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Research Article

Capillary electrophoresis immunoassay using magnetic beads

Protein A-coated magnetic beads (0.3 μm) have been trapped in a small portion of a neutrally coated capillary (50 μm id). Anti-β-lactoglobulin (β-LG) antibodies have then been immobilized on the beads through strong affinity with protein A to subsequently capture β-LG from model or real samples. Once the immunocomplexes formed at physiological pH, a discontinuous buffer system has been used to release the partners and preconcentrate them by transient ITP. The antigens and antibodies have finally been separated by CZE and detected by UV absorbance. An LOQ of 55 nM has been achieved. This methodology has been applied to quantify native β-LG in pasteurized and ultra-high-temperature-treated bovine milk. All the described procedures, including immunosorbent preparation, sample extraction, cleanup, preconcentration, and separation are completely automated on a commercial CE instrument. As this CE immunoassay method is simple, rapid, selective, and sensitive, it should be a practical and attractive technology for the analysis of complicated biological samples.

Keywords:
Immunocapture / Isotachophoresis / Magnetic beads / β-Lactoglobulin
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1 Introduction

Since CE combines high separation efficiency, low sample consumption, speed, and compatibility with a wide range of detectors, this technique has played a major role in many forefront fields such as genome sequencing, proteomic, and metabolic research.

In the context of complex biological sample analysis, sample preparation that may include desalting, immunodepletion of major components or concentration of a subclass of compounds are often mandatory to further analyze the compounds of interest. In the field of electrokinetically driven separation, online immunoaffinity CE (IA-CE) has opened the way to more straightforward protocols [1–5]. It involves the online hybridization of two technologies: immunoaffinity extraction and CE. The method permits the online capture of target molecules on a solid support that may contain immobilized antigens, antibodies, or receptors. Then, the captured analyte molecules are released from the solid support by changing the composition of the running buffer and separated by CE. This online combination limits the pretreatment of complicated samples because selective sample extraction, cleanup, and even enrichment can be achieved simultaneously prior to CE. So far, different approaches have been developed to prepare the immunosorbent phase. Open tubular [6–11], packed [12–16], and monolithic [17–19] capillaries are mostly used to immobilize the antibodies. For instance, Phillips' group described an immunoaffinity CE technique with Fab fragment covalently immobilized on the walls of a fused-silica capillary [9]. After functionalization of one-third of the capillary, four kinds of neuropeptides and their tracers were captured, released, separated by CE, and finally detected by LIF. As an alternative to open tubular columns, a short packed bed of controlled-porous glass beads, containing covalently immobilized Fab fragment was integrated within a capillary by Guzman [13]. Gonadotropin-releasing hormone in serum and urine was extracted and detected with this technique. The monolithic column format that is currently under constant progress also showed its potential application as a support material for IA-CE. For example, a Protein G containing monolithic preconcentrator has been used in a CE system for the extraction and preconcentration of IgG from human serum [19].

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Abbreviations: HPC, hydroxypropylcellulose; **IA-CE,** immunoaffinity CE; **β-LG,** β-lactoglobulin; **MB,** magnetic bead; **t-ITP,** transient ITP; **UHT,** ultra-high-temperature

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All these approaches have contributed to the development of CE, making it an easy and versatile technique for complex systems, but still with some limitations. In open tubular configuration, the amount of antibodies immobilized on the capillary surface is restricted. This induces a low capacity and limits the efficiency of immunoextraction. The use of packed beds offers a better loading capacity, however the column preparation is laborious and requires the use of frits to maintain the beads in the capillary. As monolithic columns are synthesized *in situ*, it represents a very interesting alternative when narrow capillaries or microchannels have to be functionalized. Nevertheless, the synthesis procedure is time-consuming and has to be well established to provide reproducible results [20]. As briefly described, the different methodologies derived from LC to perform IA-CE represent very interesting options but alternative approaches must be developed to overcome the existing drawbacks and complement the current technologies.

In that context, magnetic trapping may represent an interesting alternative. For many years, magnetic beads (MBs) have been widely used in chemical and biological engineering. For example, selective separation and collection of target analytes using MBs is regularly used in immunoassay protocols [21, 22]. Also, the good compatibility of MBs with microfluidic platforms has been demonstrated through the fabrication of a microreactor allowing efficient and very fast tryptic digestion of protein [23].

An early report on the online application of MBs in CE has been published by Rashkovetsky *et al*. in 1997 [24]. They reported the magnetic trapping of functionalized MBs (2.8 μm) into a fused-silica capillary for enzymatic reaction and immunocapture. In this last case, sheep Ig covalently coated MBs have been used to capture a mouse mAb. After capture, the mouse antibody was released, preconcentrated by ITP, and quantified by UV detection. Although this fritless approach for online IA-CE was promising, only feasibility was demonstrated. Since this pioneering work, other applications of MBs in CE were presented for electrochromatography [25, 26], protein capture [27, 28], and affinity preconcentration [29]. However, to the best of our knowledge, no research about MBs-based IA-CE was reported.

In the present work, protein A-coated MBs were trapped into a neutrally coated capillary as a support for antibody. After bounding the antibody of interest on the MBs, the corresponding antigen was immunocaptured and preconcentrated online. Then, the immunological complex was dissociated under acidic conditions and the respective antibody and antigen were stacked by transient ITP (t-ITP) and further separated by CZE. Taking β-lactoglobulin (β-LG) as a model analyte, optimized conditions for MBs trapping, immunocapture, t-ITP, CZE separation, nonspecific adsorption, and reproducibility were investigated in detail. β-LG is the most abundant whey protein in milk, but it is also one of the major allergens in bovine milk [30]. Its detection and quantification is thus important in the control of food quality. For these reasons, reliability and practicability of the developed IA-CE method were evaluated by determining the native β-LG concentration in different milk samples.

2 Materials and methods

2.1 Chemicals

Protein A-coated superparamagnetic beads of uniform size (mean diameter 0.3 μm) were purchased from Ademtech (France). Rabbit antibovine β-LG polyclonal antibody (1 mg/mL in PBS) was obtained from GeneTex (San Antonio, TX, USA). β-LG from bovine milk (90%), BSA (98%), and polyoxyethylene-sorbitan monolaurate (Tween-20) were purchased from Sigma–Aldrich (Buchs, Switzerland). Hydroxypropylcellulose (HPC) was from Acros (Chemie Brunschwig AG, Basel, Switzerland). Acetic acid (99.5%) was purchased from Fluka (Buchs, Switzerland) and ammonium acetate (98%) from Merck (Darmstadt, Germany). All buffer and sample solutions were prepared with water produced by an alpha Q-Millipore System (Zug, Switzerland). Commercial milk samples of different types (pasteurized and skimmed bovine milk powder (35 g protein/100 g), ultra-high-temperature (UHT)-treated bovine liquid milk and liquid soy milk) were purchased from a local supermarket.

Throughout the experiments, the following buffers were used: binding and leading buffer (ammonium acetate 100 mM, pH 8.0); sample buffer (binding buffer with 0.5% BSA and 0.1% Tween), elution buffer and separation buffer (10% v/v acetic acid). The suspension of MBs was prepared daily by sonicating and diluting 20 times the commercial solution with distilled water. Anti-β-LG polyclonal antibody (100 μg/mL) was also daily prepared by diluting the stock solution with binding buffer. β-LG (2.5 mg/mL) was prepared in sample buffer, aliquoted, and stored at −20°C. All the milk samples were obtained after centrifugation and filtration through 20-μm Nalgene filter units (VWR, Dietikon, Switzerland).

2.2 Apparatus

All experiments were carried out on a PACE MDQ system (Beckman Coulter, Nyon, Switzerland) equipped with a photo-DAD, an autosampler, and a power supply able to deliver up to 30 kV. Fused-silica capillaries (50 μm id, 375 μm od, 30 cm effective length, 40 cm total length) were obtained from BGB Analytik AG (Böckten, Switzerland) and coated with HPC following the procedure described by Shen *et al*. [31] to limit as much as possible the EOF and sample adsorption. The magnetic field was applied by two round magnets (Nd-Fe-B, 5 mm diameter, 2 mm thickness, SuperMagnete, Zürich, Switzerland), which were placed directly around the capillary at a distance of 11 cm from the inlet. To arrange the magnets around the capillary, the cooling system of the apparatus was disabled and the coolant tube removed.

2.3 Procedure for online immunocapture and separation

Step 1: MBs trapping; a uniform suspension of MBs was injected into the capillary for 3 min at 34.5 mbar.
Step 2: Antibody immobilization; anti-β-LG antibodies (100 μg/mL) were injected for 10 min at 34.5 mbar.
Step 3: Antigen immunocapture; β-LG (standard solutions or samples) were injected for a given period of time at 34.5 mbar.
Step 4: Washing; the binding buffer was injected at 34.5 mbar for 10 min to remove unbound proteins and totally fill the capillary. This binding buffer was used as the leading buffer for ITP stacking.
Step 5: Reverse rinsing; a plug of separation buffer was injected at 34.5 mbar from the outlet vial.
Step 6: Release and separation of the antigens and antibodies; 15 kV was applied from anode to cathode with electrode compartments filled with separation buffer. As electrophoresis occurs, the pH in the zone of MBs gradually diminishes till a value where the immunocomplexes are dissociated. The antigen and the antibody are then released from the MBs, stacked by t-ITP and finally separated by CZE.
Step 7: MBs removal; the trapped MBs are removed at the end of each analysis by flushing at high pressure (1379 mbar) with separation buffer. The different steps are illustrated in Fig. 1.

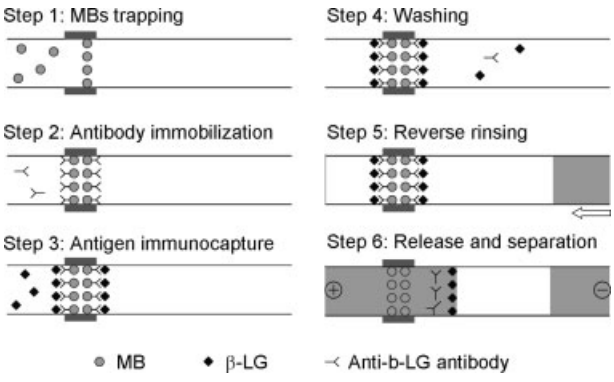

Figure 1. Schematic presentation of the procedure for online immunocapture and separation.

2.4 Quantification of β-LG

The developed procedure was first tested with standard β-LG solutions (0.001–2.5 mg/mL). Three repetitions at each concentration were performed. A calibration curve was then constructed by plotting the corrected peak area (A/t) as a function of the concentration. In order to fit in the linear range of the calibration curve, milk samples were first diluted with sample buffer. One-tenth diluted pasteurized skimmed milk powder (5 mg/mL) and 1/200 diluted UHT liquid milk were analyzed. 1/10 diluted soy milk was taken as a control. Again, three repetitions have been performed. The β-LG concentration was then calculated for each sample by taking into account the mean value of the repetitions, the calibration curve and the dilution factor.

3 Results and discussion

3.1 MB trapping and stability in capillary

MBs with different diameters (2.8, 1, and 0.3 μm) were investigated. While the largest beads exhibit the highest magnetic susceptibility, the smallest ones present the lowest sedimentation rate. Consequently, MBs presenting a mean diameter of 0.3 μm have been chosen. This kind of beads stays as a rather uniform suspension for a considerable time, which is of importance in order to obtain a suitable reproducibility. Furthermore, the small size of MBs provides a high surface area to volume ratio, which not only reduces the diffusion distance for all steps but also increases the density of binding sites for immobilization of antibody. Moreover, as shown in Fig. 2, small MBs can be trapped in the capillary by the magnetic field by using reduced pressure and easily removed by applying a high pressure. As the UV absorption of MBs is proportional to the amount trapped in the capillary, Fig. 2 shows an increase in trapped MBs when injection time increased. Although more MBs are advantageous for the following immunoaffinity step, it induces a higher resistance to the flow and thus requires long washing times. In an extreme case, it can even lead to a blocking of the capillary resulting in current breakdown. In our experiments, a 3 min MB injection time has been chosen because it provides a good compromise between loading capacity, speed, and current stability.

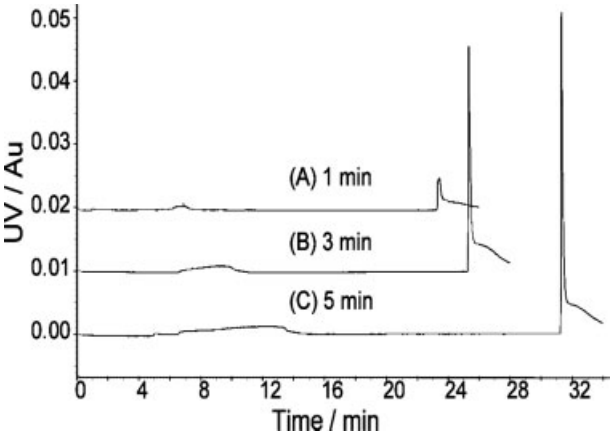

Figure 2. UV response corresponding to the high-pressure removal of the magnetically trapped MBs. Conditions: HPC-coated capillary, total/effective length 40/30 cm × 50 μm id, UV absorbance at 200 nm. Injection sequence: protein A-coated MBs (0.3 μm) injection: 34.5 mbar for (A) 1 min, (B) 3 min, (C) 5 min; washing with water: 34.5 mbar for (A) 22 min, (B) 22 min, (C) 26 min; MB removal step with water: 1379 mbar for 2 min.

For its potential use as a support for immunocapture, it is important to make the trapping of MBs stable. To achieve this, a required condition is to make the magnetic forces dominate over the competing forces [24], which is pressure and electric field in our system. Due to the very small diameter of the used MBs, their magnetic susceptibility is rather limited. Consequently, to avoid the competing forces outweighing the magnetic force, the experimental conditions had to be optimized. In our setup, MBs removal was observed at a pressure of 69 mbar (Fig. 3a). A 34.5 mbar pressure was thus used for all the injection and rinsing steps. At the end of each run, a high pressure of 1379 mbar was performed in order to remove the trapped MBs from the capillary. Then, it was important to know if the MBs can sustain the electric field without being removed. To test the stability of the MBs under electric field, a voltage ranging from 10 to 25 kV was applied during 20 min after the injection of the MBs in the capillary. Subsequently, the capillary was rinsed and the MBs removed by applying a high pressure. As shown in Fig. 3b, no significant bead losses were observed because the amount of removed MBs at the end of each analysis was similar. For all the following electrophoresis experiments, a voltage of 15 kV was applied.

3.2 Online immunoaffinity and separation

Protein A-coated MBs were used for the immobilization of anti-β-LG antibody. Specific binding happened at the Fc part of the antibody, thus controlling its orientation and making its paratopes fully available to react with its antigen. In a first step, to assure a maximum loading capacity to the immunosorbent, the antibody injection was optimized to work at saturation of the MBs. To proceed to this optimization, the antibody was injected for a given period of time. Then, after the washing step that permits to remove the unbound antibodies, the bound ones were released and electrophoretically transported toward the detector by the previously described procedure. As can be seen in Fig. 4a, the quantity of bound antibodies increased with the injection time until 5 min and then remained stable. To provide a maximum loading capacity to the immunosorbent, 10 min was thus chosen for the injection of the antibody.

After having loaded the anti-β-LG antibody into the capillary at a saturated level (injection for 10 min), the antigen was injected for a given period of time. To allow the different immunoreactions to occur, all the protein samples were dissolved in binding buffer (100 mM ammonium acetate, pH 8.0). After the immunoaffinity step, unbound proteins were washed by rinsing with binding buffer for another 10 min. Then, separation buffer (10% v/v acetic acid, pH 2.0) was introduced from the outlet vial to fill a determined portion of the capillary. The further voltage application while the separation buffer was placed in both electrode compartments allowed the dissociation of the different immunocomplexes, the ITP preconcentration and finally the CZE separation of the antibody and antigen. As shown in Figs. 4b and 5, results demonstrate the successful capture and separation of anti-β-LG antibody and β-LG. Thanks to the miniaturized format of the capillary, all the immunoaffinity reactions happened rapidly at room temperature and no incubation time was needed.

Another interesting feature of the developed system, in addition to allow an online immunoselection, is its ability to preconcentrate a protein of interest. As can be seen from Figs. 4b and 5, when the sample presents a low concentra-

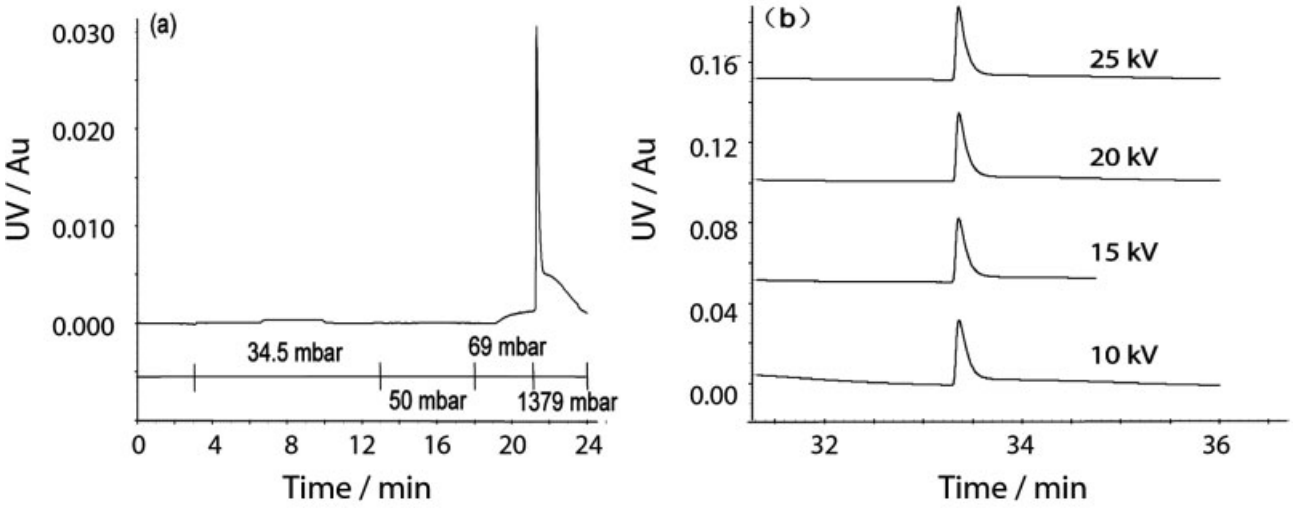

Figure 3. Stability of trapped MBs under applied pressure (a) and voltage (b). Conditions: HPC-coated capillary, total/effective length 40/30 cm × 50 μm id, UV absorbance at 200 nm. Injection sequence: protein A-coated MBs (0.3 μm) injection: 34.5 mbar for 3 min; (a) washing with binding buffer: 34.5 mbar for 10 min, 50 mbar for 5 min, 69 mbar for 3 min and 1379 mbar for 2 min; (b) removal of trapped MBs by high-pressure (1379 mbar) application after applying different voltages (10, 15, 20, 25 kV) for 20 min.

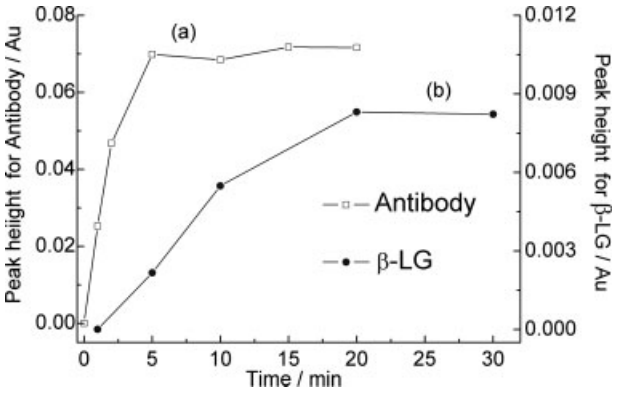

Figure 4. (a) Plot of peak height of antibody *versus* injection time. Anti-β-LG antibody, 100 μg/mL. (b) Plot of peak height of β-LG *versus* injection time. β-LG, 1 μg/mL; antibody injection for 10 min.

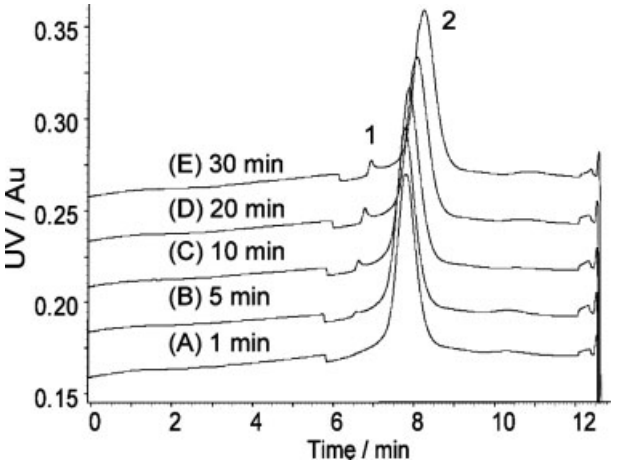

Figure 5. Sample preconcentration by increasing the percolating time of the sample. Peak 1, β-LG; Peak 2, anti-β-LG antibody. Conditions: HPC-coated capillary, total/effective length 40/30 cm × 50 μm id, UV absorbance at 200 nm. Injection sequence: protein A-coated MBs (0.3 μm) injection: 34.5 mbar for 3 min; anti-β-LG antibody (100 μg/mL in binding buffer) injection: 34.5 mbar for 10 min; β-LG solution (1 μg/mL in sample buffer) injection: 34.5 mbar for (A) 1 min, (B) 5 min, (C) 10 min, (D) 20 min, (E) 30 min; washing with binding buffer: 34.5 mbar for 10 min; reverse injection of separation buffer: 34.5 mbar for 5.5 min; elution and separation: applied voltage, 15 kV; MB removal step with separation buffer: 1379 mbar for 2 min.

tion, the injection time can be varied in order to provide a suitable sensitivity for further analysis and/or quantification. As an illustration, it is shown for β-LG that an injection time of 1 min is not sufficient for detecting this protein if its concentration in the sample is 1 μg/mL. However, the sensitivity can be linearly improved by increasing the sample injection time to 20 min. Thus, depending on the sample to be studied, as different sensitivity levels could be achieved, the experimental conditions can be tuned to fulfill the requirements.

3.3 Stacking by ITP

With the present experimental conditions, the binding (ammonium acetate) and the separation (acetic acid) buffers have been chosen as they constitute a well-known buffer system for conducting cationic ITP at acidic pH. As it is demonstrated in Fig. 6, adjusting the reverse rinsing step duration permitted to set the magnitude of the t-ITP preconcentration. As a longer reverse rinsing duration reduced the ammonium acetate zone length, the amount of leading ions and thus the stacking effect were concomitantly decreased. In addition to t-ITP, as the binding and the separation buffer present very different pH values, preconcentration by dynamic pH junction should also occur. While a strong stacking brings a high efficiency and sensitivity, meanwhile, it leads to some drawbacks. First, as the stacking is increased, the resolution is decreased because the available time for CZE separation is reduced. Second, in extreme stacking conditions, the local protein or antibody concentration can reach the respective limit of solubility and consequently induce precipitation. Ultimately, this can provoke the blocking of the capillary. A good compromise had thus to be found. From Fig. 6, it could be concluded that a 1 min reverse rinsing time constituted the best condition, however, the current was not very stable under these conditions. For this reason, a 2 min reverse rinsing time has been chosen for the next experiments.

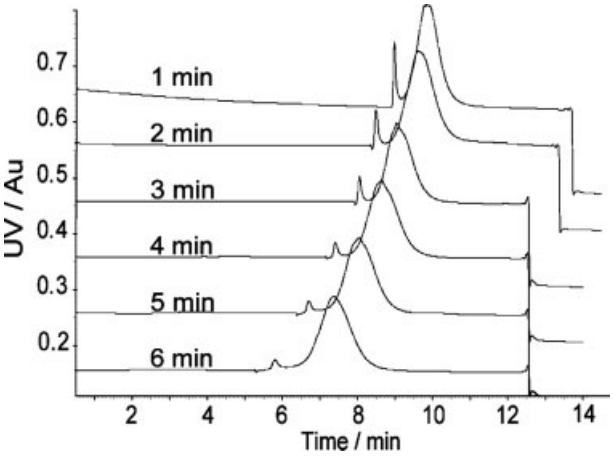

Figure 6. Variation of the t-ITP magnitude by varying the reverse rinsing time of separation buffer. Peak 1, β-LG; peak 2, anti-β-LG antibody. Conditions: β-LG solution (1 μg/mL in sample buffer) injection: 34.5 mbar for 2 min; other conditions are the same as in Fig. 5.

3.4 Nonspecific adsorption

Another important problem in any immunoaffinity methodology stems from the nonspecific adsorption of proteins on the immunosorbent phase. It usually decreases the selectivity of a method, especially when complex real sam-

ples have to be analyzed. From this point of view, it was very important to minimize as much as possible these interactions. However, Van der Waals, hydrophobic and electrostatic interactions always exist between proteins and different surfaces, coating a fused-silica capillary with a polymer by covalent bonding or physical adsorption is usually the most effective way to diminish these interactions in CE. Different additives could also be used to avoid the nonspecific retention of compounds. Both approaches were applied in our method. HPC-coated capillaries as well as 0.1% Tween in sample solutions have been used. Also, to mimic real conditions, all sample solutions used in the following experiments contained a 0.5 mg/mL concentration of BSA. It is proven in Fig. 7 that the chosen experimental conditions keep the nonspecific adsorption contribution at an acceptable level.

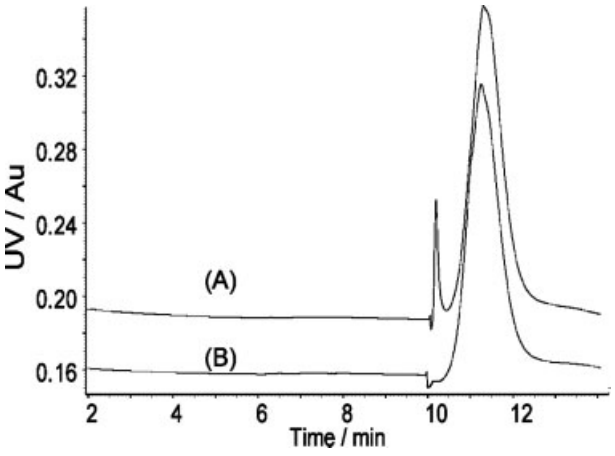

Figure 7. Nonspecific adsorption assessment. Conditions: (A) β-LG solution (2.5 mg/mL in sample buffer) injection: 34.5 mbar for 2 min. (B) Control sample (BSA 0.5 mg/mL in sample buffer) injection: 34.5 mbar 2 min; reverse rinsing of separation buffer: 34.5 mbar for 2 min; other conditions are the same as in Fig. 5.

3.5 Quantification of β-LG in milk

To further assess the native β-LG concentrations in different milk samples, a calibration curve has been constructed. The results obtained are shown in Fig. 8. A linear evolution of the corrected area was observed for β-LG concentrations ranging from 1 to 17.5 μg/mL ($R^2 = 0.98$). A trend toward saturation was observed for concentrations above 17.5 μg/mL. For each point of the calibration curve, an RSD between 2.6 and 6.9% has been calculated. This rather good repeatability was likely due to the renewal of the MBs before each run. Also, before each MB injection, the suspension was homogenized by gentle mixing to minimize the effects of sedimentation. Under the used experimental conditions, an LOQ of 1 μg/mL has been determined for β-LG. This corresponds approximately to a protein concentration of 55 nM. However, as demonstrated in Fig. 5, an LOD in the very low nanomolar range can be expected by increasing the sample injection time.

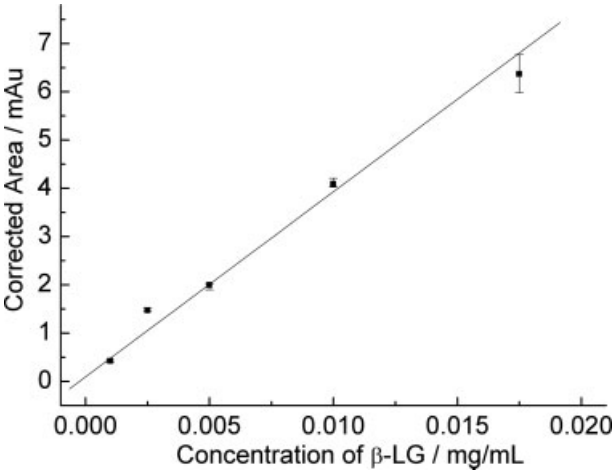

Figure 8. Calibration curve for β-LG quantification. Conditions: except the β-LG concentration, conditions are the same as in Fig. 7.

The developed procedure was then applied to quantify the concentration of native β-LG in two different commercially available milks. The corresponding electropherograms are shown in Fig. 9. As samples, two bovine milks with different processing treatments have been analyzed. Soy milk, as a control sample, has also been considered in the study. Taking into account the calibration curve, it has been determined that the skimmed milk powder presents a native β-LG concentration of 12.77 ± 0.58 mg/g and the UHT milk a concentration equal to 0.47 ± 0.01 mg/mL. As compared to other references that mention the concentration of β-LG in raw milk [32, 33], the concentrations that we have determined are lower. Now, it is well known that β-LG is highly sensitive to temperature [34]. Indeed, in the latter work, it has for example been shown that the native concentration of β-LG decreases from 3.5 to less than 0.5 mg/mL after 90 s at 95°C. Thus, if we consider that the two bovine milks, which have been analyzed had undergone pasteurization or UHT process (135°C for a short time), and that the used antibodies may recognize conformational but not linear epitopes, the obtained results appear fully reasonable. For soy milk, as expected, no β-LG was detected. As this sample presents a high total protein concentration, this confirms that nonspecific adsorption is not a significant problem with the developed protocol. All these results clearly demonstrate that this MBs-based IA-CE methodology is effective and reliable for real biological sample analyses. As compared to other immunosorbent previously reported in the literature, the main advantage of the use of MBs in IA-CE is undoubtedly the possibility to renew the stationary phase after each analysis. Indeed, as it has been demonstrated, the MBs can be easily washed out from the capillary by simply applying a high-pressure rinse. Under these conditions, if the nonspecific adsorption on the capillary walls is significantly avoided, different samples can be analyzed successively in

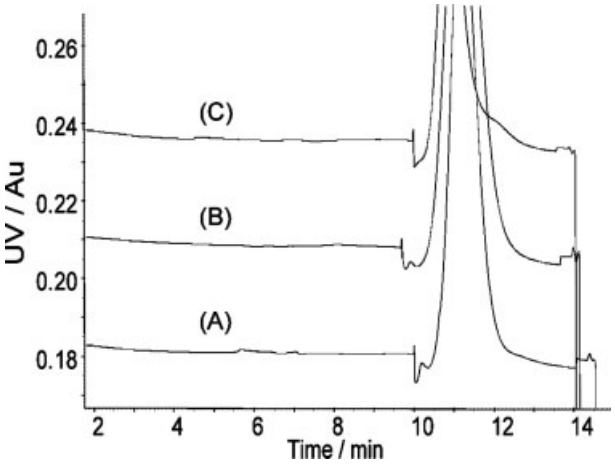

Figure 9. Electropherograms demonstrating the detection of β-LG from milk samples after online immunocapture. Conditions: (A) pasteurized skimmed milk sample (5 mg/mL, 1/10 diluted); (B) UHT milk sample (1/200 diluted); (C) soy milk (1/10 diluted, control). Reverse rinsing time, 2 min; other conditions are the same as in Fig. 6.

the same capillary without any memory effects. Also, if the concentrations of different antigens in a given sample have to be determined by IA-CE and if the respective functionalized MBs are available, one can envisage proceeding successively with the different analyses in one single capillary. In that context, a higher throughput can be reached with the MBs-based IA-CE system. From another point of view, it is well known that it can be difficult in some cases to recover bound analytes from their capture antibody. If MBs are used for the immunoaffinity step, harsh elution conditions, which can denature the capture antibodies, can be used given that a new set of antibody will be used for the following experiment. A higher recovery rate and a higher sensitivity could then be expected.

4 Concluding remarks

Protein A-coated MBs (0.3 μm) have been trapped in a neutrally coated capillary for conducting immunocapture of β-LG. Thanks to the affinity of protein A toward different mammalian Igs, a polyclonal anti-β-LG antibody, raised in rabbit has been immobilized on the surface of the MBs. As protein A binds the Fc part of the antibodies, the reactivity of anti-β-LG antibody was fully preserved in spite of its immobilization on the immunosorbent MBs. In addition, the miniaturized format of the experimental setup provided a high density of binding sites and rapid mass transfers that allowed capturing β-LG online without the need of any incubation time. After immunocapture at pH 8, the partial-filling of the capillary with different buffers and the subsequent application of a voltage led successively to the dissociation of the different immunocomplexes, the t-ITP preconcentration of the antibody and antigen and their final separation by CZE. The developed method was applied to the quantification of β-LG in both standard solutions and commercial milk samples. Excellent reproducibility (RSD = 2.6–6.9%) and trustworthy results confirmed the effectiveness and reliability of the method. As compared to other online IA-CE techniques, a distinct advantage of the described approach is the simple and automated preparation of an immunoaffinity support that can be renewed after each analysis.

An LOQ of 55 nM has been achieved in this work but the LOD can further be lowered through large sample volume percolation. Then, if the sensitivity still needs to be enhanced, the use of fluorescence detection could represent a very interesting alternative however a labeled secondary antibody would have to be used if native fluorescence is not employed. In that case, the sensitivity of the method would certainly be improved but this would make the immunoassay more complex and the detection of the native antigen impossible.

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5 References

[1] Guzman, N. A., Stubbs, R. J., *Electrophoresis* 2001, *22*, 3602–3628.

[2] Guzman, N. A., *Anal. Bioanal. Chem.* 2004, *378*, 37–39.

[3] Guzman, N. A., Phillips, T. M., *Anal. Chem.* 2005, *77*, 60A–67A.

[4] Amundsen, L. K., Sirén, H., *Electrophoresis* 2007, *28*, 99–113.

[5] Delaunay-Bertoncini, N., Hennion, M.-C., *J. Pharm. Biomed. Anal.* 2004, *34*, 717–736.

[6] Phillips, T. M., Chmielinska, J., *J. Biomed. Chromatogr.* 1994, *8*, 242–246.

[7] Ensing, K., Paulus, A., *J. Pharm. Biomed. Anal.* 1996, *14*, 305–315.

[8] Phillips, T. M., Kennedy, L. M., De Fabo, E. C., *J. Chromatogr. B* 1997, *697*, 101–109.

[9] Phillips, T. M., *Anal. Chim. Acta* 1998, *372*, 209–218.

[10] Little, M. K., Crawley, C. D., *Anal. Chim. Acta* 2002, *464*, 25–35.

[11] Phillips, T. M., Smith, P., *Biomed. Chromatogr.* 2003, *17*, 182–187.

[12] Cole, L. J., Kennedy, R. T., *Electrophoresis* 1995, *16*, 549–556.

[13] Guzman, N. A., *J. Chromatogr. B* 2000, *749*, 197–213.

[14] Dalluge, J. J., Sander, L. C., *Anal. Chem.* 1998, *70*, 5339–5343.

[15] Thomas, D. H., Rakestraw, D. J., Schoeniger, J. S., Lopez-Avila, V., Van Emon, J., *Electrophoresis* 1999, *20*, 57–66.

[16] Guzman, N. A., *Electrophoresis* 2003, *24*, 3718–3727.

[17] Bedair, M., El Rassi, Z., *J. Chromatogr. A* 2004, *1044*, 177–186.

[18] Faure, K., Delaunay, N., Alloncle, G., Cotte, S., Rocca, J.-L., *J. Chromatogr. A* 2007, *1149*, 145–150.

[19] Armenta, J. M., Gu, B., Humble, P. H., Thulin, C. D., Lee, M. L., *J. Chromatogr. A* 2005, *1097*, 171–178.

[20] Augustin, V., Jardy, A., Gareil, P., Hennion, M.-C., *J. Chromatogr. A* 2006, *1119*, 80–87.

[21] Moaddel, R., Marszall, M. P., Bighi, F., Yang, Q. *et al.*, *Anal. Chem.* 2007, *79*, 5414–5417.

[22] Hahn, Y. K, Jin, Z., Kang, J. H., Oh, E. *et al.*, *Anal. Chem.* 2007, *79*, 2214–2220.

[23] Slovakova, M., Minc, N., Bilkova, Z., Smadja, C. *et al.*, *Lab Chip* 2005, *5*, 935–942.

[24] Rashkovetsky, L. G., Lyubarskaya, Y. V., Foret, F., Hughes, D. E., Karger, B. L., *J. Chromatogr. A* 1997, *781*, 197–204.

[25] Okamoto, Y., Ikawa, Y., Kitagawa, F., Otsuka, K., *J. Chromatogr. A* 2007, *1143*, 264–269.

[26] Wang, Y., Zhang, Z., Zhang, L., Li, F. *et al.*, *Anal. Chem.* 2007, *79*, 5082–5086.

[27] Kaneta, T., Inoue, J., Koizumi, M., Imasaka, T., *Electrophoresis* 2006, *27*, 3218–3223.

[28] Kaneta, T., Takahashi, M., Imasaka, T., *Electrophoresis* 2007, *28*, 2262–2266.

[29] Okamoto, Y., Kitagawa, F., Otsuka, K., *Anal. Chem.* 2007, *79*, 3041–3047.

[30] Wal, J. M., *Allergy* 2001, *56*, 35–38.

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

[32] Farrell, H. M., Jr., Jimenez-Flores, R., Bleck, G. T., Brown, E. M. *et al.*, *J. Dairy Sci.* 2004, *87*, 1641–1674.

[33] Bordin, G., Cordeiro Raposo, F., de la Calle, B., Rodriguez, A. R., *J. Chromatogr. A* 2001, *928*, 63–76.

[34] Lieske, B., Konrad, G., Faber, W., *Int. Dairy J.* 1997, *7*, 805–812.

