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Received August 29, 2007
Revised November 9, 2007
Accepted December 2, 2007

Research Article

Electrokinetic supercharging for highly efficient peptide preconcentration in capillary zone electrophoresis

Electrokinetic supercharging has been integrated in CZE for the development of a highly sensitive methodology for protein tryptic digest analysis. A careful choice of the experimental conditions led to sensitivity enhancement factors between 1000 and 10 000 whilst maintaining a satisfactory resolution. Peptides in the low nanomolar concentration range have been detected despite the use of the poorly sensitive UV absorbance detection mode. The buffer system used in this study is fully suitable for coupling CE to MS.

Keywords:
Capillary zone electrophoresis / Electrokinetic supercharging / Stacking / Transient isotachophoresis / Tryptic digest DOI 10.1002/elps.200700643

1 Introduction

CZE is today recognized as a very powerful tool for protein or peptide separation [1]. Its major advantages are high efficiency, resolution, and speed. However, as compared to HPLC, CZE generally presents a lower sensitivity. This lack of sensitivity stems from the very small inner diameter of the separation capillary. Indeed, while the use of capillaries presenting inner diameter of 50 μm or even less allows the application of very high electric field strength to achieve efficient and fast separations without inducing any significant Joule heating, the short optical path length impedes the sensitivity of the UV detection. Other detection mode such as MS, and especially LIF usually permits to reach better sensitivities. However, MS is expensive and LIF often requires the derivatization of the analytes to be detected. In comparison, UV is certainly the most universal and cheapest detection method. Therefore, it is often used in spite of its intrinsic low sensitivity, and is an interesting alternative to LIF and MS if no structural elucidation has to be performed.

To circumvent the sensitivity problem of UV detection, online preconcentration methodologies have been developed in the past. The common aim of preconcentration methods is to increase the injected amount of analytes whilst maintaining the separation resolution at a correct level. When no preconcentration or stacking mechanism is integrated, the sample is typically introduced in the separation capillary by a hydrodynamic injection of a short sample plug representing less than 1% of the total capillary volume. Such small sample plug ensures the achievement of a highly efficient separation but this approach is only suitable when rather concentrated samples are considered. Indeed, if low concentration samples have to be studied, the injected quantity might be too low. If so, to afford the detection of the analytes, one can use large hydrodynamic or electrokinetic injections. For example, hydrodynamic injection leading to the filling of more than 30% of the total capillary volume could be used. However, if such an injection allows the introduction in the capillary of a suitable amount of analytes, care should be taken in order to reach a satisfactory efficiency. If not, only a moderate gain in sensitivity will be obtained, but with a dramatic decrease in resolution. To accomplish both a suitable resolution and sensitivity, the analytes must experience at a given location a sudden decrease in their mobility in order to be concentrated in a zone sharper than the initial sample zone. Different mechanisms can be used to induce this concentration process [2–7].

The most widely spread and simple preconcentration method is certainly the field-enhanced sample stacking (FESS). It is based on electrokinetic phenomena and allows, if an appropriate buffer is chosen, the injection of a large sample plug by hydrodynamic injection whilst maintaining a good resolution.

According to Ohm's law, the current density in CE has to remain constant at any point of the capillary during an electrophoretic separation. If the capillary is filled with different solutions, the local electric field strengths will take different

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Abbreviations: EKS, electrokinetic supercharging; **FESI,** field-enhanced sample injection; **FESS,** field-enhanced sample stacking; **HPC,** hydroxypropylcellulose; **SEF,** sensitivity enhancement factor; **t-ITP,** transient ITP

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values depending on the zone conductivities. Therefore, at a given pH, if the sample matrix is presenting a lower conductivity than the BGE, the analytes will migrate faster in the sample than in the BGE zone. Preconcentration of the analytes will thus occur at the boundary between the sample and the BGE zones where a sharp change in electric field exists. FESS is really easy to perform, however, the maximum analyte amount that can be loaded is limited. Indeed, the higher the sample volume is, the lower the resolution is because of the decrease in the available separation length. Moreover, if uncoated capillaries are used, problems linked to the coexistence of two different EOFs will bring an additional source of dispersion resulting in a decrease in resolution [8]. For these different reasons, it is difficult to reach with FESS integration sensitivity enhancement factors (SEFs) higher than 100. In order to achieve higher SEFs, field-enhanced sample injection (FESI) can be used. FESI is based on the same electrokinetic phenomena but an electrokinetic injection instead of a hydrodynamic one is used to introduce the analytes into the capillary. If the electrophoretic mobility of the analytes is high enough, this allows the injection of more analytes and thus the achievement of a higher sensitivity. Furthermore, problems linked to the coexistence of different EOFs are reduced given that the sample plug volume is decreased [8]. FESI usually yields SEFs between few hundreds and few thousands depending on the BGE and the analytes. ITP is another preconcentration method based on electrokinetic phenomena. Compared to FESS and FESI where only the front boundary is stabilized, the sample is here sandwiched between two different BGEs that allow the stabilization of both sample plug boundaries [9, 10].

When ITP is used as a preliminary step to CZE, we would rather speak about transient ITP (t-ITP) because the buffer system as well as the initial filling of the capillary is chosen so as to permit in a first step the preconcentration of the analytes by t-ITP and then, after destacking, their separation by CZE. The term sample self-stacking can also be used to characterize a t-ITP preconcentration step induced by the presence of a major component in the sample solution [11].

Other partial-filling techniques enable preconcentration by sweeping or dynamic pH junction. The first uses interaction between the analytes and a pseudostationary phase to induce the preconcentration, and the second is based on pH differences along the separation path [12–19]. Although a high sensitivity enhancement can be reached when one of the above-mentioned methods is integrated in CZE, even higher SEFs can be achieved when combining these techniques. For example Quirino and Terabe [20] have demonstrated that SEFs as high as several hundreds of thousands are reachable if FESI is used under sweeping condition. Also, Hirokawa and co-workers [21–23] have introduced few years ago the electrokinetic supercharging (EKS) methodology. In EKS, analytes are introduced into the separation capillary with an electrokinetic injection under conditions that are suitable for isotachophoretic stacking. So far, EKS has been applied to rare-earth chlorides [21], DNA fragments [23], and proteins denaturated by SDS [22].

In this study, after assessing the capabilities of t-ITP for enhancing the sensitivity of a tryptic digest analysis in CZE, we demonstrate that EKS allows a further improvement in sensitivity with a good separation resolution for the analysis of complex peptide mixtures.

2 Materials and methods

2.1 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.

2.2 Tryptic digestion

β-Lactoglobulin (3.2 mg/mL) was dissolved in a 75 mM ammonium bicarbonate buffer (pH 7.7) and placed in boiling water 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.

2.3 CE

As acidic BGEs have been chosen, neutrally coated fused silica capillaries have been used to avoid any significant peptide adsorption on the capillary walls. To be able to perform fast CZE separations, acetic acid (10% v/v) that presents a rather low conductivity has been chosen. CZE experiments were carried out with a PACE MDQ system (Beckman Coulter, Munich, Germany) equipped with a DAD, an autosampler, and a power supply able to deliver up to 30 kV. Fused-silica capillaries (50 μm id, 375 μm od, 50 cm effective length, 60 cm total length) were obtained from BGB Analytik AG (Böckten, Switzerland) and coated with hydroxypropylcellulose (HPC) in the laboratory following the procedure described earlier by Shen and Smith [24]. As it has been reported in previous articles, the steadiness of these neutrally coated capillaries is rather high. Indeed, it can be used over a wide pH range without being dramatically deteriorated and used over a long period of time [24–26]. In our experiments, the state of the capillary was checked regularly by analyzing a simple protein tryptic digest by CZE. Usually, a given neutrally coated capillary can be used for 20–40 analyses.

Between different separations in the same BGE, a water and a buffer rinse were successively performed. Depending on the cases, as specified in the different figure captions, samples were introduced into the capillary by hydrodynamic or electrokinetic injection.

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The SEF has been calculated by $\mathrm{SEF} = \left(\frac{h'}{h}\right) f$ where h' is the peak height of the preconcentrated analyte, h the peak height of the nonpreconcentrated analyte when detected with a conventional injection, and f is the dilution factor. For all experiments, the dilution medium has been water. As very long electrokinetic injections have been performed in this study, a fresh sample solution has been used for each experiment. Indeed, if the same sample is used for several experiments, sample depletion can occur and BGE ions can enter the sample solution. If not taken into account, these phenomena alter the reproducibility of the technique.

3 Results and discussion

3.1 t-ITP integration

In a first step, we assess the capabilities of t-ITP to enhance the sensitivity of tryptic digest analyses by CZE. As the aim is to develop a procedure suitable for MS hyphenation, acetic acid has been chosen as BGE. It additionally presents the advantage of being a potential terminating electrolyte for performing cationic ITP at acidic pH. Therefore, with this BGE, only a given amount of a potential leading ion is needed to induce a t-ITP step. The leading ion can whether be present in the sample or it can be added to the sample matrix. Also, partial-filling techniques can be implemented to inject in front of the sample plug a zone of leading electrolyte (LE). In ITP, the leading ion of the LE must present an electrophoretic mobility higher than those of the analytes to be preconcentrated. Inorganic ions, such as Li^+, Na^+, or NH_4^+ for example can all be used here as leading ions. Usually, the choice of leading ion is made as a function of the analyte mobility. Indeed, at a given leading electrolyte concentration, the closer the leading ion electrophoretic mobility to that of the analyte to be stacked, the more efficient is the t-ITP process. However, as the aim of our study is to develop a sensitive electrophoretic method suitable for future coupling to MS, we chose to work with the volatile ammonium ion in spite of its high electrophoretic mobility. As a standard protein proteolytic digest, we have chosen to work with a tryptic digest of medium complexity, the one of β-lactoglobulin.

The interest of integrating a t-ITP preconcentration step in CZE is to inject a large sample volume without decreasing dramatically the resolution as compared to a classical CZE analysis in the absence of stacking. Here, an ammonium acetate buffer (pH 4.0, ionic strength = 115 mM) has been chosen and the results are shown in Fig. 1. The electropherogram A corresponds to the classical CZE analysis of the β-lactoglobulin tryptic digest when no t-ITP is integrated. In that case, the sample concentration is high enough (87 μM) to allow the peptide detection even if the sample is injected as a narrow plug presenting a volume equivalent to 0.75% of the total capillary volume. Then, if the sample is diluted 50 times (electropherograms B–E), higher injection volumes

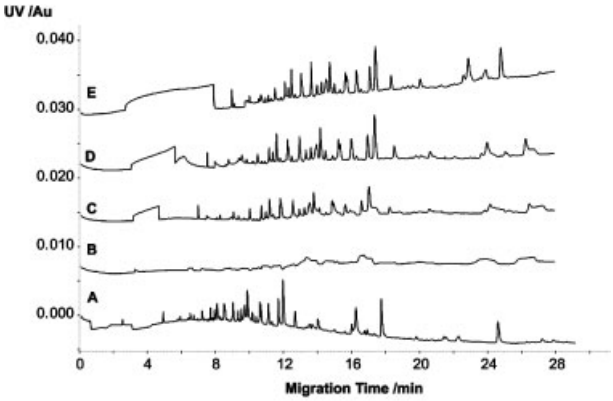

Figure 1. t-ITP integration with a sample plug representing 20% of the total capillary volume. Effect of the LE plug length. HPC-coated capillary, total/effective length 60/50 cm × 50 μm id. Voltage, 30 kV; temperature, 25°C; UV absorbance at 200 nm. Sample: tryptic digest of β-lactoglobulin at 87 (A) or 1.75 μM (B–E). Sample injection: 30 mbar 10 s (A) or 83 mbar 100 s (B–E). LE: 115 mM ionic strength ammonium acetate pH 4. (A) No LE plug injection; (B) no LE plug injection; (C) LE plug injection: 28 mbar 30 s; (D) LE plug injection: 28 mbar 60 s; (E) LE plug injection: 62 mbar 60 s.

have to be used. In the case of the experiments reported in Fig. 1, sample plug volumes representing about 20% of the total capillary volume have been injected for the analysis of low concentration samples. If experimental conditions compatible with FESS integration (electropherogram B) are used, we can see that the buffer used is not suitable for providing an efficient stacking. Indeed, no sharp peaks but rather crenels of low intensity are detected. On the contrary, if a plug of ammonium acetate is injected in front of the sample plug, the electropherogram pattern is strongly improved as a function of the leading electrolyte plug length. Even a really short LE plug representing 2% (electropherogram C) of the total capillary volume leads to a strong improvement of the separation efficiency that increases both, the sensitivity and the resolution of the separation. If such a short plug is suitable to achieve an efficient stacking of the fast moving peptides, it can be seen on the electropherogram C that the slow migrating peptides are not yet presenting a suitable peak shape. Therefore, the leading electrolyte plug has to be further increased. Though, this is only valid to some extent because the longer the length of the LE plug will be, the longer the t-ITP step will last and the shorter the time available for the CZE separation. Thus, when t-ITP is integrated in CZE, depending on the analyte mobilities, a compromise has to be found to provide at the same time an appropriate resolution and a high sensitivity. In the case of Fig. 1, most of the peptides are efficiently stacked when an LE plug representing approximately 4% of the total capillary volume is injected in front of the sample plug (electropherogram E). In that case, depending on the peptides, SEF between 50 and 100 can be achieved whilst maintaining a suitable resolution.

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To assess further the capabilities of t-ITP for protein tryptic digest preconcentration, we then investigated if a sample plug volume superior to 20% of the total capillary volume could be injected without decreasing dramatically the separation resolution. The results of Fig. 2 correspond to analyses where 37% of the total capillary volume was filled by the sample. As before, to be used as a reference, the classical separation of the nondiluted sample (87 μM; electropherogram A) is reported. A 50 times diluted sample (1.74 μM) has then been analyzed under FESS (electropherogram B) and t-ITP (electropherograms C–E) conditions. Again, we can see on electropherogram B that the separation is not satisfactory if no LE plug is injected in front of the sample plug. Then, as a function of the LE plug concentration, we can observe the usefulness of the t-ITP step to improve the separation efficiency. As in the case of Fig. 1, the fast peptides undergo the t-ITP concentration process before the slow ones. Indeed, when a short plug of 115 mM ionic strength LE is suitable for concentrating the fast peptides, a higher concentration of LE is required to stack the slow ones. This is due to the fact that the magnitude of the t-ITP concentration is proportional to the difference between the electrophoretic mobility of the leading ion and that of the peptide to be concentrated. At a given LE concentration, the closer the electrophoretic mobility of the leading ion from that of the peptide to stack, the longer and thus the more efficient the t-ITP process will be. Consequently, the higher the difference between the leading ion electrophoretic mobility and that of the peptide to stack, or the higher the amount of peptide, the higher the required amount of LE will be. This is confirmed by comparing the electropherogram E of Fig. 1 to electropherogram C of Fig. 2. Indeed, in the case of a sample plug volume of approximately 240 nL (20% of the total capillary volume), a LE plug of about 24 nL was suitable to preconcentrate most of the tryptic peptides from β-lactoglobulin. Then, if the sample plug volume is increased to 440 nL, we see (electropherogram C, Fig. 2) that the same LE plug is not sufficient to accomplish an efficient stacking of the slow peptides. In fact, a 24 nL plug of a three times more concentrated LE (345 mM ionic strength) is required (electropherogram E). Under these conditions, SEF between 100 and 200 can roughly be evaluated from the electropherograms of Fig. 2. Then, if the sensitivity of the analysis had to be further increased, a longer sample plug would have to be introduced into the capillary. As the sensitivity would be increased, this would certainly lead to a decrease in the separation resolution. Thus, to reach higher sensitivity enhancements at comparable resolutions, we switched to electrokinetic injections that combine higher loading capabilities and longer distance for CZE separation.

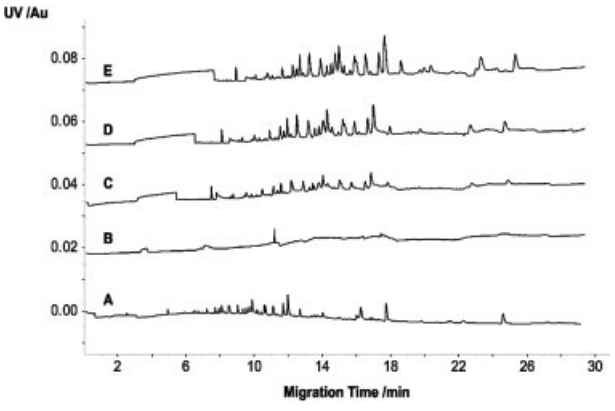

Figure 2. t-ITP integration with a sample plug representing 37.5% of the total capillary volume. Effect of the LE concentration. HPC-coated capillary, total/effective length 60/50 cm × 50 μm id. Voltage, 30 kV; temperature, 25°C; UV absorbance at 200 nm. Sample: tryptic digest of β-lactoglobulin at 87 (A) or 1.75 μM (B–E). Sample injection: 30 mbar 10 s (A) or 152 mbar 100 s (B–E). LE injection: 28 mbar 30 s. (A) No LE plug injection; (B) no LE plug injection; (C) LE: 115 mM ionic strength ammonium acetate, pH 4; (D) LE: 230 mM ionic strength ammonium acetate, pH 4; (E) LE: 345 mM ionic strength ammonium acetate, pH 4.

3.2 EKS

After the injection of a diluted sample by hydrodynamic injection under suitable conditions for t-ITP preconcentration (Fig. 3, electropherogram B), electrokinetic injections of different magnitudes have been subsequently performed (Fig. 3, electropherograms C, D). As before, the electropherogram A is used as a reference and corresponds to the standard CZE analysis of a nondiluted sample (87 μM) of the tryptic digest of β-lactoglobulin. The electropherogram B of Fig. 3 relates the results obtained when conventional t-ITP is

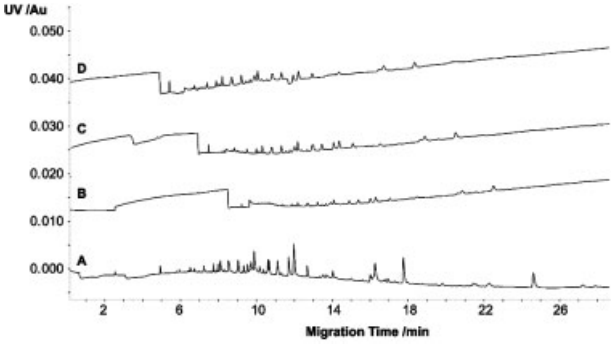

Figure 3. t-ITP integration with a sample plug representing 37.5% of the total capillary volume. Sensitivity enhancement by the subsequent use of an electrokinetic injection. HPC-coated capillary, total/effective length 60/50 cm × 50 μm id. Voltage, 30 kV; temperature, 25°C; UV absorbance at 200 nm. Sample: tryptic digest of β-lactoglobulin at 87 μM (A) or 174 nM (B–D). LE (345 mM ionic strength ammonium acetate, pH 4) plug injection: 28 mbar 45 s. (A) No LE plug injection. Sample injection: 30 mbar 10 s. (B) Sample injection: 152 mbar 100 s. (C) Sample injection: 152 mbar 100 s and 20 kV 4 min. (D) Sample injection: 152 mbar 100 s and 20 kV 8 min.

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used as a preconcentration step after having introduced the diluted sample (174 nM) by hydrodynamic injection. In this experiment, the sample plug represents, as in Fig. 2, 37% of the total capillary volume. With such a low sample concentration, we can see that the use of a hydrodynamic injection, even as large, does not authorize a suitable detection of the peptides. However, if an electrokinetic injection is further carried out (electropherograms C, D), the peak heights are increased while the resolution is not significantly reduced. This simple set of experiments demonstrates the power of electrokinetic injection as compared to hydrodynamic ones. Moreover, even if the fast peptides present a higher peak height increase when electrokinetic injection is subsequently used, a gain in sensitivity can still be noticed for the slow-migrating peptides.

Having verified the high power of electrokinetic injection, we have investigated the application of EKS to take full benefit of this kind of injection for the development of a highly sensitive peptide mapping methodology. To reduce as much as possible the sample zone, no preliminary hydrodynamic injections of the sample have been performed in the next experiments. To integrate EKS, the separation capillary was successively filled with different solutions. In a first step, the capillary is rinsed and filled with the BGE (10% acetic acid), a zone of leading electrolyte is then introduced in the capillary. Finally, before proceeding to the electrokinetic injection of the sample, a short plug of BGE (about 1.4% of the total capillary volume) is introduced into the capillary. As very large electrokinetic injections are used to inject the sample, the short BGE plug is used to ensure that the stacking of the peptides occurs inside and not at the far end of the capillary.

Here, we have chosen to work with a more concentrated leading electrolyte than the one that has been used previously. Indeed, preliminary experiments showed that the use of an ammonium acetate buffer presenting a very high ionic strength of 935 mM and a pH of 9.3 allows the achievement of longer electrokinetic injections and consequently a better sensitivity. The better stacking achieved under these conditions is undoubtedly due to both, the higher concentration of leading ion and the pH difference existing between the leading electrolyte and the BGE used. Indeed, in addition to the higher difference in electric field strength at the boundary between the short BGE plug and the leading electrolyte plug, the pH difference induces, as in dynamic pH junction, a variation of the peptide migration behaviors. Moreover, given that the EOF in HPC-coated capillaries is neither significant at acidic pH nor at basic pH, the focusing process is not hindered by the coexistence of different EOFs in the same capillary [25].

After having chosen the leading electrolyte to be used, the length of the LE plug had to be optimized. Indeed, it must be carefully tuned to allow both an efficient stacking and the achievement of an appropriate resolution. The electropherograms reported in Fig. 4 demonstrate the importance of this optimizing step. The electropherograms show the separations as a function of LE plug length when EKS is integrated. To perform this optimization step, a very low sample concentration (43.5 nM) has been considered given the extensive electrokinetic injections that have been used to introduce the peptides into the capillary. As can be seen in Fig. 4, it is crucial to carefully define the LE plug length when EKS has to be integrated. Indeed, a suitable compromise has to be found between the stacking capabilities and the final resolution of the separation. For example, if the LE plug length is too large, all peptides will remain stacked together till the detection point where a high sensitivity but a low resolution will be observed (electropherogram A). On the contrary, if the LE plug is too short, a poor stacking will result in a low sensitivity analysis (electropherogram C). If the LE plug is even shorter, the preconcentration process will not be efficient enough to stack properly the peptides during the electrokinetic injection. In that case, the fast moving peptides are even not detected at all (electropherograms D and E). From Fig. 4, we can conclude that the best compromise is obtained when 18% of the capillary is filled with the LE (electropherogram B). Under these conditions, as a very intense peak is detected after the ammonium zone, we can guess that several of the fast moving peptides are still stacked together at the detection point. However, about 30 peaks are still detected under these conditions while about 45 peaks can be counted on the reference electropherogram (Fig. 3, trace A). Consequently, even if some peptides are not separated under these conditions, it still appears

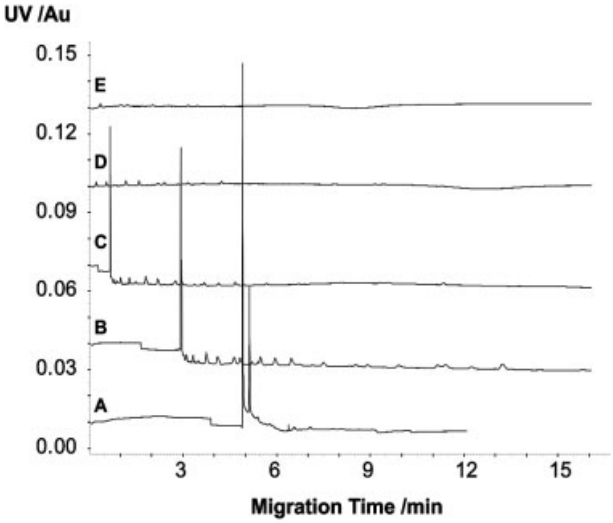

Figure 4. EKS integration. Effect of the LE plug length. HPC-coated capillary, total/effective length 60/50 cm × 50 μm id. Voltage, 30 kV; temperature, 25°C; UV absorbance at 200 nm. LE: 935 mM ionic strength ammonium acetate pH 9.3. BGE plug injection: 30 mbar 20 s. Sample: tryptic digest of β-lactoglobulin at 43.5 nM. Sample injection, 30 kV 20 min. (A) LE plug injection: 83 mbar 100 s; (B) LE plug injection: 83 mbar 90 s; (C) LE plug injection: 83 mbar 80 s; (D) LE plug injection: 83 mbar 70 s; (E) LE plug injection: 83 mbar 60 s.

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that most of the peptides are suitably stacked and separated. Anyhow, when a rather complex mixture as a protein tryptic digest is analyzed with t-ITP integration, the accurate compromise is very difficult to find because such a mixture is presenting analytes with highly heterogeneous electrophoretic mobilities at a given pH. Thus, developing a method for achieving both suitable sensitivity and resolution for the fast moving peptides would certainly lead to a lack of sensitivity of the slow moving peptides. The other alternative is to develop a method that would allow the detection of a maximum number of peptides. In the latter case, it could lead to a lack of resolution between the fast moving peptide. However, if MS is the detection mode used, it could bring a further resolution of the stacked peptides. For this reason, we have developed a method for the detection of most of the peptides of the tryptic digest, even if those of very high electrophoretic mobility were detected while still stacked together.

When FESI is performed in CZE to enhance the sensitivity analysis, a short water plug is usually injected into the capillary before proceeding to the sample electrokinetic injection [27]. It is used to ensure the fast migration of the analytes across the water plug and their further efficient stacking at the boundary between this zone and the BGE zone where a sharp change in electric field exists. In the previous experiments, we used a BGE plug instead of the water plug. In Fig. 5, one can see the importance of injecting such a short plug before proceeding to the sample injection.

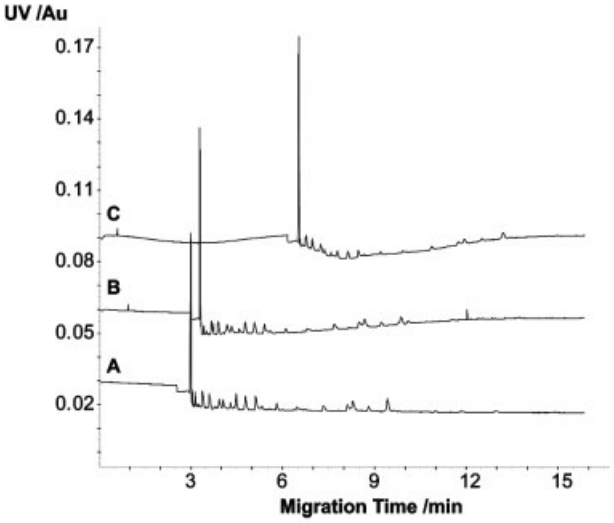

Figure 5. EKS integration. Effect of the injection of a short water or BGE plug before the sample electrokinetic injection. HPC-coated capillary, total/effective length 60/50 cm × 50 μm id. Voltage, 30 kV; temperature, 25°C; UV absorbance at 200 nm. LE (935 mM ionic strength ammonium acetate pH 9.3) injection: 83 mbar 90 s. Sample: tryptic digest of β-lactoglobulin at 43.5 nM. Sample injection: 30 kV 20 min (A–C). (A) BGE plug: 30 mbar 20 s; (B) water plug: 30 mbar 20 s; (C) no BGE or water plug injection.

Indeed, it reports the evolution of the UV trace depending on the nature of the low-conductivity plug. Electropherograms A and B correspond respectively to the use of a BGE or a water plug and the trace C shows the separation when no low conductivity plug is injected before the start of the electrokinetic injection. If we first compare traces A and B, we can see that no significant difference is induced by the use of a water instead of an acetic acid plug. However, if no low-conductivity plug is injected before the electrokinetic injection (electropherogram C), it appears that the general sensitivity is significantly lowered while the migration time is increased. The decrease in sensitivity can be explained by a possible loss of peptides during the electrokinetic injection given that the stacking process should occur at the extremity of the capillary. Indeed, it has recently been shown that protein losses due to sedimentation of the enriched protein zone might occur during electrokinetic injection under zone-sharpening conditions [28].

After setting up the conditions to integrate EKS in CZE for protein tryptic digest analysis, we have assessed the sensitivity of the developed method. To do this, we have considered different samples of β-lactoglobulin tryptic digest presenting molar concentration ranging from 87 μM to 5.4 nM. A scheme depicting the different injection steps of the developed method is shown in Fig. 6. The corresponding experiments are shown in Fig. 7. For comparison, the electropherogram corresponding to the standard CZE analysis of the nondiluted sample (87 μM) is also reported in the figure. Though, given that the preconcentration process significantly alters the separation pattern, it is difficult to attribute the peaks on the traces obtained after EKS to those obtained

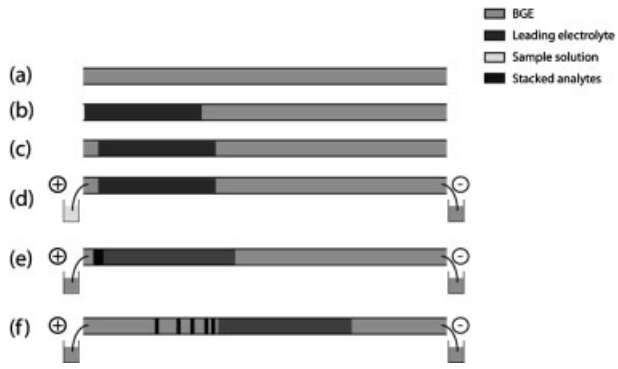

Figure 6. Schematic depicting the different steps of the developed preconcentration methodology. (a) Filling of the separation capillary with the acidic BGE; (b) hydrodynamic injection of LE; (c) hydrodynamic injection of a short BGE plug; (d) electrokinetic injection of the analytes. Focusing occurs simultaneously by dynamic pH junction and t-ITP; (e) a vial containing the BGE is placed at the anode and a voltage is applied. As the front boundary of ammonium is not stabilized, the zone length is increased while the overall concentration of ammonium is decreased. As long as the contact between the sample zone and the ammonium containing zone is preserved, t-ITP preconcentration is maintained; (f) no more contact between sample and ammonium, t-ITP is over and the stacked peptides are separated by CZE.

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under classical conditions. Nevertheless, the group of peaks detected between 7.5 and 9.5 min after EKS preconcentration might certainly be recognized as the group of peaks detected between 16 and 18 min under classical CZE conditions (electropherogram A). Taking into account the height of the signals on the trace A as well as those on the other signals with the corresponding dilution factors, the achieved SEF could be assessed. For example, if we compare the electropherogram A to the electropherogram E, it appears that the general peak height is rather comparable. Thus, given that the sample analyzed in E is 2000 times more diluted than the one analyzed in A, we can roughly estimate a general sensitivity enhancement of 2000. In fact, it seems that the slow moving peptides present a SEF below 2000 while the fast moving ones show a SEF above 2000. Indeed, by examining the electropherogram C, the general height of the peaks detected between 2.5 and 5 min is comparable or even higher than the one of several peptides that are detected between 5 and 10.5 min on trace A. Now, if we consider that the sample in C is 8000 times more diluted than the one in A, we might guess that SEFs in the range of 10 000 can be reached with the integration of EKS. However, given the pattern differences, the only way to evaluate accurately the magnitude of the SEF reached by EKS integration would be the MS detection. Still, according to the electropherograms presented in Fig. 7, the LOD of the method can be assessed. Indeed, it appears that the fast moving peptides are still detected at a sample concentration of 5.4 nM while a concentration above 10 nM is required to detect the slow moving peptides. However, from the results presented in Fig. 7, it is clear that the developed methodology is suitable for analyzing peptides at the low nanomolar range. More precisely, fast moving peptides will be detected at a concentration of a few nM while a concentration of about 50 nM will be the minimum for very slow-migrating peptides. These are those detected after 10 min on the electropherograms E and F. On the reference trace A, the corresponding peptides are those detected between 20 and 24 min. By taking into account the separation conditions, we can calculate the corresponding electrophoretic mobilities to be approximately ranging from 5 to 8 $cm^2 \cdot V^{-1} \cdot s^{-1}$. Consequently, this method can be applied to a wide range of peptides presenting highly heterogeneous electrophoretic mobilities at a given pH. Then, if MS is hyphenated to this methodology in the future, we can expect a strong improvement in the LOD. Indeed, as an example, we can mention that An *et al.* [29] have recently demonstrated that the only integration of t-ITP in CZE could provide a detection limit as low as 0.1 nM for several peptides of the ovalbumin tryptic digest when MS is used as detection.

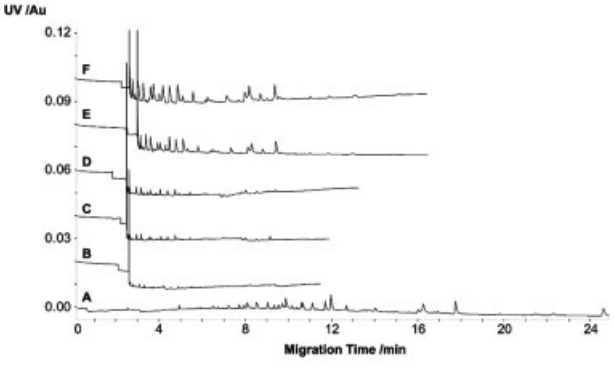

Figure 7. EKS. Effect of the sample concentration. HPC-coated capillary, total/effective length 60/50 cm × 50 μm id. Voltage, 30 kV; temperature, 25°C; UV absorbance at 200 nm. LE (935 mM ionic strength ammonium acetate pH 9.3) plug injection: 83 mbar 90 s. BGE plug injection: 30 mbar 20 s. Sample: tryptic digest of β-lactoglobulin at different concentrations. Sample injection: 30 mbar 10 s (A) or 30 kV 20 min. (B–F). Sample concentrations are: (A) 87 μM; (B) 5.4 nM; (C) 10.9 nM; (D) 21.7 nM; (E) 43.5 nM; and (F) 87 nM.

4 Concluding remarks

After having shown that t-ITP represents an interesting tool to enhance the sensitivity of tryptic digest analysis by CZE, we have demonstrated that the integration of EKS affords a further improvement in the detection limit. Indeed, in spite of performance discrepancies, it has been proven that fast as well as very slow migrating peptides could be efficiently preconcentrated. Indeed, it has appeared that SEFs ranging approximately from 1000 to 10 000 can be reached. The SEF magnitude depends on the electrophoretic mobilities of the considered peptides. This led to detection limits in the low nanomolar range in spite of the use of the low sensitive UV absorbance detection. Moreover, given that the proposed technique is applicable to a wide range of peptides and that the electrolytes used are compatible with MS, it could be positively considered in the future for the development of a peptide mapping platform.

Now, as in this study the optimized leading buffer was presenting a pH totally different than the one of the BGE, a mechanism similar to the one of dynamic pH junction should occur simultaneously to the one of t-ITP. Thus, taking into account the conductivity and pH differences existing inside the separation capillary when integrating the proposed methodology, the preconcentration process should be rather complex. Consequently, in order to fully take advantage of the proposed procedure, it should be interesting to perform in the future a precise study of the involved mechanisms.

The authors have declared no conflict of interest.

5 References

[1] Kraly, J., Fazal, M. A., Schoenherr, R. M., Bonn, R. *et al.*, *Anal. Chem.* 2006, *78*, 4097–4110.

[2] Chien, R. L., Burgi, D. S., *Anal. Chem.* 1992, *64*, 1046–1050.

[3] Beckers, J. L., Boček, P., *Electrophoresis* 2000, *21*, 2747–2767.

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[4] Quirino, J. P., Terabe, S., *J. Chromatogr. A* 2000, *902*, 119–135.

[5] Shihabi, Z. K., *J. Chromatogr. A* 2000, *902*, 107–117.

[6] Urbanek, M., Křivánková, L., Boček, P., *Electrophoresis* 2003, *24*, 466–485.

[7] Monton, M. R. N., Terabe, S., *J. Chromatogr. B* 2006, *841*, 88–95.

[8] Burgi, D. S., Chien, R. L., *Anal. Chem.* 1991, *63*, 2042–2047.

[9] Křivánková, L., Pantůčková, P., Boček, P., *J. Chromatogr. A* 1999, *838*, 55–70.

[10] Foret, F., Kleparnik, K., Gebauer, P., Boček, P., *J. Chromatogr. A* 2004, *1053*, 43–57.

[11] Gebauer, P., Thormann, W., Boček, P., *J. Chromatogr.* 1992, *608*, 47–57.

[12] Giordano, B. C., Newman, C. I. D., Federowicz, P. M., Collins, G. E., Burgi, D. S., *Anal. Chem.* 2007, *79*, 6287–6294.

[13] Kim, J. B., Britz-McKibbin, P., Hirokawa, T., Terabe, S., *Anal. Chem.* 2003, *75*, 3986–3993.

[14] Quirino, J. P., Terabe, S., Boček, P., *Anal. Chem.* 2000, *72*, 1934–1940.

[15] Imami, K., Monton, M. R. N., Ishihama, Y., Terabe, S., *J. Chromatogr. A* 2007, *1148*, 250–255.

[16] Kim, J. B., Okamoto, Y., Terabe, S., *J. Chromatogr. A* 2003, *1018*, 251–256.

[17] Monton, M. R. N., Imami, K., Nakanishi, M., Kim, J. B., Terabe, S., *J. Chromatogr. A* 2005, *1079*, 266–273.

[18] Monton, M. R. N., Otsuka, K., Terabe, S., *J. Chromatogr. A* 2003, *985*, 435–445.

[19] Nesbitt, C. A., Lo, J. T. M., Yeung, K. K. C., *J. Chromatogr. A* 2005, *1073*, 175–180.

[20] Quirino, J. P., Terabe, S., *Anal. Chem.* 2000, *72*, 1023–1030.

[21] Hirokawa, T., Okamoto, H., Gaš, B., *Electrophoresis* 2003, *24*, 498–504.

[22] Xu, Z., Ando, T., Nishine, T., Arai, A., Hirokawa, T., *Electrophoresis* 2003, *24*, 3821–3827.

[23] Xu, Z., Nishine, T., Arai, A., Hirokawa, T., *Electrophoresis* 2004, *25*, 3875–3881.

[24] Shen, Y., Smith, R. D., *J. Microcol. Sep.* 2000, *12*, 135–141.

[25] Poitevin, M., Morin, A., Busnel, J.-M., Descroix, S., Hennion, M.-C., Peltre, G., *J. Chromatogr. A* 2007, *1155*, 230–236.

[26] Busnel, J. M., Lion, N., Girault, H. H., *Anal. Chem.* 2007, *79*, 5949–5955.

[27] Chien, R. L., Burgi, D. S., *J. Chromatogr.* 1991, *559*, 141–152.

[28] Hjerten, S., Mohabbati, S., Westerlund, D., *J. Chromatogr. A* 2004, *1053*, 181–199.

[29] An, Y., Cooper, J. W., Balgley, B. M., Lee, C. S., *Electrophoresis* 2006, *27*, 3599–3608.

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