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Separation and quantitation of milk whey proteins of close isoelectric points by on-line capillary isoelectric focusing—Electrospray ionization mass spectrometry in glycerol–water media

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ABSTRACT

On-line coupling between CIEF and ESI/MS based on the use of bare fused-silica capillaries and glycerol–water media, recently developed in our laboratory, has been investigated for the separation of milk whey proteins that present close p*I* values. First, a new rinsing procedure, compatible with MS detection, has been developed to desorb these rather hydrophobic proteins (α-casein (α-CN), bovine serum albumin (BSA), lactoferrin (LF)) from the inner capillary wall and to avoid capillary blockages. Common hydrochloric acid washing solution was replaced by a multi-step sequence based on the use of TFA, ammonia and ethanol. To achieve the separation of major whey proteins (β-lactoglobulin A (β-LG A), β-lactoglobulin B (β-LG B), α-lactalbumin (α-LA) and BSA, which possess close p*I* values (4.5–5.35), CIEF parameters *i.e.* carrier ampholyte nature, capillary partial filling length with ampholyte/protein mixture and focusing time, have been optimized with respect to total analysis time, sensitivity and precision on p*I* determination. After optimization of sheath liquid composition (80:20 (v/v) methanol–water + 1% HCOOH), quantitation of β-LG A, β-LG B, α-LA and BSA was performed. The limits of detection obtained from extracted ion current (EIC) and single ion monitoring (SIM) modes were in the 57–136 nM and 11–68 nM range, respectively. Finally, first results obtained from biological samples demonstrated the suitability of CIEF–MS as a potential alternative methodology to 2D-PAGE to diagnose milk protein allergies.

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1. Introduction

Since twenty years, food allergy has increased substantially and some recent studies have shown that 5% of children and 3% of adults are concerned [1]. Studies on food allergies are complicated since their prevalence depends on age, country and consuming habits. A large number of foods have been described to cause allergy, among them, hen egg, peanut and milk are the most common in western countries [2,3]. For children younger than 2 years, cow milk allergy is the second cause of food allergies and its prevalence is ranging from 2 to 3% [4]. Since milk is the main food of infant, this pathology is a significant healthy problem. Bovine milk contains about 3–3.5% of proteins which can be divided by acidic precipitation into two main classes: caseins (80%) and whey proteins (20%). Casein fraction constitutes the colloidal phase of milk and is subdivided into α-, β-, γ- and κ-casein. Whey proteins involve α-lactalbumin (α-LA), β-lactoglobulin isoforms (β-LG A and β-LG B), together with BSA, lactoferrin (LF) and immunoglobulins [5]. In addition to these, other twenty minor proteins are also present in cow milk. Most of milk proteins involve an allergic response [6,7] but studies revealed that caseins, α-LA and β-LG are the main allergens [8–11].

Many techniques are available for the analysis of milk proteins [4]. As far as the identification of allergens is concerned, ELISA is the main immunoassay-based method currently employed. Specificity and sensitivity of the enzymatic reaction between antibodies and their recognized antigens constitute two main advantages of this method but they were counterbalanced by long procedure required to obtain antibodies and stability problem. Separation methods have also been performed to analyze milk proteins, among which reversed-phase liquid chromatography [11–13] and electrophoresis [14–16]. 2-D PAGE is the most commonly used method to separate proteins [17,18]. In the first dimension, proteins are separated according to their isoelectric point along a pH gradi-

Abbreviations: α-CN, α-casein; α-LA, α-lactalbumin; β-LG, β-lactoglobulin; BSA, bovine serum albumin; Myo, Myoglobin; LF, lactoferrin; Rnase, ribonuclease A; EIC, extracted ion current; MeOH, methanol; EtOH, ethanol.

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ent by IEF, and according to their relative molecular mass (*Mr*) by SDS-PAGE in the second dimension. It has been successfully applied for milk proteins analysis, including low-abundance protein identification [19], their stability evaluation under various heat treatment and storage conditions [20] and allergen identification [21]. In this latter application, 2-D PAGE separation was followed by immunoblotting analysis using sera from milk-allergic patients to identify allergens. Despite high resolution, 2-D PAGE is difficult to use routinely because it requires many manual operating steps for protein separation and detection.

Electrophoresis in a capillary format is an alternative to the traditional 2-D electrophoresis because it allows to perform fast and automated analyses with high resolution and small sample and buffer requirements. First separations of main milk proteins were carried out in CZE in an uncoated fused-silica capillary using 4 M urea in a phosphate buffer [22]. The presence of urea in the BGE allowed the efficient separation of caseins, as it prevents the formation of aggregates. However, the separation of proteins by CZE was often hampered by protein adsorption to the negatively charged fused-silica surface, leading to distorted peak shapes and non-reproducible results [23]. These problems were overcome by simultaneously using hydrophilic coated capillary, extreme pH buffer and polymeric additives [24,25]. These methods were applied and modified for the analysis of caseins and whey proteins in dairy products such as milk [26–28], milk powder [29] and cheese [30,31]. Another application consisted in the quality control of products commercialized as 100% vegetal [32]. This CE method allowed to determine bovine whey proteins in soybean dairy-like products to prevent the consumption of the animal proteins by allergic people. Separation of casein and whey proteins has also been performed by MEKC in less than 2 min using an uncoated fused-silica capillary and SDS [33]. Busnel et al. [34] developed an original way to improve detection thresholds of proteins. A transient isotachophoresis (*t*-ITP) step was integrated in carrier ampholyte-based capillary electrophoresis (CABCE). Caseins and whey proteins from skimmed and dried milk were partially resolved in less than 8 min and the authors estimated that *t*-ITP/CABCE allowed to detect protein concentration at the level of few tens $mg\,L^{-1}$.

CIEF is a high-resolution technique for the separation of ampholytes, such as proteins, as a function of their p*I*. Several reviews on CIEF have been published, covering a full spectrum of applications [35,36]. CIEF experiments are classically performed in capillaries coated with a neutral and hydrophilic polymer, using dynamic or permanent coating [36–39]. The purpose is double: (1) to suppress or at least lower EOF and (2) to avoid irreversible protein adsorption to the capillary wall. To our knowledge, only two recent articles deal with the CIEF-UV analysis of milk proteins. Somma et al. [40] described the successful separation of cow and buffalo caseins by CIEF as a preliminary step before the characterization of their peptides produced from plasmin activity. In the CIEF method developed by Poitevin et al. [41], "narrow pH cut" added to commercial ampholyte mixtures improved the difficult separation between β-LG isoforms and α-LA, which possess very close p*I* values.

Recently, we demonstrated the feasibility of on-line coupling between CIEF and ESI/MS detection for the analysis of model proteins [42]. This coupling was inspired by a previous work featuring an original CIEF protocol which does not require the use of a modified bare fused-silica capillary and an anticonvective gel [43]. Indeed, gel was replaced by glycerol. Thanks to its rather high viscosity, glycerol could both play the role of anticonvective medium and considerably reduce EOF, making possible CIEF experiments in an unmodified capillary. This method takes into account different constraints associated to the coupling of CIEF with ESI/MS. First, the electrical continuity required for the electrophoretic separation has been maintained thanks to a discontinuous electrolyte, filling the separation capillary: 50–60% capillary length were filled with the catholyte whereas the remaining 40–50% length were filled with the proteins/ampholytes mixture. The compatibility of fluids entering the MS detector constitutes the second main difficulty. Hence, anolyte and catholyte solutions, which are classically composed of phosphoric acid and sodium hydroxide, respectively, have been replaced by formic acid and ammonia. Finally, replacing gel by glycerol allowed the total compatibility of the fluid arising from CIEF to enter MS. Under known conditions, ESI/MS offers the potential to identify a protein unequivocally from the envelope of the multi-charged ions. Therefore, CIEF–ESI/MS provides equivalent information as 2-D PAGE with some added advantages as speed, automation and sensitivity.

The aim of this study was to investigate this new on-line CIEF–ESI/MS method for the qualitative and quantitative analysis of milk whey proteins of close p*I* values, that present high propensity to capillary wall adsorption. First, particular interest was focused on the optimization of post-conditioning sequence compatible with MS detection. Indeed, a suitable rinsing procedure is essential to remove residual adsorption of protein to the inner capillary wall, leading to more repeatable results. Then, different sheath liquid compositions were compared with respect to sensitivity. The influence of CIEF conditions (ampholyte nature and concentration, discontinuous capillary filling length, focusing time), as well as the quantitative method performances (*e.g.* limits of detection and quantitation, linearity, repeatability, trueness) are also discussed in this paper. Finally, this method was applied to the determination of whey proteins in a biological sample, *i.e.* a rabbit serum.

2. Materials and methods

2.1. Chemicals and reagents

Myoglobin (horse heart, p*I* 6.30, *Mr* 16.9 kDa) and proteins from bovine milk: α-lactalbumin (α-LA, p*I* 4.50, *Mr* 14.2), β-lactoglobulin A (β-LG A, p*I* 5.25, *Mr* 18.4 kDa), β-lactoglobulin B (β-LG B, p*I* 5.35, *Mr* 18.4 kDa), bovine serum albumin (BSA, p*I* 4.90, *Mr* 66.0 kDa), α-casein (α-CN, p*I* 4.60, *Mr* 23.6 kDa), lactoferrin (LF, p*I* 8.30, *Mr* 78.0 kDa) were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Ribonuclease A (Rnase, p*I* 9.45, *Mr* 13.6 kDa), carrier ampholytes (pH 3–10), 1 M phosphoric acid and 1 M sodium hydroxide were obtained from cIEF 3-10 Kit (Beckman Coulter, Fullerton, CA, USA). Narrow pH gradient ampholyte mixtures, Servalyt pH 4–6 (Serva Electrophoresis GmbH, Heidelberg, Germany) and Ampholine pH 4–6 (Amersham Pharmacia Biotech, Uppsala, Sweden) were also used. Rabbit serum was kindly provided by the group of H. Sénéchal (ESPCI ParisTech, France). All solvents and reagents were of analytical grade. Lysine, glutamic acid and ammonium hydroxide (25%) were purchased from Merck (Darmstadt, Germany). Methanol (MeOH) and ethanol (EtOH) were supplied from VWR (Fontenay-sous-Bois, France). TFA, acetic acid, formic acid, hydrochloric acid and glycerol were purchased from Sigma–Aldrich-Fluka (Saint-Quentin Fallavier, France). All background electrolytes and sample solutions were prepared using purified water produced by an Alpha Q system (Millipore, Bedford, MA, USA).

2.2. CE apparatus

CIEF experiments were performed using a HP^{3D}CE apparatus (Agilent Technologies, Massy, France) equipped with a diode-array detector, an autosampler and a power supply able to deliver a voltage up to 30 kV. The cassette temperature was set at 25 °C. Data were collected and analyzed using the HP Chemstation software

(version 1990–2002, Rev A.09.03). Separations were carried out in a bare fused-silica capillary (80 or 100 cm length, 50 μm i.d., 375 μm o.d.) obtained from Photon Lines (Marly-le-Roi, France). Prior to use, new capillaries were activated using the following three-step sequence: 1 M NaOH, 0.1 M NaOH and H_2O, for 15 min each, by applying a pressure of 950 mbar. When not in use, capillary was rinsed by the procedure described previously and air-dried.

2.3. Mass spectrometry detection

An Agilent Series 1100 MSD single quadrupole mass spectrometer (Agilent Technologies) equipped with an orthogonal ESI source was used in the positive ionization mode. Nitrogen was used as nebulizing (NG) and drying gas (DG). In the optimized conditions, the temperature of NG and DG was set to 100 °C (pressure 55 kPa) and 350 °C (flow rate 7 L min^{-1}), respectively. Optimized spray and skimmer voltages were 4.5 kV and 100 V, respectively. CE was coupled to the ESI interface using an Agilent Technologies triple coaxial tube nebulizer held at ground potential. The optimized coaxial sheath liquid was a mixture of MeOH/H_2O (80:20, v/v) containing 1% HCOOH. It was delivered at a flow-rate of 6 μL min^{-1} by a 1100 series isocratic pump (Agilent) equipped with a splitter (1:100). Signal acquisition of glutamic acid, lysine (*Mr* 147) and BSA (*Mr* 1478) was performed in the selected ion monitoring mode (SIM). The scan mode (*m*/*z* 1000–2500) and the extracted ion current (EIC) signals were recorded for the detection of proteins. For the quantitation of whey proteins, the following relative molecular masses were acquired: 1670 for β-LG A, 1663 for β-LG B, 1576 for α-LA, 1370 for Rnase and 1061 for Myo. Peak width and dwell time were set to 0.3 min and 880 ms, respectively.

2.4. Sample and ampholyte preparation

Stock solutions of each protein were dissolved in a 30:70 (v/v) glycerol–water mixture at a concentration of 4 mg mL^{-1}, aliquoted and stored at −20 °C. For the preparation of running sample, an aliquot was taken from the freezer, each day, and thawed at room temperature.

After optimization, electrolyte was composed of 1% (v/v) Beckman ampholyte mixture (pH 3–10) supplemented with 1% (v/v) Ampholine mixture (pH 4–6) in the glycerol–water medium. Whey proteins and p*I* marker proteins (Rnase and Myo) were mixed with the electrolyte at final concentrations between 380 and 11,300 nM, according to proteins. Prior to loading, the electrolyte was vortexed for 10 s and centrifuged for 10 min at 5000 rpm in an Eppendorf centrifuge 5414 (Roucaire, Courtaboeuf, France) to homogenize and avoid air bubbles.

As described in our previous work [42], anolyte is composed of 1 mM glutamic acid and 50 mM formic acid in the glycerol–water medium. Catholyte is a mixture of 1 mM lysine–100 mM ammonia diluted in the same medium. Glutamic acid and lysine are detectable by MS and allow to visualize the limits of the pH gradient zone.

2.5. CIEF–ESI–MS experiments

All CIEF separations were performed in a 30:70 v/v glycerol–water medium and using a bare fused-silica capillary [42]. In CIEF–ESI/MS experiments, the polyimide coating on the external capillary surface was removed from the last 20 mm of the capillary outlet to increase stability of the electrospray and prevent capillary blockage. In the optimized following conditions, 100 cm length capillaries were flushed for 6 min at 950 mbar with the catholyte. Taking into account the viscosity of glycerol–water mixture, the proteins/ampholytes solution was injected over a 50 cm capillary length from the inlet (1.45 min, 950 mbar). Focusing was performed at a constant voltage (30 kV) for 16 min. Mobilization to the cathodic side was achieved by applying a pressure of 50 mbar while keeping voltage constant (30 kV) [42], in order to prevent dispersion effect. After each separation, the capillary was also rinsed by successive flushes of H_2O (5 min), 100 mM TFA (8 min), H_2O (5 min), 1 M NH_4OH (8 min), H_2O (5 min), EtOH (8 min) and H_2O (5 min) under 950 mbar, in order to release adsorbed proteins from the capillary wall.

3. Results and discussion

CE separation of milk proteins is a difficult task due to their strong adsorption to the inner capillary wall and the low water solubility of caseins, especially α-, β- and κ-CN. In view of improving these methods, we assessed the potential of CIEF–MS in glycerol-based media that was previously investigated in our group with model proteins [42]. Preliminary experiments showed that different contents of glycerol (60–90%, v/v) in water did not allow the total solubility of caseins except for α-CN. As a consequence, our attention was paid to the separation and the quantitation of whey proteins. α-casein, which is as hydrophobic as BSA, was added to the mixture of whey proteins to develop the conditioning protocol of the capillary. Our first step was focused on the set-up of a new rinsing procedure that would be effective for protein desorption and be compatible with MS detection.

3.1. MS-compatible post-run cleaning of unmodified bare fused-silica capillary

Regeneration of a capillary after each analysis is a crucial step, because after aging, proteins have been found to be more difficult to remove from the capillary wall [44–46] leading to non-reproducible results. This holds especially true for the case of this new CIEF–ESI/MS design performed in bare fused-silica capillaries. Also, we examined conditions allowing the regeneration of the capillary wall without having to withdraw the capillary outlet tip from the ESI needle. To this end, the separation of a standard mixture of hydrophobic milk proteins (BSA, α-CN and β-LG A) was investigated by CIEF-UV (capillary length: 80 cm; effective length: 71.5 cm) using 1.5% (v/v) Beckman ampholyte mixture (pH 3–10) in a 30:70 (v/v) glycerol–water mixture. In our previous work [42], we showed that a low HCl concentration was suitable for desorbing model proteins from capillary wall and did not interfere with MS detection. A capillary rinse with water (3 min) followed by 10 mM HCl (5 min) and water (3 min), all under 950 mbar (2.8 displaced capillary volumes) was thus performed. However, with the most hydrophobic proteins, the first results for detection time repeatability showed that HCl was not efficient in removing the more hydrophobic proteins (BSA, α-CN) from the capillary wall. The protein migration times increased irreversibly from injection to injection until capillary blockage. Therefore, a new protocol for capillary rinsing was developed in order to remove adsorbed proteins while preserving MS compatibility. Based on Popa's works [47], the multi-step rinsing sequence consisting in H_2O (3 min), 1 M H_3PO_4 (5 min), H_2O (3 min), 1 M NaOH (5 min), H_2O (3 min) was efficient to desorb whey proteins from the inner capillary wall. The repeatability of migration times ($n = 6$) was between 0.9 and 1.7%, according to proteins. Phosphoric acid was particularly effective for high protein concentration and strong adsorbed proteins such as α-CN and BSA [45,48]. Due to the very poor volatility of H_3PO_4 and NaOH, and ion suppression effects involved by H_3PO_4 [49], these two solutions were replaced by 100 mM TFA and 1 M NH_4OH, respectively. Decreasing TFA concentrations below 100 mM led to poorer migration time precision and capillary blockage was observed again.

By applying this procedure to the CIEF–MS protocol, instability or inhibition of ESI/MS signal were randomly observed, probably

due to the presence of residual TFA in the system, which is known to inhibit protein ionization because of ion pair formation [50]. Therefore, an EtOH washing step was included at the end of the post-conditioning sequence to rinse out TFA residues from both the capillary and the ESI-source. Finally, the retained protocol was as follow: H_2O (5 min), 100 mM TFA (8 min), H_2O (5 min), 1 M NH_4OH (8 min), H_2O (5 min), EtOH (8 min), H_2O (5 min) all under 950 mbar corresponding to 22 displaced capillary volumes. RSD on migration times, calculated from six consecutive injections and repeated on three consecutive days, varied from 0.58 to 2.64%, depending on the studied protein. The effectiveness of this washing protocol may well result from the application of successive acidic and basic solutions and ion pairing formation.

3.2. Sheath liquid composition

Since CIEF–ESI/MS experiments preclude the use of non-volatile additives (tween 20, urea. . .) which usually improve protein solubility, attention was paid to the optimization of the parameters influencing detection sensitivity. Particularly, sheath liquid parameters, including viscosity, volatility, conductivity, polarity and surface tension, affect the MS response. For these reasons, the influence of sheath liquid composition (nature and content of organic modifier, nature of carboxylic acid) was studied. Concerning carboxylic acid nature, the effect of TFA on the MS response of six milk proteins was evaluated and compared to that of the two most widely used carboxylic acids (CH_3COOH, HCOOH) for MS detection. Each protein sample (176 μg mL^{-1}) was dissolved individually in a 30:70 (v/v) glycerol/water mixture containing 1.3% (v/v) Beckman ampholyte mixture (pH 3–10). Another solution containing only carrier ampholyte (1.3%, v/v) was prepared and considered as a blank sample. After capillary was rinsed with water for 5 min, protein or blank sample was infused for 60 s (50 mbar) and a 30 mbar pressure was applied. The sheath liquid – 1% carboxylic acid in MeOH–water (50:50 v/v) – was added at a flow-rate of 6 μL min^{-1}. Proteins were detected in IEC after blank subtraction. All results are summarized in Fig. 1a. 1% TFA in the sheath liquid produced a dramatic loss of the ESI/MS signal as compared to acetic or formic acid. Especially, the signal intensity of β-LG A was divided by a factor of twenty. These results were in accordance with literature. Indeed, Hubert et al. [50] reported that despite its volatility, 13 mM TFA added to the BGE had a strong signal suppression effect on the ESI/MS signal intensity of proteins, together with an increase in baseline noise. Furthermore, these results confirm that attention should be paid during multi-step post-conditioning to eliminate any trace of TFA used to remove irreversibly adsorbed protein on the capillary wall. Varying the content of formic acid from 0.65 to 2% did not reveal significant improvement in sensitivity (data not shown). Slightly better results were obtained by using 2% (v/v) formic acid, but this was counterbalanced by a faster fouling of the ionization chamber. Therefore, a concentration of 1% (v/v) formic acid into the sheath liquid was selected.

The influence of MeOH content in the sheath liquid (SL) on the MS signal was also studied. The experiments were performed with SL composed of 20, 50 or 80% MeOH in water (v/v) and a constant formic acid concentration (1%). In all cases, the performances of the ESI interface were improved by adding a high content of organic modifier in the SL (Fig. 1b). Indeed, the weak surface tension (22.7 mN m^{-1}) and the high volatility of MeOH allows better ion desolvation from charged droplets.

3.3. Ampholyte composition

The carrier ampholyte mixture has a critical role in stabilizing pH gradient and in protein focusing. Strong diminution of carrier ampholytes concentration in a CIEF experiment has strong consequences on focusing completion since pH gradient stability is directly affected by the ampholyte type and concentration [51,52]. In standard CIEF-UV experiments, carrier ampholytes concentrations of 1–5% (v/v) are currently used. The coupling between CIEF and MS requires lower ampholyte concentrations to avoid ion suppression. Indeed, it has been reported that the addition of 0.2% (v/v) carrier ampholyte can lead to 45% peptide signal loss [48]. In this study, the capillary (L = 80 cm) was partially filled (50%) with Beckman ampholytes (pH 3–10) at various concentrations, ranging from 0.5 to 2% (v/v). Increasing ampholytes concentration led to a decrease in migration time but no improvement in protein resolution was observed (results not shown). Considering resolution, sensitivity and repeatability, a compromise was found and the final concentration of carrier Beckman ampholytes mixture (pH 3–10) was set at 1% (v/v). In order to achieve a better separation of the acidic whey proteins (β-LG A, β-LG B, α-LA) which possess very close p*I*s (5.25; 5.35 and 4.50, respectively), the Beckman ampholyte mixture (pH 3–10) was supplemented with Ampholine or Servalyt ampholyte mixture (pH 4–6), at various concentrations ranging from 1 to 3% (v/v, glycerol–water medium). Fig. 2 presents the plot of p*I* as a function of detection time for 6 proteins (β-LG A, β-LG B, α-LA, LF, Rnase and Myo): Rnase (p*I* 9.45) and Myo (p*I* 6.30) were added as p*I* markers in order to evaluate the method performances over the whole pH gradient range. The addition of Ampholine ampholyte mixture (pH 4–6) to Beckman ampholyte mixture (pH 3–10) induced a modification of the pH gradient time span (limits of pH gradient zone) which increased from 14.4 to 18.2 min upon increasing Ampholine content from 1 to 3% (v/v) (data not shown). A break in the slope at pH 6 was observed (Fig. 2a) corresponding to a change in ampholyte composition in the pH 4–6 zone. This break led to smaller slopes in the pH 4–6 range. As a consequence, the

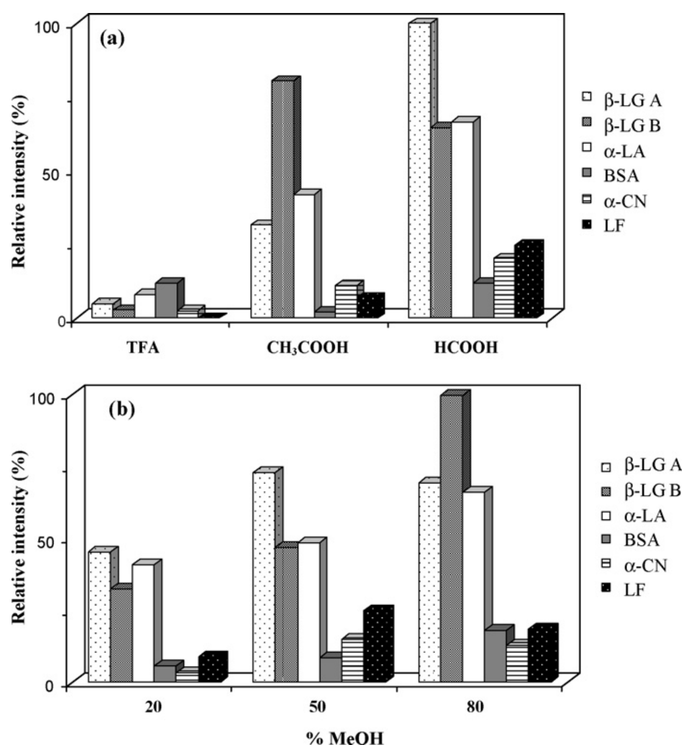

Fig. 1. Influence of sheath liquid composition on the MS response of six milk proteins. (a) Carboxylic acid nature added at 1% (v/v) in 50:50 (v/v) methanol–water mixture. (b) Methanol content in the hydro-organic sheath liquid containing 1% (v/v) formic acid. Abundance scale obtained in EIC mode was normalized using the most intense $[(M+nH^+)/n]^{n+}$ ion as 100% for each optimized parameter. Protein concentration: 176 μg mL^{-1}; injection: 50 mbar, 60 s; mobilization: 30 mbar; sheath liquid flow-rate: 6 μL min^{-1}.

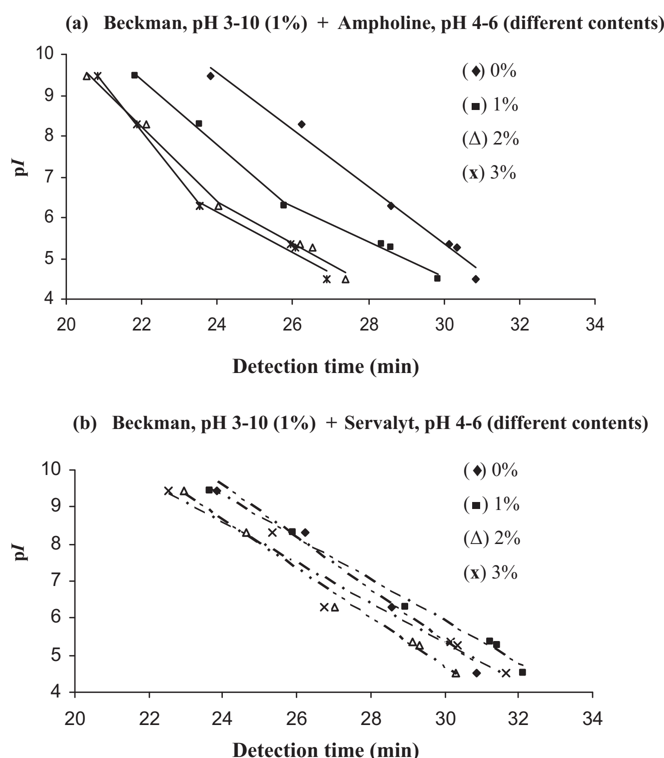

Fig. 2. Protein p*I* as a function of detection time using different carrier ampholyte compositions. Bare fused-silica capillary: 80 cm × 50 μm i.d. Carrier ampholyte: (a) Beckman (pH 3–10) (1%, v/v) + Ampholine (pH 4–6) in 30:70 (v/v) glycerol–water; (b) Beckman (pH 3–10) (1%, v/v) + Servalyt (pH 4–6) in 30:70 (v/v) glycerol–water. Ampholyte (pH 4–6) concentration (♦) 0% (v/v); (■) 1% (v/v); (△) 2% (v/v); (×) 3% (v/v). Anolyte: 1 mM glutamic acid/50 mM formic acid (pH 2.35) in 30:70 (v/v) glycerol–water. Catholyte: 1 mM lysine/100 mM ammonia in 30:70 (v/v) glycerol–water. Protein sample: 5.9 μM Rnase, 4.0 μM LF, 4.7 μM Myo, 4.4 μM β-LG B, 4.3 μM β-LG A and 5.6 μM α-LA in carrier ampholyte. Capillary length filled by ampholytes/proteins mixture: 40 cm. Focusing: 30 kV for 6 min. Cathodic mobilization: 50 mbar, 30 kV. Temperature: 25 °C. Sheath liquid: 80:20 (v/v) MeOH/H_2O + 1% HCOOH. Flow-rate: 6 μL min^{-1}. MS conditions: positive ionization mode; nebulization gas: nitrogen (100 °C, 55 kPa); drying gas: nitrogen (350 °C, 7 L min^{-1}); ESI voltage: 4500 V; fragmentor: 100 V. Least-squares regression straight lines: (a) (♦) $y_{(pH\ 3-10)} = -0.70x + 26.28$, $R^2 = 0.98$ (■) $y_{(pH\ 4-6)} = -0.43x + 17.40$, $R^2 = 0.97$, $y_{(pH\ 6-10)} = -0.802x + 27.044$, $R^2 = 0.994$; (△) $y_{(pH\ 4-6)} = -0.51x + 18.58$, $R^2 = 0.96$, $y_{(pH\ 6-10)} = -0.903x + 28.085$, $R^2 = 0.991$; (×) $y_{(pH\ 4-6)} = -0.50x + 18.14$, $R^2 = 0.94$, $y_{(pH\ 6-10)} = -0.89x + 27.45$, $R^2 = 0.96$; (b) mean of four least-squares regression straight lines: $y = -0.62x + 23.79$ (mean $R^2 = 0.98$). In all regression line equation, *x* referred to protein detection time and *y* to p*I* value.

resolution between β-LG isoforms, the p*I*s of which are very close (5.25 and 5.35), was enhanced from 1.6 to 2.1 by adding 1% (v/v) Ampholine (pH 4–6) to Beckman (pH 3–10) ampholyte mixture. Although higher concentration (*e.g.* 3%, v/v) of Ampholine (pH 4–6) further improved protein resolution, these conditions were not retained, since they favored MS signal suppression and increased background noise. Whatever the concentration of Servalyt (pH 4–6) added to Beckman (pH 3–10) ampholyte mixture, no flattening of pH gradient in the pH 4–6 range occurred (Fig. 2b). The mean value of straight line regressions expressing p*I* as a function of detection time was $y = -0.62x + 23.79$ ($R^2 = 0.98 \pm 0.02$). This difference in behavior between Servalyt and Ampholine ampholyte mixtures can be explained by Righetti's studies on ampholyte characterization [53]. Although Servalyt (pH 4–6 range) appeared to be outstanding in terms of complexity (199 *Mr*-different compounds, instead of 80 for Ampholine) for a total of 1302 isoforms (325 for Ampholine), the amount of focusing species was very poor, about 20%, as opposed to 50% for Ampholine. Indeed, only 13% carrier ampholytes in the Ampholine solution are considered as "bad focusing species" compared to up to 30% for Servalyt one.

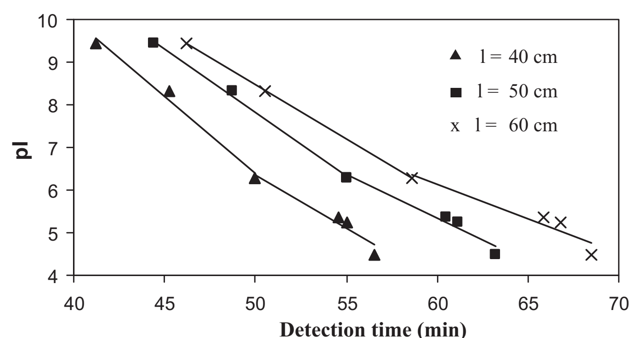

Fig. 3. Protein p*I* as a function of detection time for variable capillary lengths filled with the ampholytes/proteins mixture. Experimental conditions: see Fig. 2 except for total capillary length, 1 m, and capillary length filled with the ampholytes/proteins mixture: (▲) 40 cm, (■) 50 cm, (●) 60 cm, corresponding to filling times of 56 s, 87 s and 126 s under a pressure of 950 mbar. Least-squares regression straight lines: (▲) $y_{(pH\ 4-6)} = -0.25x + 19.12$, $R^2 = 0.94$; $y_{(pH\ 6-10)} = -0.36x + 24.32$, $R^2 = 0.98$; (■) $y_{(pH\ 4-6)} = -0.21x + 17.84$, $R^2 = 0.95$, $y_{(pH\ 6-10)} = -0.298x + 22.741$, $R^2 = 0.998$; (●) $y_{(pH\ 4-6)} = -0.16x + 16.14$, $R^2 = 0.93$, $y_{(pH\ 6-10)} = -0.2521x + 21.0710$, $R^2 = 0.9997$. In all regression line equation, *x* referred to protein detection time and *y* to p*I* value.

Eventually, we decided to use 1% (v/v) Ampholine (pH 4–6) added to 1% Beckman (pH 3–10) as the optimal ampholyte composition.

3.4. Capillary length filled with ampholytes/proteins mixture and focusing time

The effect of the length filled with the ampholytes/proteins mixture was studied by varying the filling time of the capillary with this mixture. To maximize the gain in resolution, the capillary total length was increased from 80 to 100 cm. By taking into account the medium viscosity and the total capillary length (1 m), this time was calculated for an applied pressure of 950 mbar so that the ampholytes/proteins mixture occupies the first 40, 50 or 60 cm length of the capillary. The focusing time was set to 16 min. Fig. 3 shows the plots of protein p*I*s in terms of detection time for Rnase, LF, Myo, β-LG A, β-LG B and α-LA. Good linearities were observed above and after the breaking point, whatever the experimental conditions. The slope value was slightly smaller for a longer ampholyte zone, allowing an easier p*I* determination. For example, in pH 4–6 range, the slopes were −0.25 and −0.16 with ampholyte zones of 40 cm and 60 cm length, respectively. These results can be explained on considering the span time: the longer the ampholyte zone, the longer the span time (34, 57, 82 min for 40, 50, 60 cm ampholyte zone length, respectively) and the larger the difference in detection time between β-LG isoforms. However, resolution between β-LG isoforms remained constant (mean *R*s value = 2.23)

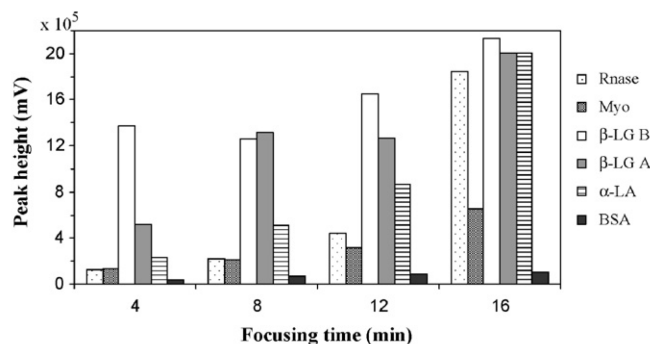

Fig. 4. Influence of focusing time on protein peak height. Conditions: see Fig. 2 except for total capillary length, 1 m, and capillary length filled with ampholytes/proteins mixture, 40 cm; BSA (3.1 μM).

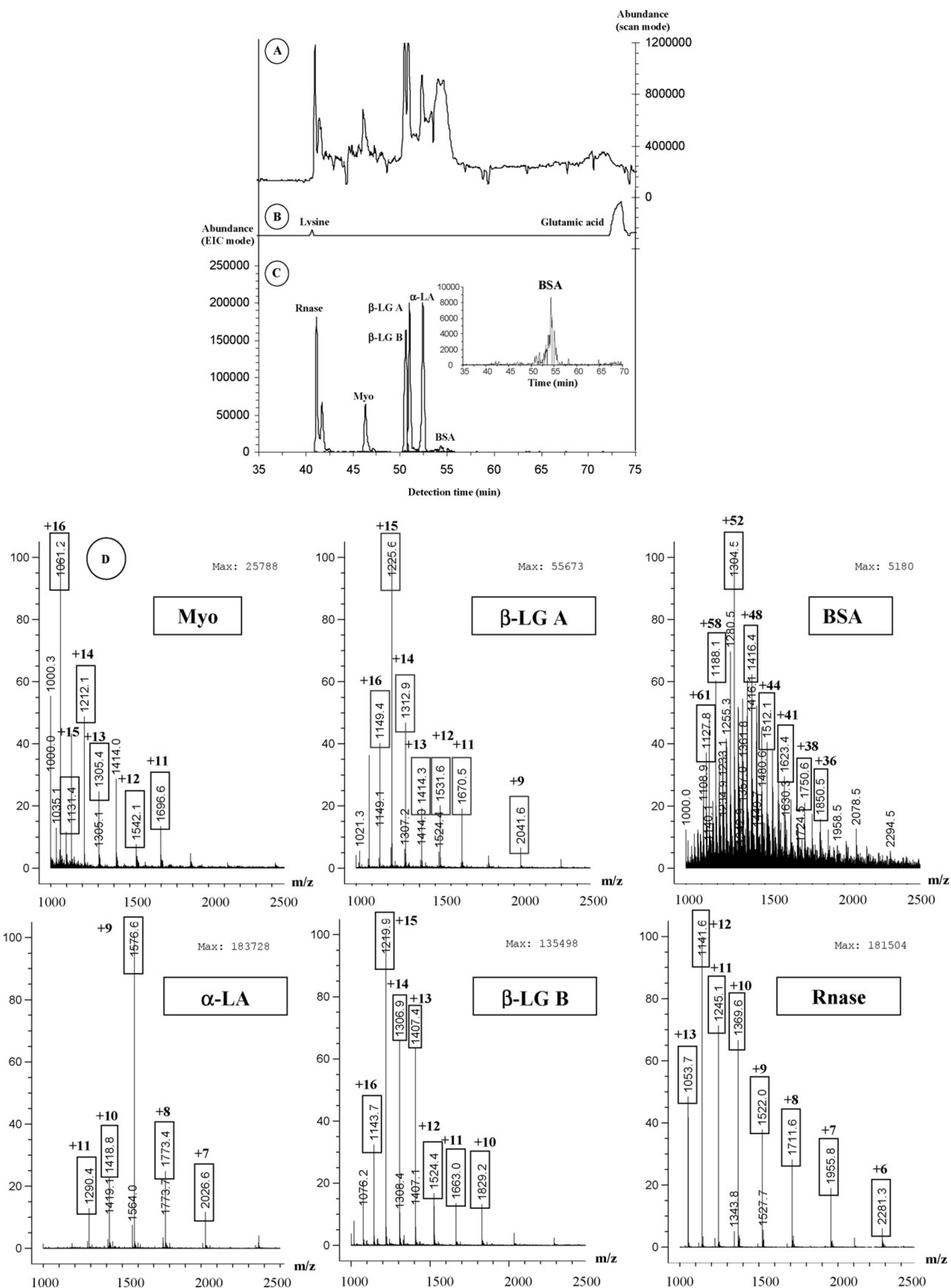

Fig. 5. On-line CIEF–ESI/MS separation of standard whey protein mixture. (A) TIC electropherogram from scan mode (m/z 1000–2500); (B) SIM (m/z 147) electropherogram of pH gradient markers; (C) reconstructed EIC electropherogram from each protein: Rnase (m/z 1245), Myo (m/z 1061), β-LG B (m/z 1307), β-LG A (m/z 1313), α-LA (m/z 1773), BSA (m/z 1478); (D) protein mass spectra extracted from the peaks in (A). Bare fused-silica capillary: 1 m × 50 μm i.d. Separation electrolyte: Beckman (pH 3–10) (1%, v/v) and Ampholine (pH 4–6) (1%, v/v) ampholyte mixture in 30:70 (v/v) glycerol–water containing Rnase (5.9 μM), LF (4.0 μM), Myo (4.7 μM), β-LG B (4.4 μM), β-LG A (4.3 μM), α-LA (5.6 μM), BSA (3.1 μM). Anolyte: 1 mM glutamic acid/50 mM formic acid (pH 2.35) in 30:70 (v/v) glycerol–water. Catholyte: 1 mM lysine/100 mM ammonia in 30:70 (v/v) glycerol–water. Focusing: 30 kV for 16 min. Cathodic mobilization: 50 mbar, 30 kV. Cassette temperature: 25 °C. Capillary length filled by ampholytes/proteins mixture: 40 cm; sheath liquid and MS conditions: see Fig. 2.

Table 1
Limits of detection (LOD) and of quantitation (LOQ) obtained for whey proteins by CIEF–ESI/MS.

Protein	m/z (amu)	EIC mode		SIM mode	
		LOD[a] (nM)	LOQ[b] (nM)	LOD[a] (nM)	LOQ[b] (nM)
β-LG A	1670	132	441	40	132
β-LG B	1663	136	454	68	226
α-LA	1576	73	242	11	37
BSA	1478	57	191	19	63

[a] Limit of detection (S/N = 3).
[b] Limit of quantitation (S/N = 10).

due to larger peak width and detection time was longer. Consequently, a 40 cm ampholyte zone length was selected.

The influence of focusing time on protein detection time was also studied in the 4–16 min time range. Indeed, it is well-known that sensitivity depends on focusing time. During this focusing step, the establishment of the pH gradient and then, the focusing of the proteins occurs. In most cases, increasing focusing time led to thinner protein peak. This was coupled with an increase in peak height (Fig. 4) and an improvement in detection thresholds. For the ensuing study, we decided to work with a focusing time of 16 min in order to maximize signal-to noise ratio while retaining reasonable analysis time.

Fig. 5 represents the CIEF–MS separation of the standard whey protein mixture, with the TIC electropherogram shown in Fig. 5A, the SIM electropherogram of lysine and glutamic acid (m/z = 147) in Fig. 5B, allowing the limits of pH gradient zone to be determined, and the reconstructed EIC electropherogram in Fig. 5C, built from the more abundant and/or the more selective ions observed in the mass spectra (Fig. 5D). All proteins were separated in less than 60 min with good resolution. Nevertheless, two main problems were encountered: (i) a low sensitivity was obtained for LF, probably due to its poor solubility and (ii) BSA appeared difficult to focus, as it gave a 5 min-broad peak (see insert Fig. 5C). Moreover, considering its pI value (pI = 4.90), BSA should have migrated between β-LG B (pI = 5.35) and α-LA (pI = 4.5). This atypical behavior could be explained by adsorption to the capillary wall. To reduce this phenomenon, an additional experiment was performed in 40/60 (v/v) glycerol–water mixture, but no improvement in BSA peak shape was noticed.

3.5. Quantitative aspects and method pre-validation

Detection limits, linearities and precisions obtained for the studied whey proteins β-LG A, β-LG B, α-LA and BSA by the CIEF–ESI/MS method were evaluated.

The limits of detection (LOD) and of quantitation (LOQ) were established in EIC and SIM modes under the optimized electrophoretic conditions described in the experimental section. LODs and LOQs were determined as mean values of three determinations of extrapolated concentrations corresponding to S/N of 3 and 10, respectively. The m/z values selected for quantitation were those described in the experimental section. Results are presented in Table 1. As expected, much lower LODs were obtained in SIM mode, varying from about 10 to 70 nM, depending on the protein. EIC mode, however, allowed to detect an unlimited number of proteins in the same run, whereas only 4 SIM signals can be recorded simultaneously with our MS instrument. The LOD of α-LA obtained from this work in SIM mode was similar to that previously obtained by t-ITP/CABCE-UV [34]. Depending on protein structure, LODs from this work were between 10 and 100 times lower than those determined by CZE-UV [13,25,54,55] but ca 5 times higher than those obtained by CZE-LIF [56]. Nevertheless, CIEF–ESI/MS analysis avoids the tedious sample treatment of protein derivatization and offers the potential to identify β-LG isoforms unambiguously thanks to the specific envelope of the multi-charged ions, in a detection range that is relevant for allergy purposes.

The linearity of the method was evaluated from five concentration levels of a standard mixture containing β-LG A, β-LG B, α-LA and BSA whey proteins, supplemented with two internal standards (Rnase, Myo) and injected in triplicate. The calibration curves were plots of whey protein to internal standard (IS) peak area ratios versus protein concentration. All protein signals were recorded in EIC mode, except for BSA, which was recorded in SIM mode. BSA signal acquired in EIC mode suffered from difficult peak integration due to badly defined peak shape (see Fig. 5). The concentrations chosen for calibration plots were in the range 0.55–8.9 μM for β-LG A and β-LG B, 0.7–1.13 μM for α-LA and 0.38–6.11 μM for BSA, whereas Rnase and Myo concentrations were kept constant at 5.85 and 4.55 μM, respectively. In both cases, results of Cochran's test ($0.55 < C_{exp} < 0.86$) were higher than the theoretical value ($C_{th} = 0.54$), which showed the non-homogeneity of variances all over the calibration range at the 95% confidence level. Hence, the linear least-squares regression was weighted by a factor (1/concentration) to provide a proper fit. Rnase was found to be a less convenient IS than Myo. One explanation could be a worse focusing of Rnase (pI = 9.45) due to a detection time closer to the basic pH gradient frontier, which induced poorer precision on peak area. Myo was thus the preferred IS. Except for BSA, regressions were found to be linear all over the concentration range studied, with correlation coefficients higher than 0.992 (Table 2).

Table 2
Results of linearity and repeatability studies.

Protein	m/z	Concentration range (μM)	Equation[a]	R^2	Repeatability (RSD %)[b]	
					t_M	$A_{protein}/A_{Myo}$
β-LG A	1670	0.55–8.8	$y = 0.0003x - 0.096$	0.992	0.74	10.11
β-LG B	1662	0.54–8.6	$y = 0.0002x - 0.039$	0.997	0.70	8.68
α-LA	1576	0.71–11.3	$y = 0.0002x - 0.043$	0.994	0.58	12.53
BSA	1348	0.38–6.11	$y = 0.0002x - 0.079$	0.97	0.86	15.21

[a] Where y was the area ratios ($A_{protein}/A_{Myo}$) and x the protein concentration.
[b] The RSD values ($n = 6$) calculated for migration times (t_M) and areas ratios ($A_{protein}/A_{Myo}$) for 4.55 μM Myo, 3.23 μM β-LG A, 3.31 μM β-LG B, 4.23 μM α-LA and 0.76 μM BSA.

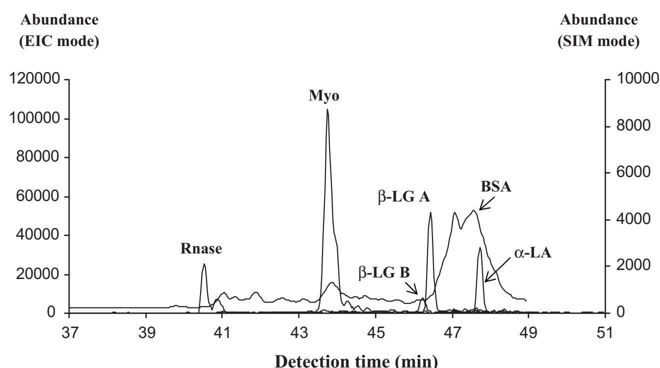

Fig. 6. On-line CIEF–ESI/MS analysis of rabbit serum spiked with whey proteins: 0.17 μM β-LG B, 0.55 μM β-LG A, 1.41 μM α-LA, 4.55 μM Myo and 5.88 μM Rnase. Experimental conditions: see Fig. 5. Detection of β-LG A, β-LG B, α-LA and Myo in EIC mode; detection of BSA in SIM mode.

The method repeatability was evaluated as the RSD value calculated from six consecutive injections of a mixture of standard whey proteins, β-LG A (3.23 μM), β-LG B (3.31 μM), α-LA (4.23 μM) and BSA (0.76 μM) supplemented with Myo as IS, at a concentration of 4.55 μM. The RSD values calculated for detection times and peak area ratios (whey protein/Myo) ranged from 0.58 to 0.86% and from 8.7 to 15.2%, respectively (Table 2). The good precision on detection time allowed us to confirm the suitability of the capillary post-conditioning protocol. RSD values on peak area ratios reflected the limited precision of the method at this concentration level.

3.6. Application to biological samples

In order to test this method with a real sample, a rabbit serum was analyzed, without pre-treatment. The selection of serum as biomatrix was performed so as to evaluate matrix effect in quite extreme conditions. A 1 μL-volume of 10 times-diluted serum was added to 48 μL of glycerol–water medium containing 0.5 μL Ampholine (pH 4–6) and 0.5 μL Beckman ampholyte (pH 3–10). The sample was analyzed in triplicate and a protein having similar p*I* value and MS spectrum as BSA was detected and estimated at a concentration of 748 ± 55 mM, using the BSA calibration line. This protein could correspond to rabbit serum albumin. Finally, we tried to demonstrate the trueness of the method by spiking the rabbit serum with β-LG B (0.17 μM), β-LG A (0.55 μM), α-LA (1.41 μM), Myo (4.55 μM) and Rnase (5.88 μM) [57] (Fig. 6). Results were expressed in terms of relative bias (%) and were −15.6, −1.4 and −11.9% for β-LG A, β-LG B, α-LA, respectively. Whatever the protein, these biases never exceeded the acceptable value of ±20%.

4. Concluding remarks

Whey proteins of bovine milk β-LG A, β-LG B, α-LA and BSA, which possess close p*I* values have been successfully separated and quantified by coupling CIEF with an ESI–MS detector. Protein adsorption to the capillary wall was overcome using a specific rinsing procedure with TFA, ammonia and EtOH. Proteins were reproducibly focused and separated in an uncoated bare-fused silica capillary. The addition of narrow-p*I* range ampholyte mixture (pH 4–6) to a wider-p*I* range ampholyte mixture (pH 3–10) was a simple way to increase resolution between β-LG A, β-LG B and α-LA which possess very close p*I* values (5.25, 5.35 and 4.50, respectively). The sensitivity of single quadrupole MS detection allowed to reach detection thresholds ranging from 10 to 70 nM in SIM mode, in a range that is relevant for allergy purposes. Repeatabilities on peak areas (RSD) at S/N of the order of 100 were in the range 8–15%. The developed method was demonstrated to be suitable for the analysis of whey proteins in serum. This technique should be an alternative to 2D-PAGE for the determination of milk allergens in biological samples.

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