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Capillary Electrophoresis of α-Lactalbumin in Milk Powders

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A quick and simple method for the extraction and analysis of α-lactalbumin in milk protein powders is presented, which permits accurate duty classification of commercial products as required under the U.S. Harmonized Tariff System. An acetic acid buffer medium is utilized to extract the whey proteins, which are then analyzed by capillary electrophoresis using an electrokinetic injection onto an uncoated column. The running buffer is composed of a 50 mM borate buffer at pH 8.0. The separation is rapid (<5 min) and displays good limits of detection (±0.01 mg mL^{-1}). Overall, the method provides a simple means for extracting and analyzing α-lactalbumin in milk powders.

KEYWORDS: Capillary electrophoresis; milk protein powders; whey proteins; α-lactalbumin

INTRODUCTION

Milk powders and their components are utilized in a variety of food products. Traditional uses incorporate milk powders as gelling additives in baked goods and comminuted meat products and as nutritional supplements for essential amino acids in beverages and cereals (*1*). Recent advances in milk processing (*1*) have created new applications, particularly in the field of bioactive products; for example, isolated and purified whey components are used in infant formulas (α-lactalbumin), therapeutics (milk peptides with opioid activity), and dental applications (lactoperoxidase—inhibition of pathogen/microorganism growth). These advances in milk/whey processing, in conjunction with the financial potential in emerging markets, have created increasing competition in the domestic and international trade arena.

As with any imported commodity, milk powder formulations are subject to duties or tariffs as specified in the Harmonized Tariff Schedule of the United States (HTSUS), wherein the dutiable classification of each commodity is explicitly defined, and every product so labeled must strictly adhere to the definition. A milk protein concentrate (MPC) is defined as, "any complete milk protein (CMP) concentrate, casein plus lactalbumin, that is 40% or more protein by weight" (*2*); therefore, chemical analyses must confirm the presence of casein, lactalbumin, and 40% protein content. Caseins account for 80% of the protein content in liquid milk and are easily detected in the corresponding powder (AOAC method 927.03 for casein). However, the identification of lactalbumin is more difficult due to its inherently low levels in milk (3.7−5.0% of the total milk protein)—a problem that is further exacerbated in the milk powder because of degradation/denaturation caused by processing (*3*−*7*). Therefore, analyses of milk powders may give a poorly separated, indistinguishable, or absent α-lactalbumin peak.

Previous studies have presented the isolation of the major whey proteins (bovine serum albumin, α-lactalbumin, *β*-lactoglobulin A and B, and immunoglobulins) via preliminary precipitation of casein in liquid milk samples by acidification or through the action of rennet enzymes. The remaining supernatant (containing the whey proteins) has then typically been analyzed using gel electrophoresis (*8*−*12*), high-performance liquid chromatography (HPLC) (*3*, *4*, *13*−*18*), or capillary electrophoresis (CE) (*5*, *10*−*12*). Additional studies focused on genetic variants (*19*), product processing and stability (*1*, *3*, *4*, *7*, *12*, *15*), method development (*3*, *5*, *10*, *11*, *13*, *14*, *16*−*22*, *24*), and physicochemical characterization (*4*, *6*, *7*, *23*, *25*−*27*).

Prior studies utilizing gel electrophoresis showed poor analyte sensitivity (*4*, *10*, *15*) and long analysis times. Analogous work using HPLC employed a reversed phase silica-based column, C_4 or C_{18}, with a gradient mobile phase mixture employing acetonitrile and water containing trifluoroacetic acid (*3*, *13*, *14*, *16*). The resulting separations showed poor to good resolution between the whey proteins with run times averaging 30 min. Work using CE (*5*, *10*, *12*, *20*−*22*) showed shorter run times relative to gel electrophoresis and HPLC, with good to excellent resolution among the whey proteins. Various running buffers were utilized, including borate, phosphate, sulfate, and urea. However, all of the previous CE studies employed a hydrodynamic injection, and most focused only on the separation of whey proteins in liquid milk.

Several papers analyzed powdered milk (*5*, *7*, *12*, *26*), nonfat dry milk (*3*, *4*, *7*), or whey protein concentrates/isolates (WPC/WPI) (*6*, *11*, *25*). Our experience with MPCs revealed minimal solubility in water at neutral pH but good solubility in acidic and basic media. In duplicating the work of the above cited literature, our CE analyses of the water-extracted powders displayed broad peaks with little differentiation among the whey/casein proteins. Herein, we present an improved methodology,

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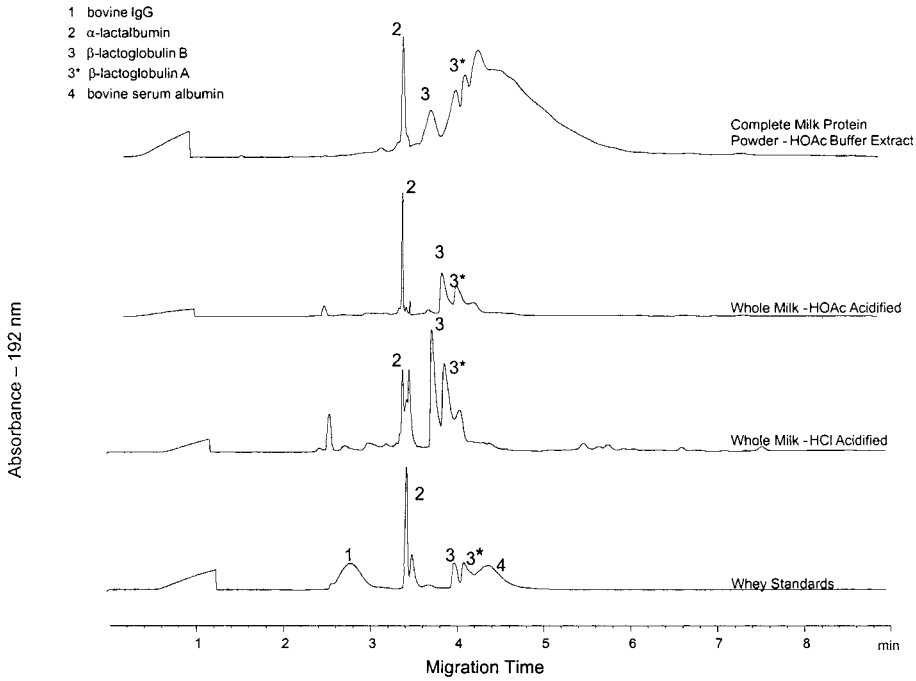

Figure 1. Whey proteins in various samples.

Table 1. Milk Powder Protein Content

product	identifier	protein content (% w/w)
CMP	CMP	≥85
MPC	MPC-42	42
nonfat dry milk	nonfat	33.0
TMPC (USCS)	TMPC	≥80
whole milk	whole	22

which provides a rapid analysis of extracted α-lactalbumin (derived from milk powders) using CE with an electrokinetic injection.

MATERIALS AND METHODS

Chemicals. Boric acid, sodium tetraborate, citric acid, sodium citrate, glacial acetic acid, phosphoric acid, sodium acetate, hydrochloric acid, and sodium hydroxide were obtained from Fisher Scientific (Springfield, NJ). Commercially purified whey protein standards, including α-lactalbumin, bovine serum albumin, *β*-lactoglobulin A and B, and bovine immunoglobulin G, were supplied by Sigma-Aldrich (St. Louis, MO). A borate buffer (0.3 M and pH 8.6) containing a proprietary additive was also obtained from Sigma. All reagents and standards were reagent grade or better. The water used to prepare solutions was purified through a Barnstead/Thermolyne Easypure (Dubuque, IA) reverse osmosis unit (18 MΩ cm resistivity).

Samples. Liquid whole milk was obtained in half pint cartons from Cloverland/Green Springs Farms (Baltimore, MD). Powdered milk samples were received from the U.S. Customs San Francisco Laboratory (USCS) and from Milky Whey, Inc. (Missoula, MT). **Table 1** details the sample abbreviations and the stated or nominal protein content of each product, obtained from elemental nitrogen analysis (N × 6.38).

Sample Preparation. Locally purchased liquid milk served as the baseline sample for method development. The whey proteins were isolated by acidifying the sample with either acetic acid (HOAc) or hydrochloric acid (HCl) until the casein precipitated (approximate pH of 4.5). The sample was then centrifuged at 2000*g* for 15 min, and the resulting supernatant was removed and placed in a polyethylene vial for immediate whey protein analysis or for storage in a refrigerator set at 0 °C.

Milk powders were prepared by placing approximately 250 mg of the product in a 15 mL centrifuge tube, adding 5 mL of 50 mM HOAc, pH 4.6, extraction fluid, and vortexing the mixture for 1 min. The solution was then centrifuged at 2000*g* for 15 min, and the resulting supernatant was filtered through a 0.45 *μ*m poly(tetrafluoroethylene) syringe filter and handled as above.

CE. All experiments were run using an Agilent Capillary Electrophoresis Unit 3^D CE with positive polarity (+ inlet to − outlet). The method run time was approximately 12 min, including column preconditioning. Various running buffer media were tried, including acetate, citrate, and phosphate buffers, over a wide pH range. The best buffer proved to be a 50 mM borate solution at pH 8.0, with a Sigma-Aldrich 0.3 M borate buffer (pH 8.6) with a proprietary additive being a suitable replacement. Capillary columns from Agilent (Palo Alto, CA), Polymicro Technologies (Phoenix, AZ), and Supelco (Bellefonte, PA) were utilized. The final CE method protocol was as follows: 32 cm uncoated 50 *μ*m Agilent silica capillary (23.5 cm effective); 50 mM, pH 8.0, borate buffer; 15 kV (+ polarity: + inlet to − outlet); 30 kV s electrokinetic injection; 15 °C cassette temperature; absorbance at 192 nm. Column preconditioning: 0.25 min, 0.10 M NaOH; 0.50 min, H_2O; 0.25 min, 0.20 M HCl; 0.25 min, buffer media.

RESULTS AND DISCUSSION

The goals of the study were to develop an improved CE method and a better extraction protocol for whey proteins in milk powders. CE provides several significant advantages over HPLC. Because proteins are charged molecules, CE gives a faster separation and higher resolution. The use of an aqueous buffer as the "running" background electrolyte also eliminates organic wastes. Another advantage is the ability to do an electrokinetic injection, which concentrates the sample and enables a lowered detection limit (no solvent evaporation or boil down is required). Previous studies using CE (*5*, *11*, *12*, *19*, *20*−*22*) employed a hydrodynamic injection (which does not preconcentrate the sample), thereby limiting the analyte detectability.

The solubility of milk powders is dependent on various factors, including preheating of the original liquid milk sample, lactose and protein content, and storage conditions. Prior studies solubilized dry milk either in water or in the column eluent/

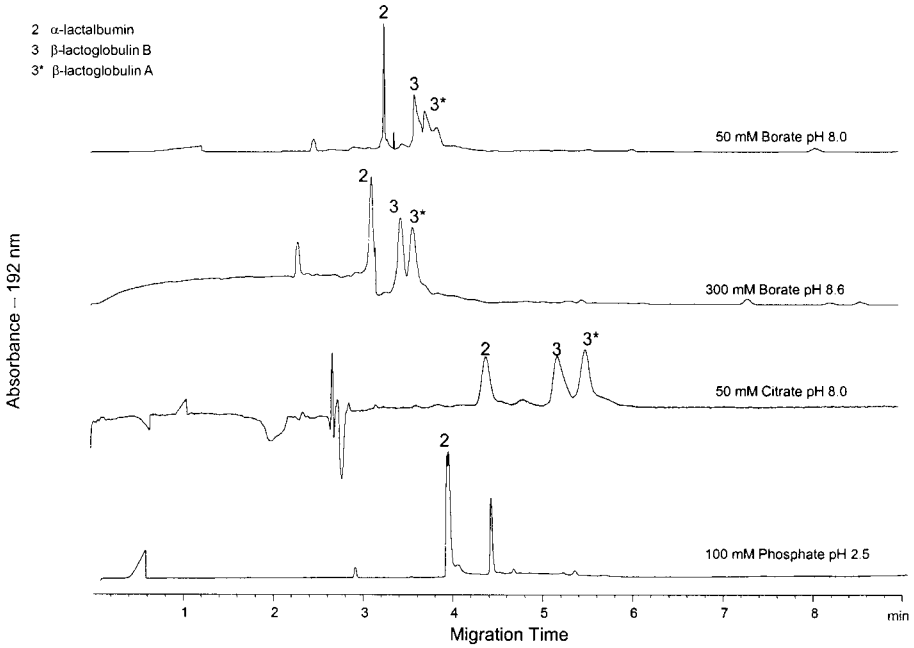

Figure 2. Whey protein separation of an HCl-acidified liquid milk in different running buffers.

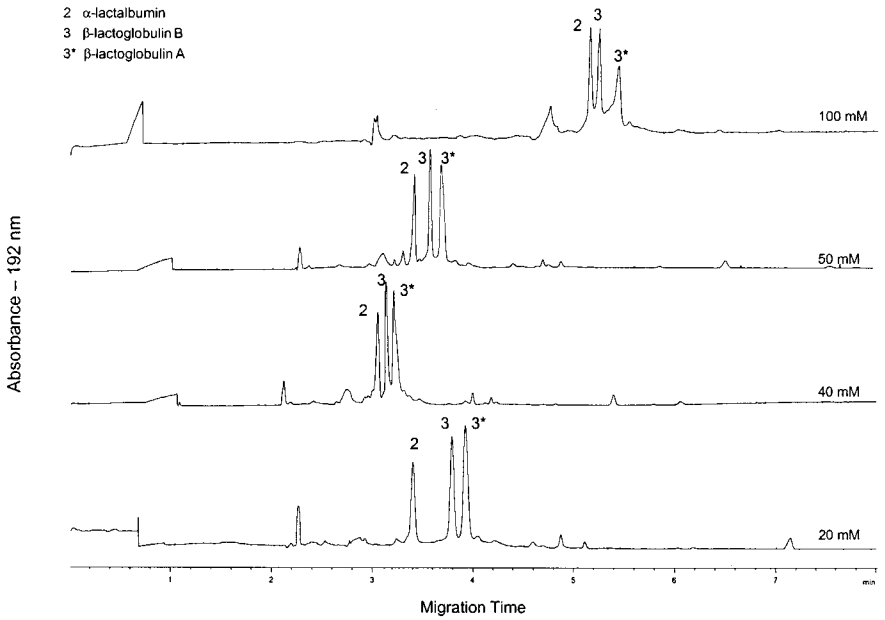

Figure 3. Whey protein separation of a HCl-acidified liquid milk in borate buffers (pH 9.25) at different concentrations.

buffer, with dialysis employed to increase purity. We observed minimal solubility of the milk powders in water, except for nonfat dry milk, dry whole milk, and some WPC and WPI samples.

CE. For method development, we used whey protein standards and samples derived from the supernatant of HCl- or HOAc-acidified (casein-precipitated) liquid milk: knowledge of the respective species migration times (from the standards) and relative concentrations (from the acidified samples) aided in the protein identifications and comparisons to the observed milk powder extracts. Although several articles (*10*, *11*) have detailed the utility of capillary gel electrophoresis in the separation of proteins, free zone electrophoresis was chosen to simplify the procedure. Minimal band broadening (from adsorption) was observed with the uncoated capillary column. **Figure 1** portrays, respectively, the separation of whey proteins from standards, a liquid milk sample acidified with acetic acid or hydrochloric acid, and a CMP powder extract. The traces are each set in coincidence to the α-lactalbumin peak for the purpose of more direct comparison (also done in later **figures**). The protein elution sequence was constant in the order immunoglobulin (IgG), α-lactalbumin, *β*-lactoglobulin B, *β*-lactoglobulin A, and bovine serum albumin. The optimal operating parameters are described in the Experimental Section and are similar to those previously reported (*22*).

Figure 1 shows that a larger amount of *β*-lactoglobulin is present following HCl acidification as compared to HOAc acidification. However, the latter procedure also showed a single α-lactalbumin peak (note that two forms exist—*apo*, stable in acidic pH, and *holo*, stable in basic pH) (*23*). **Figure 1** also displays the broad peak typically observed in the elution of the *β*-lactoglobulin components, which may be due to the presence of associated whey and casein proteins or denatured protein fragments (*3*−*7*).

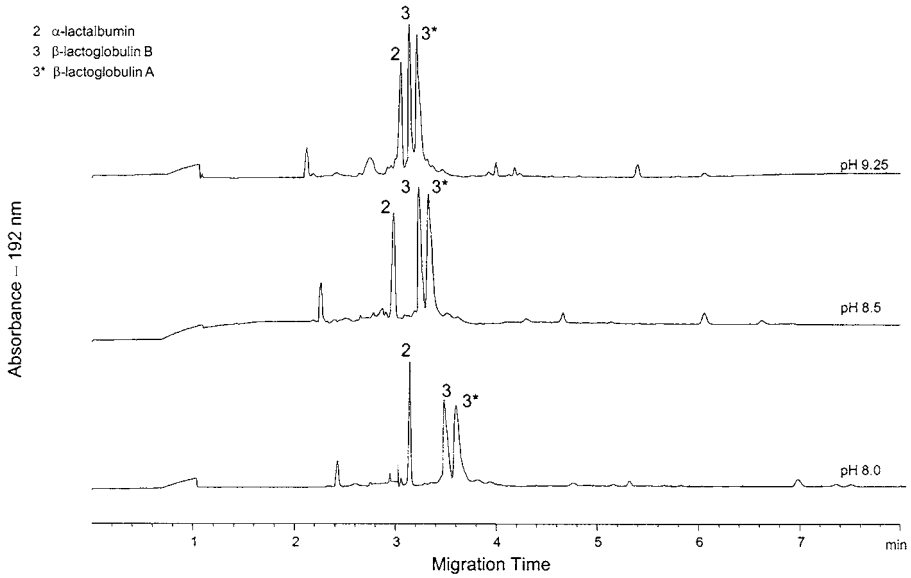

Figure 4. Whey protein separation of a HCl-acidified liquid milk at various pH values (40 mM borate buffer).

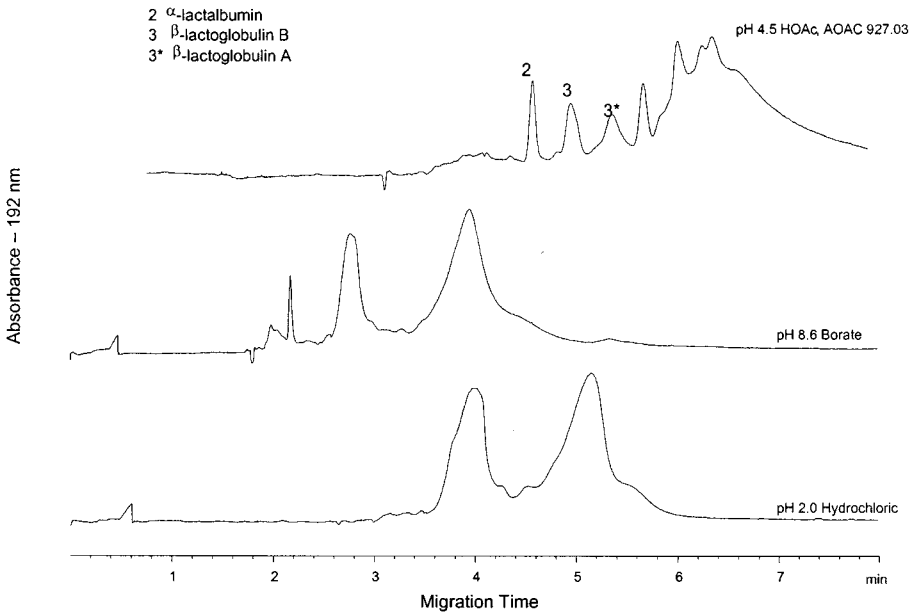

Figure 5. Solubility/extraction of a milk protein powder (TMPC) into various fluids.

Figures 2−4 show the optimization of the CE protocol using the supernatant of a HCl-acidified liquid milk sample. **Figure 2** compares the analysis of whey proteins under different running buffers. A basic borate buffer confers a more expeditious and improved separation relative to phosphate or citrate buffers. **Figures 3** and **4** depict, respectively, the effect of borate buffer concentration and pH on the resolution and runtime. The results suggest that a pH 8.0 or 8.5, 50 mM borate running buffer solution works best. The optimal CE method therefore utilizes a 32 cm, uncoated 50 μm silica capillary (23.5 cm effective) column with a pH 8.0, 50 mM borate running buffer.

A concentration of 0.05 mg mL^{-1} of α-lactalbumin (2 orders of magnitude below the native level of α-lactalbumin in liquid milk, 1.2−1.7 mg mL^{-1}) can be detected. The instrumental method detection limit was approximated as 0.01 mg mL^{-1} (3σ at $n = 11$ of a 0.05 mg mL^{-1} standard). The within day and day to day precision in peak area were <8 and <5% rsd, respectively, over the concentration range of 0.05−5 mg mL^{-1}. A standard calibration curve displayed linearity in the same range with a regression analysis providing a correlation coefficient of 0.998.

The daily precision in the migration times averaged <2% rsd. Over the course of the study, migration times for α-lactalbumin ranged from 3.22 to 6.12 min with less than <5% rsd. Better precision in the migration time may be obtained by keeping the power constant, instead of the voltage or the current. In this way, changes in the reproducibility of the capillary electroosmotic flow can be minimized from the effects of variances in factors such as the ambient temperature, the buffer strength and pH, and the column length and internal diameter.

Independent of the variability in migration time, baseline resolution between α-lactalbumin and β-lactoglobulin was consistently observed (as seen in the **figures**). The clear and distinct separation provides a more important figure of merit for the method.

The sequential development of the CE protocol (prior to the extraction protocol) facilitated the optimal conditions for the separation, enabling the direct comparisons of the extractions

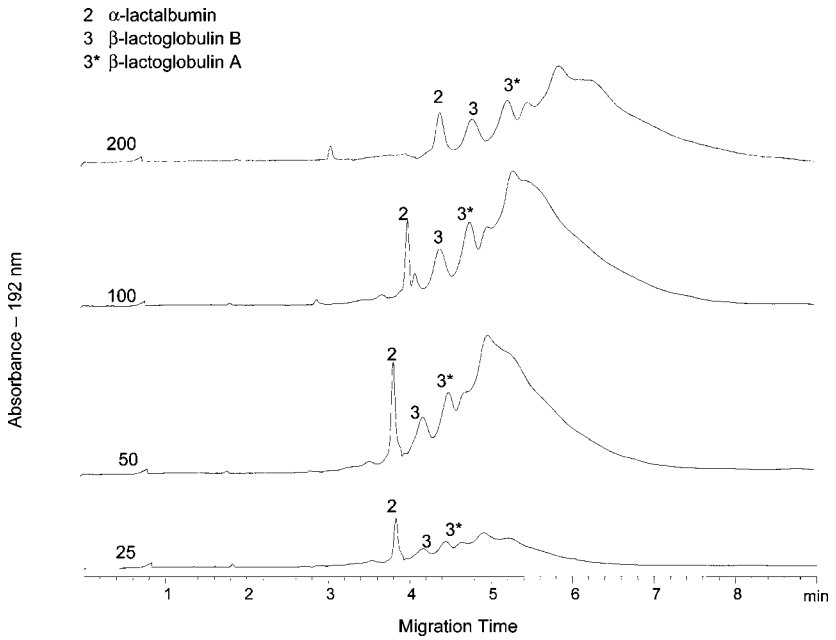

Figure 6. Extraction efficiency and [HOAc] (mM) at pH 4.6 on a CMP powder.

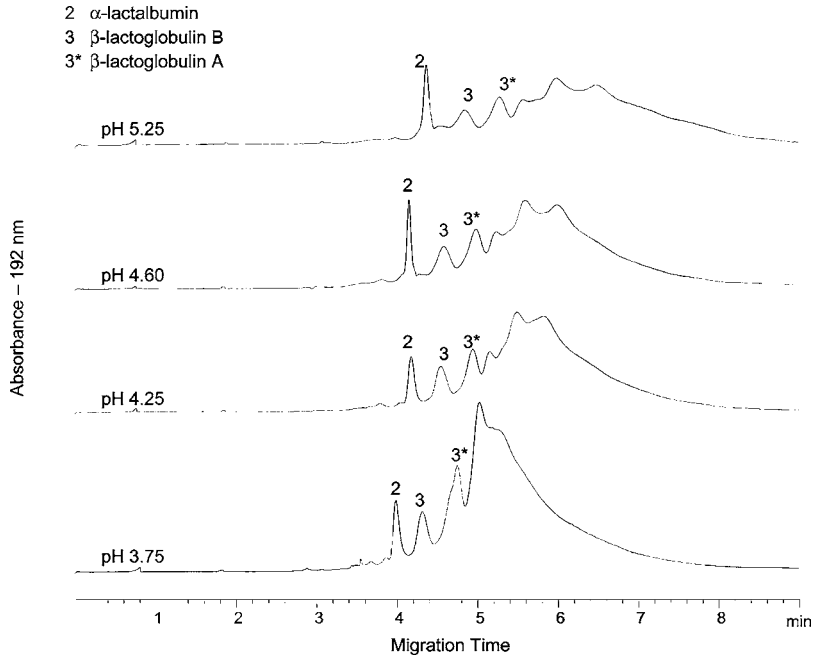

Figure 7. Extraction efficiency and pH (50 mM, HOAc) on a CMP powder.

done with various solutions and concentrations. The final CE methodology therefore allowed comparisons to improve the extraction of the whey proteins.

Whey Protein Extraction. As previously detailed, casein is readily separated from α-lactalbumin and other whey proteins through acidification. At a pH of 4.6, casein precipitates, leaving the whey proteins in solution; however, the addition of more acid resolubilizes the casein. Various acid solutions (acetic, boric, citric, hydrochloric, and phosphoric) were used to precipitate casein. As previously noted (*3*), the use of HOAc and HCl as precipitating reagents provided relatively casein-free supernatants. Additional studies using organic modifiers or other additives (acetonitrile, methanol, sodium dodecyl sulfate, and β-mercaptoethanol) to increase the solubility or the extraction of α-lactalbumin were not successful. **Figure 5** exhibits the CE separations of pH 2.0, 4.5, and 8.6 milk powder extracts, showing the better delineation of whey proteins in the pH 4.5 HOAc buffer.

The extraction protocol derives from a variation of the HOAc acidification methods for milk protein, such as AOAC methods 927.03 for casein and 925.24 for α-lactalbumin. However, instead of being precipitated, α-lactalbumin was analyzed directly in the supernatant solution after casein precipitation. **Figures 6** and **7** depict the extraction of α-lactalbumin from a CMP powder as a function of HOAc concentration and pH, respectively. The results indicate that the optimal extraction medium for α-lactalbumin is 50 mM HOAc at a pH of 4.6.

The final extraction protocol was selected based on a comparison between the addition of 5 mL of a 50 mM HOAc buffer (pH 4.6) solution to 0.25 g of a powdered sample and the dropwise acidification using a 0.2 M HOAc solution to 0.25 g of the same sample in 5 mL of water. The final liquid volume

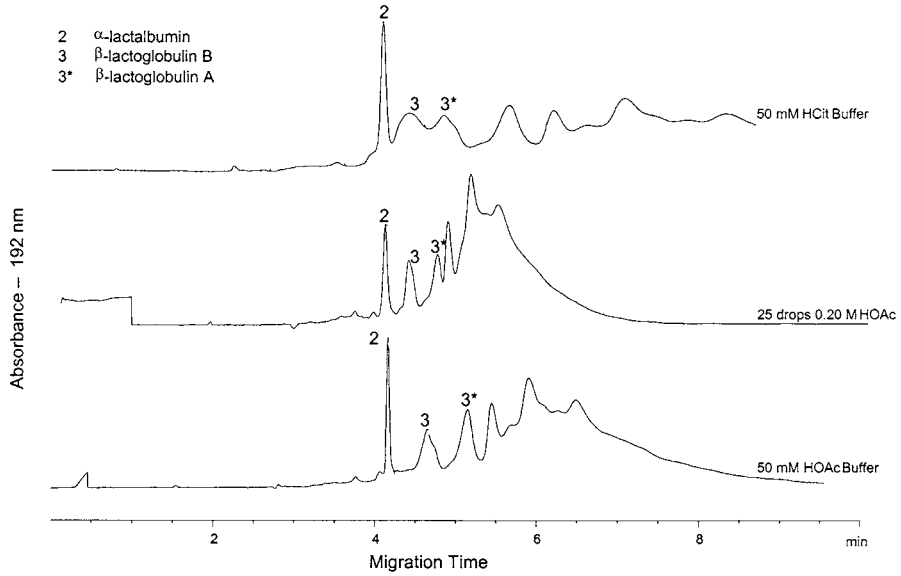

Figure 8. Comparison of extraction fluids on a CMP powder.

Table 2. Comparison of Extraction Efficiency of α-Lactalbumin from Various Milk Protein Powders[a]

	[α-LA] (mg mL^{-1})		% α-LA (w/w sample)	
product	HOAc buffer	22 drops of 0.20 M HOAc	HOAc buffer	22 drops of 0.20 M HOAC
CMP	0.91	0.71	1.82	1.42
MPC-42	0.56	0.37	1.11	0.74
nonfat	0.83	0.47	1.66	0.94
TMPC	0.49	0.25	0.98	0.50
whole	0.27	0.30	0.53	0.61

[a] Acidified liquid whole milk, whey supernatant: 1.39 mg mL^{-1}.

from the dropwise HOAc addition was approximately 5.5 mL. **Figure 8** shows the CE traces from the extraction of CMP powder by an HOAc buffer, HOAc dropwise addition, and a citric acid (HCit) pH 4.6 buffer. **Table 2** compares the amount of α-lactalbumin extracted from various milk powders using the above two protocols. The table also contains the observed concentration of α-lactalbumin in liquid whole milk. The combined data from the comparison reveal the HOAc buffer as the better extraction medium, yielding higher concentrations for four of the five samples tested. However, the data also show the values for α-lactalbumin to be below the level found in liquid milk (3.7−5.0 wt %), suggesting a degeneration due to processing of the liquid milk.

In summary, the use of a buffered HOAc−acid extraction fluid for milk protein powders provides an improved and more convenient mechanism for isolating the whey protein fraction from the rest of the sample. When coupled with the rapid and sensitive separation of the whey proteins through the improved CE methodology, the whole procedure significantly enhances the analyses of milk powders relative to previous methods.

CONCLUSION

The extraction process plays a critical role in the determination of α-lactalbumin content in milk powders. A 50 mM solution of acetic acid buffered at pH 4.6 has been shown to be effective in extracting α-lactalbumin in various milk powders. This study has also illustrated a fast, efficient, and sensitive separation of these whey extracts using CE and a borate buffer (50 mM, pH 8.0).

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