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Carrier ampholytes-based capillary electrophoresis as an alternative to capillary zone electrophoresis in classical background electrolytes

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Abstract

Carrier ampholytes (CAs), originally designed for isoelectric focusing (IEF), have been used as background electrolytes (BGE) in capillary zone electrophoresis (CZE). Their main electrophoretic properties, relatively high buffering capacity and low electric conductivity allowed fast (less than 2 min) and high efficient (500,000 theoretical plates/m) separation of a test mixture of proteins under very high electric field strength (more than 1000 V/cm). The results obtained in such buffers have been compared to those obtained in more classical sodium—phosphate and sodium—*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonate) background electrolytes. High ionic strength classical buffers were necessary to achieve the separation of the proteins contained in the test mixture. This induced a significant Joule heating and temperature increase inside the capillary whereas a negligible Joule heat was produced in carrier ampholyte buffers even at the above electric field strength (higher than 1000 V/cm).

Keywords: Carrier ampholytes; Background electrolyte; CZE; CABCE; Protein

1. Introduction

Low conductivity background electrolytes (BGE) have been introduced for capillary zone electrophoresis (CZE) in 1995 by Hjertén et al. [1]. They proposed several compounds to be used as low conductivity buffers in CZE. Among others, amino acids and carrier ampholytes (CAs) have been tested. Blanco et al. [2] studied the band spreading mechanism in this kind of buffers and found by computation results in accordance with [1]. Whereas CAs have not been, as far as we know, further considered as buffers in CZE, amino acids have been much more studied and have been proven to be an alternative to more classical buffers in several cases. As an example of the benefits linked to the isoelectric properties of such compounds, one can mention that the peptide map of bovine β-casein was performed in less than 10 min in an aspartic acid (Asp)-based buffer, at a pH close to its isoelectric point (pI) (pH ∼ pI ∼ 2.77) whereas in a 80 mM phosphate (pH 2) common buffer, it was done in 80 min [3]. Other amino acids have been tested as isoelectric buffers, cysteic acid [4], histidine, the His-Gly dipeptide [5] or iminodiacetic acid [6,7].

From these studies, the use of amino acids has shown several advantages, but other authors have carried out more theoretical studies of the use of amphoteric molecules as BGE in CZE which have led them to some scepticism [8,9].

The problem with amino acids is that only few of them exhibit a sufficient buffering capacity to be used as BGE in CZE [10]. CAs, originally designed for isoelectric focusing (IEF) may represent a better alternative, from a buffering capacity point of view, given that CAs are able to establish a pH gradient in IEF. A preparative IEF fractionation of a wide 3–10 pH range home-made CA mixture provided us 25 "narrow pH cuts" of CAs. Each obtained fraction has already been characterized according to its heterogeneity, conductivity and buffering capacity [11]. Experimental results led us to the conclusion that most of the "narrow pH cuts" had suffi-

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cient buffering capacity to be used as BGE in CZE in addition to low conductivity.

Moreover, we have to notice that "narrow pH cuts" of CAs have been used as BGEs in "zone electrophoresis" (ZE) since 1976 [12] in the second step of the electro-titration curve (ETC) method in slab gels. This technique is robust and has been used in the past to optimize ZE pH conditions [12], protein charge determination prior to ion exchange chromatography, protein interactions [13] or to determine protein isoelectric points with high precision [14]. Thus, ETC could be seen as the precursor method to carrier ampholytes-based capillary electrophoresis (CABCE) as well as a good experimental proof that CAs may be used as BGEs in ZE.

In this paper, a test mixture of well-behaved proteins was used in order to evaluate the capabilities of CABCE to separate a protein mixture. We present the separation of the same protein test mixture, first by the ETC technique in slab gel, and then we show that CABCE is a good method to separate these well-behaved proteins. We made the comparison between CABCE in the "narrow pH cuts" as previously prepared and CZE in classical buffers. The results obtained in CA-based buffers were compared to those obtained with phosphate and sodium—*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonate) (HEPES) BGEs which are common buffers in CZE. High ionic strengths were needed to achieve a well resolved separation with both classical buffers. This induced an important Joule heating whereas nearly no Joule effect and almost baseline resolution were shown in BGE-based on CAs. The results obtained in this study show that CA-based buffers are a valuable alternative to classical buffers when high speed electrophoretic separation is required.

2. Materials and methods

2.1. Chemicals

Agarose-IEF was purchased from Amersham Biosciences (AB) (Orsay, France). Anolyte and catholyte solutions were obtained from Serva (Coger, Paris, France). All chemicals used were of analytical reagent grade and obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Buffer solutions compositions were computed by Phoebus software and were prepared, as sample solutions, with water produced by an alpha Q Millipore system (Molsheim, France) and filtered through 0.2 μm Sartorius filter units (VWR, Strasbourg, France) before their use in capillary electrophoresis. Sodium HEPES ([HEPES] = 120 mM, [NaOH] = 100 mM), pH 8.1, and sodium phosphate buffers ($[NaH_2PO_4]$ = 3.6 mM, $[Na_2HPO_4]$ = 37.2 mM), pH 7.9, have been prepared at a high ionic concentration which corresponds respectively to 100 and 115 mM ionic strength.

BDH pI marker kit, containing nine proteins with pI in the range 5.65–8.3, used as protein test mixture both in CABCE, CZE and ETC methods, was purchased from VWR (Strasbourg, France). Its composition is given in Table 1.

Table 1
Composition of the protein test mixture

Proteins	Isoelectric point	Identification
Azurin (*P. aeruginosa*)	5.65	A
Trifluoroacetylated Myoglobin Met (porcine)	5.90	B
Myoglobin ferro (porcine)	6.15	C
Myoglobin Met (porcine)	6.45	D
Trifluoroacetylated Myoglobin Met (equine)	6.90	E
Myoglobin ferro (equine)	7.00	F
Myoglobin Met (equine)	7.30	G
Trifluoroacetylated Myoglobin Met (sperm whale)	7.65; 7.70	H
Myoglobin Met (sperm whale)	8.30	I

2.2. Capillary electrophoresis

The CZE and CABCE 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) presenting a 50/375 μm internal/external diameter, 35/26.5 cm total/effective length, were used. In each CZE or CABCE experiment, the anode was set at the injection end of the separation capillary.

Each new capillary was activated by the following three-step sequence: 10 min 1 M NaOH rinse, 10 min 0.1 M NaOH rinse, then 10 min water rinse. Between different separations in the same BGE, a buffer rinse was only performed. 0.1 M NaOH, water and the new buffer were successively flushed into the capillary when different buffers have been used in the same capillary. Hydrodynamic injection (30 mbar, 3 s) has been used for sample injection. The theoretical plate number (N) was calculated by the HP Chemstation software following the general formula:

$$N = 5.54\left(\frac{t_m}{w_{1/2}}\right)^2,$$

where t_m is migration time, and $w_{1/2}$ is peak width at half height.

2.3. Electro-titration curve in agarose gel

ETC of the BDH pI mixture ($5.65 \leq pI \leq 8.3$) was performed in a 1.2% (m/v) agarose gel containing 12% (m/v) sorbitol and 2% (m/v) 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 CAs across the gel on the Multiphor II apparatus (AB) with cooling to 15 °C, at a constant power of 2 W for 10 min, followed by constant power, 3 W, for 50 min. After this focusing step, the gel was rotated by 90° and 25 μL of the protein test mixture was applied into the sample trench. ETC was then run at a

constant voltage, 600 V for 15 min. Coomassie blue staining was then performed to detect the proteins in the gel.

3. Results and discussion

3.1. Electro-titration curve of the protein test mixture

An ETC of the protein test mixture (for its composition see Table 1) was performed in order to show the protein migration as a function of the pH (Fig. 1). This identifies each peak obtained in CABCE and CZE by their pI obtained by ETC. The protein test mixture analyzed by ETC was first submitted to IEF in the first step of the ETC. This allows us to draw the slope of the pH gradient across the gel (not shown). The intersections of the protein curves with the sample trench are giving the pI of the proteins present in each curve separated in the second step of the ETC. The intersections of the protein curves with the sample trench are aligned with the protein bands obtained by IEF in the first step of the ETC. Thus, the second ETC step did not act on the pH gradient and we can consequently consider the second step of the ETC as the result of multiple juxtaposed carrier ampholytes-based electrophoretic (CABE) separations.

3.2. Carrier ampholytes-based capillary electrophoresis

A preparative IEF fractionation of a wide pH range (3–10) CA solution has provided us 25 potential BGEs for capillary electrophoresis. These amphoteric fractions have different conductivities and buffering capacities. "Narrow pH cuts" presenting the most basic and acidic pH are rather more conductive and some may not be buffering enough. But, as a result of their physico-chemical study [11], we can consider that we have 20 potential low conductivity buffers of different pH available. This is really useful in order to optimize protein separations based on protein charges. The example of CABCE separation of the protein test mixture is meaningful (Fig. 2).

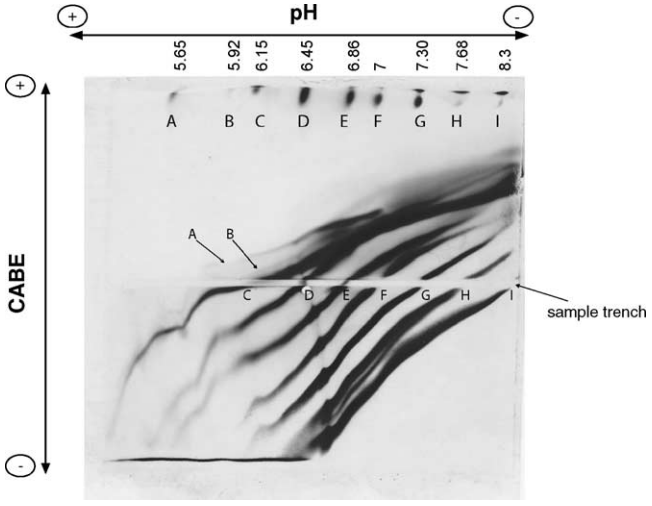

Fig. 1. ETC of the protein test mixture. In the upper part of the gel, the IEF separation of the protein test mixture allowed us to identify the protein present in each curve. One microlitre of the protein test mixture (each protein at 5 mg/mL) is deposited on the top of the gel for the IEF. Twenty-five microlitres of the protein test mixture (each protein at 2.5 mg/mL) is deposited on the sample trench before the execution of the ETC second step. For protein identification, see Table 1.

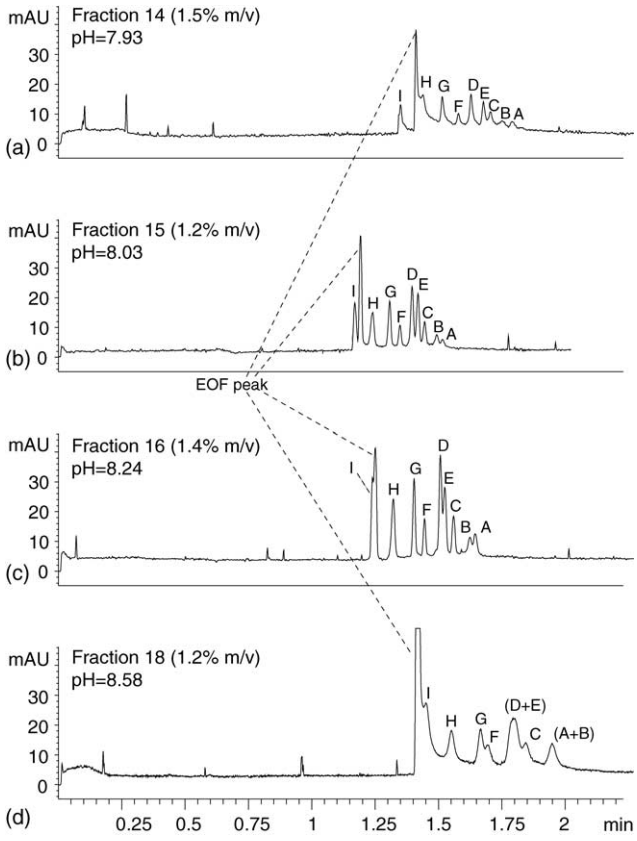

Fig. 2. Protein test mixture analyzed by CABCE in four different "narrow pH cuts". Fused silica capillary, total/effective length 35/26.5 cm × 50 μm i.d.; voltage, 30 kV; temperature, 25 °C. UV absorbance at 214 nm. Hydrodynamic injection (30 mbar, 3 s). Sample, protein test mixture (each protein at 1 mg/mL) diluted in the considered "narrow pH cut". For protein identification, see Table 1.

In order to avoid a significant adsorption of the test proteins to the negatively charged bare fused silica capillaries, all the test proteins, with the exception of protein I, were analyzed as anions at pH above their pI. By this way, the proteins are electrostatically repulsed from the negatively charged capillary walls [15]. As the proteins are analyzed in anionic mode in presence of a strong electroosmotic flow (EOF), the protein with longest migration time behind the EOF peak has in fact the greatest electrophoretic mobility and the first peak emerging after the EOF peak represents the slowest protein.

Once the concentration of each CA fraction has been individually optimized to achieve the best resolution, we can see that the use of the amphoteric fraction no. 15 (pH 8.03) (Fig. 2b) gives a quasi baseline separation of the nine test

mixture proteins whereas Fig. 2d in amphoteric fraction no. 18 (pH 8.58) shows poor resolution. It illustrates that protein mobilities are very sensitive to even slight pH shifts of the BGE.

It has to be emphasized that the low conductivity of the "narrow pH cuts" always allowed us to work at maximum voltage (30 kV). The current induced by such high voltage was very low. Depending on the considered amphoteric fraction, it was comprised between 3 μA and less than 6 μA in a capillary of a total length ranging from 29.3 to 35 cm. Thus, advantages linked to the use of amphoteric fractions appear obvious when considering the detection time. For example, the nine proteins contained in the test mixture have been analyzed in 90 s in amphoteric fraction no. 15. Their detection profile showed symmetrical peaks. The separation efficiency reached for some of them values of 500,000 theoretical plates/meter.

Another peculiarity of CABCE in our "narrow pH cuts" is the presence of a peak, marked as the "EOF peak" on the electropherograms in Figs. 2 and 3. This peak is produced when protein samples are injected diluted into amphoteric BGE. This same peak is also produced by benzyl alcohol and dimethylformamide (DMF), routinely used as neutral electroosmotic flow markers in CZE. The injection of water induces a negative peak at the same migration time (data not shown). Thus, we considered this peak as a system peak of our CA BGE migrating with mobility very close to the electroosmotic mobility. That is why we noted the considered system peak as the "EOF peak".

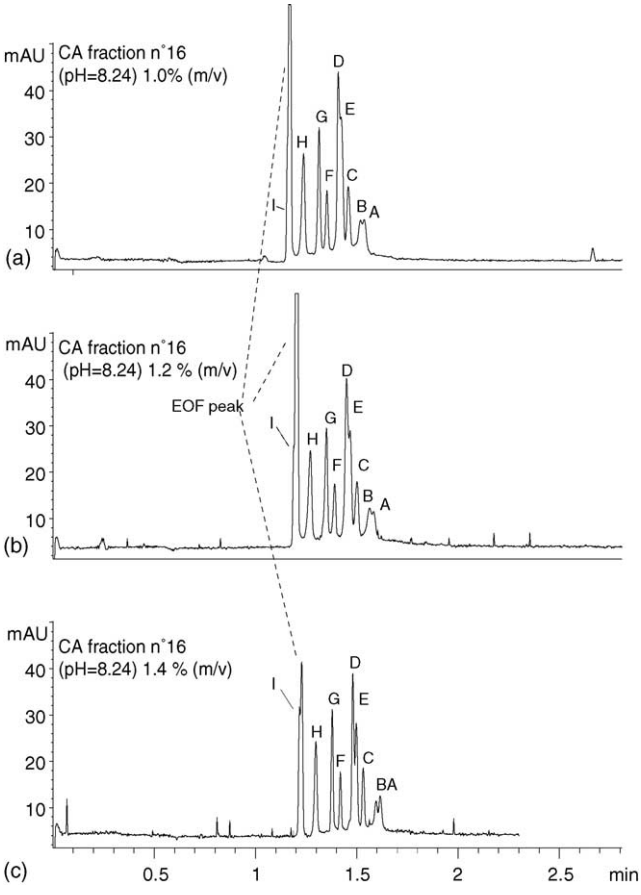

Fig. 3. Influence of the CA concentration on the separation resolution BGE: "narrow pH cut" no. 16 (pH 8.24), concentration of the "narrow pH cut" indicated on the figure. Fused silica capillary, total/effective length, 35/26.5 cm × 50 μm i.d.; voltage, 30 kV; temperature, 25 °C. UV absorbance at 214 nm. Hydrodynamic injection (30 mbar, 3 s). Sample, protein test mixture (each protein at 1 mg/mL) diluted in the considered "narrow pH cut". For protein identification, see Table 1.

We can see on Fig. 2 that the EOF mobility is not, as expected, increasing with the pH, but the CA "narrow pH cuts" present different amphoteric species, heterogeneity, buffering capacity and conductivity [11]. We can thus expect that each CA fraction induces a different zeta potential in a same bare fused silica capillary. Moreover, the CA fractions may present different viscosity. Thus, different electroosmotic flow can be expected when different CA narrow pH cuts, even with close pH values are used.

In the Fig. 3, the influence of the amphoteric fraction concentration on the resolution is shown for the "narrow pH cut" no. 16. Indeed, an increase of the resolution is clearly observed between peaks D and E and between peaks A and B. This is certainly due to an increase of the ionic strength of the BGE. The increase of the observed current (3.4 μA (a), 3.8 μA (b) and 4.11 μA (c)) and the slight increase of migration time confirm this hypothesis.

The repeatability of the protein test mixture separation by CABCE has been assessed in two different BGEs based on CAs, the no. 14 (1.2% (m/v)), and the no. 16 (1% (m/v)). In both cases, four separations have been performed successively with only buffer rinses between runs. In both cases, the protein effective mobilities showed a relative standard deviation (RSD) between 0.4 and 1.6% while a RSD below 1% was observed for the EOF mobility. Besides, the corrected areas show a RSD between 1.9 and 9.5%. Thus, CABCE experiments exhibit an acceptable repeatability.

Another important aspect of CABCE is linked to the UV absorbance spectra of CAs. 200 nm, the optimal detection wavelength for sensitive protein detection cannot be used as CAs are strongly absorbing. We found that the detection at 214 nm was a good compromise. Thus, we cannot expect the best sensitivity in CABCE when working with UV absorbance detection. Other detection modes like laser induced fluorescence or even conductivity detection (based on the conductivity changes in the protein zones in comparison with the conductivity of the surrounding CA-based BGE) have to be considered in the future.

3.3. Protein test mixture analyzed by CZE in classical buffers

The protein test mixture (Table 1) has been analyzed by CZE in two classical buffers, sodium HEPES and sodium phosphate buffers. As in the CABCE experiments, with the

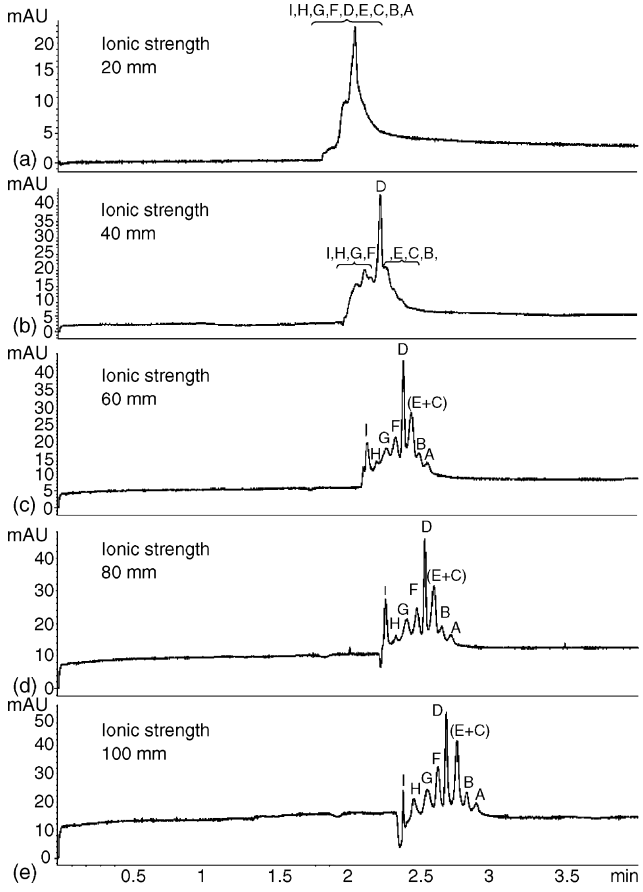

Fig. 4. Protein test mixture analyzed by CZE in sodium HEPES BGE (pH 8.1) at different ionic strengths. Fused silica capillary, total/effective length, 35/26.5 cm × 50 μm i.d.; voltage, 15 kV; temperature, 25 °C. UV absorbance at 214 nm. Hydrodynamic injection (30 mbar, 3 s). Sample, protein test mixture (each protein at 0.4 mg/mL) diluted in buffer. For protein identification, see Table 1.

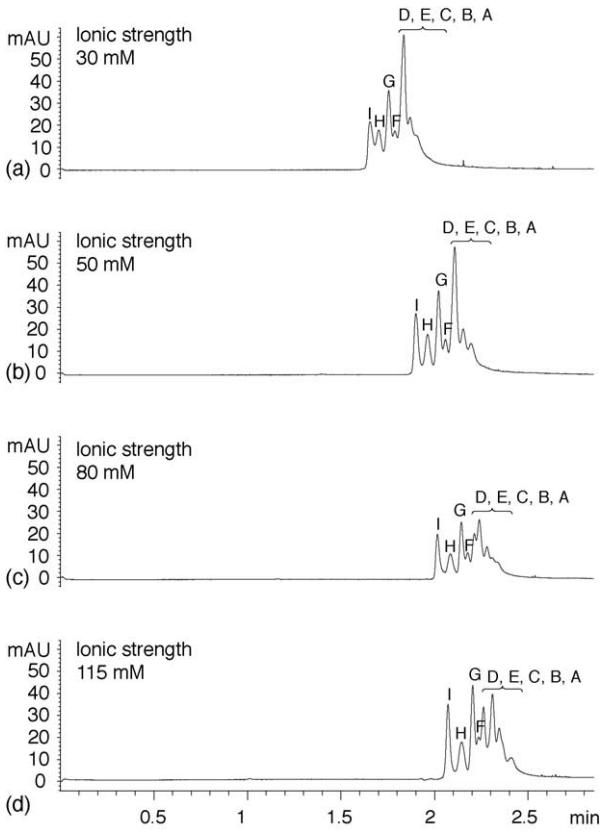

Fig. 5. Protein test mixture analyzed by CZE in sodium phosphate BGE (pH 7.9) at different ionic strengths. Fused silica capillary, total/effective length, 35/26.5 cm × 50 μm i.d.; voltage, 16 kV; temperature, 25 °C. UV absorbance at 214 nm. Hydrodynamic injection (30 mbar, 3 s). Sample, protein test mixture (each protein at 0.4 mg/mL) diluted in buffer. For protein identification, see Table 1.

exception of protein I, the test proteins were analyzed in counter-electroosmotic mode. The pH of the two classical BGEs was chosen to allow the comparison with the CABCE separation in amphoteric fraction no. 15. Sodium HEPES, pH 8.1, and sodium phosphate buffers, pH 7.9, have been prepared at a high ionic concentration which corresponds respectively to 100 and 115 mM ionic strength. Then, the protein test mixture separation was carried out in each buffer, used at different dilutions. It appears that the test separation was not easy to be resolved in its nine components and that high ionic strengths were necessary to provide nice separation in HEPES/NaOH (Fig. 4). But, two proteins (E and C) are still migrating together in this BGE. Co-migration also occurred in the phosphate buffer when high ionic strengths were used. Even a 115 mM ionic strength phosphate buffer (Fig. 5) did not produce a good separation of the proteins contained in the test mixture. The non-growing resolution within the BGE ionic strength used can also be caused by the increased Joule heat generated in BGEs with high conductivity.

3.4. Joule heating in the different background electrolytes

Joule heating in capillary electrophoresis is known to act on the p*K*a values of weak electrolytes present in BGE or sample, on the solution viscosity and consequently on the electrophoretic mobilities of all ions of the BGE and of the sample and on the electroosmotic flow. Variation of temperature inside the capillary due to Joule heating influences also the separation efficiency and reproducibility of the analysis [16]. Moreover, when working with biological samples, the proteins may be deteriorated by the temperature increase induced by the Joule effect. Thus, BGEs have to be properly chosen in order to minimize the Joule heat effect. In Section 3.3, we have demonstrated that highly concentrated classical buffers were needed to separate the protein test mixture. In this part, we will compare the Joule heating generated in the different BGEs used in this study. To see if the use of low conductivity buffers, as “narrow pH cut” of CAs is really advantageous as compared to classical buffers, we have followed the increase of electric current with the increasing electric field strength. On Fig. 6, for each BGE, one can find the ex-

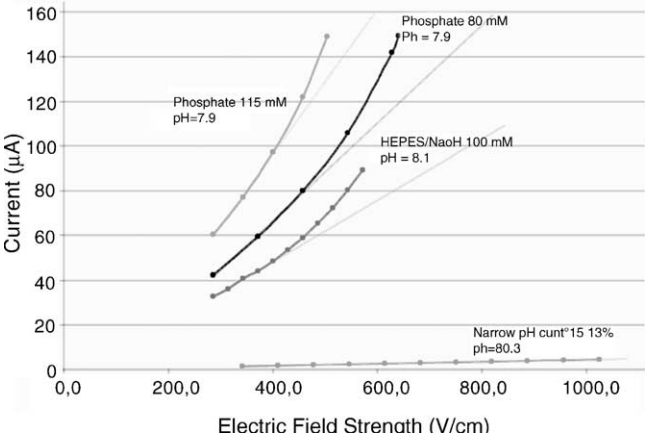

Fig. 6. Ohm's plot for the different studied buffers. Bare fused silica capillary: 35 cm × 50 μm i.d. for the classical buffers; 29.3 cm × 50 μm i.d. for the narrow pH cut no. 15 at 1.3% (m/v).

perimentally determined dependence of electric evolution of current on the applied electric field strength in continuous line and the theoretical expected evolution of the electric current in the absence of Joule heating in dotted line. Positive deviation from linearity is obvious in the case of classical buffers for electric field strength above 400 V/cm. Considering the "narrow pH cut" no. 15 (1.2% m/v), it appears that the Ohm's plot is linear till electric field strength as high as 1024 V/cm. Electric field strength above this value has not been tested because the capillary electrophoresis equipment used did not allow it.

4. Conclusion

Through the different experiments performed in this study, some results of the previous physico-chemical study [11] have been confirmed. Indeed, it has appeared that some of the "narrow pH cuts" of CAs provided by preparative IEF fractionation of a wide pH range 3–10 CA mixture are suitable BGE for capillary electrophoresis. Thanks to their low conductivity, high electric field strengths could be applied without the increased Joule heating, which allowed to separate all proteins contained in the test mixture within a short period of time. Moreover, good resolution of the nine test mixture proteins, rather high efficiency (up to 500,000 theoretical plates/meter) and quite symmetrical peak shape have been obtained. When compared to CZE experiments in classical buffers, it emerges that these classical buffers have to be highly concentrated (around 100 mM ionic strength) to afford comparable resolution to those obtained by CABCE. The high ionic concentration of the CZE buffers involved significant Joule heating as soon as the electric field strength was higher than 400 V/cm while the use of "narrow pH cuts" of CAs allows the application of electric field strength above 1000 V/cm. In view of the obtained results, we could expect that even higher electric field strengths could be applied but we did not have the possibility to confirm this hypothesis because we were limited by our CE equipment.

Due to their physico-chemical properties, some CAs may be better BGEs in CABCE than others. We expect to select the good from the bad ones by improving the CA preparation mainly by further reducing the heterogeneity of each "narrow pH cut" of CAs.

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