



Sensitive identification of milk protein allergens using on-line combination of transient isotachopheresis/micellar electrokinetic chromatography and capillary isoelectric focusing in fused silica capillary with roughened part

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ABSTRACT

A method for on-line concentration of milk proteins from large sample volumes using combination of transient isotachopheresis (tITP) and micellar electrokinetic chromatography (MEKC) in fused silica capillary with an inner roughened part has been developed. The method utilizes reversible dynamic adsorption of proteins onto a thin layer of PEG 4000 on the roughened surface of the capillary. In addition, the tITP/MEKC method was combined with capillary isoelectric focusing (CIEF) for on-line concentration, separation, identification and sensitive determination of proteins in skimmed milk. The method allows analysis of up to 50 μL of sample. This study has focused on the four important whey proteins, bovine serum albumin (BSA), α -lactalbumin (α -LA), and two genetic variants of β -lactoglobulin (β -LG A and β -LG B). The proteins were identified on the basis of their migration times and characteristic $p\text{I}$ values. The $p\text{I}$ values of BSA, α -LA, β -LG A, and β -LG B were determined as 4.7, 4.4, 5.1, and 5.2, respectively. Limits of detection for BSA, α -LA and both β -LG variants were found as 1.2, 1.0 and 1.0 pg mL^{-1} , respectively. The linearity of calibration curves was characterized by the $R^2 = 0.9982$. The method provided highly reproducible results as the relative standard deviations of the migration times and peak areas of the examined proteins did not exceed 1.6%.

1. Introduction

Capillary electrophoresis (CE) belongs to the powerful analytical techniques suitable for rapid separation and characterization of bio-analytes, e. g., proteins, from different matrices (Astorga-Wells & Swerdlow, 2003; Pero-Gascon, Benavente, Minic, Berezovski, & Sanz-Nebot, 2020). A short optical path length in separation capillary in the case of UV-Vis detection and the small volumes (~ 100 nL) of the injected samples contribute to the insufficient detection limit (LOD) of analytes (Horká, Karásek, Roth, & Slais, 2017; Urbanek, Krivankova, & Bocek, 2003). Fluorescence detection, sample clean-up and/or different concentration techniques that enable analysis of the large sample volumes were used to improve LOD and to minimize sample manipulation. On-line combination of CE techniques such as different variations of sample stacking and sweeping (Breadmore et al., 2019; Horká et al., 2017; Šlampová, Malá, & Gebauer, 2019), changing the capillary geometry (Benavente, Medina-Casanellas, Giménez, & Sanz-Nebot, 2016; Breadmore et al., 2019; Horká et al., 2017; Pont, Pero-Gascon, Gimenez, Sanz-Nebot, & Benavente, 2019; Ramautar, Somsen, & de Jong, 2016;

Stolz et al., 2019; Šlampová et al., 2019; Urbanek et al., 2003), combination of solid phase extraction with CE (SPE-CE) where micro-cartridge with an appropriate sorbent is often integrated in-line near the inlet of the separation capillary (Benavente et al., 2016; Pero-Gascon et al., 2020; Pont et al., 2019; Ramautar et al., 2016) or various chromatographic techniques (Benavente et al., 2016; Breadmore et al., 2019; Pont et al., 2019; Ramautar et al., 2016) are among the concentration techniques.

CE has also been proven a suitable technique for separation, identification, and quantification of bovine milk proteins (Cartoni, Coccioli, Jasionowska, & Masci, 1999; Horká et al., 2009; Veledo, de Frutos, & Diez-Masa, 2005). Bovine serum albumin (BSA), α -lactalbumin (α -LA), and β -lactoglobulin (β -LG) are among the most frequently studied proteins found in a bovine milk. BSA, a major blood serum protein, has many functions including maintaining osmotic pressure and pH of blood and binding a diverse range of molecules (Farrell et al., 2004). Due to the extraordinary binding properties, BSA has a wide range of clinical, pharmaceutical, and biochemical applications. In addition, BSA has also been identified as an allergen (Chruszcz et al., 2013). α -LA is the second

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most abundant whey protein that plays, beside the nutritional significance, a key role in the lactose synthesis. β -LG is the major whey protein which biological function is still unclear (Farrell et al., 2004). Several genetic variants of this protein have been described, with variants A and B being the most common (Farrell et al., 2004; Lauer & McManigill, 1986). β -LG is the main allergen in bovine milk (Veledo et al., 2005).

Many methods have been developed to detect food allergens (Bianchi, Giannetto, & Careri, 2018). In particular, these methods include polymerase chain reaction assays (PCR) with specific primers and probes (Eischeid & Stadig, 2018; Mayer, Bürger, & Kaar, 2012; López-Calleja et al., 2007), ultra-performance liquid chromatography-Triple Quadrupole MS (UPLC-TQMS) (Chen et al., 2016), sandwich enzyme-linked immunosorbent assay with covalently bound monoclonal antibody and gold probe for sensitive and rapid detection of bovine β -LG (He et al., 2018), electrochemical disposable platform based on composite polymer coupled with an aptamer for sensitive detection of β -LG (Lettieri, Hosu, Adumitrachioaie, Cristea, & Marrazza, 2020), aptamer-based biosensors (Tran et al., 2013) etc. The most often used method for detection of allergenic proteins is enzyme-linked immunosorbent assay (ELISA) (López-Calleja et al., 2007) based on specific antibody-antigen reactions. The minimal LOD for β -LG 0.01 ng mL^{-1} was reached using surface enhanced Raman scattering immunosorbent assay-based method (Su et al., 2019). Nevertheless, the above-mentioned methods are quite expensive, laborious and mainly time-consuming.

Several CE modifications were employed to improve α -LA and β -LG separation and/or reduce the LOD. The peaks of milk proteins from cow's milk were well resolved, e.g., by using sodium borate at pH 9.2 as background electrolyte in methyl-silanized capillaries (Cartoni et al., 1999). Free zone CE was used to separate and identify A, B, and C variants of β -LG (Paterson, Hill, & Otter, 1995). Laser-induced fluorescence (LIF) detection with on-capillary derivatization was utilized in CE analysis of trace amounts of β -LG ($\sim 3.7 \text{ ng mL}^{-1}$) in a commercial hypoallergenic formula (Veledo et al., 2005). Fluidic pre-concentrator device was used for off-line CE separation of proteins including α -LA, β -LG A, and β -LG B (Astorga-Wells & Swerdlow, 2003). The chromophoric yellow nonionogenic surfactant (HOPAB) was used in CE separations for sensitive detection of BSA (detectable amount $0.1 \mu\text{g mL}^{-1}$, i. e. 5 pg in 50 nL of the injected sample) at 326 nm (Horká et al., 2009). Fused silica (FS) capillary with two segments of different internal diameters and surfaces, prepared by etching with supercritical water (SCW; Karasek, Horka, Slais, Planeta, & Roth, 2018), was also used for BSA concentration (Horka et al., 2017). LOD of the method was determined as 60 ng mL^{-1} (0.2 ng of BSA in $3.7 \mu\text{L}$ of the injected sample). The combination of transient isotachophoretic stacking (tITP) and sweeping of charged bioanalytes in micellar electrokinetic chromatography (MEKC) was applied to the analyses of large sample volumes (Horka et al., 2017; Horká, Šalplachta, Karásek, Ružička, & Roth, 2020).

The bovine milk proteins, namely β -LG A and β -LG B, were also separated according to their isoelectric points (pIs) (Andersen, Pepaj, Trones, Lundanes, & Greibrokk, 2004). The pI values of BSA, α -LA, β -LG A, and β -LG B have been reported as 4.7–4.9 (Farrell et al., 2004; Horká et al., 2009), 4.2–4.6 (Farrell et al., 2004), 5.13 and 5.23 (Fredriksson, 1972), respectively.

This study focuses on sensitive determination of BSA, α -LA, β -LG A, and β -LG B in skimmed milk (SM) samples. The main task was to investigate the possibility to on-line concentrate the discussed proteins in FS capillary with a roughened part from the large sample volumes with low analytes content. Combination of tITP and MEKC methods was used for this purpose. Furthermore, on-line combination of tITP/MEKC method with capillary isoelectric focusing (CIEF) was also used for concentration of milk proteins from the large sample volumes and their identification according to their migration times and characteristic pIs. CIEF serves as a simple and powerful tool for protein pI determination and their concentration (Horká, Ružička, Holá, & Šlais, 2006; Horká, Ružička, Horký, Holá, & Šlais, 2006).

2. Material and methods

2.1. Chemicals

High resolution ampholyte, pH 2–4, ampholyte pH 3–4.5, 2-morpholino-ethanesulphonic acid monohydrate, 3-morpholino-propanesulphonic acid and *N*-[tris-(hydroxymethyl)-methyl]-3-amino-2-hydroxypropanesulphonic acid were purchased from Fluka Chemie GmbH (Buchs, Switzerland). The solution of synthetic carrier ampholytes (Biolyte, pH 3–10) was obtained from Bio-Rad Labs (Hercules, CA). *N*-(2-acetamido)-2-aminoethanesulphonic acid and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid were obtained from Merck (Darmstadt, Germany). *L*-aspartic acid was obtained from LOBA Chemie (Vienna, Austria), other spacers (Horká, Ružička, Holá et al., 2006) as well as the non-ionogenic detergent Brij 35, (3-glycidyloxypropyl)trimethoxysilane (GOTMS), acetone, ethanol (EtOH), hydrochloric acid (HCl), sodium hydroxide (NaOH), α -LA (M_r 14 178, pI 4.2–4.6 (Farrell et al., 2004)), β -LG A (M_r 18 363, pI 5.13), β -LG B (M_r 18 277, pI 5.23 (Fredriksson, 1972)) and BSA (M_r 66 369, pI 4.7–4.9 (Farrell et al., 2004; Horká et al., 2009)) were purchased from Sigma (St. Louis, MO). Polyethylene glycols (PEG 4000 and PEG 10,000, M_r 4000 and 10,000, respectively) were obtained from Aldrich (Milwaukee, WI). The low-molecular pI markers, pI = 2.7, 4.0, 5.3, 7.2 (Šťastná, Trávníček, & Šlais, 2005) and 4-morpholinyl acetic acid (Horká, Ružička, Holá et al., 2006) were synthesized at the Institute of Analytical Chemistry of the CAS. All chemicals were of electrophoresis or analytical grade purity.

2.2. Modification of the smooth FS capillary and preparation of etched FS capillaries

The smooth FS capillary was modified by GOTMS according to the procedure described before (Horká et al., 2001; Horka et al., 2017). The experimental setup for preparation of etched capillaries has already been described (Karasek et al., 2018). The authors have newly developed a method that enables the repeatable and targeted creation of various surface structures differing not only in the depth of the structure but also in its morphology. It is thus possible to create not only structures with dimples or a porous character, but also a regular structure formed by conical shapes, the resulting geometric parameters of which can be influenced in a targeted manner. This is made possible by the special design of the device, which allows the simultaneous counter-current movement of the capillary and the flowing water and where the supercritical water is formed only in one, fixed point. Structuring were carried out at 400°C , 440 bar and 1000 nL of water flow. For the purposes of this work, capillaries were made with a “cone structure” which height were about 400 nm and a base diameter 250 nm. Commercial FS capillaries, 100 μm ID and 360 μm outer diameter were delivered by Agilent Technologies (Waldbronn, Germany). Purification of water consisted of a single distillation followed by reverse osmosis (Ultra Clear UV, SG Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel, Germany). The resultant value of water conductivity was kept below $0.055 \mu\text{S/cm}$, the total concentration of organic compounds was below 3 ppb, and the dissolved gases were removed by stripping with a gentle stream of helium (purity 4.8, Siad Czech spol. s r.o.).

3. Preparation of skimmed milk sample

The SM was prepared from bovine milk (3.5% fat) according to the published procedure (He et al., 2018) with a minor modification. The bovine milk was first centrifuged at $10,000 \times g$ for 10 min at 4°C using Eppendorf 5804 R (Eppendorf AG, Hamburg, Germany) to remove fat (the top layer) from the milk. Then the supernatant was subjected to the isoelectric precipitation at pH 4.6 by adding 1 mol L^{-1} HCl, and subsequently centrifuged again at $10,000 \times g$ for 10 min at 4°C to remove caseins. The supernatant was finally neutralized with 1 mol L^{-1} NaOH.

The prepared SM was used (either undiluted or diluted with distilled water) for the sensitive separation of milk proteins.

4. Capillary electrophoretic equipment and procedures

A laboratory-made apparatus (Horká et al., 2018) at a constant voltage (−20 kV on the detector side) supplied with a Spellman CZE 1000 R high-voltage unit (Plainview, NY) was employed for the electrophoretic experiments. Smooth FS capillary (100 μm ID, 360 μm OD) and FS capillary with roughened part prepared by etching with SCW were used for separations. The FS capillaries were 500 mm long with detection window created at the distance of 350 mm from the anodic end of the capillary. The FS capillaries with roughened part were etched in the length of 250 mm from the anode side. The ends of the capillaries and the electrodes were placed in 3-mL glass vials filled with background electrolyte (BGE). The non-etched part of the capillary was orientated toward the cathode side. Detection was performed at 280 nm by a LCD 2082 on-column UV–Vis detector (Ecom, Prague, Czech Republic) connected to the detection cell by optical fibers (Polymicro Technologies, Phoenix (AZ)). The samples and rinsing fluids were injected using a single-syringe infusion pump (Cole-Parmer, Vernon Hills (IL)) attached to the either anodic or cathodic end of the capillary at a flow rate of maximum 5 $\mu\text{L s}^{-1}$. The samples were either milk proteins, α -LA, BSA, β -LG A and β -LG B, dissolved in physiological saline solution (PSS) or the prepared SM sample. With regard to the amount of proteins dissolved in PSS, the ratio of β -LG A to β -LG B was always 2:3 in order to distinguish both genetic variants in the electropherograms. Clarity Chromatography Station (version 2.6.3.313, DataApex, Prague, Czech Republic) was used for acquisition and processing of the detector signals. The LOD was determined on the basis of signal-to noise approach. Determination of the signal-to-noise ratio was performed by comparing measured signals from samples with low concentrations of the individual proteins with blank sample. Series of low-concentration protein samples and the blank sample were analyzed for that purpose. A signal-to-noise ratio of 3 was used for determining the LOD. Each experiment was repeated eight to ten times.

CIEF separations were carried out in the FS capillary with 4×10^{-2} mol L^{-1} sodium hydroxide as a catholyte and 0.1 mol L^{-1} orthophosphoric acid as an anolyte, both with the addition of 5% (v/v) EtOH and 0.3% (w/v) PEG 10,000 (smooth FS capillary) or 5% (v/v) EtOH and 0.5% (w/v) Brij 35 (etched FS capillary). Segmental injection was employed in CIEF analyses (Horká, Růžicka, Holá et al., 2006; Horká, Růžicka, Horký et al., 2006). In the case of smooth FS capillary, the individual segments were injected at 70 nL s^{-1} from the anodic side. The first segment (250 nL) was solution of the selected simple ampholytic electrolytes (spacers), 1.5×10^{-4} mol L^{-1} , dissolved in the catholyte. The second segment (100 nL) was sample containing BSA, α -LA, (50 $\mu\text{g mL}^{-1}$ each), and β -LGs (50 $\mu\text{g mL}^{-1}$ both). The third segment (350 nL) was 5% (w/v) aqueous solution of the commercial carrier ampholytes Biolyte pH 3–10, ampholytes pH 3.0–4.5, and pH 2.0–4.0, mixed in the 1:2:5 ratio. This segment also contained low-molecular-mass pI markers (pI 2.7, 4.0 and 5.3; 25 $\mu\text{g mL}^{-1}$ each) for monitoring of the pH gradient used (2.7–5.3). Before each analysis, the capillary was rinsed with 5 μL of EtOH from the cathodic side and then back-flushed with 5 μL of the catholyte, both at 5 $\mu\text{L min}^{-1}$.

CZE (capillary zone electrophoresis) or tTTP/MEKC separations were carried out in both the smooth FS capillary and the FS capillary with roughened part. The BGE was composed of $2\text{--}5 \times 10^{-3}$ mol L^{-1} phosphate buffer, pH 7, and the additives, 0–5% (v/v) EtOH, 0–0.6% (w/v) PEG 10,000 and 0–0.6% (w/v) Brij 35. Before each analysis, the capillary was rinsed with 0–50 μL of EtOH at 0.1–2.5 $\mu\text{L s}^{-1}$, then with 50 μL of water or the water/EtOH mixture (1:1) at 1 $\mu\text{L s}^{-1}$, and finally with 0–20 μL of 0–0.6% (w/v) PEG 10,000 or PEG 4000 at 70 nL s^{-1} from the cathodic side. After the rinsing procedure, the sample was injected into the capillary from either the anodic side (0–200 nL, analyte separation, 70 nL s^{-1}) or the cathodic side (50 μL , analyte adhesion and pre-

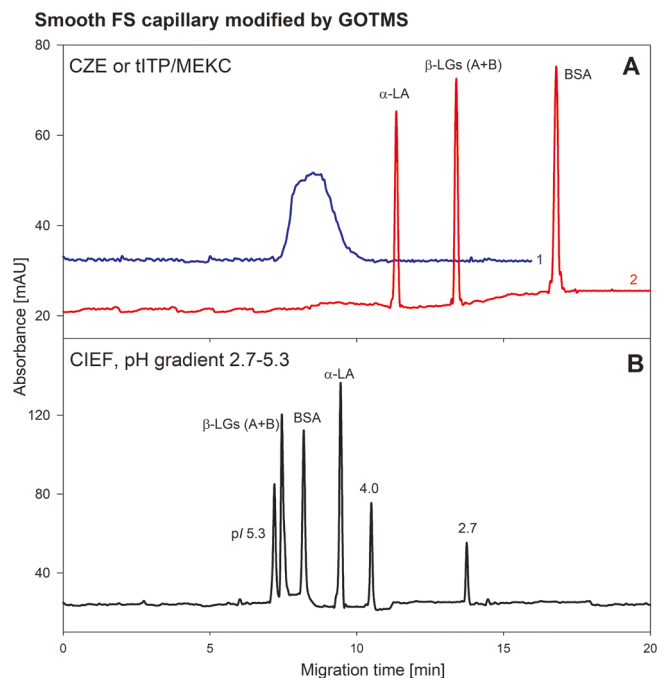


Fig. 1. CE (A) and CIEF (B) separation of α -LA, β -LGs (variants A and B, mixed in the 2:3 ratio) and BSA in the smooth FS capillary. Volume of injected samples: 100 nL at 70 nL s^{-1} ; proteins dissolved in PSS; (A) BGE: 5×10^{-3} mol L^{-1} phosphate buffer, pH 7; (A1) BGE without additives; protein concentration: 100 $\mu\text{g mL}^{-1}$ of each (BSA, α -LA, and β -LGs); (A2) BGE with additives (5% EtOH, 0.6% Brij 35, 0.6% PEG 10,000); protein concentration: 10 $\mu\text{g mL}^{-1}$ of each (BSA, α -LA, and β -LGs); (B) catholyte: 4×10^{-2} mol L^{-1} sodium hydroxide; anolyte: 0.1 mol L^{-1} H_3PO_4 ; additives: 5% EtOH and 0.3% PEG 10,000; protein concentration: 50 $\mu\text{g mL}^{-1}$ each of each (BSA, α -LA, and β -LGs).

concentration) at 0.25–1 $\mu\text{L s}^{-1}$. The sample was the selected milk proteins dissolved in PSS to the concentration of 0–1 mg mL^{-1} or the prepared SM sample (undiluted or diluted up to 5×10^9 with distilled water). The capillary was finally filled with 0–12 μL of phosphate buffer (4×10^{-3} mol L^{-1} , pH 7, containing 0–5% (v/v) EtOH, 70 nL s^{-1}) from the cathodic side.

In the case of the on-line combination of tTTP/MEKC analysis with CIEF performed in the FS with the roughened part, the electrophoretic separations were stopped 10 s after detection of the BSA zone. Under the optimized separation conditions, the zones of selected milk proteins, from α -LA to BSA, were located behind the detection window and the catholyte end of the capillary. In order to perform CIEF analysis, the segment of the simple ampholytic electrolytes (250 nL, 1.5×10^{-4} mol L^{-1} , dissolved in the catholyte) and 3.75 μL of 4×10^{-2} mol L^{-1} NaOH were injected into the capillary from the cathodic side at 70 nL s^{-1} . Then, 350 nL of the aqueous solution of carrier ampholytes Biolyte pH 3–10 (5% (w/v)) and low-molecular-mass pI markers (pI 2.7, 4.0, 5.3 and 7.2, for monitoring of the used pH gradient 2.7–7.2) were injected into the capillary from the anodic side again at 70 nL s^{-1} . The experimental conditions for both tTTP/MEKC and CIEF analyses are described in detail in the previous paragraphs.

5. Results and discussion

5.1. Separation of selected milk proteins in smooth FS capillary

First of all, the selected bovine milk proteins were separated in the commonly used smooth FS capillary for subsequent evaluation of the adsorption capacity of the etched FS capillary (with the part of roughened inner surface). The examined proteins, α -LA, β -LG (variants A, B, mixed in the ratio 2:3) and BSA, were dissolved in PSS. The prepared

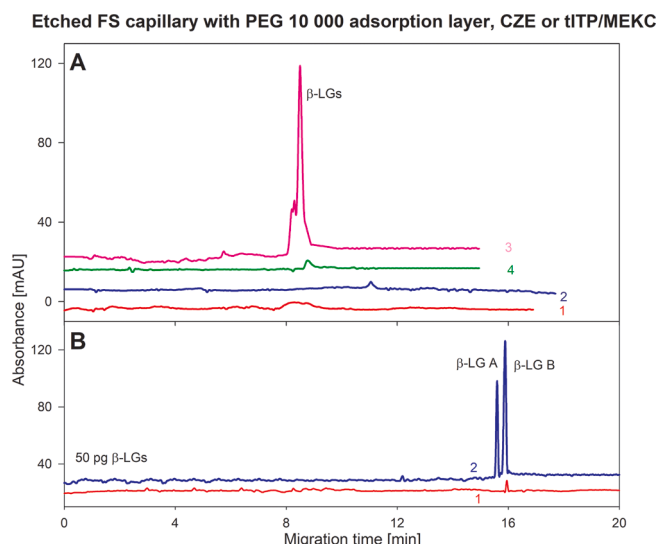


Fig. 2. CZE or tITP/MEKC of β -LGs on the etched FS capillary (A, B) with PEG 10,000 used as an adsorption layer. Conditions and designations: see Fig. 1; BGE: 2×10^{-3} mol L $^{-1}$ phosphate buffer, pH 7; (A) Volume of injected sample (anodic side): 200 nL; proteins dissolved in PSS; BGE: (A1) without additives; (A2–4) with additives: (A2) 0.1% Brij 35; (A3, A4) 0.3% Brij 35; total protein (β -LGs) concentration: (A1, A2, A4) 0.1 mg mL $^{-1}$; (A3) 0.01 mg mL $^{-1}$; rinsing procedure: (A2–4) 10 μ L of 0.5 (w/v) PEG 10,000 (70 nL s $^{-1}$); (A3) capillary filled with 4×10^{-3} mol L $^{-1}$ phosphate buffer, pH 7 (70 nL s $^{-1}$); (A4) 10 μ L of water/EtOH (1:1, 1 μ L s $^{-1}$) between separation runs. (B) BGE additives: 0.5% (w/v) Brij 35, 5% (v/v) EtOH; capillary rinsed with EtOH (50 μ L, 0.1 μ L min $^{-1}$), water (50 μ L, 1 μ L s $^{-1}$), 0.3% PEG 10,000 (20 μ L, 70 nL s $^{-1}$); volume of injected sample (cathodic side): 50 μ L (0.4 μ L s $^{-1}$); total protein (β -LGs) concentration in PSS: 50 pg (20 pg β -LG A and 30 pg β -LG B); capillary filled with 4×10^{-3} mol L $^{-1}$ phosphate buffer, pH 7, with 5% (v/v) EtOH (70 nL s $^{-1}$).

sample (100 nL) was separated by CE methods in the smooth FS capillary modified with GOTMS (Horká et al., 2001; Horka et al., 2017). In the first step, the protein sample (10 ng of each, BSA, α -LA, and β -LGs) was injected into the capillary and analyzed by CZE employing BGE without additives. Under these experimental conditions, the proteins adsorbed onto the capillary inner surface, which resulted in unresolved broad peak (Fig. 1A, electropherogram 1). It was not known, how much proteins, if any, remained adsorbed onto the capillary surface after CZE. Therefore, only PSS (100 nL) was injected into the capillary. The tITP/MEKC analysis employing BGE with additives, EtOH, Brij 35 and PEG 10,000, was performed. Nevertheless, no detectable amount of proteins was desorbed from the capillary surface during the analysis. In the next step, the protein sample (1 ng of each, BSA, α -LA, and β -LGs) was injected into the capillary and analyzed using the BGE with additives. Three narrow peaks of the examined proteins were detected because of co-migration of both β -LG variants (Fig. 1A, electropherogram 2).

The selected milk proteins (5 ng of each, BSA, α -LA, and β -LGs, injected from the anodic side) were also separated by CIEF in the pH gradient pH range 2.7–5.3 according to their pI values (Fig. 1B). The pH range was traced by the pI markers 2.7, 4.0 and 5.3. As in the previous tITP/MEKC analysis, β -LG A and β -LG B (2 ng and 3 ng) migrated within one zone and thus only three narrow peaks of the examined proteins were detected. The pI values of α -LA, β -LBs, and BSA were determined as 4.4, 5.2, and 4.7, respectively. Determined pI values corresponded to the values reported in the literature (Farrell et al., 2004; Fredriksson, 1972; Horká et al., 2009). Relative standard deviations (RSDs) of the migration times of the analytes were up to 1.1%.

5.2. Separation of β -LGs, genetic variants A and B, in the FS capillary with the roughened part

With respect to our previous experience with SCW-etched FS

capillaries (Karasek et al., 2018), there was an assumption that the proteins could adsorb on the roughened part of the capillary (Horka et al., 2017; Horká et al., 2020). That could be utilized for both enhanced separation of proteins and concentration of milk proteins from the large sample volumes. The next experiments were performed with β -LGs (variants A and B in the 2:3 ratio) dissolved in PSS to different concentrations (Fig. 2). The sample (200 nL) was injected into the capillary from the anodic side and analyzed by CE. The BGE (2×10^{-3} mol L $^{-1}$ phosphate buffer, pH 7) was used either without (CZE, Fig. 2A, electropherogram 1) or with the additives (tITP/MEKC, Fig. 2A, electropherograms 2–4). The etched FS capillary provided almost no detection response compared to smooth FS capillary when the BGE was used without additives (electropherograms 1, Fig. 1A (injected 30 ng of proteins) vs. Fig. 2A (injected 20 ng of proteins)). This suggests that, under the same separation conditions, the adsorption capacity of the etched surface is significantly higher in comparison with the smooth capillary surface. Irreproducible protein adsorption onto the inner capillary wall was observed when the capillary was not rinsed before an electrophoretic run.

Protein adsorption increased when the capillary was rinsed with 10 μ L of 0.5% (w/v) PEG 10,000 before injection of the sample (Fig. 2A, electropherogram 2) although 0.1% (w/v) Brij 35 was used as an BGE additive. Regarding the electropherogram 3 in Fig. 2A, the capillary was rinsed again with 10 μ L of 0.5% (w/v) PEG 10,000 before injection of the β -LGs (0.8 ng of β -LG A and 1.2 ng β -LG B) from the anodic side. Subsequently, the capillary was filled with 4 μ L of 4×10^{-3} mol L $^{-1}$ phosphate buffer (volume of the capillary) from the cathodic side. Moreover, the concentration of Brij 35 in the BGE was increased to 0.3% (w/v). The injected and adsorbed proteins (including proteins from previous analyses, electropherograms 1 and 2) were separated in the tITP/MEKC mode. The detection response was inadequate to the total amount of injected β -LGs (2 ng). This was confirmed by the next analysis (Fig. 2A, electropherogram 4), where 20 ng of β -LGs were injected into the capillary. The capillary was rinsed with 10 μ L of water/EtOH in mixture (1:1, 1 μ L s $^{-1}$), and subsequently with 10 μ L of 0.5% (w/v) PEG 10,000 before sample injection and filling the capillary with the phosphate buffer. Although the concentration of Brij 35 in the BGE was the same as in the previous analysis (electropherogram 3), the detection response was significantly lower.

On the basis of performed experiments, the separation conditions were optimized (Fig. 2B), especially the rinsing procedure, to allow injection of larger sample volume into the capillary. The capillary was first rinsed with 50 μ L of EtOH (0.1 μ L s $^{-1}$), then with 50 μ L of water (1 μ L s $^{-1}$) and finally with 20 μ L of 0.3% (w/v) PEG 10,000 (70 nL s $^{-1}$) to form an adsorption layer on the roughened surface of the FS capillary. After rinsing of the capillary, 50 μ L of β -LGs in PSS (20 pg β -LG A and 30 pg β -LG B) was injected into the capillary from the cathodic side at 0.4 μ L s $^{-1}$ in order to adhere the proteins onto the inner capillary wall. The capillary was subsequently filled with 4 μ L (volume of the capillary) of 4×10^{-3} mol L $^{-1}$ phosphate buffer with 5% (v/v) EtOH (70 nL s $^{-1}$) to increase the conductivity of the inside electrolytes. The analysis of the sample was then performed in the tITP/MEKC mode with BGE composed of 2×10^{-3} mol L $^{-1}$ phosphate buffer with the additives, 0.5% (w/v) Brij 35 and 5% (v/v) EtOH. The schematic overview of the injection procedure is given in Fig. 3. Two electropherograms of the performed tITP/MEKC analyses are depicted in Fig. 2B. Narrow zone (electropherogram 1) or zones (electropherogram 2) of the analyzed proteins were detected in both cases. Although the separation conditions including protein concentration were the same, significantly different detection responses were obtained (electropherogram 1 vs. 2). Adsorption of the excessive amount of PEG 10,000 onto the roughened part of the etched FS capillary can consequently result in the significantly increased adsorption of proteins as shown in electropherogram 1. Therefore, the capillary must be thoroughly washed with 50 μ L of EtOH at 2.5 μ L min $^{-1}$ and then immediately with 50 μ L of water at 1 μ L s $^{-1}$ after approximately ten analyses. This procedure ensures achieving reproducible results and

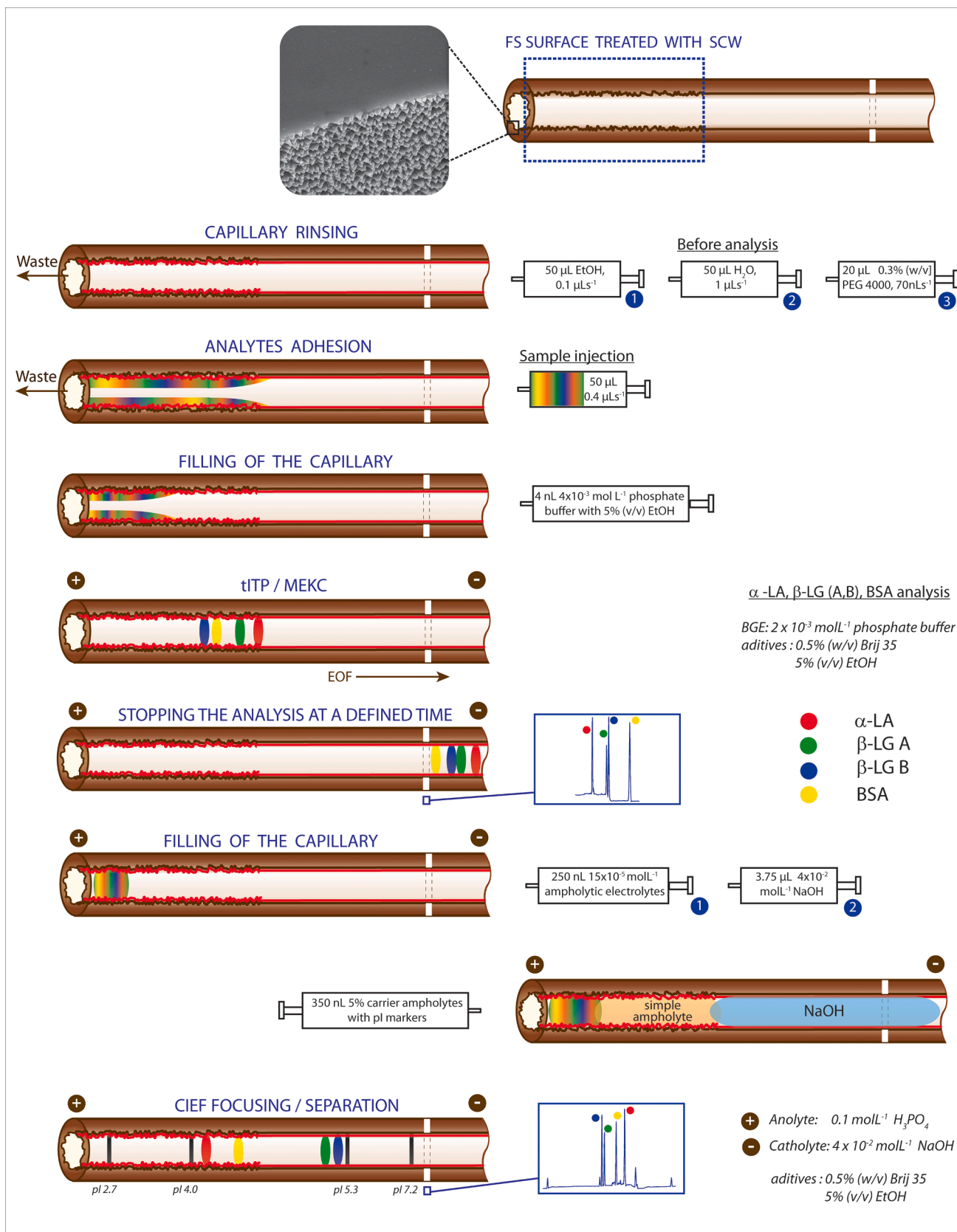


Fig. 3. The schematic overview of the on-line combination of tITP/MEKC and CIEF procedures.

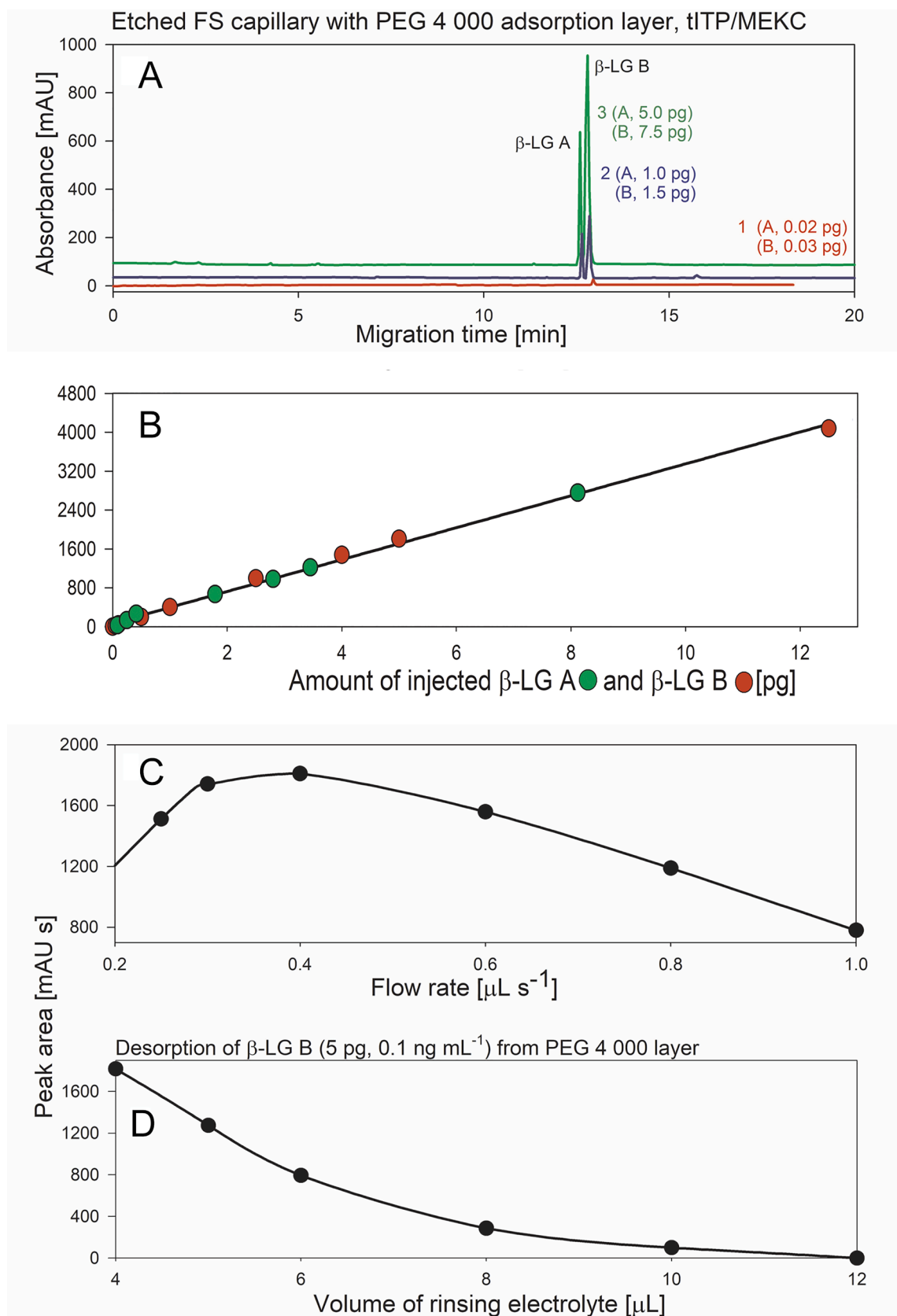


Fig. 4. tITP/MEKC of β -LGs in the etched FS capillary (A), calibration curve (B) for the separation of genetic variants β -LG, A (green points) and β -LG, B (red points), dependence of the peak area on the sample injection flow rate (C) and decrease in the peak area during the rinsing of the capillary with phosphate buffer with 5% EtOH (D), PEG 4000 used as an adsorption layer. Conditions and designations, see Figs. 1 and 2; capillary rinsed with 0.3% PEG 4000 (20 μL); sample volume: 50 μL ; (D) capillary rinsed with $4 \times 10^{-3} \text{ mol L}^{-1}$ phosphate buffer, pH 7, with 5% (v/v) EtOH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

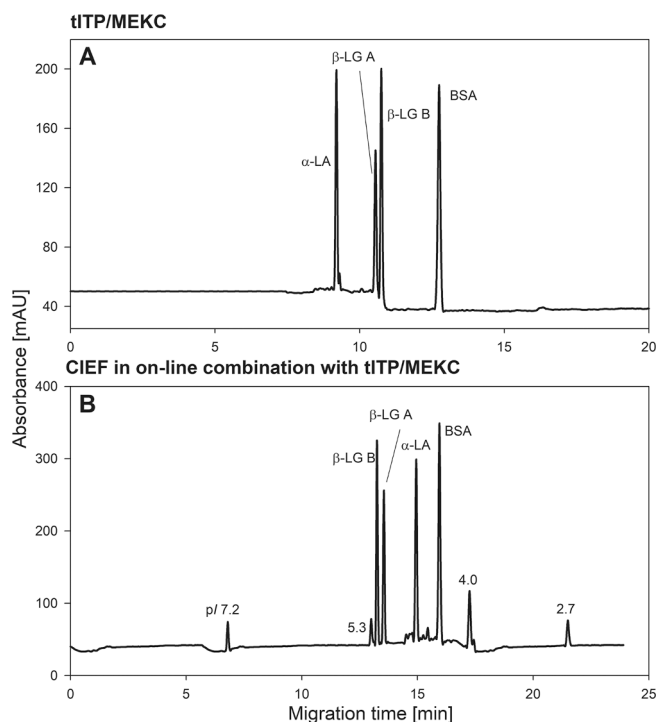


Fig. 5. On-line combination of tITP/MEKC (A) with CIEF (B), electropherograms of the tITP/MEKC and CIEF runs depicted separately. Conditions and designations, see Figs. 1–4; volume of injected sample (cathodic side): 50 μL containing 2.5 pg of each BSA, α -LA and β -LGs (50 pg mL^{-1} , dissolved in PSS); CIEF – additives in the catholyte and the anolyte: 5% EtOH, 0.5% Brij 35; injection from the cathodic side: 250 nL of simple ampholytic electrolytes and 3.75 μL of $4 \times 10^{-2} \text{ mol L}^{-1}$ NaOH; injection from the anodic side: 350 nL of carrier ampholytes and pI markers.

separation of both genetic variants of the β -LGs, A and B, as shown in electropherogram 2. The peak areas approximately corresponded to the amount ratio of both variants.

Subsequently, PEG 10,000 was substituted with PEG 4000, when lower adsorption to the etched inner surface of the capillary wall was observed during analyses due to the different physical properties of this amphiphilic polymer. The capillary was rinsed with 20 μL of 0.3% (w/v) PEG 4000 before sample injection. Other separation conditions were the same as in the previous analyses shown in Fig. 2B. Both variants of β -LG (mixed in the ratio 2:3) were dissolved in PSS to different concentrations and 50 μL of each sample was injected into the capillary from the cathodic side. Results of tITP/MEKC analyses are shown in Fig. 4A, electropherograms 1–3. Separation of the β -LG variants were achieved in the samples with concentrations 0.25 ng mL^{-1} and 50 pg mL^{-1} (electropherograms 2 and 3, respectively). The narrow peaks of both proteins were detected and the peak areas corresponded to the amount ratio of A and B variants (2:3). The calibration curve, shown in Fig. 4B, is characterized by the $R^2 = 0.9982$ for both variants (β -LG A – green points, β -LG B – red points). The RSDs of the peak areas and the migration times were 1.3% and 1.2%, respectively, proving high reproducibility of the analyses. LOD for both β -LG A and β -LG B was determined as 0.05 pg in 50 μL of the injected sample (1 pg mL^{-1}), linear range from 0.05 to 12.5 pg (1–250 pg mL^{-1}). Achieved LODs were approximately ten times lower than the values reported in the literature (Su et al., 2019) where minimal LOD 10 pg mL^{-1} for β -LGs was reached using surface enhanced Raman scattering immunosorbent assay-based method. The increased volume of the injected sample in our experiments allows significantly higher detection sensitivity than other CE methods. For example, in our published study (Horká et al., 2009), LOD of 0.1 $\mu\text{g mL}^{-1}$ (5 pg in 50 nL of the injected sample) was achieved in CE of proteins labeled by HOPAB.

Optimal flow rate, 0.4 $\mu\text{L s}^{-1}$, at which the sample was injected into the capillary with respect to the adsorption rate of proteins onto the PEG 4000 layer, was defined in our preliminary experiments. To verify this value, dependence of the β -LG B peak area on the sample injection rate was measured, and the result is shown in Fig. 4C. The optimal sample injection rate was found to be in the range from 0.3 to 0.4 $\mu\text{L s}^{-1}$ providing practically the same results.

It was also investigated whether some amount of proteins remain adsorbed on the PEG 4000 layer after injection of the increased volume of the electrolyte (phosphate buffer with EtOH). As mentioned above, the capillary was first rinsed using optimized procedure followed by sample injection and finally filled with phosphate buffer with EtOH before tITP/MEKC analysis. The capillary (4 μL) was rinsed with 4–12 μL of $4 \times 10^{-3} \text{ mol L}^{-1}$ phosphate buffer with 5% (v/v) EtOH. As shown in Fig. 4D, significant decrease of protein peak area was observed with increase in the injected electrolyte volume over 4 μL . It can be assumed that, unlike the experiments with PEG 10,000, sufficient desorption occurs during tITP/MEKC analysis.

5.3. Separation of milk proteins by tITP/MEKC and by on-line combination of tITP/MEKC with CIEF

The optimized conditions for the tITP/MEKC were used for the separation of milk proteins (2.5 pg of each, BSA, α -LA, and β -LGs, in 50 μL of PSS). The results are shown in Fig. 5A. Narrow peaks of all proteins were detected including the genetic variants of β -LGs, A and B (peak areas corresponded to the amount ratio of A and B variants, 2:3). The sample was injected into the capillary at the flow rate of 0.3 $\mu\text{L s}^{-1}$. The LODs for BSA and α -LA were determined as 0.06 and 0.05 pg (1.2 and 1 pg mL^{-1}), respectively.

Then, an on-line tITP/MEKC analysis in combination with CIEF was then performed to determine the pI of concentrated and separated proteins in the previous tITP/MEKC run. The experimental conditions (Fig. 3) were selected so that the proteins were separated with tITP/MEKC, detected (Fig. 5A), and subsequently analyzed by CIEF (Fig. 5B). The tITP/MEKC and CIEF runs are shown in the separate electropherograms (Fig. 5A and B) for better clarity of the obtained results. Migration time of α -LA (eluted first) from the anode to the detection window was 548 s and from the detection window to the cathode additional 234 s (total migration time, 782 s). Migration time of BSA (eluted last) from the anode to the detection window was 760 s. The tITP/MEKC analysis was stopped in the time of 770 s. The capillary was then filled from the cathodic side with 250 nL of simple ampholytic electrolytes ($15 \times 10^{-5} \text{ mol L}^{-1}$, dissolved in the catholyte) and 3.75 μL of $4 \times 10^{-2} \text{ mol L}^{-1}$ NaOH. Finally, 350 nL of 5% (w/v) aqueous solution of the commercial carrier ampholytes Biolyte pH 3–10 and the pI markers (2.7, 4.0, 5.3 and 7.2) was injected from the anodic side. In the CIEF run, $4 \times 10^{-2} \text{ mol L}^{-1}$ NaOH and 0.1 mol L^{-1} phosphoric acid were used as the catholyte and the anolyte, respectively, both with the additives, 5% (v/v) EtOH and 0.5% (w/v) Brij 35. The linear pH gradient was reached, which was characterized by the $R^2 = 0.9979$. Very narrow peaks of all the examined proteins and the pI markers were detected (Fig. 5B). The pI values of α -LA and BSA corresponded to those obtained upon analysis in the smooth capillary (Fig. 1B). The β -LG variants, A and B, were separated and their pI values were determined as 5.1 and 5.2, respectively, which agrees with the Refs. (Farrell et al., 2004; Fredriksson, 1972). The RSDs of the migration times were 1.4%.

5.4. Analysis of proteins in skimmed milk by on-line combination of tITP/MEKC with CIEF

Undiluted and diluted SM sample were first separated only by tITP/MEKC under the conditions defined in the previous experiments (shown in Fig. 4A and A). Result of the analyses are shown in Fig. 6A. The adsorption layer of PEG 4000 was overloaded by the injection of 50 μL of the undiluted SM sample (electropherogram 1), and thus, the individual

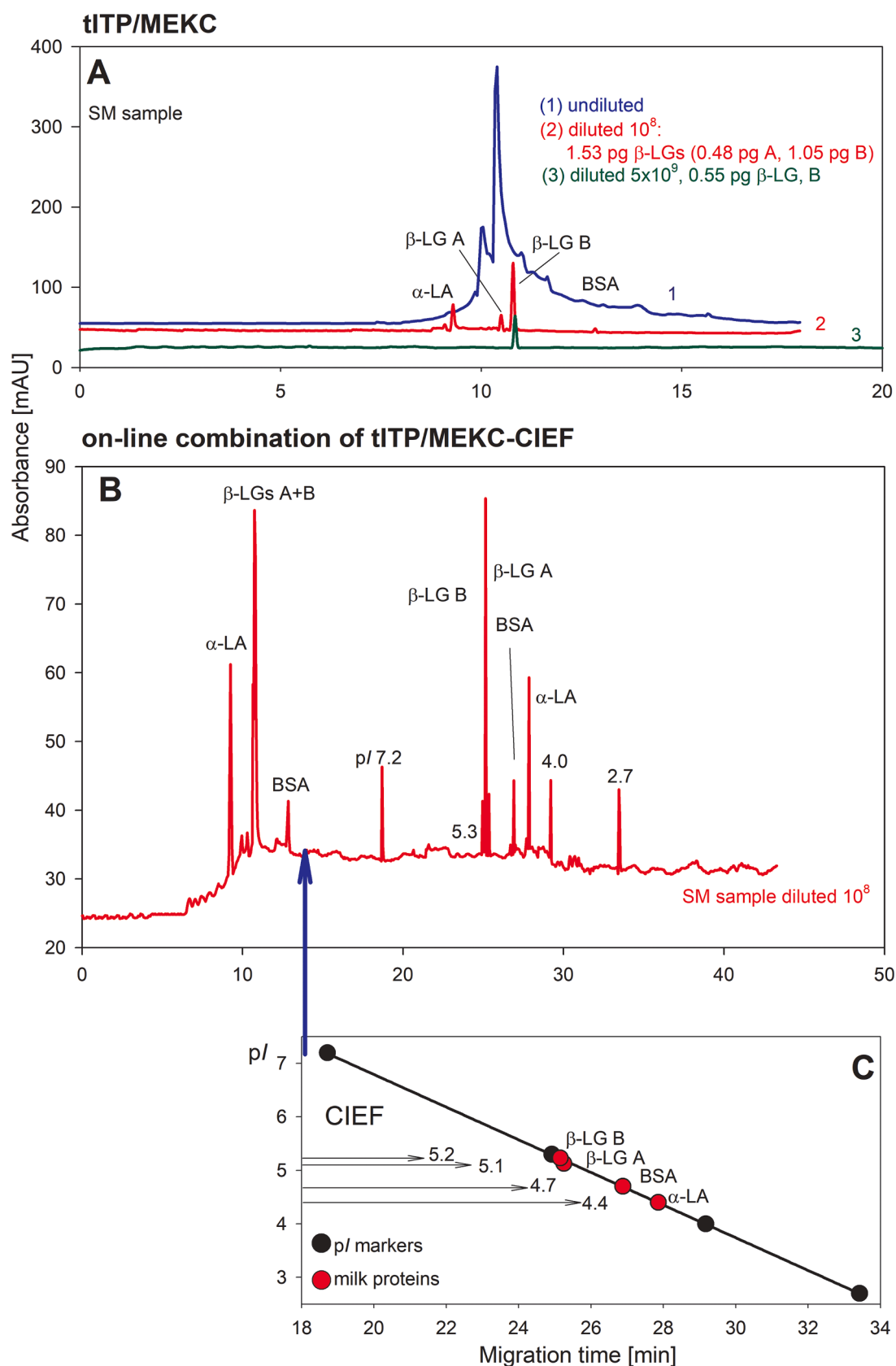


Fig. 6. tITP/MEKC separation of SM (A), on-line combination of tITP/MEKC with CIEF of diluted SM (B) and linearity of the pH gradient in the CIEF mode (C). Conditions and designations, see Figs. 1–5. Volume of injected sample: 50 μ L of SM sample; (A1) undiluted; (A2, B) 10^8 times diluted with water; (A3) 5×10^9 times diluted with water.

proteins were not separated. For the following experiments, the SM sample was diluted with distilled water (10^8 times, electropherogram 2, and 5×10^9 times, electropherogram 3). In the case of the 10^8 times diluted sample, the proteins were completely separated including both genetic variants of β -LG (electropherogram 2). The amounts of β -LG A and β -LG B in this sample were determined as 0.48 pg and 1.05 pg, respectively. That represents 3.06 g L^{-1} of both β -LGs in the undiluted SM sample which is in good agreement with the value reported in literature (Farrell et al., 2004). Only one peak, β -LG B, was detected when the SM sample was 5×10^9 times diluted (Fig. 6A, electropherogram 3). The amount of β -LG B was determined as 0.55 pg (2.2 g L^{-1} in the undiluted SM sample). RSDs of the migration times and peak areas were 1.4% and 1.6%, respectively.

The SM sample diluted 10^8 times was then on-line separated by tTTP/MEKC-CIEF. The course of this analysis is shown in the electropherogram depicted in Fig. 6B. The experimental conditions were the same as in the analysis shown in Fig. 5B. The end of tTTP/MEKC (blue segment) analysis is indicated by a blue arrow in Fig. 6B, CIEF analysis (red segment) was started after injection of all the necessary solutions (simple ampholytic electrolytes, $4 \times 10^{-2} \text{ mol L}^{-1}$ NaOH, commercial carrier ampholytes, and pI markers) into the capillary. Very narrow peaks of all the analyzed proteins and pI markers were detected. The linear pH gradient was reached again ($R^2 = 0.9998$) as shown in Fig. 6C. The RSDs of the peak areas were around 1.6%.

6. Conclusions

tTTP/MEKC method was developed for the analysis of large volumes ($50 \mu\text{L}$) of protein samples in FS capillary with inner roughened part. The proteins were reversibly dynamically adsorbed onto thin layer of polymeric adsorbent (in our experiments PEG 4000) formed on the roughened part of the etched FS capillary. The great advantage is that the other sample components are removed from the capillary before the analysis itself, and the proteins are subsequently desorbed during the tTTP/MEKC analysis. The optimization of adsorption conditions (choice of capillary surface roughness, suitable adsorbents, type of rinsing electrolyte, etc.) can thus be targeted to a certain group of monitored analytes. In addition to the tTTP/MEKC method, on-line combination of tTTP/MEKC with CIEF was developed for the sensitive on-line analysis of proteins in skimmed milk. The proteins were concentrated, separated, identified according to their migration times and characteristic pI values and their concentrations in the analyzed samples were determined. CIEF step was involved to improve protein identification. The method also allows sensitive protein determination, is not expensive, laborious nor time-consuming, and is ready for automation in near future and for off-line combination with, e.g., matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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