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ORIGINAL PAPER

# Analysis of α-lactalbumin, β-lactoglobulin A and B in whey protein powder, colostrum, raw milk, and infant formula by CE and LC

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**Abstract** Two analytical methods, capillary electrophoresis (CE) with UV detection and high-performance liquid chromatography (HPLC) with photodiode array detection, were developed for the determination of α-lactalbumin (α-Lac), β-lactoglobulin A (β-Lg A), and β-lactoglobulin B (β-Lg B) in whey protein powder, colostrum, raw milk, and infant formula. The CE analysis was performed in a bare fused silica capillary (57 cm×50 μm, effective length 50 cm) with a separation buffer consisting of 0.5 mol $L^{-1}$ boric acid, 0.025 mol $L^{-1}$ hydroxypropyl-β-cyclodextrin, and 0.8 g $L^{-1}$ poly(ethylene oxide) 4,000,000 at pH 9.10. The HPLC analysis was performed on a CAPCELL PAK $C_8$ SG 300 (200×4.6 mm, 5-μm particles) column with a gradient mobile phase mixture consisting of acetonitrile and water containing trifluoroacetic acid. The CE method is suitable for the simultaneous determination of α-Lac, β-Lg A, and β-Lg B in whey powder, colostrum, and raw bovine milk, whereas the HPLC method can only be used for quantitative assays of β-Lg A and β-Lg B due to the co-elution of lactoferrin with α-Lac. Recoveries for both methods over the concentration range were between 90.7% and 116.8%. Whey protein powder had the highest content of the above three proteins. The contents of α-Lac in infant formula increased with the raise of age stage. Infant formula with the same age stage but different brands and countries had significant differences in the content of α-Lac.

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**摘要** 本文建立了紫外检测−毛细管电泳 (CE-UV) 及二极管阵列检测−高效液相色谱 (HPLC) 测定乳清蛋白粉、初乳、原料奶及婴儿配方奶粉中α-乳白蛋白 (α-Lac) 、β-乳球蛋白A (β-Lg A) 及β-乳球蛋白B (β-Lg B) 的新方法 CE法以石英毛细管柱 (57 cm×50 μm, 有效长度50 cm) 为分离柱、以0.5 mol $L^{-1}$硼酸、0.025 mol $L^{-1}$羟丙基β-环糊精及0.8 g $L^{-1}$ 聚乙烯醇4,000,000为分离缓冲液 (pH 9.10)。HPLC法以CAPCELL PAK C8 SG 300 (200×4.6 mm, 5 μm)为色谱分离柱, 以含乙腈及三氟乙酸的流动相梯度淋洗方式实现了上述三个蛋白的分离与测定。CE方法适合于乳清蛋白粉、牛初乳及原料奶样品中α-Lac、β-Lg A及, β-Lg B的同时分离与测定, 而HPLC法中因乳铁蛋白干扰α-Lac, 仅能定量其中的β-Lg A 及 β-Lg B。两种方法在测定的浓度范围内回收率在90.7–116.8%间。乳清蛋白粉中上述三个蛋白的含量最高, 婴儿配方奶粉中α-Lac含量随着年龄阶段数的增加而增加, 而且同一阶段内不同厂家不同国别婴儿配方奶粉中α-Lac含量差异也较大。

**Keywords** Capillary electrophoresis · High-performance liquid chromatography · α-Lactalbumin · β-Lactoglobulin · Infant formula

**关键词** 毛细管电泳 · 高效液相色谱 · α-乳白蛋白 · β-乳球蛋白 · 婴儿配方奶粉

## 1 Introduction

Fresh bovine milk, which typically contains 3.0–3.5% proteins, is an excellent source of essential nutritional components for humans. Bovine milk proteins can be grouped into casein (approx. 80% of total protein) and whey protein (approx. 15% of total protein) fractions. The whey proteins are mainly composed of α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg; Strickland et al. 2001). β-Lg usually has two isoforms, namely, β-lactoglobulin A (β-Lg A) and β-lactoglobulin B (β-Lg B). Approximately 3% of the bovine milk protein is composed of α-Lac. Human milk has far lower total protein content, 0.9% (Chatterton et al. 1998). However, the most abundant protein in human milk is α-Lac, which constitutes 10–20% of the total protein (Jackson et al. 2004). The level of α-Lac in bovine milk is lower than that in human milk by an order of magnitude. Clinical research has demonstrated that this highly nutritive protein plays a central biochemical role as the regulatory subunit for lactose synthesis in the rapidly growing neonate. Having 72% sequence homology with human α-Lac (Santos and Ferreira 2007), bovine α-Lac is now widely added to infant formula to benefit formula-fed infants. As a potential allergen to human infants, β-Lg accounts for 9% of the bovine milk protein. Its content in hypoallergenic infant formula is lower than that in cow's milk by three orders of magnitude (Dolnik 2008). Therefore, it is necessary to establish reliable analytical methods for the analyses of α-Lac, β-Lg A, and β-Lg B.

At present, capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) have become the dominant techniques for the analysis of whey proteins or protein genetic variants in milk and milk products. HPLC usually requires a gradient mobile phase mixture employing acetonitrile and water containing trifluoroacetic acid on a silica-based column ($C_4$ or $C_8$), which is time-consuming and laborious for routine purposes (Bonfatti et al. 2008; Jackson et al. 2004; Santos and Ferreira 2007). In addition, irreversible adsorption of the proteins

on the stationary phase often makes the column rapidly lose separation efficiency. CE methods for the analysis of milk proteins published from 1990 to 1996 have been reviewed (Recio et al. 1997). The most recent applications of CE to the analysis of milk proteins were summarized elsewhere (García-Cañas and Cifuentes 2008). CE methods generally use inorganic salts with or without polymeric additives on coated or bare capillaries to complete the separation of milk proteins with shorter run time than HPLC. Unfortunately, β-Lg A and β-Lg B cannot be well separated and quantified (Bütikofer et al. 2006; Clément et al. 2006; García-Ruiz et al. 1999). Although some separations of α-Lac, β-Lg A, and β-Lg B have been reported, no quantitative data were given (Gonzalez et al. 2003; Kinghorn et al. 1996; Patel et al. 2007). In addition, the high content of bovine serum albumin (BSA) in pasteurized milk makes the quantitation of β-Lg B inaccurate (Recio et al. 1995).

The aim of this work was to develop two kinds of methods, CE and HPLC, for the analysis of α-Lac, β-Lg A, and β-Lg B in whey protein powder, bovine colostrum, raw milk, and infant formula. The two methods were totally different in principle and were validated, respectively. Then, they were cross-validated prior to application in real samples. The key factors that affect CE separation, such as the concentration of additives and the concentration and pH of the running buffer, were investigated in detail. The main factors affecting HPLC separation were also studied.

## 2 Materials and methods

### 2.1 Reagents and samples

Boric acid ($H_3BO_3$) was obtained from the Chemical Plant of Kai Yuan in the Tie Ling (Liaoning, China). Cesium hydroxide monohydrate ($CsOH{\cdot}H_2O$, 99.5%) was purchased from Across Organics (USA). Lithium hydroxide monohydrate ($LiOH{\cdot}H_2O$) was purchased from Beijing Xinhua Chemical Reagent Plant (Beijing, China). Sodium chloride (NaCl, ≥99.5%) was purchased from Beijing Yili Fine Chemical Ltd. Corp. (Beijing, China). Acetic acid (HAc, 99%), potassium hydroxide (KOH), and sodium hydroxide (NaOH) were purchased from Beijing Chemical Reagent Company (Beijing, China). Polyethylene glycol (PEG) 20000 and PEG 35000 were bought from Fluka (Germany). Hydroxypropyl-β-cyclodextrin (HP-β-CD, $M_W$=1,540 g $mol^{-1}$); poly(ethylene oxide) (PEO; $M_w$=600,000, 900,000, and 4,000,000 g $mol^{-1}$); α-lactalbumin (α-Lac, ≥85%); bovine lactoferrin (90%); β-Lg A (90%); and β-Lg B (90%) were obtained from Sigma-Aldrich (USA). Albumin bovine serum fraction V (BSA, ≥98%) was from Beijing BIODEE Biotechnology Co. Ltd. (Beijing, China). Five samples of bovine colostrum and five samples of bovine raw milk were obtained from the local dairy. Four samples of whey protein powder, nine samples of infant formula with the same brand but for different age stages, and 19 samples of infant formula with the same stages but different brands and countries were collected from the local market.

A stock solution of PEO 4,000,000 at 2 g $L^{-1}$ was prepared by adding 0.2 g of PEO 4,000,000 to 100 mL pure water in a 100-mL SCHOTT reagent bottle with a cap. The bottle was put into a water bath at 50 °C until PEO 4,000,000 was

dissolved. The resultant homogeneous solution was kept at room temperature. HPLC-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA, >99%) was from Merck-Schuchardt (Berlin, Germany). The water for the preparation of all solutions was made by a Millipore Milli-Elix/RiOs ultrapure water system (Bedford, MA, USA). All chemicals were of analytical grade or higher purity unless otherwise stated. The protein stock solutions for HPLC and CE analyses were prepared by dissolving 10 mg α-Lac, 10 mg β-Lg A, and 10 mg β-Lg B in 1.5-mL vials containing 1 mL water. The working solutions for the proteins were prepared by serial dilution of the stock solutions with 0.05 mol $L^{-1}$ HAc.

## 2.2 Apparatus

*CE system* A P/ACE 5000 system (Beckman Instruments, Fullerton, CA, USA) electrophoresis apparatus equipped with a UV detector was controlled by a Pentium/100-MHz personal computer. All the data were collected and analyzed using the P/ACE Station software.

*Capillary column* A bare fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with 50-μm i.d. and total length of 57 cm (50 cm to the detection window) was used. A constant voltage of 14 kV was applied (current about 80 μA) for the separation. The detection wavelength was set at 214 nm. New capillaries were preconditioned by successively flushing with 1 mol $L^{-1}$ NaOH for 5 min, $H_2O$ for 5 min and running buffer for 5 min. Before each run, the capillary was flushed for 5, 2, and 1 min with 1 mol $L^{-1}$ NaOH, $H_2O$, and the running buffer, respectively. Hydrodynamic injection was carried out under $N_2$ pressure at 0.5 psi for 5 s. The injection volume was approx. 5.2 nL. All electrophoresis runs were performed at a temperature of 25 °C.

*CE buffer* Running buffer contained 0.5 mol $L^{-1}$ $H_3BO_3$, 0.025 mol $L^{-1}$ HP-β-CD, and 0.8 g $L^{-1}$PEO 4,000,000, pH 9.10 (adjusted with 1 mol $L^{-1}$ CsOH). Sample buffer was 0.05 mol $L^{-1}$ HAC (pH 2.98).

*pH meter* The pH meter used was model F-33 pH/mV Meter (Beijing Yiyuan Electrical Instrument Technique Corp., Beijing, China).

*Centrifugation* Centrifugation intrument used was a Universal 32 (Hettich, Germany).

*HPLC system* A Waters 2690-996 high-performance liquid chromatography system (Milford, MA, USA) equipped with a 600 gradient pump was used together with a Waters Millennium 2010 Chromatography Manager workstation (version 2.15) for instrument control as well as data acquisition and processing.

*HPLC conditions* An analytical column of CAPCELL PAK $C_8$ SG300 (250×4.6 mm, 5 μm) from Shiseido Fine Chemicals was used. A Waters 996 photodiode array detector (PDA) was used and the detection wavelength set at 214 nm. The column temperature was 30 °C. The mobile phase gradient ratios ranged from 32A/68B to

50A/50B (where A was acetonitrile/0.5 mol $L^{-1}$ NaCl/TFA=50:450:0.5; B was acetonitrile/0.5 mol $L^{-1}$ NaCl/TFA=500:450:0.95). The flow rate was 1.0 mL $min^{-1}$. The column was equilibrated in 50% A and followed by a series of linear gradients as follows: 0.01–35 min, 50–68% B; 35–37 min, 68% B; 37–39 min, 68–50% B; 39–42 min, 50% B. The injection volume of standard and sample solutions was 10 μL by a Waters 717 plus autosampler. In CE analysis, the corrected peak area (peak area divided by migration time) versus concentration had a good linear relationship; therefore, the corrected peak area was used for quantification (Grob et al. 2003). For HPLC analysis, peak area versus concentration had a good linear relationship, and the peak area was used for quantification.

### 2.3 Sample pretreatment

Accurately weighed samples were dissolved in 0.05 mol $L^{-1}$ HAc and vortex-mixed. The solution was then centrifuged at 9,000 rpm for 10 min and the supernatant used directly for CE analysis. For HPLC analysis, the supernatant was filtered prior to injection.

## 3 Results and discussion

### 3.1 CE method development

#### *3.1.1 Optimization of CE conditions*

Boric acid is able to complex with diol groups, which can facilitate the analysis of sugars and glycoproteins (Landers et al. 1993). It has been successfully utilized for the separation of another glycoprotein, IgG, in bovine colostrum products in our laboratory (Zhao et al. 2007). Thus, boric acid was chosen for the current work.

α-Lac, β-Lg A, and β-Lg B have molecular weights of 14.178, 18.363, and 18.276 kDa and isoelectric points (p*I*) of 4.4, 5.1, and 5.2 (Lucy et al. 2008), respectively. Protein adsorption on bare fused silica capillary walls has always been a serious problem in CE because it causes considerable peak broadening and asymmetry, migration time irreproducibility, and loss of separation efficiency. This could be overcome by increasing the ionic strength using a running buffer pH higher than the protein's p*I* or by adding additives to the running buffer (Katariina and Vladislav 2003).

A high running buffer concentration and the use of alkali metal ions with large crystal radii as cations in the buffer have synergetic effects on increasing ionic strength. The larger the crystal radii of the metal ions, the better the peak shapes for the above three proteins and the greater the effectiveness of minimizing protein capillary wall interactions (Green and Jorgenson 1989; Wang et al. 2006). Thus, 1 mol $L^{-1}$ CsOH was utilized to adjust the running buffer pH in the present study. However, the ionic strength should not be so high that resistive heating will limit the applied voltage and consequently decrease the separation efficiency and increase the analysis time. The optimum concentration of boric acid was 0.5 mol $L^{-1}$. The optimized running buffer pH was 9.10 when 0.025 mol $L^{-1}$

HP-β-CD and 0.8 g $L^{-1}$ PEO 4,000,000 were included in the running buffer. Although β-Lg A and β-Lg B have similar p*I*'s and molecular masses, they could be baseline separated under the optimized separation conditions. The three proteins migrated in the order of molecular mass, with the smaller molecular mass proteins migrating more quickly.

Keeping the concentration of boric acid and pH constant (0.5 mol $L^{-1}$, pH 9.10), the influence of 0.025 mol $L^{-1}$ HP-β-CD and 0.8 g $L^{-1}$ PEO 4,000,000 on the separation was investigated. β-Lg B could not be separated from the adjacent unknown peaks when 0.025 mol $L^{-1}$ HP-β-CD was added alone. α-Lac, β-Lg A, and β-Lg B could be well separated from the matrix when 0. 8 g $L^{-1}$ PEO 4,000,000 was added; however, the peaks of the three proteins showed some broadening. The use of HP-β-CD in combination with PEO 4,000,000 in the running buffer has a synergetic effect on improving the resolution of α-Lac, β-Lg A, and β-Lg B from the sample matrix peaks. A sharper peak of β-Lg B could be obtained with a little decrease of peak height of the three proteins and extension of the separation time.

α-Lac, β-Lg A, and β-Lg B exist at low levels in raw bovine milk, while there is a large amount of casein. Therefore, the conditions for their effective extraction and precipitation of casein must be optimized for the analysis. Several researchers have reported the use of a sample reduction buffer (pH approximately 8.0) and a low pH running buffer (pH 3.0) to study the genetic polymorphism of milk proteins (Clément et al. 2006; Heck et al. 2009). However, for efficient sample stacking, the sample should be dissolved in a buffer of a relatively low pH and conductivity compared with that of the running buffer. If the opposite is the case, poor migration reproducibility, zone spreading, and target proteins missing may occur (Schwartz and Prichett 1996).

In the work, a 1:10 dilution of the running buffer was initially tried as a sample buffer. This was effective for both the standards and the whey protein concentrate samples since they had good solubility in the diluted running buffer. However, it could not be used to extract raw bovine milk and infant formula samples because casein cannot be precipitated at pH 9.10. With this sample buffer, a cloudy sample solution was obtained even after centrifugation at 9,000 rpm for 20 min.

At the pH of 4.6, casein will precipitate, but whey proteins will stay in solution. A solution of 0.05 mol $L^{-1}$ acetic acid buffered at pH 4.6 has been proven to be able to provide a relatively casein-free supernatant (Gutierrez and Jakobovits 2003). HAc is a well-known protein precipitator. In the present work, 0.05 mol $L^{-1}$ HAc was tested initially without pH adjustment. As a result, casein was precipitated by 0.05 mol $L^{-1}$ HAc, whereas α-Lac, β-Lg A, and β-Lg B were not precipitated and could be analyzed directly with the supernatant solution. A comparison between the addition of 3 mL of a 0.05 mol $L^{-1}$ HAc buffer (pH 4.6) solution and 3 mL of a 0.05 mol $L^{-1}$ HAc solution (without pH adjustment) to 2 mL of raw bovine milk sample is shown in Fig. 1. The peak heights in 0.05 mol $L^{-1}$ HAc were higher than those in 0.05 mol $L^{-1}$ NaAC-HAc (pH 4.6). A conclusion that can be drawn is that HAc is a good extractant for whey proteins.

A series of different concentrations (0.05, 0.1, 0.2, 0.4 mol $L^{-1}$) of HAc with a volume of 3 mL were investigated to extract α-Lac, β-Lg A, and β-Lg B from 2 mL of raw bovine milk. With the concentration of HAc being increased from 0.1 to 0.4 mol $L^{-1}$, the precipitated casein could be redissolved and give peaks that might interfere with the assay of the three proteins. Based on our experiments, 3 mL of the

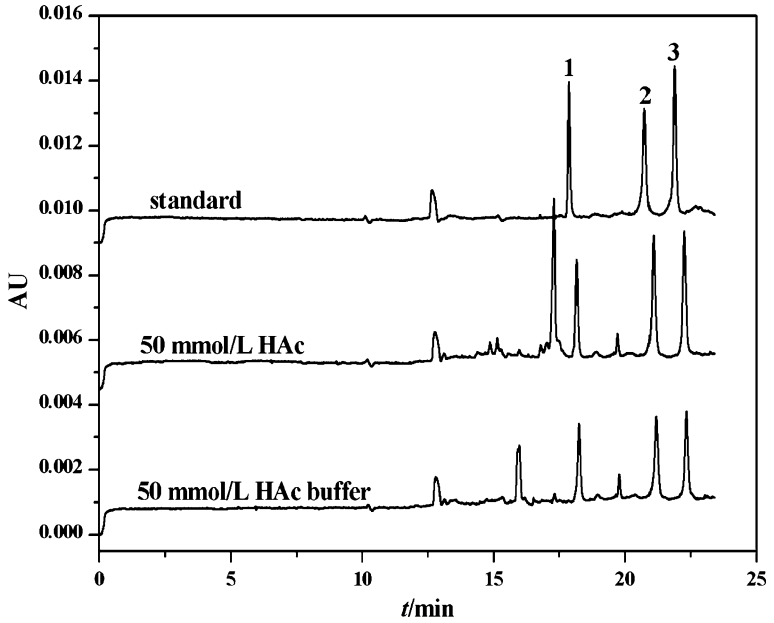


**Fig. 1** Comparison of extraction solution for a raw bovine milk sample. The CE conditions were shown in "Capillary column" and "CE buffer" in Section 2.2

0.05 mol $L^{-1}$ HAc solution is sufficient for the extraction of 2 mL of raw bovine milk. By this method, a clear, casein-free supernatant was obtained after centrifugation, which could be injected directly into the capillary for analysis.

### *3.1.2 CE method validation*

Under the optimized CE conditions for the separation and the sample pretreatment, calibration curves were established according to the procedures previously described. The corrected peak areas ($A_c$) and the concentrations ($c$, mg $L^{-1}$) of the three proteins showed a good linear relationship in the concentration range of 50–400 mg $L^{-1}$ (α-Lac: $A_c$=12.61$c$−243.6; β-Lg A: $A_c$=11.72$c$−110.5; β-LgB: $A_c$= 9.75$c$−136.76), with correlation coefficients ($r$) of 0.9983, 0.9972, and 0.9974, respectively. The LODs (S/N=3) and LOQs (S/N=10) were 10, 15, and 12 mg $L^{-1}$ and 30, 50, and 40 mg $L^{-1}$, respectively.

Standard solutions of the three proteins at 60, 100, and 200 mg $L^{-1}$ were analyzed seven times in succession to evaluate the instrumental precision. The RSD values for the corrected peak areas of α-Lac were 3.8%, 1.6%, and 1.9% and the RSD values for the migration times 0.64%, 0.38%, and 0.18%, respectively. The RSD values for the corrected peak areas of β-Lg A were 4.3%, 1.8%, and 2.5% and the RSD values for the migration times 0.79%, 0.47%, and 0.16%, respectively. The RSD values for the corrected peak areas of β-Lg B were 3.2%, 4.4%, and 1.2% and the RSD values for the migration times 0.74%, 0.42% and 0.13%, respectively.

To evaluate the precision of the CE method, seven parallel raw bovine milk samples (2.0 mL for each) were placed into seven 15-mL centrifuge tubes and pretreated as described in Section 2.3. The average values of α-Lac, β-Lg A, and β-

Lg B in the samples were 1.42, 1.92, and 2.36 mg $L^{-1}$ and the RSDs 3.5%, 3.8%, and 5.6%, respectively.

To test the accuracy of the present method, the recoveries at two levels in five replicates were evaluated by spiking 50 and 150 mg·$L^{-1}$ (α-Lac, β-Lg A, and β-Lg B) standards into raw bovine milk samples. The average recoveries at 50 mg $L^{-1}$spiking were 116.8%, 98.5%, and 108.5%, with RSD values of 7.2%, 8.6%, and 10.8%, respectively. The average recoveries at 150 mg $L^{-1}$ spiking were 111.7%, 95.6%, and 90.7%, with RSD values of 3.0%, 1.8%, and 4.2%, respectively. The electrophoregrams of the standards, raw bovine milk sample, and the spiked raw bovine milk sample are shown in Fig. 2a. α-Lac, β-Lg A, and β-Lg B were well separated from the sample matrix. The other major whey proteins (IgG and BSA) and the minor whey protein (Lf) did not interfere with the assay of the three proteins.

### 3.2 HPLC method development and validation

To further validate the above CE method, an HPLC method with PDA detection was established and optimized. The main problem encountered in the HPLC analysis of proteins is their irreversible adsorption onto the stationary phase, which causes peak broadening and tailing, especially during real sample analysis. A rugged column with high separation efficiency must be used and inorganic salts such as NaCl must be added to the mobile phase to produce a salting-out effect. For the analysis of proteins, a buffer with concentration ranging from 0.01 to 0.02 mol $L^{-1}$ in the mobile phase is necessary for most columns. CAPCELL PAK $C_8$ SG 300, different from the other columns, can tolerate inorganic salt concentration as high as 0.34 mol $L^{-1}$ NaCl and therefore was selected for the present work. The manufacturer's recommended mobile phases A and B were used (A was acetonitrile/0.5 mol $L^{-1}$ NaCl/TFA=50:450:0.5; B was acetonitrile/0.5 mol $L^{-1}$ NaCl/TFA=500:450:0.95). Figure 2b illustrates the chromatograms of the standards, raw bovine milk sample, and the spiked raw bovine milk sample. Unfortunately, α-Lac co-eluted with Lf, which interfered with α-Lac assay accuracy in real sample analysis, though only minor amounts of Lf (0.02–0.2 mg $mL^{-1}$) exist in raw bovine milk (Riechel et al. 1998). Therefore, α-Lac could not be assayed accurately by the current HPLC method. The other major whey proteins, such as IgG and BSA, did not interfere with the assay of the three proteins.

Under the optimized chromatographic conditions, the peak areas ($A$) and the concentrations ($c$, mg $L^{-1}$) of β-Lg A and β-Lg B had good linear relationships in the concentration range of 15–500 mg $L^{-1}$. The regression equation was β-Lg A: $A=445c-1370$; β-LgB: $A=328c-928$, with correlation coefficients ($r$) of 0.9998 and 0.9997, respectively. The LODs (S/N=3) and LOQs (S/N=10) were 4 and 3 mg $L^{-1}$, and 12 and 10 mg $L^{-1}$, respectively.

Method precision was evaluated by performing seven parallel analyses of the same raw bovine milk sample as that used for CE method evaluation. The average values of β-Lg A and β-Lg B in the sample were 1.82 and 2.23 mg $mL^{-1}$, with RSD values of 3.6% and 3.2%, respectively. There was no significant difference between the results of the two methods.

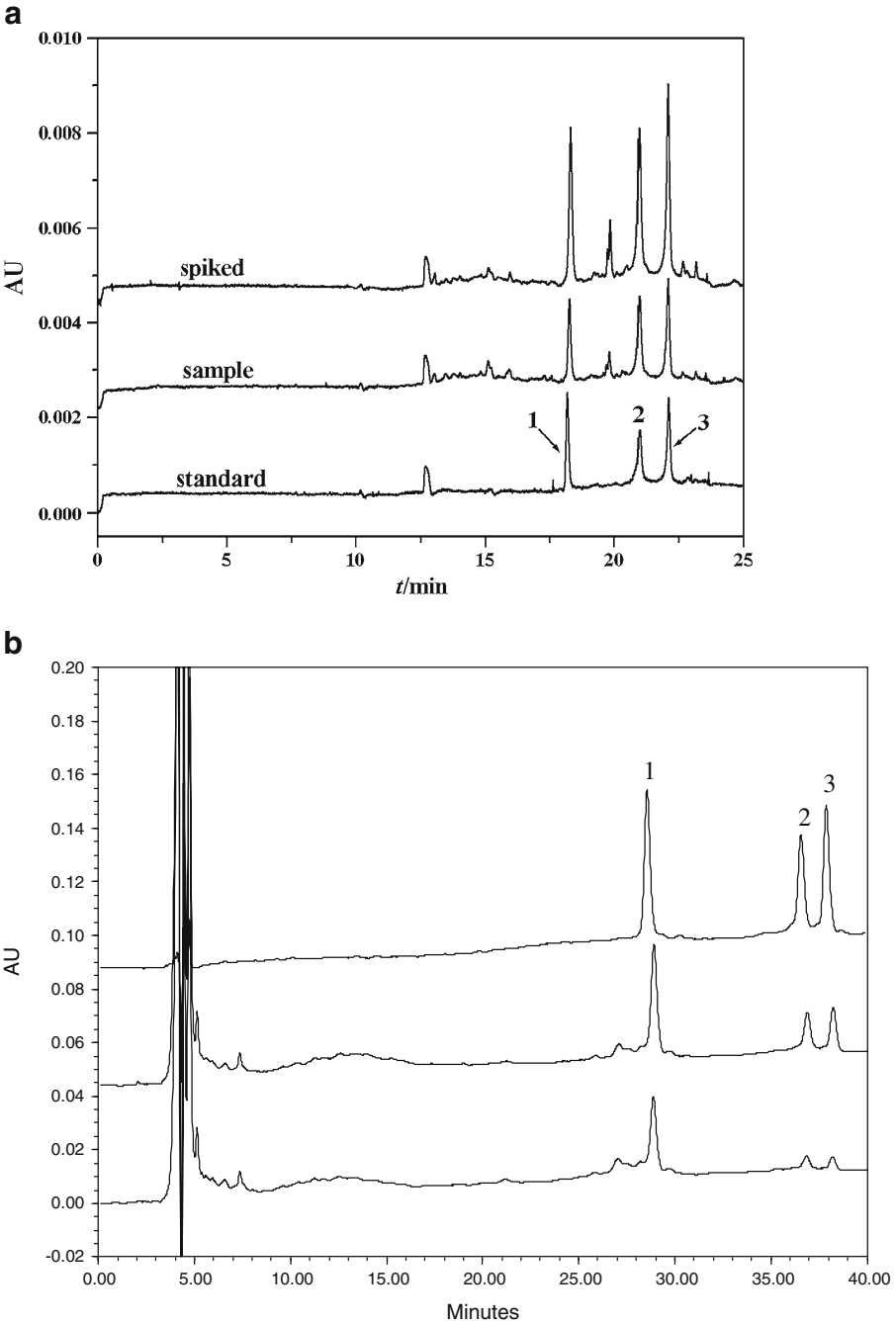


**Fig. 2** **a** Electrophoregrams of 200 mg $L^{-1}$ standard, sample, and sample spiked with 150 mg $L^{-1}$. The CE conditions were shown in "Capillary column" and "CE buffer" in Section 2.2. **b** Chromatograms of 150 mg $L^{-1}$ standard, sample, and sample spiked with 40 mg $L^{-1}$. Other chromatographic conditions were described in "HPLC conditions" in Section 2.2

**Table 1** Contents of α-Lac, β-Lg A, and β-Lg B in raw bovine milk determined by CE and HPLC

| Sample | CE (mg/mL) | | | HPLC (mg/mL) | | |
|---|---|---|---|---|---|---|
| | α-Lac | β-Lg A | β-Lg B | α-Lac | β-Lg A | β-Lg B |
| 1 | 0.91 (4.6)[a] | 1.34 (5.2) | 1.65 (6.0) | –[b] | 1.27 (2.3) | 1.78 (3.4) |
| 2 | 1.00 (3.9) | 1.63 (4.8) | 1.58 (5.6) | –[b] | 1.54 (2.2) | 1.70 (3.1) |

[a] Relative standard deviations (RSD, %) are in parentheses, ($n$=5)

[b] Could not be accurately determined

The accuracy of the present method was tested by spiking at two levels in five replicates of a raw bovine milk sample. The average recoveries at 40 mg $L^{-1}$ spiking were 97% and 104.5%, with RSD values of 5.0% and 7.5%, respectively. The average recoveries at 200 mg $L^{-1}$ spiking were 102.1% and 105.3%, with RSD values of 1.5% and 3.6%, respectively.

### 3.3 Cross-validation

Two raw bovine milk samples were pretreated and analyzed by both the HPLC and the CE methods, with five replicates. The results were given in Table 1. The values for β-Lg A and β-Lg B were quite similar for the two methods. The $t$ test is usually used to determine whether two methods yield the same results within experimental error. The $t$ values were all less than the tabulated $t$ values at 95% confidence, indicating no significant difference between the two methods.

**Table 2** Results of α-Lac, β-Lg A, and β-Lg B in whey protein powder, bovine colostrum, and bovine raw milk

| Sample | | α-Lac (mg/100 g) | β-Lg A (mg/100 g) | β-Lg B (mg/100 g) |
|---|---|---|---|---|
| Whey protein power | 1 | 2,120 | 3,610 | 4,800 |
| | 2 | 1,380 | 1,480 | 2,310 |
| | 3 | 1,350 | 2,100 | 3,340 |
| | 4 | 3,470 | 3,010 | 4,100 |
| Bovine colostrum | 1 | 310 | 500 | –[a] |
| | 2 | 188 | 244 | 217 |
| | 3 | 210 | 260 | 250 |
| | 4 | 150 | 300 | 230 |
| | 5 | 120 | –[a] | 280 |
| Bovine raw milk | 1 | 140 | 250 | 200 |
| | 2 | 130 | 230 | 200 |
| | 3 | 130 | 270 | 200 |

[a] Not obtained

**Table 3** Results of α-Lac in infant formula of different age stages

| Sample | Stage | α-Lac (mg/100 g) |
|---|---|---|
| 1 | I | 37 |
| | II | 103 |
| | III | 140 |
| 2 | I | 90 |
| | II | 110 |
| | III | 260 |
| 3 | I | 110 |
| | II | 217 |
| | III | 285 |

### 3.4 Real sample analysis

Since the established CE method was judged to be the better choice, it was used for real sample analysis in the following work. The contents of the α-Lac, β-LgA, and β-LgB in whey protein powder, bovine colostrum, and bovine raw milk were listed in Table 2. Whey protein powder had the highest content of the above three proteins among the three samples. Sample 1 of bovine colostrum contained only β-Lg B and no peak of β-Lg A was observed, whereas sample 5 contained only β-Lg A. This extreme phenomenon had also been reported by Kim et al. (1996). β-Lg A and β-Lg B in infant formula were interfered by the matrix; therefore, only α-Lac could be

**Table 4** Results of α-Lac in infant formula from domestic and imported products with the same age stage

| Stage | Country | Sample | α-Lac (mg/100 g) |
|---|---|---|---|
| I | Domestic | 1 | 83 |
| | | 2 | 143 |
| | | 3 | 167 |
| | | 4 | 212 |
| | Imported | 5 | 91 |
| | | 6 | 21 |
| | | 7 | 314 |
| | | 8 | 162 |
| II | Domestic | 9 | 234 |
| | | 10 | 150 |
| | Imported | 11 | 276 |
| | | 12 | 103 |
| | | 13 | 43 |
| III | Domestic | 14 | 310 |
| | | 15 | 126 |
| | Imported | 16 | 307 |
| | | 17 | 202 |
| | | 18 | 89 |
| | | 19 | 19 |

accurately assayed. The contents of α-Lac in imported infant formula for different age stages were shown in Table 3. The contents of α-Lac in domestic or imported infant formula with the same age stage were listed in Table 4. It was found that the contents of α-Lac in infant formula increased with the age stage raised. Infant formula with the same age stages but different brands and countries had significantly different α-Lac contents. The contents of α-Lac in imported formula were not always higher than those in domestic products.

## 4 Conclusions

$H_3BO_3$ (0.5 mol $L^{-1}$) in combination with PEO 4,000,000 polymer (0.8 g $L^{-1}$) and HP-β-CD (0.025 mol $L^{-1}$) in the running buffer could successfully improve the resolution and greatly suppress the undesirable adsorptions of α-Lac, β-Lg A, and β-Lg B to the capillary wall. The composition of the sample extraction solution played a critical role in the determination of the three proteins in raw bovine milk. The CE method offered excellent resolution of α-Lac, β-Lg A, and β-Lg B, with shorter run time than HPLC. More importantly, the CE method can be used for the simultaneous quantification of α-Lac, β-Lg A, and β-Lg B in whey protein, bovine colostrum, and raw bovine milk.

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