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Capillary Electrophoresis as a Second Dimension to Isoelectric Focusing for Peptide Separation

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Capillary zone electrophoresis and carrier ampholytes based capillary electrophoresis have been used as a second separation step to Off-Gel isoelectric focusing for the analysis of complex peptide mixtures. A tryptic digest of four proteins (bovine serum albumin, *β*-lactoglobulin, horse myoglobin, cytochrome *c*) has been chosen as a peptide test mixture. After assessment of different modes of capillary electrophoresis as a second dimension to Off-Gel isoelectric focusing, the optimized two-dimensional platforms provide a degree of orthogonality comparable to state-of-the-art multidimensional liquid chromatography systems as well as a practical peak capacity above 700.

Today, two approaches are mainly used for proteomic studies. The oldest one is the top-down proteomic approach that corresponds to what has been done for decades using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Briefly, once the proteins have been separated by their isoelectric point (pI) and molecular weight, each protein of interest is excised from the gel, digested by an enzyme (usually trypsin), and identified by peptide mass fingerprinting. Although 2D-PAGE is still the most resolutive separation methodology when complex protein mixtures have to be analyzed, this technique suffers from several limitations. In addition to the long analysis time, strongly hydrophobic proteins as well as those of very high molecular weight or extreme pI are difficult to analyze by 2D-PAGE. These limitations can be overcome in the context of the bottom-up approach in which proteins are first submitted to proteolysis. Then, the resulting peptide mixture is analyzed by LC−MS/MS.

In the context of both approaches, it is well-known that no one-dimensional separation technique is able to provide a peak capacity high enough to resolve the complex mixtures to be analyzed. Thus, multidimensional separation methodologies have to be developed. In order to provide the highest peak capacity, the combined methods should present separation mechanisms as orthogonal as possible. If the two combined methods are fully orthogonal, the resulting peak capacity should be equal to the product of individual peak capacities.[1]

In shotgun proteomics, strong cation-exchange (SCX) HPLC is often combined to reversed-phase (RP) HPLC.[2,3] But, as has been shown in a recent study, other HPLC modes could also be considered: for example, Gilar et al. showed that the combination of two RP-HPLC separations, if performed at two significantly different pHs (2.6 and 10.0), can be an interesting two-dimensional separation method with rather orthogonal separation principles.[4]

Although chromatographic techniques are widely used, it has already been shown that capillary electrophoresis (CE) can represent a very interesting alternative. Indeed, in comparison to HPLC, CE provides higher efficiencies and separation speeds. Particularly, capillary zone electrophoresis (CZE) possesses high-speed capabilities and, as such, appears particularly suitable as a second dimension in a two-dimensional separation scheme. For these reasons, as early as 1990, RP HPLC has been coupled online to CZE for peptide separation.[5] Similarly, size exclusion chromatography has been assessed as a first separation dimension before CZE.[6,7]

In the context of developing online two-dimensional separation platforms, the speed and the high efficiency of CZE usually afford achievement of a high sampling rate between the first and the second dimension. If offline two-dimensional separation methodologies are developed, the use of CZE as a second dimension, in comparison to the use of HPLC, is also interesting because it permits us to reduce the total analysis time. From another point of view, other electrokinetic techniques, such as isoelectric focusing (IEF) and capillary isoelectric focusing (CIEF), present appropriate characteristics to be used as a first separation step in multidimensional methods. Indeed, IEF is a steady-state method that provides at the same time high resolving and concentrating power. Its capabilities, as a first separation step were first assessed when hyphenated to RP-HPLC.[8,9] In comparison to multidimen-

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sional separation based only on chromatographic techniques, the use of CIEF increases both the method sensitivity and dynamic concentration range.

To fully take advantage of CE, two-dimensional methods, fully based on electrokinetic separation methodologies, have been developed. Micellar electrokinetic chromatography has been coupled to CZE[10,11] or capillary gel electrophoresis (CGE).[12] In both cases, high-resolution separations have been obtained in a reduced period of time. However, in the context of developing two-dimensional separations, CIEF, due to its high loading capacity, resolution, and concentrating power, appears as the most suitable first dimension. So far, CIEF has been coupled to CGE[12] and to CZE.[10] A very accomplished work is certainly the one published by Mohan et al.[13,14] In these studies, a microdialysis junction has been employed to hyphen CIEF and CZE. Moreover, the presence of carrier ampholytes (CAs) in the fractions eluted from the CIEF dimension has been used to induce a transient isotachophoresis (t-ITP) step in the second dimension. This afforded the stacking of the CIEF fractions at the beginning of the CZE dimension and consequently led to a high resolution and a high sensitivity. After demonstrating the effectiveness of the 2D separation methodology with UV detection, the system was coupled online with electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry for the analysis of the tryptic digest of the cytosolic fraction of *Shewanella oneidensis*.[14] In this work, a total of nine fractions, ranging from pH 3.8 to pH 10.8 were sampled from the CIEF and further analyzed by t-ITP-CZE. This platform allowed the identification of 1174 unique proteins corresponding to a 26.5% proteome coverage in less than 3 h.

A few years ago, Off-Gel electrophoresis (OGE) was introduced to fractionate proteins or peptides by IEF at the micropreparative scale.[15,16] In comparison to gel IEF, the separated compounds can be recovered in solution while taking advantage of the reproducibility of the immobilized pH gradient (IPG) technology. As in CIEF, CAs can be used in OGE, but at a much lower concentration as their purpose is mainly to increase the protein or peptide solubility at a pH close to their pI. Thus, OGE can be more easily coupled to another separation method than CIEF. Moreover, in contrast to CIEF, neither anolyte nor catholyte is needed to establish the pH gradient in OGE. Therefore, subsequent analyses are not hindered by extreme pHs or high conductivities. For these different reasons, OGE has already been used as a first dimension before LC−MS/MS for proteomic studies.[17,18]

In this work, CE has been assessed as a subsequent separation step to OGE for complex peptide mixture separation. The tryptic digest of a four-protein mixture has been considered as a test sample. IEF fractionation by OGE has been performed on a wide pH range (3−10), and different experimental conditions to perform CE have been tested. Also, to know whether CZE can really be considered as a method complementary to OGE, some significant OGE fractions have also been analyzed by RP-HPLC. Finally, the orthogonality of the combined electrophoretic methods as well as the practical peak capacity of the proposed two-dimensional separation platform has been assessed.

MATERIALS AND METHODS

Chemicals. All chemicals used were of analytical reagent grade and obtained from Sigma-Aldrich (Schnelldorf, Switzerland). All buffer and sample solutions were prepared with water produced by an alpha Q Millipore system (Zug, Switzerland) and filtered through 0.2-μm Nalgene filter units (VWR, Dietikon, Switzerland) before their use in CE.

CA-Based Buffer Preparation. Three mixtures (pH range 2−4, pH range 4−9, pH range 9−11) of Servalyts from Coger (Paris, France) were mixed in equal volumes to have a wide pH range of CAs. The CA narrow pH cuts were then fractionated according to the protocol described by Busnel et al.[19] Briefly, a preparative gel (Sephadex G-75) IEF at 50% (v/v) CAs in distilled water was achieved. The fractionation was performed for 24 h at constant power (5 W). The preparative gel presented the following dimensions: 12.5 cm long, 12.5 cm wide, and 0.5 cm thick. After the IEF, 25 gel fractions were collected with a spatula and the CAs were eluted from each fraction with distilled water. The CA fraction stock solutions, collected after the preparative IEF, were simply diluted in distilled water before their use as background electrolytes (BGEs) in CE.

Tryptic Digestion. Each protein (1−3 mg.mL^{-1}) was dissolved in a 75 mM ammonium bicarbonate buffer (pH 7.7) and heated for 5 min. Then, trypsin was added to the protein solution (enzyme to protein ratio w:w = 1:100), and the digestion was carried out overnight at 37 °C.

Off-Gel Electrophoresis. OGE separations were performed with a previously described apparatus.[16] In this study, we used an OGE device allowing the use of 13-cm IPG strip (Amersham Biosciences, Otelfingen, Switzerland) and collection of 20 fractions. Briefly, the separation was carried out as follows. After dispensing 80 μL of sample in all wells, the potential was fixed at 500 V during 1 h, 1000 V for 1 h, 2000 V for 1 h, and finally 3500 V during 2 h. The current limit was set at 1 mA. At the end of the fractionation, the remaining volume in each well was collected and further analyzed without any particular treatment.

Capillary Electrophoresis. As acidic BGEs were chosen for CE separations, positively or neutrally coated fused-silica capillaries were used to avoid significant peptide adsorption on the capillary walls. To be able to perform fast CE separations, two different BGEs presenting a rather low conductivity were chosen. Namely, a classical CZE BGE (10% acetic acid) and a CA-based BGE were assessed. CZE and carrier ampholytes based capillary electrophoresis (CABCE) experiments were carried out with a

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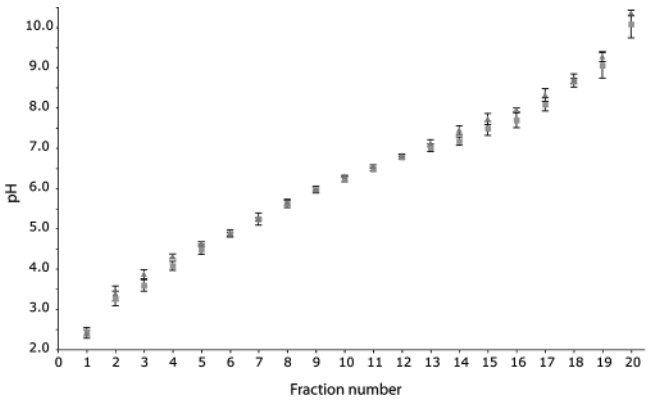

Figure 1. Established pH gradient in OGE as a function of CA concentration. Square: CAs at 0.6% (v/v), Circle: CAs at 1.2%.

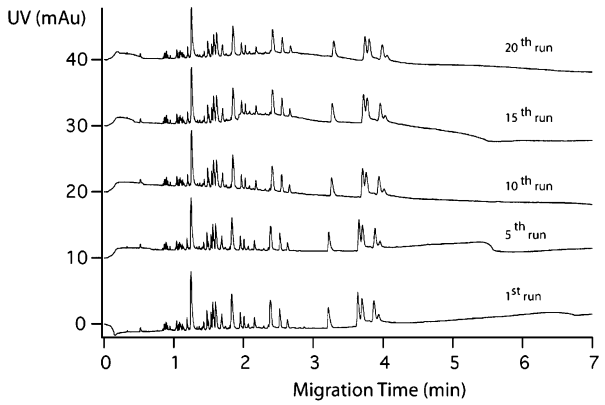

Figure 2. Assessment of the HPC coating stability (HPC-coated capillary, acetic acid 10%). Conditions: voltage 30 kV, observed current 23 μA, temperature 25 °C, UV absorbance at 200 nm. Sample: tryptic digest of cytochrome *c* at 0.4 g.L^{-1}.

PACE MDQ system (Beckman Coulter, Munich, Germany) equipped with a diode-array detector, an autosampler, and a power supply able to deliver up to 30 kV. Data were then extracted and treated using IGOR software (Wavemetrics, Portland, ME). Fused-silica capillaries (50-μm i.d., 375-μm o.d, 20.5-cm effective length, 30.5-cm total length) were obtained from BGB Analytik AG (Böckten, Switzerland) and coated with hydroxypropyl cellulose (HPC) in the laboratory following the procedure described earlier by Shen et al.[20] For positively coated capillaries, after an intensive rinse with 1 M NaOH, 0.5% chitosan (high molecular weight) solution containing 0.1% acetic acid was flushed into the capillary and allowed to react for 10 min. This procedure was repeated every five runs. Between different separations in the same BGE, a water and a buffer rinse were successively performed. Samples were injected by hydrodynamic injection (30 mbar, 6 s).

Liquid Chromatography. An Ultimate system (Dionex LC Packings, San Francisco, CA) equipped with C18 pepmap 100 precolumn (Dionex, 0.5-cm length, 300-μm i.d., 3-μm particles, 100-Å pore size) and column (Dionex, 15-cm length, 300-μm i.d., 3-μm particles, 100-Å pore size) was used for chromatographic separation (flow, 4 μL/min; solvent A, 0.1% FA/2% ACN; solvent B, 0.085% FA/80% ACN). A 70-min gradient was applied (0−30% solution B in 45 min, 30−50% B in 15 min, and 50−100% B in 10 min) while the separation was carried out at room temperature. The injected volume was 1 μL.

RESULTS AND DISCUSSION

pH Gradient in OGE-IEF. The pH gradient established in OGE-IEF was characterized in terms of pH and reproducibility. The 13-cm 3-10 IPG strips were used with an OGE setup allowing the recovery of 20 fractions. In OGE, the pH gradient is established by the IPG but the separation media can contain a given amount of CA that is used to increase the protein or peptide solubility at pHs close to their pI. In order to evaluate both the reproducibility of the pH gradient and the influence of CA concentration on the established pH in our cell design, we considered two CA concentrations (0.6 and 1.2% v/v) and performed four OGE fractionations at each concentration. Figure 1 shows the OGE fraction pH as a function of the well number.

Depending on the considered OGE fraction, the pH shows a relative standard deviation (RSD) between 0.3 and 5.4% (n = 4). Most of the OGE fractions (75%) even present a pH RSD below 2%. It can also be seen in Figure 1 that the CA concentration does not influence significantly the pH fraction. Consequently, OGE-IEF, in addition to be a useful fractionation technique can also be considered as a useful characterization tool. Additionally, the pI information has already been found as important data for validating peptide identifications.[17,18]

CE as a Subsequent Separation Step to OGE-IEF Fractionation. After assessing the reproducibility of the pH gradient establishment in OGE, the orthogonality of CZE and IEF was investigated. The test peptide mixture theoretically contains 123 peptides. Given the limited stability of capillary coating at basic pH, CZE separations were performed at acidic pH. Positively (Figure S-1 Supporting Information (SI)) or neutrally coated capillaries were used to decrease as much as possible the adsorption risk. Two different kinds of BGEs were used to perform CE separations, a classical acetic acid buffer (10% solution, pH 2.2) and a narrow pH cut of CAs (pH 3.25). During the OGE fractionation, CAs were used at 1% (v/v). To roughly evaluate the repeatability of the proposed method, two repetitions of the whole experiment (OGE fractionation and CE separations) were performed for each of the tested conditions.

CZE Analysis of OGE-IEF Peptide Fractions Using a Neutrally Coated Capillary and an Acetic Acid Buffer. Positively coated capillaries used for CZE at acidic pH are known to decrease the adsorption of peptides and proteins; however, neutrally coated capillaries usually provide a better resolution.[21] For this reason, HPC-coated capillaries were used to reach higher efficiency and resolution. In this section, acetic acid at 10% (v/v) was still used as BGE.

In a first step, a CZE run time of 7 min was shown to be sufficient for the analysis of each OGE fraction (data not shown). In order to assess the stability of the HPC coating, a simple peptide mixture corresponding to the tryptic digest of cytochrome *c* was separated 20 times. Several runs extracted from this sequence are shown in Figure 2. In spite of several baseline perturbations, the HPC coating reaches an acceptable repeatability in terms of resolution and migration times. Thus, this coating was selected

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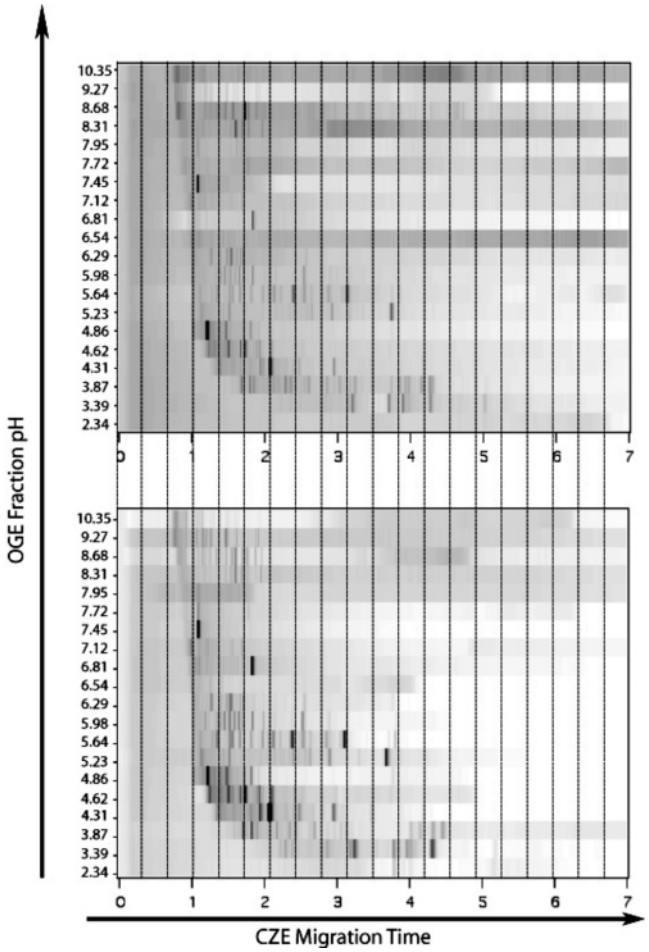

Figure 3. Fractions from OGE-IEF analyzed by CZE (HPC-coated capillary, acetic acid 10%). Conditions: voltage 30 kV, observed current 23 μA, temperature 25 °C, UV absorbance at 200 nm. Concentration of each tryptic digest before the OGE fractionation, 0.02 g.L^{-1}.

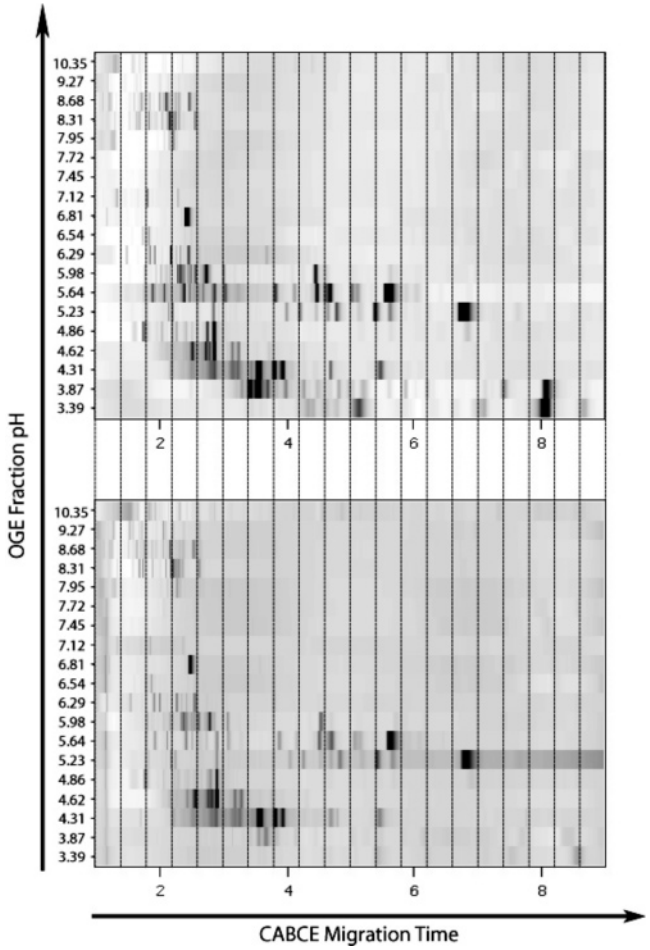

Figure 4. Fractions from OGE-IEF analyzed by CABCE (neutrally coated capillary, CA narrow pH cut, pH 3.25). Conditions: voltage 30 kV, observed current 5.5 μA, temperature 25 °C, UV absorbance at 200 nm. Concentration of each tryptic digest before the OGE fractionation, 0.05 g.L^{-1}.

to perform the CZE separation of the fractions obtained from the OGE-IEF of the peptide test mixture.

Two repetitions of the two-dimensional separation of the peptide mixture are shown in Figure 3. The results were treated as images comparable to what is observed with gel electrophoresis. Moreover, for comparison purposes, the images to be compared were aligned and horizontally separated into 20 zones presenting the same width. Thus, the band positions can be easily compared from one image to another. For several fractions, the standard electropherograms are shown in Figures 5−7.

In comparison to what can be observed with positively coated capillaries (Figure S-1 SI), the use of a neutrally coated capillary strongly improves the resolution. Indeed, ~150 bands can be observed on each image presented in Figure 3. However, only 123 peptides are supposedly present in the test mixture. Different parameters could explain this fact. First, the focusing time might have been too short to completely focus all peptides into sharp zones. Second, it could be linked to the titration curve of each considered peptide. If a peptide presents a titration curve with a flat slope around its pI, it will not be able to focus into a sharp band and will thus be collected in several fractions.[22] Also, the number of observed bands could be superior to the one of predicted peptides because of the occurrence of missed cleavages during proteolysis.

Besides, as in Figure S-1, we can clearly note the similarity of the patterns of the images in Figure 3. Indeed, if we omit that bands are showing different intensities in each image, it has to be noted that, for most fractions, the same bands can be observed at similar migration times from one image of Figure 3 to the other. For example, in fractions of pH 6.81 and 7.45, the same patterns are observed in both experiments. Even for the more populated OGE fraction of pH 5.64, we can detect more than 15 bands in both experiments at similar migration times. Moreover, the repeatability of the proposed two-dimensional method can be verified by noting that, on both images, one of the bands observed in the fraction of pH 5.64 is also observed in the neighboring fraction of pH 5.23. This peptide is migrating in ~90 s and might be presenting a titration curve with a shallow slope around its pI.

However, in spite of the depicted similarity, the band intensities are not comparable from one image to the other. Such a discrepancy cannot be explained by a lack of injection repeatability in CE. Consequently, the variation can only be attributed to fraction collection from OGE. Indeed, during the OGE fractionation, water was added to compensate for liquid evaporation in

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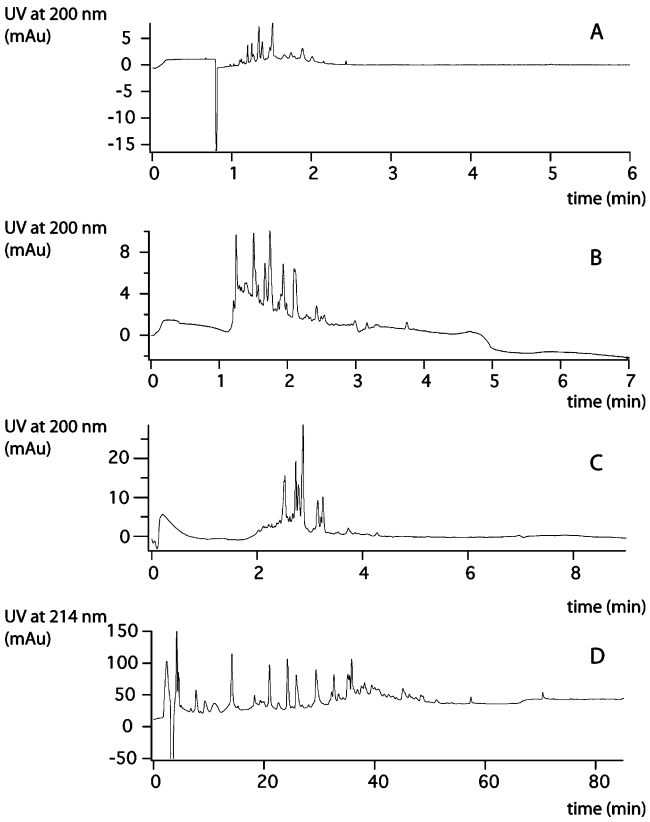

Figure 5. Analysis of one OGE peptide fraction (pH 4.62) by different techniques. (A) CZE in acetic acid with positively coated capillary. (B) CZE in acetic acid with neutrally coated capillary. (C) CABCE analysis at pH 3.25 in neutrally coated capillary. (D) RP-HPLC.

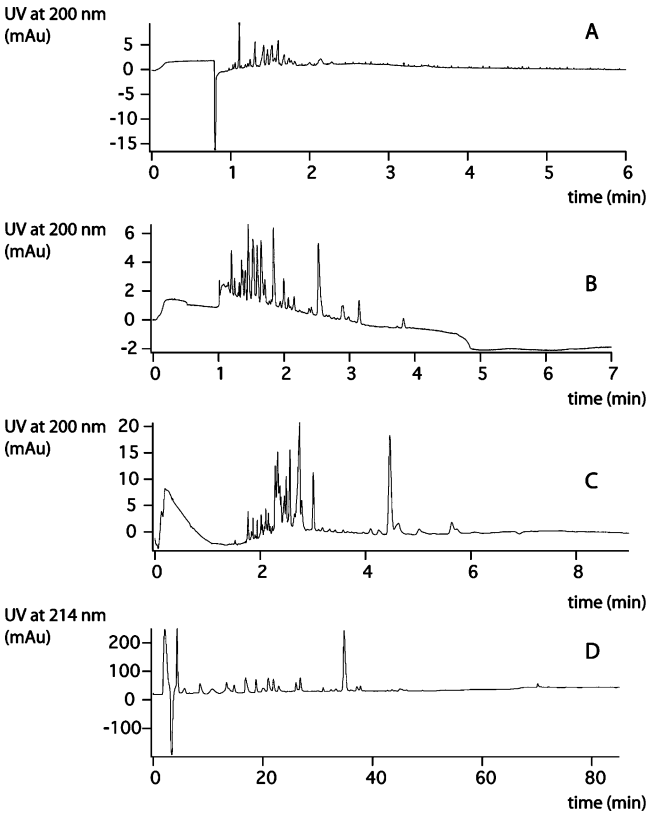

Figure 6. Analysis of one OGE peptide fraction (pH 5.98) by different techniques. (A) CZE in acetic acid with positively coated capillary. (B) CZE in acetic acid with neutrally coated capillary. (C) CABCE analysis at pH 3.25 in neutrally coated capillary. (D) RP-HPLC.

the different wells. As a consequence, the volume collected at the end of the experiment can be different from one fraction to another. Also, from an OGE fractionation to another, the same fraction can be collected under different volumes. Consequently, so far, the usefulness of the described two-dimensional method, for quantitative or comparative purposes, is limited.

Depending on the steepness of their titration curve, different peptides presenting close pIs can exhibit different charge states at a given pH and thus strongly different electrophoretic mobilities. Conversely, proteins or peptides can, in some cases, present the same electrophoretic mobility while presenting different pIs. These two cases can be found while observing the 2D maps presented in Figure 3. For example, the OGE fraction presenting a pH of 5.23 contains peptides showing a broad range of electrophoretic mobilities. Indeed, one can notice that this fraction is composed of peptides migrating as fast as those detected in the most basic fractions in addition to slow-migrating peptides, as those observed in the most acidic fractions. This simple example proves that IEF and CZE can be considered as complementary techniques for the development of two-dimensional separation methodologies.

CABCE Analysis of the OGE-IEF Peptide Fractions Using a Neutrally Coated Capillary and an Acidic Narrow pH Cut of CAs. CABCE was recently introduced for protein or peptide separations.[23,24] It has been shown to be a suitable method for providing efficient and fast separations due to the extremely low conductivity of narrow pH cuts of CAs used as BGE. As has previously been shown,[19] if a wide pH range mixture of CAs is fractionated by preparative IEF, a large number of CA narrow pH cuts with rather high buffering capacity and low conductivity could be prepared and then used as BGE in CE. Unfortunately, even if the large majority (20 over 25) of the prepared fractions have suitable properties to be used as buffer in CE, those of extreme pH, either acidic or basic, present too high a conductivity to be used as low-conductivity BGE. In practice, it appears that the most acidic CA fraction that can be used in CABCE presents a pH of 3.25. This fraction was chosen to perform the second-dimension separation. The results obtained for the peptide test mixture are shown in Figure 4. We have to note that, as the pH of the most acidic OGE fraction is below the one of the buffer used, its analysis has not been carried out in this system.

On the one hand, as in the previously shown 2D maps, a strong similarity can be noted between the two experiments shown in Figure 4. On the other hand, the band intensity is different from an image to another and some bands are not even seen in the bottom image that is showing a lower general intensity. As in Figure 3, ~150 bands can be counted on each image presented in Figure 4. Thus, the use of a CA-based buffer, in comparison to an acetic acid buffer, does not afford a better separation. Moreover, as the pH is higher, the electrophoretic mobilities of the peptides are lower and thus the total analysis time is increased. As a result,

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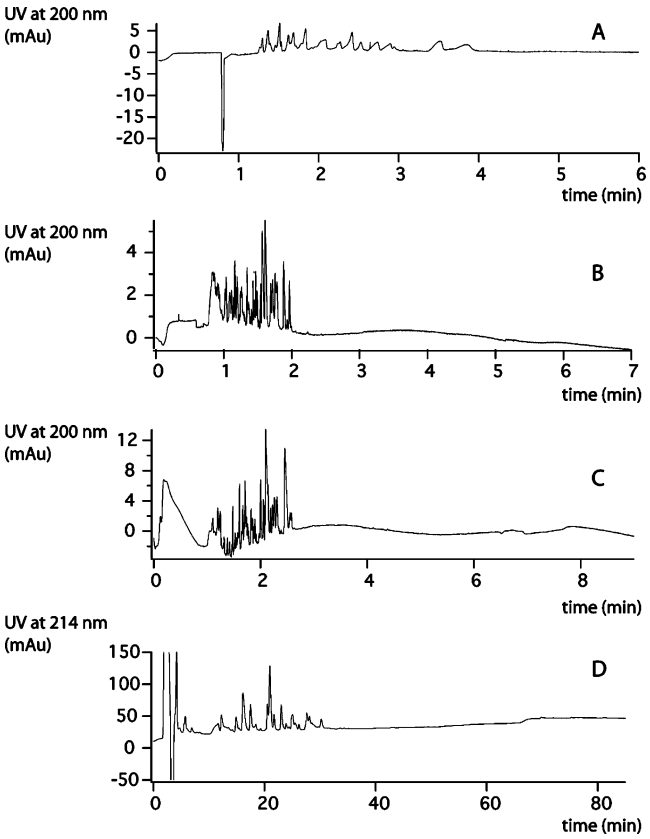

Figure 7. Analysis of one OGE peptide fraction (pH 8.68) by different techniques. (A) CZE in acetic acid with positively coated capillary. (B) CZE in acetic acid with neutrally coated capillary. (C) CABCE analysis at pH 3.25 in neutrally coated capillary. (D) RP-HPLC.

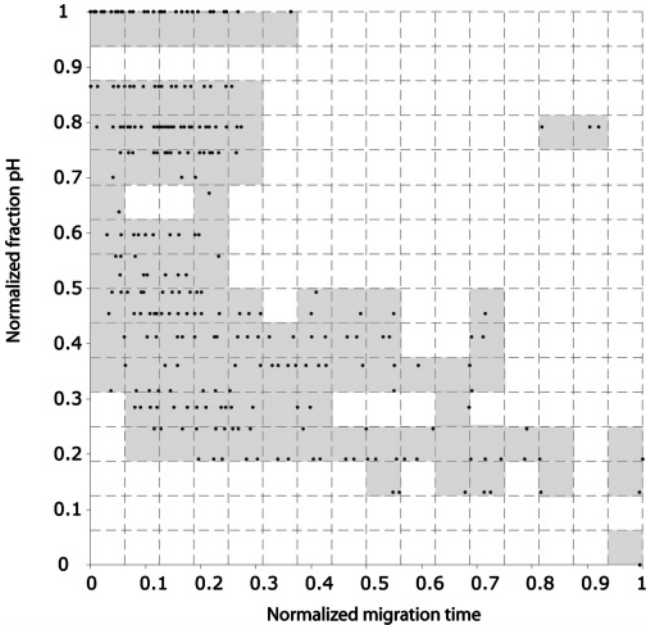

Figure 8. Peptide test mixture analyzed by OGE-IEF followed by CZE. Normalized 2D plot. Experimental conditions as in Figure 3.

we can conclude that the use of CABCE, in comparison to CZE, is not beneficial. However, in future works, if the pH of the BGE has to be precisely tuned to increase the resolution between several comigrating peptides, the large choice of low-conductivity CA-based BGEs between pH of 3.25 and 9.5 could represent an interesting parameter.

RP-HPLC as a Subsequent Separation Step to OGE-IEF. Through the results reported in the previous sections, it has been demonstrated that CZE or CABCE can be considered as a complementary technique to OGE-IEF. In spite of this, to fully assess the significance of this combination, it is important to compare the results obtained by CE to those that can be obtained by RP-HPLC. Indeed, this type of chromatography affords peptide separation according to their hydrophobicity, a parameter theoretically strongly independent from the pI. To this end, we selected several OGE fractions to also be analyzed in RP-HPLC. These fractions have been chosen as a function of their average complexity. Namely, we chose the fractions with the following pH: 4.62, 5.98, and 8.68. In order to favor high-resolution analysis by RP-HPLC, a rather slow gradient (see conditions in Materials and Methods section) was used for the OGE fraction analysis. Figures 5−7 that relate the analysis of some OGE fractions by different methods show the significant cases encountered in this study. From a general point of view, this evaluation did not permit us to demonstrate the superiority of CE or HPLC over the other. Indeed, depending on the considered fraction, the electrophoretic analysis, as compared to the chromatographic one, seemed less (Figure 5), as much (Figure 6), or more (Figure 7) informative. However, some conclusions still arose from this comparative study. First, CE has appeared greatly faster than HPLC. Indeed, through the different examples, the required analysis time for an electrophoretic analysis could be 10−20 times shorter than the one required for a chromatographic separation. Second, as it could be expected, the general analysis sensitivity is higher in HPLC than in CE. This difference is due to both a shorter UV path length and a smaller injection volume. Indeed, when 1 μL is injected in HPLC, only a few nanoliters are injected in CE. However, in order to increase the sensitivity of an electrophoretic analysis, online preconcentration methods can be integrated in CZE. These protocols usually afford a sensitivity improvement of a few orders of magnitude. In the case of the BGEs used in this study, it has been shown that both acetic acid[13,14] and CA-based BGE[25] are buffers in which a t-ITP step, for example, can be integrated to improve the sensitivity. Thus, in future studies, it would undoubtably be interesting to integrate preconcentration methods such as t-ITP in the CE dimension to improve the sensitivity.

Orthogonality and Practical Peak Capacity Evaluation. In order to quantify the separation performances that can be reached with the proposed two-dimensional separation system, the orthogonality of the two dimensions and the practical peak capacity of the proposed two-dimensional method were evaluated. Recently, a geometric approach for assessing the orthogonality and the practical peak capacity of a two-dimensional method was proposed by Gilar et al.[26] In this study, the orthogonality of different LC modes was evaluated. We followed the same approach to quantify the separation performances of the best combination presented above (i.e., Off-Gel followed by CZE in neutrally coated capillaries with acetic acid as background electrolyte). To this end, the experiment corresponding to the top image of Figure 3 has been

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(26) Gilar, M.; Olivova, P.; Daly, A. E.; Gebler, J. C. *Anal. Chem.* **2005**, *77*, 6426−6434.

considered. Approximately 150 bands can be graphically counted on this image, but a precise analysis of each electropherograms reveals that 255 peaks are in fact detected during these analyses. This high number of peaks (compared to a theoretical number of 123 peptides) can be explained by the presence of miscleavages during tryptic digestion, as well as spreading of the same peptides over different Off-Gel fractions.[22] As described in the article from Gilar et al., the fraction pH as well as the migration times observed in the second dimension was normalized so that these values range from 0 to 1 in each dimension. Then, the obtained values were plotted on a normalized 2D separation space containing 256 bins (Figure 8).

The orthogonality O can thus be calculated using the equation (eq 1) proposed by Gilar et al.[26]

$$O = \frac{\sum \text{bins} - \sqrt{P_{\max}}}{0.63 P_{\max}} \quad (1)$$

When considering the 2D plot of the normalized data, Σbins is the number of bins containing data points and $P_{\max}$ is the total number of bins. As explained in the cited paper, the surface used for 2D separation statistically varies from 10 (nonorthogonal system) to 63% (ideally orthogonal separation) of the total separation space. In the case of OGE-IEF coupled to CZE, 91 bins over 256 contain data points. Consequently, the orthogonality of this system can be estimated to 47%. As a comparison, the reference method for peptide analysis that consists of the hyphenation of SCX and RP-HPLC presents an orthogonality of the same magnitude (53%).[26]

Finally, after assessing the orthogonality of OGE-IEF and CZE, the practical peak capacity of the proposed method can be calculated from the eq 2.[26]

$$N_p = P_1 P_2 \frac{\sum \text{bins}}{P_{\max}} \quad (2)$$

N_p represents the practical peak capacity of the studied two-dimensional method and P_1 and P_2 are the respective peak capacities of the first and second dimensions. In our case, the peak capacity of the OGE-IEF is 20 given that the used cell design allows the recovery of 20 fractions. Concerning the CZE, the average base bandwidth has been found to be ~0.042 min over a separation window (time between the detection of the fastest and the slowest peptide) of ~4.2 min. This results in a peak capacity of 100. Consequently, by using the eq 2, we find a practical peak capacity of 711. Taking into account that Off-Gel cell design allowing the recovery of more fractions can be used and that the effective capillary length in CZE is only 20.5 cm, there is a large space for improvement of the practical peak capacity of the proposed two-dimensional separation method.

CONCLUSIONS

OGE-IEF allows efficient and reproducible peptide fractionation according to their pI. Its micropreparative scale provides fraction volumes large enough to perform subsequent analyses. In this study, it has been demonstrated that CZE or CABCE can be positively considered as a second separation step to OGE-IEF. Indeed, the orthogonality of the two hyphenated methods has been found to be rather good. Also, as compared to RP-HPLC, CZE has appeared as or even more informative in the context of the studied peptide mixture. Moreover, CE enables a drastic decrease of the analysis time in comparison to RP-HPLC. Finally, CE is much easier to multiplex than RP-HPLC.

As mentioned above, the IEF separation setup and fraction collection methodology still have to be improved to allow for quantitative recovery of fractionated peptides. In particular, it will be necessary in further studies to control or compensate for the liquid evaporation during OGE runs in order to properly control the concentration of peptides injected in the second dimension.

The practical peak capacity of the separation platform introduced in this work has been found to be ~700 but can easily and strongly be enhanced by increasing both the number of recovered fractions after OGE and the capillary length in CZE. The proposed two-dimensional method thus allows an efficient separation of complex peptide mixtures. Then, if MS detection is successfully hyphenated to the CE separation and if an online preconcentration method is integrated in CE to provide high sensitivity, we can guess that the proposed method could represent a valuable alternative to two-dimensional HPLC.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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