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# Multi-dimension microchip-capillary electrophoresis device for determination of functional proteins in infant milk formula

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ABSTRACT

To improve resolution of important minor proteins and eliminate time-consuming precipitation of major protein with associated analyte co-precipitation risk, a multi-dimension strategy is adopted in the 2D microchip-CE device to isolate major proteins on-chip, enrich minor proteins in capillary before their separation in CE for UV quantitation. A standard fluorescent protein mixture containing FITC-BSA, myoglobin and cytochrome as specific pI markers has prepared to demonstrate capability of the device to fractionate minor proteins by IEF. The results using a standard protein mixture with profile resembling infant milk formula show a complete isolation of high abundance proteins by a 2-min 1D IEF run. The subsequent t-ITP/CZE run by on-chip high voltage switching delivers a high stacking ratio, realizing 60 folds enrichment of isolated protein fractions. All five important functional proteins (LF, IgG, α-LA, β-LgA and β-LgB) known to fortify infant milk formula are isolated and determined using two consecutive t-ITP-CZE runs within a 18-min total assay time, a significant saving compared to several hours conventional pretreatment. For a 100 g infant milk formula sample, working ranges of 20–8000 mg, repeatability 3.8–5.3% and detection limits 2.3–10 mg have been achieved to meet government regulations. Method reliability is established by 100% recoveries and agreeable results within expected ranges and labeled values. The capability of the device for field operation, rapid assay with quick results, label-free universal detection, simple operation by aqueous dissolution before injection, and the demanding matching in 2D separation based on isolated fractions at specified pI ranges, closely matched migration time and baseline-resolved peak shape makes the device a general tool to detect unknown proteins and determine known minor proteins in protein-rich samples with interfering constituents.



## 1. Introduction

Human milk provides the best nutrients for infants and babies because of its balanced proportions and presence of several key functional proteins as compared to other milks [1,2]. However, if babies cannot be breast-fed, infant milk formula provides the best alternative. Although infant milk formula products are claimed to match closely to human milk, significant differences exist in several key functional proteins such as α-lactalbumin (α-LA), lactoferrin (LF), immunoglobulin G (IgG) and β-lactoglobulins (β-LgA and β-LgB). They are essential for key biological functions such as immune system development. Although α-LA, a major whey protein present in human milk, plays a key role in the production of lactose [2,3], its concentrations in most infant milk formula are relatively low (~0.8 g per 100 g infant milk formula) [2]. On the other hand, β-lactoglobulin, the major whey protein present in infant milk formula, is not found in human milk [2–4]. The other two functional whey proteins, LF and immunoglobulins, are present at significantly high levels in human milk and colostrum (1000–7000 mg/L) as compared to bovine milks (20–200 mg/L) [1,5–7]. α-LA, IgG and LF have been found adding to infant milk formula as supplements with a claim to protect newborns from infections. LF is currently regulated by the Ministry of Health of the People's Republic of China (MOH, PRC) in the new version of "Food Safety National Standards for the Usage of Nutrition Enrichment" (GB 14880-2012) as a food additive, limiting addition within 100 mg LF per 100 g infant milk formula [8]. As increasing number of manufactures have advertised elevated levels of α-LA and IgG in their products, their supplementation are expected to be regulated shortly. Thus, methods are needed for the determination of low level of functional proteins in a protein-rich sample matrix for their control and monitoring.

Methods for quantitative and semi-quantitative determination of LF, α-LA, IgG, β-LgA and β-LgB in dairy products had been reported [6,7,9–11] using various techniques such as

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high performance liquid chromatography (HPLC), Enzyme-linked immunosorbent assay (ELISA), surface plasma resonance (SPR) and 2D gel electrophoresis. Each technique is targeted on a specific or a limited number of functional proteins and a combined use of several techniques are needed for a comprehensive monitoring of required functional proteins. In addition, time-consuming sample pretreatment procedures requiring several hours operation [9,10,12,13] are needed to precipitate caseins, the major protein present in the dairy products. Loss of functional proteins due to co-precipitation with caseins had been reported [14]. To obviate the use of lengthy sample pretreatment procedures, Riechel et al. [15] had developed a high-separation efficiency capillary electrophoresis (CE) procedure to separate spiked LF from major whey proteins within 20 min in a single CE run. However, it was found that LF co-migrated with other major whey proteins like β-LgA and insufficient separation of LF from other milk proteins was obtained, although various buffer systems were tested.

Multidimensional strategies have been successfully employed in proteome research to enhance peak capacity. However, most of the proteome work is focused on profiling and identification of unknown proteins [16–18] using expensive laboratory-based instrumentation. No report has been found in the literature for quantitation of all currently known minor functional proteins in milk in a single assay. The recent advances in microfluidic devices provide a promising tool for quality control of protein in dairy industry due to its potential for field operation [19–26]. Out of the various microfluidic devices developed, the microfluidic chip-capillary electrophoresis (microchip-CE) device emerges as one with a high marketing potential due to its integration of sample pretreatment techniques on-chip with the high efficient capillary electrophoresis for separation and detection, thus enhancing the scope for application of the device developed to the determination of minor or trace levels of analytes in samples with complex and interfering matrixes. In addition, well established instrumentation at affordable low cost is commercially available from existing CE manufacturers.

In the present work, a microchip-CE device is designed and fabricated based on our previous work for urinary protein analysis, which integrated onchip desalting and stacking of samples with analysis of proteins in the embedded capillary [27]. To enable practical use for quality control purpose, all known functional proteins (α-LA, LF, IgG, β-LgA and β-LgB) as well as newly introduced proteins should be covered in an assay using economically affordable instrumentation, preferably operable in the field. Thus, on-column UV detection is used instead of the more sensitive LIF detection as the former provides a label-less detection for all five functional proteins and other newly introduced proteins, affordable in cost and available for onsite monitoring. However, a large enhancement in sensitivity is needed to detect low abundance functional proteins. Isolation or depletion of high abundance proteins is also required to facilitate analysis of low abundance proteins. The design and performance of the device developed for quantitation of minor functional proteins in infant formula are presented and discussed in Section 3.

## 2. Materials and methods

### 2.1. Chemicals, reagents and standards

All chemicals used were analytical reagent grade except otherwise stated. Lactoferrin (LF), immunoglobulin G (IgG), α-lactalbumin (α-LA), β-lactoglobulin A (β-LgA), β-lactoglobulin B (β-LgB), bovine serum albumin (BSA), α-casein (α-CN), poly(ethylene oxide) (PEO, MW = 400,000), hydroxyethyl methyl cellulose (HEMC), urea, Triton X100 (reduced form), pharmalyte (36%, W/V, carrier ampholyte for IEF operable for pH range 3–10) and three fluorescent protein markers, fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA, pI 4.7, Orange), myoglobin (pI 7.0, Brown) and cytochrome C (pI 9.6, Red) were purchased from Sigma Chemicals (St. Louis, MO, USA). N,N,N′,N′-tetramethylethylenediamine (TEMED) was purchased from Bio-Rad laboratories, Inc. All proteins were used directly as received.

DI water was used to make up the following solutions to given concentrations: 10 mg/mL standard protein stock solutions; IEF buffer solution with 2% pharmalyte, 8 M urea, 1% Triton X100, 0.1 g/L PEO, 0.5 g/L HEMC and 0.25% TEMED; Anolyte with 0.05 M HAc, 0.1 g/L PEO and 0.5 g/LHEMC (pH 3.0); Catholyte with 0.04 M NaOH, 0.1 g/L PEO and 0.5 g/L HEMC (pH 10.0); Terminating electrolyte (TE) with 0.05 M HAc, 0.1 g/L PEO and 0.5 g/L HEMC (pH 3.0), and Leading electrolyte (LE) with 0.05 M Tris–HCl, 0.1 g/L PEO and 0.5 g/L HEMC, which was also used as background electrolyte (BGE).

### 2.2. Fabricating microchip-CE device

A $CO_2$ laser engraver (V-series, Pinnacle, USA) was used to fabricate the PMMA chip with 60% power and 45% speed under computer software control (Corel DRAW 10); a hot press bonding machine (up to 500 °C and 1 MPa pressure, Guangju Machinery Company, China) to bond the capillary (Analytical Technology Lanzhou, China) embedded between two PMMA plates; and an octa-channel high voltage system (0–4500 V, Sutter Creek, CA, USA) under electronic control to hold and switch high voltages at electrodes placed in designated vials of the microchip-CE device. A microscope (Olympus, Japan) with a built-in CCD camera was used to monitor the position of the fluorescent protein spots in the microfluidic chip during operation and an UV/vis detector from CE Resources (UVV500, Singapore) used to detect the separated proteins at 280 nm. The microchip-CE device (40 mm × 27 mm) was shown in Fig. 1 with dimensions indicated at the micrograph. The channel pattern and the seven vials were ablated onto the PMMA plates by the $CO_2$ laser using procedures described previously [28]. The PMMA plates and capillary were bonded together under a pressure of 0.6 MPa at 95 °C for 25 min.

### 2.3. Procedures for sample preparation and operation of microchip-CE device

The infant milk formula sample (Augood Series 1, Ausnutria, China) was purchased from a supermarket in Shenzhen, China and 0.1 g sample was dissolved into 4 mL IEF buffer by over 30 min stirring. The solution obtained was centrifuged at 13.2 krpm for 10 min to remove insoluble matters and used as the sample solution, 10 μL of which was added into vial B. The loading of buffer and sample was monitored by a 40× microscope. All channels were firstly filled by a syringe pump from the open end of the embedded capillary with terminating electrolyte (TE). The open end of the capillary and vial A, C, D, F and G were then sealed by silicone plugs. Vacuum suction was then applied to vial E to draw sample solution from vial B to completely replace the TE buffer in channels between B and E. With vial A, B, C, D, E and F sealed, BGE (same to LE) was introduced from W to G by a syringe pump. Anolyte and catholyte were then added to vials A and F. Vials C and D were then filled by TE and vial G by BGE. Procedure for CZE separation of the standard protein mixture on microchip-CE device include: (1) filling BGE to all channels and vials, (2) loading protein sample to vial E by suction through vial B, and (3) applying high voltages at 8 designated vials for IEF, ITP and CZE run for specified time as listed in Table 1.