac{iL}{VS} \quad (1)$$

where K is the electrolyte conductivity (S/m), i the current (A), L the capillary length (m), V the voltage (V), and S is the area of a capillary cross-section (m^2).

2.5. Electro-titration curve in agarose gel

ETC of a *Dactylis glomerata* pollen extract was performed in a 1.2% agarose gel containing 12% sorbitol and 2% of a wide pH range (3–10) CA mixture (Pharmalytes) on GelBond film (11 cm × 11 cm × 0.4 cm; FMC, Marine Colloids, Portland, ME, USA). The pH gradient was first generated by isoelectric focusing of the CA across the gel on the Multiphor II apparatus with cooling to 15 °C, at a constant voltage of 200 V for 15 min followed by constant power, 1 W, for 50 min. After this focusing step, the gel was rotated by 90° and 20 μL of a *D. glomerata* pollen extract (10 mg/mL in distilled water) was applied into the sample furrow. ETC was then run at a constant voltage, 600 V for 15 min. Silver staining was then performed.

3. Results and discussion

Characterisation of the carrier ampholytes "narrow cuts" as potential electrophoretic buffers.

Table 1
pH of the CA "narrow cuts" and ΔpH measured between two neighbouring fractions

Fractions no.	pH	ΔpH
1	3.56	
2	3.69	0.13
3	4.03	0.34
4	4.36	0.33
5	4.76	0.4
6	5.16	0.4
7	5.5	0.34
8	5.87	0.37
9	6.29	0.42
10	6.62	0.33
11	7	0.38
12	7.41	0.41
13	7.66	0.25
14	8.07	0.41
15	8.23	0.16
16	8.45	0.22
17	8.63	0.18
18	8.79	0.16
19	8.97	0.18
20	9.09	0.12
21	9.21	0.12
22	9.49	0.28
23	9.85	0.36
24	10.73	0.88
25	10.95	0.22

3.1. pH study

The pH of the different fractions was measured after the isoelectric focusing (Table 1, Fig. 1).Fig. 1 shows that the increase of pH with the CA "narrow cut" number is almost linear. The mean step between each neighbouring fraction is around 0.31-pH unit but ΔpH varies from 0.12 to 0.88. This means that each CA fraction is heterogeneous and that they are all different from each other. Their heterogeneity will be studied in the next chapter. This preparative IEF produces a large choice of potential BGE: from 3.56 to 10.95.

3.2. Heterogeneity of the ampholytes in each "narrow cut" analysed by CZE

CZE analysis of each "narrow pH cut" of CA has been performed in high ionic strength (100 mM) buffers in order

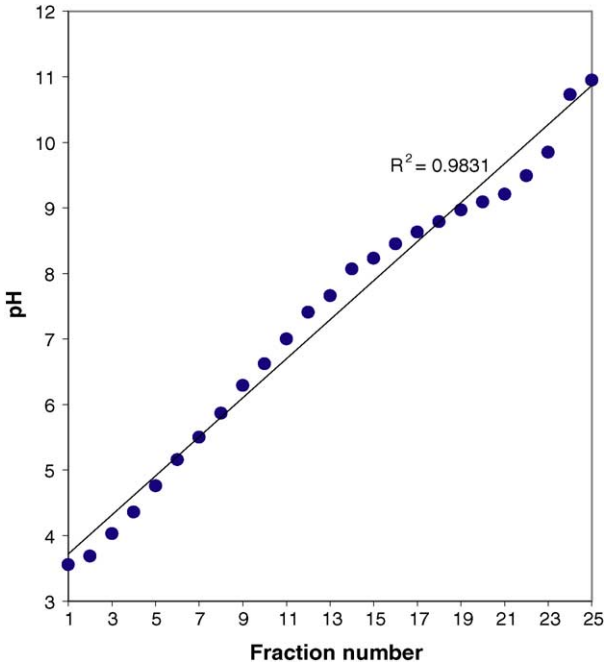

Fig. 1. pH gradient of the IEF separation of the 25 CA "narrow cuts".

to favour high-resolution analysis. Considering the pH of the different "narrow cuts", the pH of the BGE has been chosen to be higher than the pH of the studied fraction so as to separate the CA contained in each fraction in an overall negatively charged-state. Several buffers have been used (Table 2).

We have tried each buffer of the Table 2, which permits the analysis of the CA contained in each "narrow cut" in an overall negatively charged-state. The BGE, which allowed the best resolution for each fraction, are shown on Table 2. To illustrate the importance of the buffer pH for the CZE separation of the CA contained in the different "narrow cuts", the analysis of the fraction no. 17 (pH = 8.63) in three different BGE is shown on Fig. 2.

Fig. 2A shows that a BGE with a pH lower than the pH of the fraction analysed is not suitable: peaks are tailing and the resolution is poor. The electropherogram obtained in borate buffer (Fig. 2B) shows that a small difference between the buffer pH and the fraction pH (ΔpH = 0.57) is enough to have the CA in an overall negatively charged-state. The peak,

Table 2
Buffer used for the CZE analysis of each CA "narrow cut"

	Buffer pH				
	5.9	6.49	6.95	9.2	10.9
Buffer composition					
Compound 1	Citric acid 23 mM	NaH_2PO_4 45.2 mM	NaH_2PO_4 22.2 mM	Boric acid 184.9 mM	CAPS 123.6 mM
Compound 2	NaOH 55.5 mM	Na_2HPO_4 18.3 mM	Na_2HPO_4 25.9 mM	NaOH 100 mM	NaOH 100 mM
"Narrow cuts" analysed in each buffer	3, 5, 6	1, 2, 4, 7	8, 9	10–16	17–25

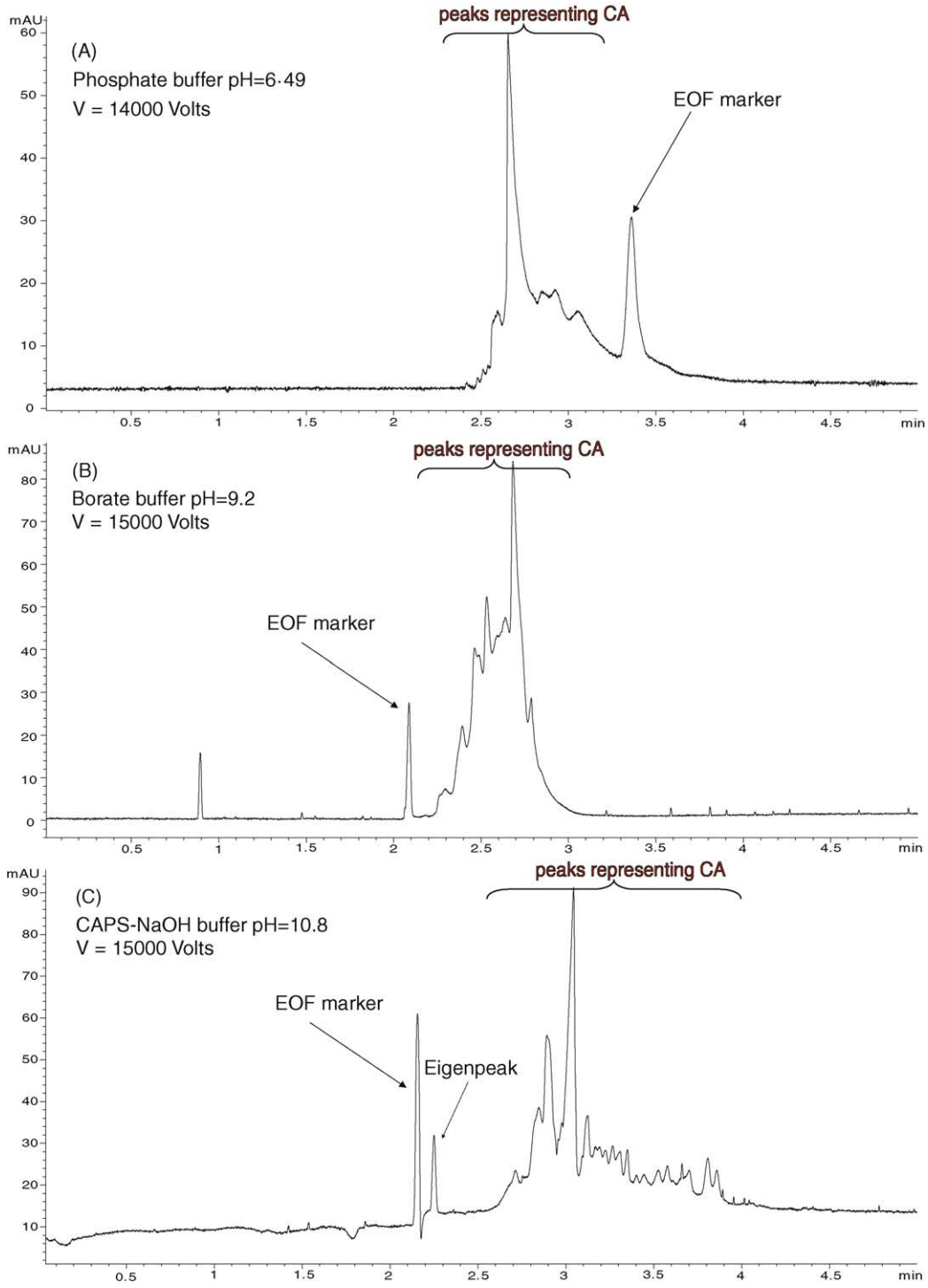

Fig. 2. Zone electropherograms of the "narrow cut" no. 17 in three different BGE. Fused silica capillary, 35 cm (detection: 26.5 cm) × 50 μm i.d. Temperature: 25 °C. UV absorbance at 200 nm. Each BGE is used at 100 mM ionic strength. Hydrodynamic injection (35 mbar, 3 s). EOF marker: benzyl alcohol (0.004%, v/v). Sample: narrow pH cut (4%, v/v).

at a migration time close to 0.9 min is a non-reproducible artefact.

An additional pH shift till 10.8 allows to increase the resolution comparing to the one obtained at pH = 9.2. The electropherogram (Fig. 2C) shows more than 20 peaks or shoulders. It has to be noticed that a peak detected after the EOF marker is present in all the CA separations performed in the CAPS-NaOH buffer, whichever the analysed "narrow cut". This peak is due to a system peak phenomenon because OH^- acts as a second co-ion at such alkaline pH [10,11]. Interest-

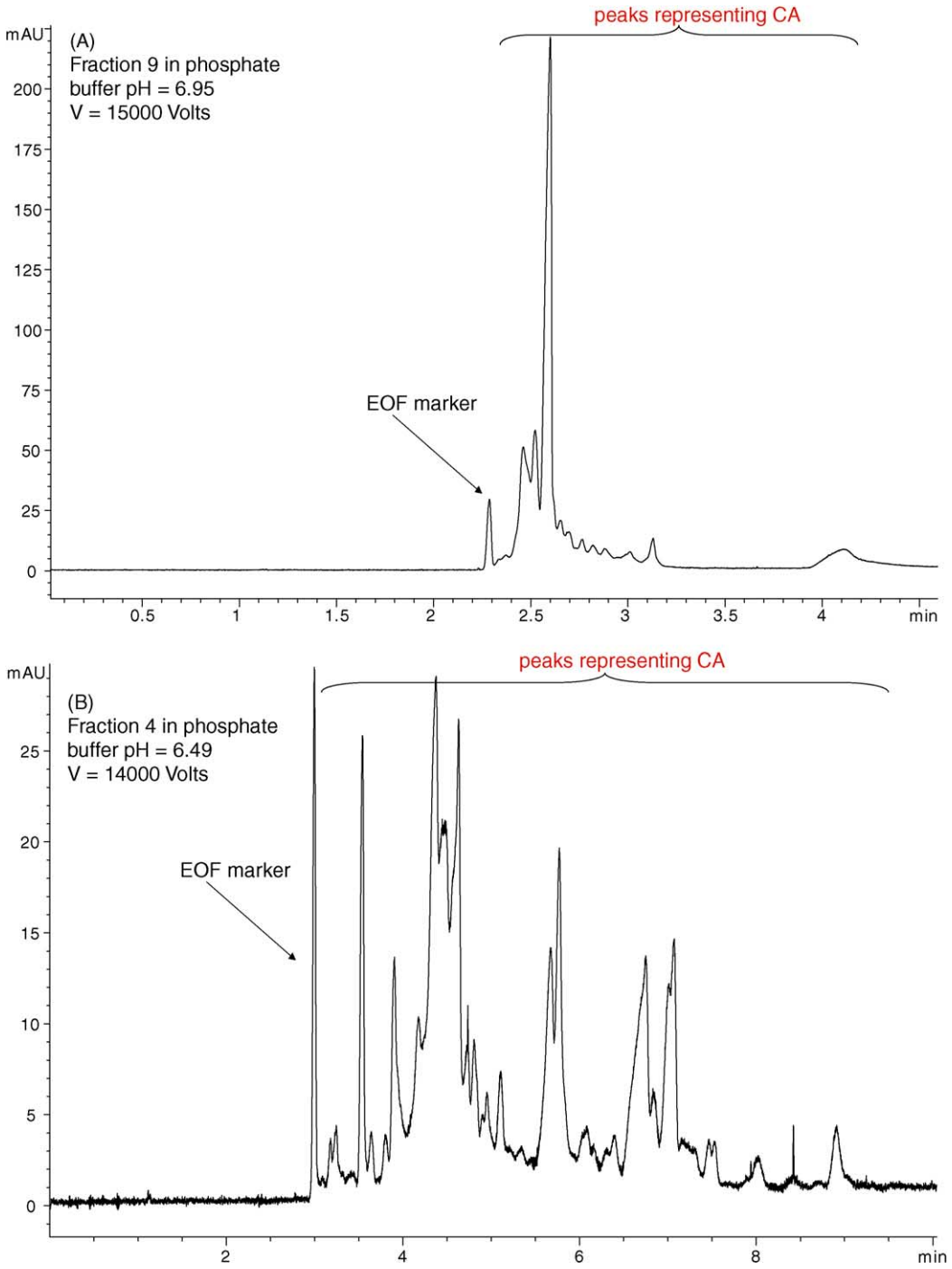

Fig. 3. Two CZE patterns of very different heterogeneity: (A) a very homogeneous (fraction 9) and (B) a very heterogeneous (fraction 4). Fused silica capillary, 35 cm (detection: 26.5 cm) × 50 μm i.d. Temperature: 25 °C. UV absorbance at 200 nm. Each BGE is used at 100 mM ionic strength. Hydrodynamic injection (35 mbar, 3 s). EOF marker: benzyl alcohol (0.004%, v/v). Sample: narrow pH cut (4%, v/v).

ingly in the different CZE analysis, two typical patterns have been observed for the CA separations. These are shown on Fig. 3.

Accordingly, it emerges from the CZE analysis of the different "narrow cuts" that several of them have a very restricted heterogeneity (around 15 peaks and shoulders) with a major peak like fraction 4 in phosphate buffer pH = 6.49 and that some others present a highly heterogeneous pattern (between 35 and 45 peaks and shoulders) without major peak as fraction 9 in phosphate buffer pH = 6.95. Considering the analysis of each "narrow cut", we can approximate the total number of CA contained in the 3–10 pH gradient to more than 700. This number is in accordance with other studies performed with other analytical techniques [12].

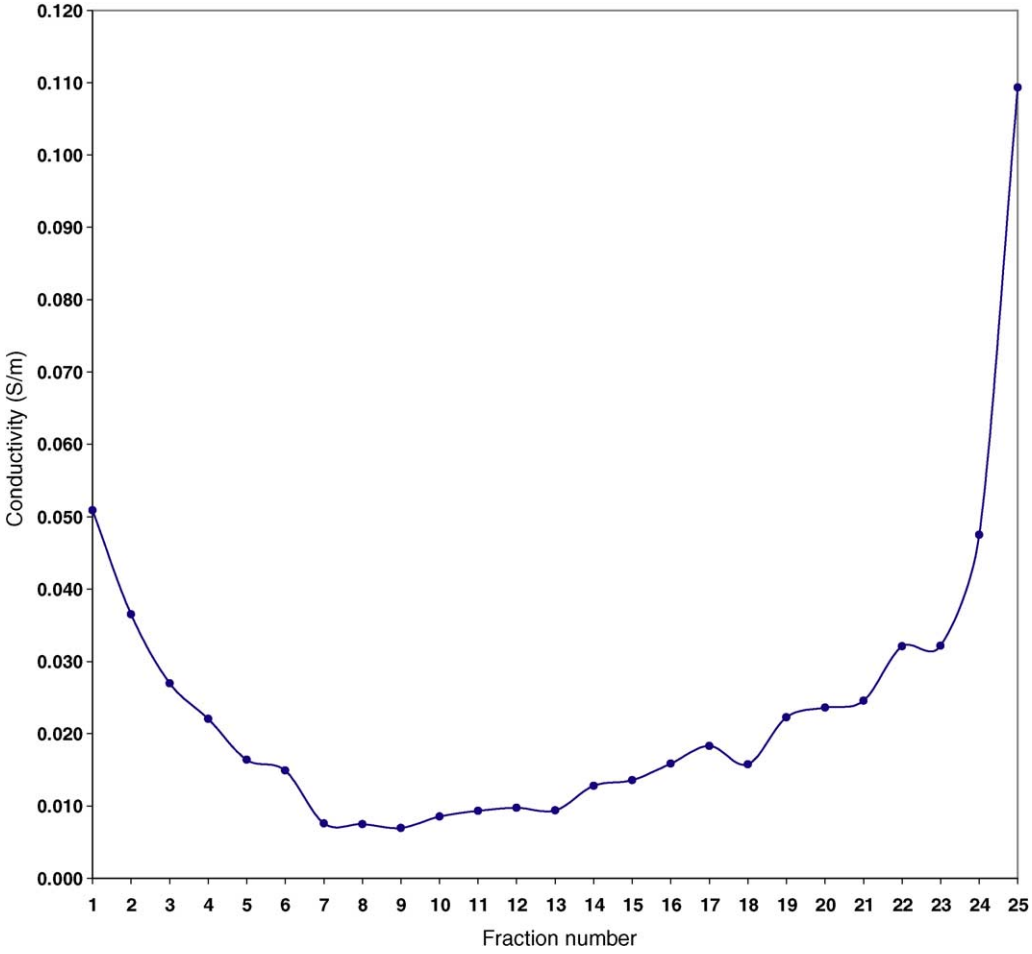

Fig. 4. Conductivities of the CA "narrow cuts" concentrated at 4% (v/v).

3.3. Conductivity study

As seen in Section 2.4, the fraction conductivities have been measured by the CE instrument, requiring a very small amount of sample (around 500 μL). For each sample, after filling the capillary with the studied "narrow cut" solution, the same solution was placed at the anode and at the cathode, then a voltage comprised between 2.5 kV and 20 kV was applied to the capillary. Voltage was increased by 2.5 kV step and the conductivity was measured for each step. The mean value was considered as the sample conductivity. The "narrow cuts" were studied at 4% (v/v) of CA in distilled water. The results obtained are shown on Fig. 4.

The "narrow cuts" which pH between 5.5 and 7.6 have the lowest conductivities (<0.010 S/m). At extreme pH (alkaline and acidic), the conductivities increase strongly. It is due to the water contribution (2) since H^+ or OH^- concentrations become significant at that extreme pH and that these ions are presenting very high mobilities. As a comparison, the conductivities of a phosphate buffer at pH = 6.95, 10 mM and 20 mM ionic strength are 0.075 S/m and 0.135 S/m, respectively. So most of the BGE based on CA are low conductivity buffers such as lysine which conductivity range 0.079–0.0057 S/m depending on the concentration (from 0.1 M to 0.001 M) [1].

3.4. Buffering capacity measurements

As it has been written in introduction, low conductivity buffers based on some amino-acids could provide very nice separation. However, not all the amino-acids are suitable buffers for CZE separation because only a few of them are presenting a sufficient buffering capacity to be used as BGE. Acido-basic titrations permit us to access to the buffering capacities of all the "narrow pH cuts". In order to limit again the sample consumption, low concentrated "narrow cuts" at 4% (v/v) in water have been titrated. NaOH at 0.1 M was added to the considered "narrow cut" aqueous solution so as to reach an alkaline pH around 12, then HCl at 0.1 M was added by micropipette injection till an acidic pH value. According to the definition, the buffering capacity (β) corresponds to the strong acid quantity needed to change the pH of the "narrow cut" solution around its initial pH value of 1 unit. The β value is expressed in mM (of strong acid)/pH. To give an example of the obtained titration curves, the one obtained for the fraction no. 13 is shown on Fig. 5.

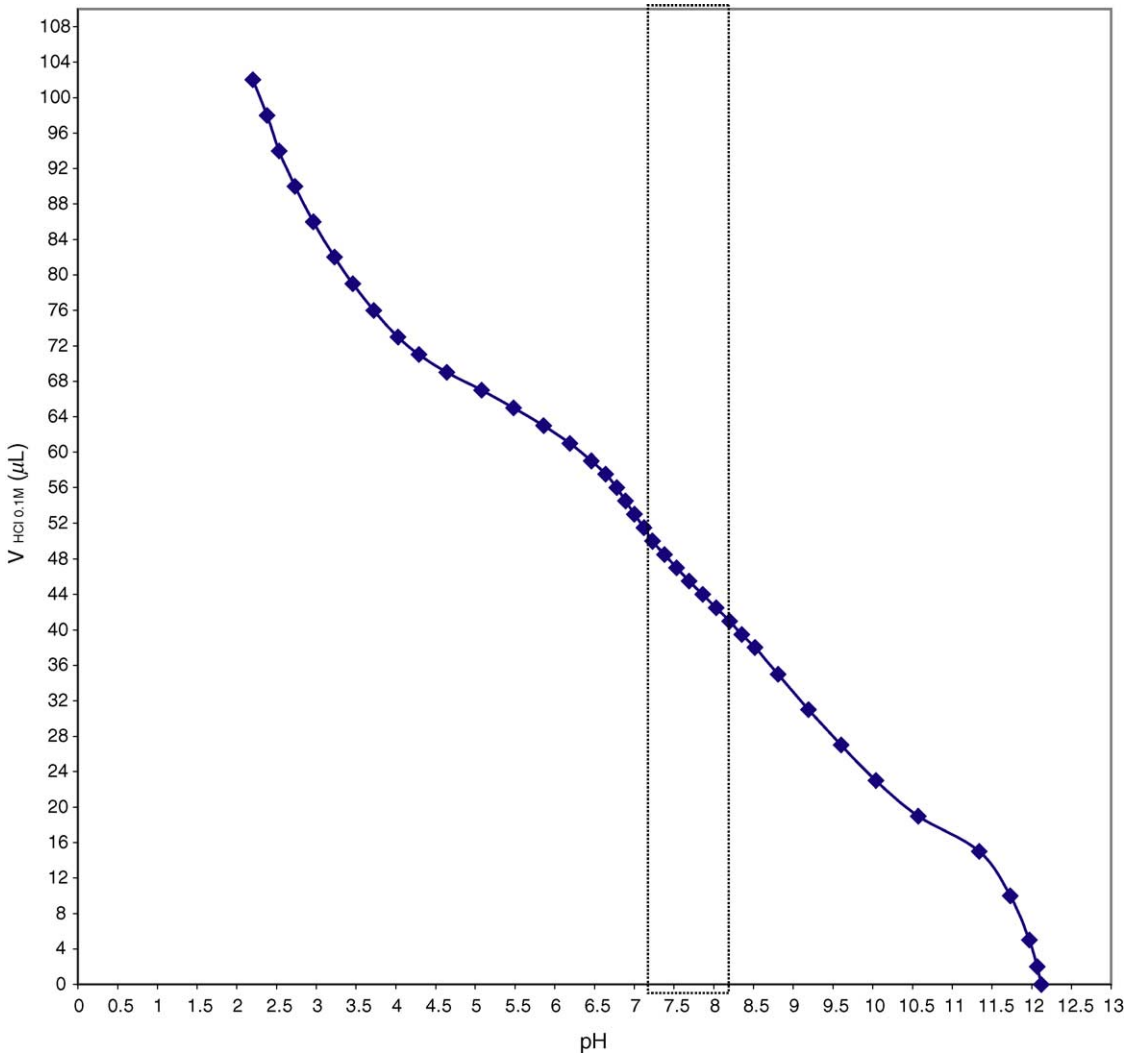

Fig. 5. Example of a CA "narrow cut" buffering capacity measurement. "Narrow cut" analysed: no. 13 at 4% (v/v). The black rectangle indicates the useful zone for calculating the buffering capacity of the considered "narrow cut".

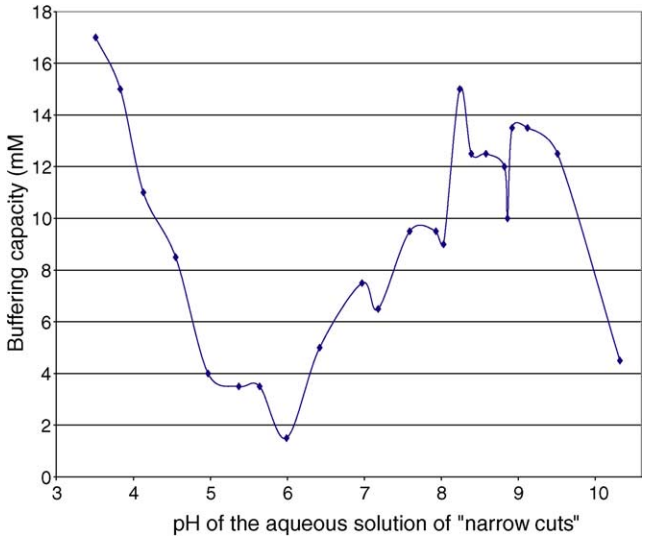

Fig. 6. Buffering capacities of the different aqueous "narrow cuts" at 4% (v/v).

After adding NaOH (0.1 M) until a pH value above 12, HCl (0.1 M) was added to the solution by successive micropipette injections of a few microliters. The initial volume and pH of the considered "narrow cut" aqueous solutions at 4% (v/v) were, respectively, 100 μL and 7.59. 9.5 μL of HCl (0.1 M) have been needed to change the solution pH from 8.1 to 7.1, thus, the buffering capacity of the "narrow cut" no. 13 at 4% (v/v) can be estimated to 9.5 mM/pH. All the results are summarized on Fig. 6.

The same experiment has been performed with a phosphate buffer presenting an ionic strength of 100 mM and a pH value of 6.95. A buffering capacity of 21 mM/pH has been measured. Even if the values observed for the potential buffers based on CA are lower than the one observed for the common phosphate buffer, the β values of the aqueous "narrow cut" solutions can be considered as comparable. Moreover, the aqueous "narrow cut" solutions can be more concentrated if needed. Furthermore, the results can be compared to a minimum β limit value given in [4]. In this study,

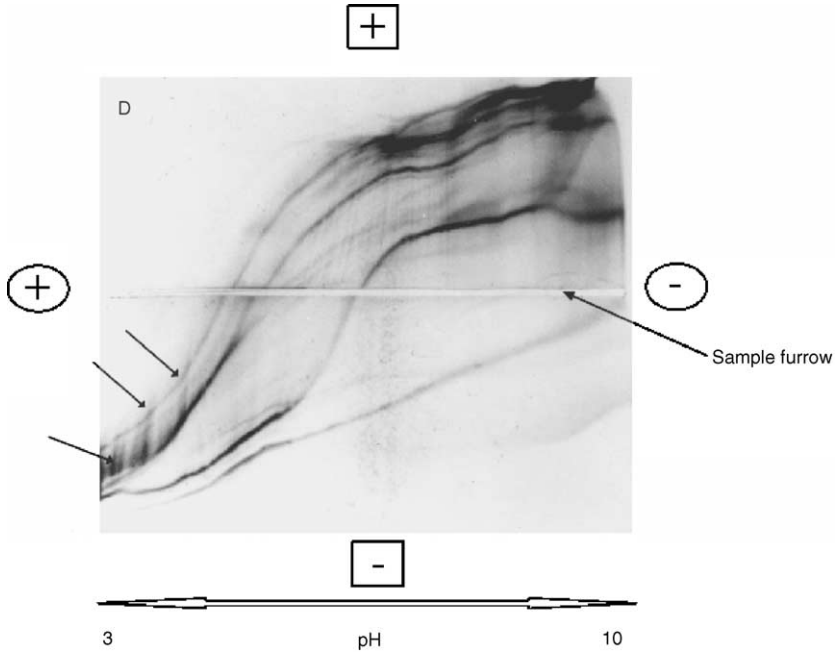

Fig. 7. Electro-titration curve of a *Dactylis glomerata* pollen extract.

the authors approximate the minimum buffering capacity to ensure a good CZE run at 10 mM/pH. Taking into account this limit, only nine of our "narrow pH cuts" at 0.4% (w/v) are presenting a sufficient buffering capacity to allow good CZE separations. But, given that the buffering capacity is proportional to the concentration, if the narrow pH cuts are used at 0.8% (w/v), 20 "narrow pH cuts" would present appropriate buffering capacity to provide good CZE run while still presenting a low conductivity. Moreover, we have to notice that Hjerten et al. [1] reported good separations in a lysine buffer, which was presenting a very low β close to 2.6 mM/pH [4].

3.5. Protein separations by the ETC technique

ETC is a three-step-experiment. First, a thin and flat gel containing CA is cast and a 3–10 pH gradient is formed as a voltage is applied by electrodes placed at both ends of the gel (horizontal axis). Electrodes are removed and then placed on the two other ends of the gel, a protein sample to be analysed is pipeted into the sample furrow, and an electric field (vertical axis), orthogonal to the previous one, is applied. Depending upon their charge, induced by their location on the pH gradient, the proteins will migrate toward the cathode or the anode. As example, ETC of a *D. glomerata* pollen extract is shown on Fig. 7. Each curve corresponds to one protein and the cross-point of this curve with the sample furrow corresponds to the p*I* of the considered protein. The second step of this experiment can be considered as a "zone electrophoresis" in a BGE based on CA. The low-observed conductivity in this stage permits the application of a 600 V voltage inducing less than 3 mA.

Proteins are nicely rapidly separated on the vertical axis in 15 min, some of them migrating over 2.6 mm/min. ETC gives more information than a single gel dimension electrophoresis like SDS–PAGE or IEF. It is easy to perform in a short period, around 3 h. The separation is mild for the protein as compared to an SDS–PAGE: enzymatic activity is preserved [9]. Although the quality of the separation obtained by ETC is high, we can notice that the resolution observed all over the gel surface is not constant. Particularly, streaks are visible in the acidic part of the gel, pointed out by arrows on Fig. 7. This phenomenon may be due to either, a lack of solubility of the considered proteins at some pH or a lack of charge at that pH. These "bad" pH are not always close to the p*I* of the considered proteins. Thus, this poor electrophoretic migration is most likely due to a poor local buffer. This CA buffer may not be conductive or buffering enough. Furthermore, interactions between some proteins and some particular ampholytes may occur interfering with their migration.

4. Conclusion

First, if we consider the results obtained by CZE for each ampholyte fractions analysed, we can approximate the total number of ampholyte peaks contained in the 3–10 pH gradient to more than 700. Moreover, some ampholyte peak may represent several ampholyte molecules. These results are in accordance with other results obtained by different methods [12]. Thus, CE appears to be a valuable tool to evaluate the ampholyte batch-to-batch reproducibility.

About the conductivity results obtained, it appears at first that the "narrow cuts" are low conductivity buffers to be compared to amino-acids such as lysine.

About the buffering capacities of the “narrow cuts”, it appears that this parameter is not a limitation to perform CZE in CA based buffers.

Thus, it emerges from this physico-chemical study that the most of the “narrow pH cuts” of CA are presenting a very low conductivity and a suitable buffering capacity to act as BGE in CZE separations. This study will be completed by experiments to show how protein samples will behave in such buffers presenting a multitude of components with very close p*I*. As seen in Fig. 7 and pointed out by arrows in the acidic part of the gel, some well defined and “ultra narrow cuts” of CA seem to be not good BGE for such protein electrophoretic separations. The CA “narrow cuts” that we have prepared are certainly including such “bad” ampholytes but are diluting them together with “good” ones into potentially new BGE for future simple and fast electrophoretic separations.

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