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# Capillary electrophoresis of Cheddar cheese[1]

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**Abstract**

Free solution capillary electrophoresis (FSCE) and micellar electrokinetic capillary chromatography (MECC) methods were developed using standard compounds. Each method was examined for its ability to simultaneously resolve caseins, peptides, and various small molecules found in Cheddar cheese. FSCE with phosphate–urea–acetonitrile run buffer separated caseins and large peptides, but this method was not robust with respect to resolution of milk proteins and peptides. Additionally, peak resolution with phosphate–urea buffer degraded after about 50 samples. MECC with borate–SDS run buffer simultaneously separated $\alpha$- and $\beta$-caseins, peptides, several free amino acids, and small aromatic molecules within 25 min. MECC was robust and electropherograms of phosphate-soluble Cheddar cheese fractions suggest that it may be a valuable tool to study the biochemistry of cheese maturation.

*Keywords:* Food analysis; Capillary electrophoresis; Micellar electrokinetic chromatography; Caseins; Peptides; Amino acids; Indole; Cresol; Skatole

## 1. Introduction

The dairy industry strives to produce high-quality, reduced-fat cheeses, but these products tend to be inferior to their full-fat counterparts. Manufacture of lower-fat products has proven difficult because reduced-fat varieties are generally more susceptible to flavor and textural defects [1]. Development of characteristic Cheddar cheese flavor and texture in reduced-fat cheese is controlled by poorly understood dynamic biochemical processes that occur during ripening. Solutions to these problems in low fat cheese require an improved understanding of the biochemical reactions that occur during the maturation process.

Casein degradation has a pronounced effect on Cheddar cheese flavor and texture [2–4]. $\alpha$-Casein and $\beta$-casein are degraded by the action of rennet, endogenous milk enzymes, and microbial proteinases, which together generate a wide variety of peptides and free amino acids [4]. The nitrogenous products from these enzymic reactions ultimately give rise to a wide variety of volatile and nonvolatile cheese flavor compounds [4–6].

Historically, analytical techniques employed to follow chemical changes in Cheddar during maturation rely on gas chromatography, mass spectroscopy, HPLC, PAGE, column chromatography, and combinations of these techniques [7]. Those studies sug-

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gest that volatile components in the lipid phase of the cheese provide some contributions to flavor [2], but the aqueous phase has a much larger impact on Cheddar cheese flavor [8]. HPLC analysis of water-soluble Cheddar cheese fractions during maturation detects a variety of compounds including large and small peptides, free amino acids, aromatic compounds, aldehydes, ketones, carbonyls, pyrazines, organic acids, fatty acids, sulfur-containing compounds, enzymes, and other molecules [9,10].

The ability to monitor and characterize the compounds formed during cheese ripening presents a significant analytical challenge. The chemical products are diverse with respect to class, size, charge, hydrophobicity, and molar ratio. As a result, efforts to follow cheese chemistry during maturation typically requires multiple analyses [7]. Unfortunately, many key flavor compounds are likely present at low levels [6], and existing methods may not detect and monitor their evolution or disappearance. For these reasons, cheese flavor research would benefit from new, highly sensitive methodology that simultaneously screens cheese extracts for many different classes of compounds. The objective of this research was to develop a robust analytical technique using capillary electrophoresis to detect some of the heterogeneous compounds in Cheddar cheese extracts in a single analytical separation.

## 2. Experimental

### 2.1. Preparation of standards

Purified caseins were prepared by the cationic FPLC method of Hollar et al. [11] from a combined herd milk sample. Whey proteins, electrophoresis-grade SDS, and other components of the standard mix were obtained from Sigma Chemical (St. Louis, MO, USA). Urea (AR grade), mono- and dibasic sodium phosphate, and dimethyl-formamide (DMF) were purchased from Mallinckrodt (Paris, KY, USA). HPLC-grade acetonitrile (ACN) was obtained from Burdick and Jackson (Muskegon, MI, USA) and a Barnstead II column purification system (Boston, MA, USA) was used to obtain double-deionized water ($ddH_2O$) for reagent preparation.

Stock solutions (10 mg/ml) of purified $\alpha$-lactalbumin, $\beta$-lactoglobulin, $\alpha_{s1}$-, $\kappa$-, and $\beta$-casein were prepared in 100 m*M* sodium phosphate buffer (pH 6.5), sterilized by passage through a 0.2-$\mu$m filter (Gelman Sciences, Ann Arbor, MI, USA), and stored at 4°C until needed. Stocks of an oligopeptide (Sigma stock number A 2152) whose sequence matched $\beta$-casein (f202–207) were suspended in $ddH_2O$ to 1 mg/ml, and frozen at −20°C until required. Stock solutions (10 m*M* each) of the amino acids phenylalanine, tryptophan and arginine were prepared in $ddH_2O$; tyrosine was suspended in 10 m*M* NaOH, 10 m*M* stocks of indole and skatole were dissolved in 50% ACN, and $\rho$-cresol 10 m*M* stock solutions were prepared in 100% methanol. Each solution was kept at 4°C until required.

### 2.2. Cheese sample preparation

Twenty grams of ground Cheddar cheese was mixed with 180 ml of 100 m*M* phosphate buffer (pH 7.2) and homogenized in a Stomacher 400 (Seward Medical, London, UK) for 5 min at room temperature. The extracts were centrifuged at 16 300 *g* for 30 min and the fat layer was removed. Residual fat was removed by sequential filtration through Whatman GF/A, no. 2, and no. 5 filter paper (Whatman International, Maidstone, UK). Some of the phosphate extract was fractionated with $M_r$ 30 000 cut-off membranes (Amicon, Beverly, MA, USA). Samples of whole extracts and $M_r$ 30 000 filtrates were stored at −20°C until needed.

### 2.3. Capillary electrophoresis (CE) equipment

Electrophoresis was performed on a P/ACE 2100 automated CE system (Beckman Instruments, Fullerton, CA, USA) equipped with a 57 cm×50 $\mu$m I.D. or 57 cm×75 $\mu$m I.D. untreated silica capillary and System Gold software (version 7.11). New capillaries were washed with 1 *M* NaOH and equilibrated with run buffer until the baseline stabilized (usually overnight); capillaries were then dedicated to the equilibration buffer. Voltages were kept within the linear range of an Ohm's law plot of voltage/current at 25°C and were ramped to the desired value in 1 min. Sample detection was achieved at 200 nm with the detector range at 0.02 AUFS and a data collection rate of 2 Hz. The polarity was set with the

positive pole at the capillary inlet. Samples (300–400 μl) were added to a 500-μl polypropylene microvial with the lid cut off, placed inside 4-ml glass autosampler vials, then sealed with a rubber cap. Similar glass vials and caps were used for electrolyte and rinse solutions during electrophoresis. Electrolytes, buffers, and wash solutions were filtered though a 0.2-μm nylon filter unit (Nalgene, Rochester, NY, USA) before use, and capillary rinses always used dedicated vials not used for electrophoresis. Data collection remained active for one column volume of a low-pressure rinse to ensure that all material eluted from the capillary.

### 2.4. Free solution capillary electrophoresis

All run buffers and sample dilution buffers were prepared fresh daily from a 200 m*M* sodium phosphate stock (pH 7.2). Samples of caseins and β-lactoglobulin were diluted to 1 mg/ml and α-lactalbumin was diluted to 0.5 mg/ml with dilution buffer [50 m*M* sodium phosphate (pH 7.2), 4 *M* urea] just before injection into a 57 cm×50 μm I.D. untreated silica capillary. Cheese extracts were filtered through 0.2-μm low-protein binding filters (Gelman) and mixed 1:1 (v/v) with double-strength dilution buffer. Immediately prior to CE, cheese extracts were centrifuged at 15 000 *g* in a micro-centrifuge for 10 min at 4°C to remove any particulate matter.

Sample electrophoresis was done at 25°C in 100 m*M* sodium phosphate (pH 7.2) containing 4 *M* urea and 20% ACN as the run buffer for 45 min at 12 kV after a 2-s pressure injection. DMF was used as an electroosmotic flow (EOF) marker and was introduced with a separate 1-s injection from a 0.01% solution in $ddH_2O$. At the end of each run, the capillary was washed at high pressure with a 2-min reverse $ddH_2O$ rinse, a 2-min forward rinse with 1 *M* NaOH, and a 2-min forward rinse with 100 m*M* sodium phosphate (pH 7.2), with the voltage ramped to 12 kV in 1 min and held for 3 min. This was followed by a 2-min high-pressure forward rinse with 100 m*M* sodium phosphate (pH 7.2). Data collection continued throughout the wash regimen to monitor the voltage and high-pressure rinses. Electrophoresis and high-pressure rinses were repeated until peaks were no longer detected.

### 2.5. MECC electrophoresis

Stock solutions were used to prepare a mixture of α-casein (50–100 μg/ml), β-casein (100 μg/ml), oligopeptide RGPFPI (25 μg/ml), and 100 μ*M* each of tyrosine, phenylalanine, tryptophan, arginine, indole, skatole, and ρ-cresol in 100 m*M* sodium borate (pH 8.5) containing 40 m*M* SDS. Levels of α-casein were varied to facilitate peak identification after electrophoresis at different conditions. Cheese extracts were filtered through 0.2-μm low-protein binding filters (Gelman) and diluted 5–15 fold in 100 m*M* sodium borate (pH 8.5) with 40 m*M* SDS, as required to bring sample peaks within range of the detector. Immediately prior to injection, cheese samples were centrifuged at 15 000 *g* for 10 min to remove any residual particulate matter.

Resolution of the ten compounds in the MECC standard mixture was optimized by electrophoresis in 100 m*M* sodium borate (pH 8.5) run buffers, which contained different levels of SDS and ACN. The buffers were prepared by mixing 400 m*M* sodium borate buffer stock (pH 8.5) with an appropriate volume of 200 m*M* SDS stock solution or HPLC-grade ACN. MECC was performed at 25°C for 25–30 min at 15 kV with a 1-s pressure injection into a 57 cm×75 μm I.D. untreated silica capillary. Methanol or ACN (10 μl/ml) was used to measure the retention time ($t_R$) of an unretained solute (EOF). A saturated Sudan III solution in ACN was added at 10 μl/ml to determine the $t_R$ of the SDS micelle [12]. After electrophoresis, the capillary was rinsed for 1 min at high pressure with 0.1 *M* NaOH followed by a 2-min high-pressure rinse with run buffer. A second 2-min high-pressure rinse with run buffer was performed prior to each new electrophoresis run.

### 2.6. Calculations

Capacity factors ($k'$) for standards separated by MECC were calculated by the formula $k' = t_r - t_0 / t_0[1 - (t_r/t_{mc})]$ [13], where $t_r$ is the elution time of a retained solute, $t_0$ is EOF, and $t_{mc}$ is the $t_R$ of the micelle. Precision for MECC in 100 m*M* borate (pH 8.5) with 40 m*M* SDS was determined by calculating R.S.D. for the retention times of three compounds in

the standard mix using retention times obtained the same day and on different days [14].

## 3. Results and discussion

The objective of this research was to develop a CE method to follow a relatively broad range of biochemical changes during cheese ripening. Chen and Zang [15] describe a phosphate–urea FSCE method which separates β-casein, α-lactalbumin, and β-lactoglobulin in milk, but does not resolve α-casein and β-lactoglobulin-B. Addition of 20% ACN to the run buffer facilitated separation of α-casein, β-casein, α-lactalbumin, β-lactoglobulin-A and β-lactoglobulin-B (Fig. 1a). Experiments to explore the utility of this method for the analysis of Cheddar cheese extracts suggested that FSCE might be used to define changes in caseins and large peptides in cheese (Fig. 1b,c). Unfortunately, this method did not detect lower-molecular-mass compounds that accumulate in ripening cheese (Fig. 1d).

Capillary performance was also a problem with the FSCE method. After 20–50 runs, peak resolution degraded and was accompanied by a gradual rise in current before leveling off. Capillary degradation can be postponed by the extensive wash procedure described in Section 2. Although FSCE with phosphate–urea–ACN run buffer might be useful for the analysis of large proteins and peptides, potential limitations in repeatability and ability to detect small molecules in cheese extracts limited its utility for cheese maturation studies.

We initiated experiments to develop an MECC method that could separate a heterogeneous mixture of α- and β-casein, oligopeptide RGPFPI, the free amino acids tyrosine, phenylalanine, tryptophan, and arginine, and the small aromatic molecules indole, skatole, and *p*-cresol. Indole, skatole, and *p*-cresol are enzymic degradation products of tryptophan and

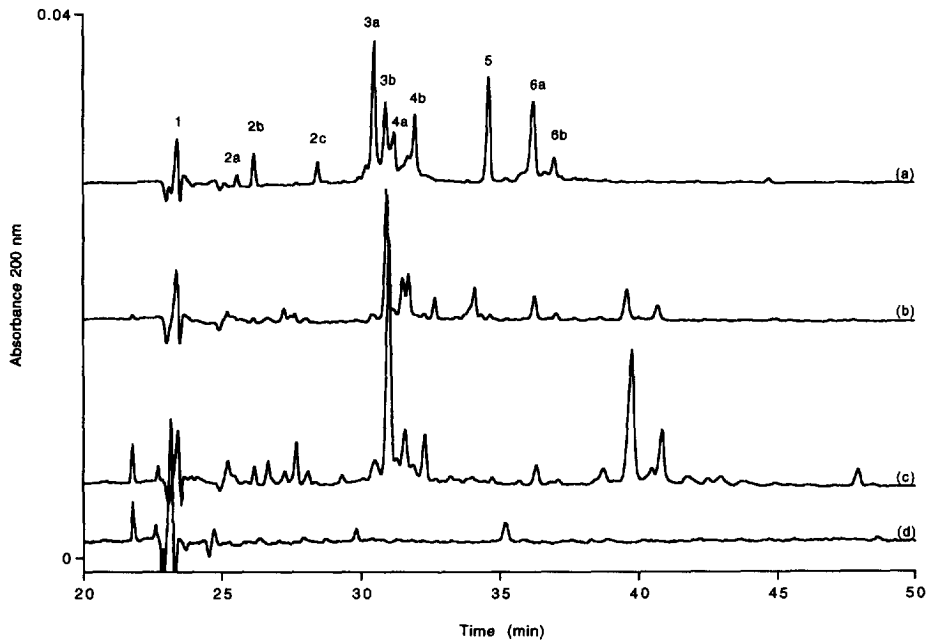


Fig. 1. Electropherograms obtained by free solution capillary electrophoresis with 100 m*M* sodium phosphate (pH 7.2), 4 *M* urea, 20% ACN run buffer. Peaks in the standard mixture (electropherogram a) are: 1=EOF marker; 2a,b,c=κ-casein; 3a,b=β-casein; 4a=β-lactoglobulin-B; 4b=β-lactoglobulin-A; 5=α-lactalbumin; 6a,b=α-casein; electropherogram (b) phosphate-soluble whole Cheddar cheese fraction collected on the day cheese was manufactured; electropherogram (c) phosphate-soluble whole Cheddar cheese fraction collected after three months of ripening at 7–10°C; electropherogram (d) $M_r$ 30 000 filtrate of a phosphate-soluble Cheddar fraction collected after three months of ripening at 7–10°C. Injection times were 2 s for all electropherograms.

tyrosine that impart unclean flavors to Cheddar cheese [16]. MECC in 100 m*M* sodium borate containing 20-, 40-, or 100 m*M* SDS resolved all ten compounds in the standard mixture (Fig. 2). MECC using borate–SDS with size-fractionated Cheddar cheese extracts showed, unlike FSCE with phosphate–urea–ACN run buffer, numerous compounds in $M_r$ 30 000 cheese filtrates (Fig. 3). This system also provided information on high-molecular-mass compounds found in whole-cheese extracts (Fig. 4).

Because $k'$ measures solute partitioning between the micelle and the mobile phase, this property was used to identify the SDS concentration which best separated molecules in the standard mix. All of the standard compounds exhibited a linear response as SDS concentration in the buffer increased (Fig. 5). As predicted by the different slopes of $k'$, the elution order of skatole and $\beta$-casein was reversed in buffer that contained 60 m*M* SDS.

The high $k'$ values for the oligopeptide indicated this molecule had the strongest interaction with the micelle. Extrapolation of $k'$ plots for small molecules in the mix showed that these lines all converged near zero, suggesting that they have a similar partitioning mechanism with SDS. Not surprisingly, $k'$ values for $\alpha$- and $\beta$-casein did not converge on zero. Their partitioning mechanism(s) was expected to differ from that of the small molecules because they are larger and could not freely enter the SDS micelle. Data from $k'$ plots and electropherograms indicated that MECC with 100 m*M* sodium borate containing 40 m*M* SDS provided a good compromise between compound resolution and run speed (Figs. 2b, 3b, and 5). As a result, this buffer system was selected for further study.

In an effort to further refine separation of compounds in the standard mix, MECC was done with 100 m*M* sodium borate buffer containing 40 m*M* SDS and 5%, 10%, or 15% ACN. Unfortunately, separation was not improved and the micelle marker failed to elute within 40 min at concentrations above 5% ACN. In addition, electropherograms of Cheddar cheese extracts showed that compounds which eluted near the micelle marker in buffer without ACN were

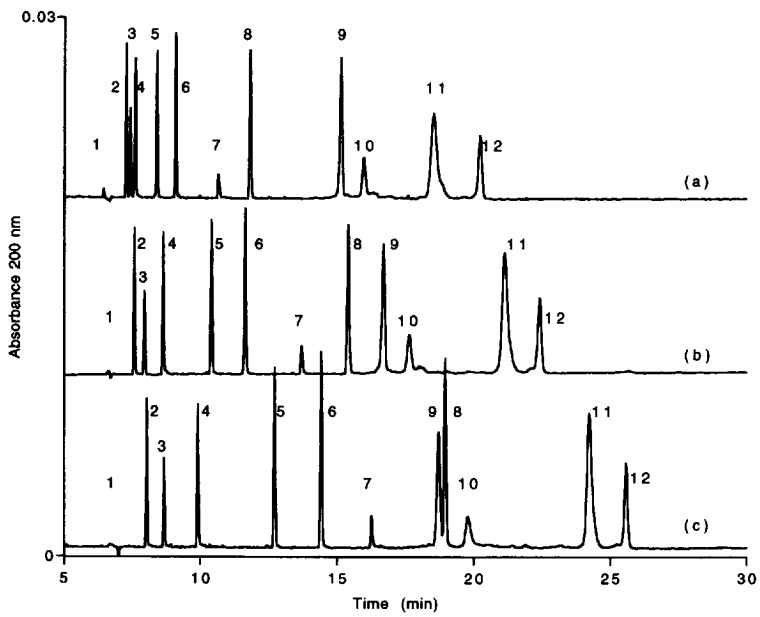


Fig. 2. Micellar electrokinetic capillary chromatography of a mixed standard in 100 m*M* sodium borate buffer (pH 8.5) that contained: (a) 20 m*M* SDS; (b) 40 m*M* SDS; and (c) 60 m*M* SDS. Peaks: 1=EOF marker, 2=tyrosine, 3=phenylalanine, 4=tryptophan, 5=*p*-cresol, 6=indole, 7=arginine, 8=skatole, 9=$\beta$-casein, 10=$\alpha$-casein, 11=oligopeptide RGPFPI, and 12=Sudan III micelle marker. The sensitivity was found to be <50 fmole for the compounds in the standard mixture.

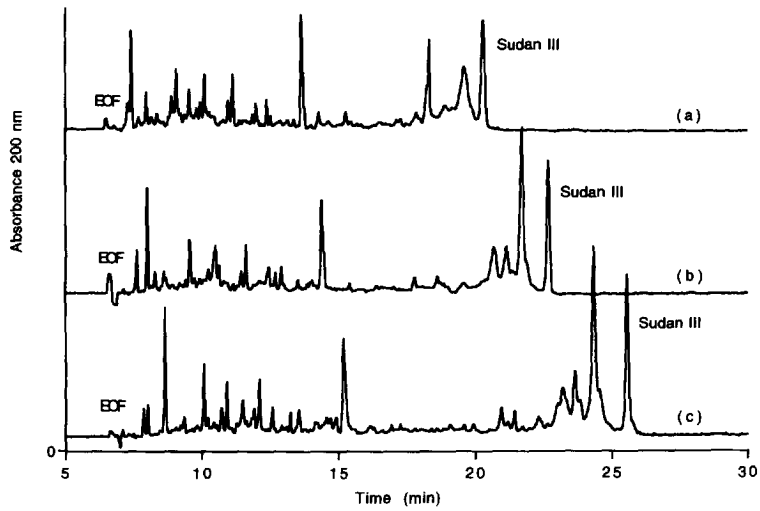


Fig. 3. Micellar electrokinetic capillary chromatography of $M_r$ 30 000 filtrate of the phosphate-soluble Cheddar cheese fraction collected after six months of ripening at 7–10°C. Electrophoresis was performed with 100 m*M* sodium borate buffer (pH 8.5) containing: (a) 20 m*M* SDS; (b) 40 m*M* SDS; and (c) 60 m*M* SDS.

poorly resolved in 5% ACN and did not elute at 10 or 15% ACN (data not shown). Our inability to detect the micelle marker or compounds normally associated with SDS when run buffers contained 10 or 15% ACN was consistent with the hypothesis that ACN may disrupt micelle formation [13].

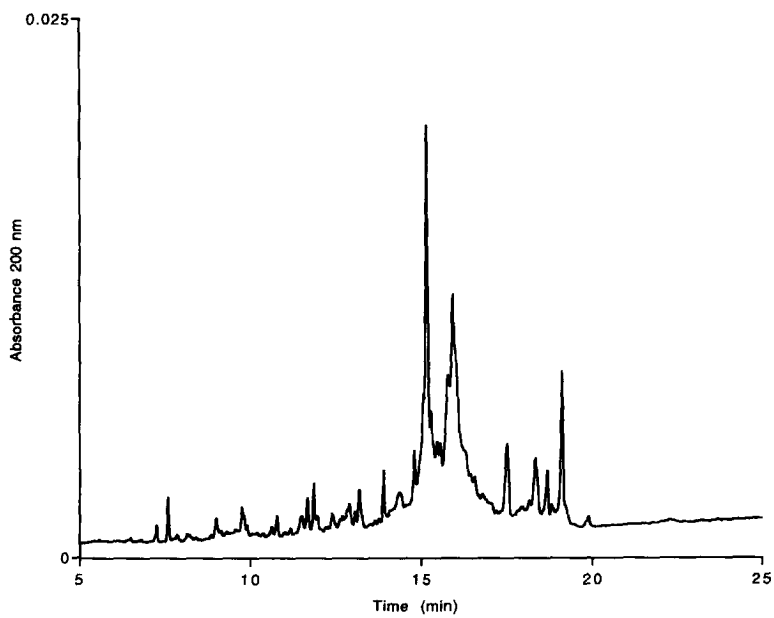


Fig. 4. Micellar electrokinetic capillary chromatography of the phosphate-soluble, unfractionated Cheddar cheese extract collected after six months of ripening at 7–10°C. Electrophoresis was performed with 100 m*M* sodium borate buffer (pH 8.5) that contained 40 m*M* SDS.

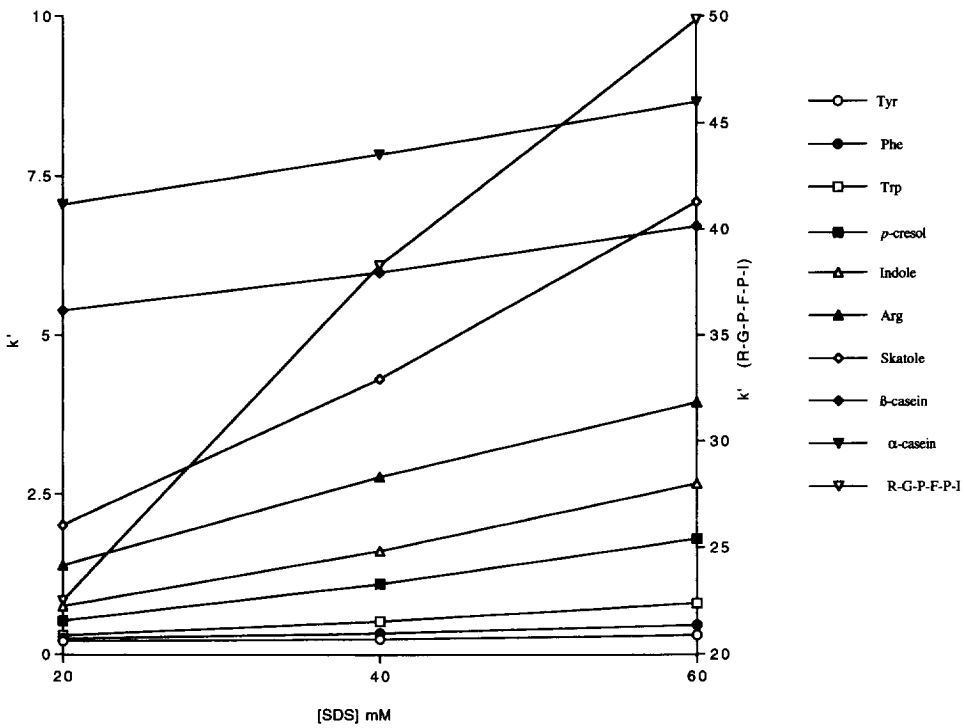


Fig. 5. Capacity factors ($k'$) for the components of standard mix separated with 100 m$M$ sodium borate buffer (pH 8.5) that contained 20, 40, or 60 m$M$ SDS. The axis on the right shows $k'$ values for the oligopeptide RGPFPI, while $k'$ values for all other compounds are represented by the left axis.

We determined the precision of the MECC method by calculating R.S.D. values for phenylanaline, indole, and the oligopeptide RGPFPI from $t_R$ obtained on the same day and over different days. R.S.D. values were generally low for data obtained within the same day and over a one-month period, indicating that the method had acceptable repeatability (Table 1). As expected, the day-to-day variation was greater than within-day variation. Analysis of within-day variation showed that repeated sampling from one set of run vials led to a progressive increase in retention times that increased variability. This effect was linked to the increased volume in the outlet vial, due to EOF which produced hydrostatic pressure and slowed the electrophoretic flow. Adjustment of the fluid level in the outlet vial returned $t_R$ to their initial values, but this step was not deemed practical for an automated operation. A more acceptable alternative was to limit the number of runs per vial pair (Table 1), an approach that we adopted in subsequent experiments.

Repeatability and peak resolution were also increased by using 1-s injections and by diluting samples in 100 m$M$ sodium borate (pH 8.5) without SDS. The resulting peaks were narrower and elution times were not noticeably affected. Sample stacking due to enhanced electrophoretic mobility in the lower-conductivity environment did not appear to influence molecular partitioning with the SDS micelle [17].

Time-course experiments to investigate the usefulness of MECC for the analysis of Cheddar cheese maturation detected rather substantive changes among $M_r$ 30 000 filtrates collected over a three-month ripening period (Fig. 6). Our laboratory has now performed several hundred runs using a single capillary without any discernible effect on cheese extract and standard mixture separation profiles.

Table 1
Precision analysis (R.S.D.) of MECC in 100 m*M* borate (pH 8.5) plus 40 m*M* SDS for separation of phenylalanine, indole, and oligopeptide RGPFPI

| Time period | Compound (R.S.D.) | | |
|---|---|---|---|
| | Phenylanaline | Indole | RGPFPI |
| Within-day (same vial pair): | | | |
| runs 1–9 | 0.284 | 0.435 | 0.950 |
| runs 1–3 | 0.074 | 0.051 | 0.273 |
| runs 4–6 | 0.074 | 0.203 | 0.354 |
| runs 7–9 | 0.073 | 0.101 | 0.350 |
| Within-day (different vial pairs) ($n=3$)[a] | 0.146 | 0.218 | 0.295 |
| Within month ($n=9$)[b] | 0.556 | 0.951 | 1.540 |

[a] Obtained from the second run of the day on nine consecutive days over one month.
[b] Data calculated from the second run for each pair.

## 4. Conclusions

For decades, efforts to accelerate or improve flavor development in Cheddar cheese have focused on the biochemical events during cheese maturation. The ability to characterize cheese maturation chemistry continues to present a significant analytical challenge. It is difficult to analyze the diverse compounds formed during this process, and the ability to simultaneously detect many different types of compounds (especially those present at very low levels) would be of considerable benefit. Capillary electrophoresis is an analytical tool that appears to have potential for robust analytical techniques to

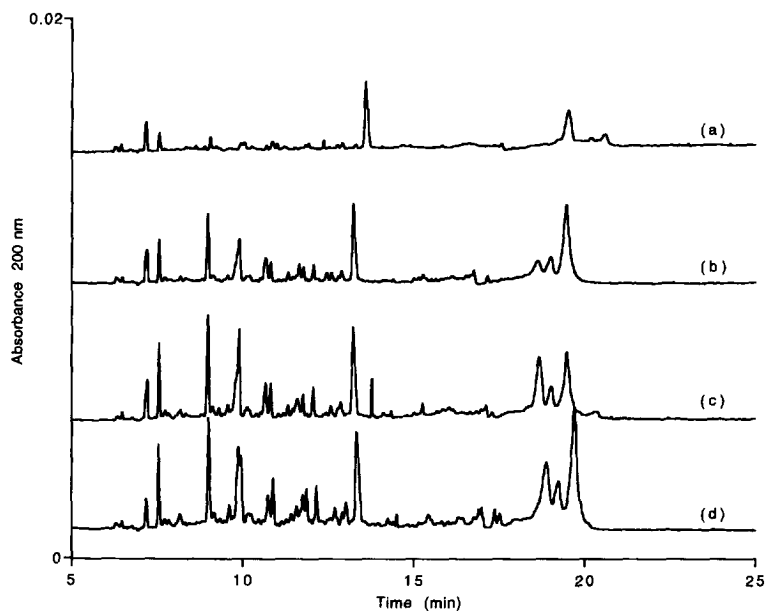


Fig. 6. Micellar electrokinetic capillary chromatography of $M_r$ 30 000 filtrates of phosphate-soluble Cheddar cheese fractions collected after: (a) the day of cheese manufacture; (b) one month of ripening; (c) two months of ripening; and (d) three months of ripening at 7–10°C. Electrophoresis was performed with 100 m*M* sodium borate buffer (pH 8.5) that contained 40 m*M* SDS.

simultaneously detect many of the heterogeneous compounds found in ripening cheese. This study investigated FSCE and MECC to simultaneously resolve caseins, peptides, and various small molecules found in Cheddar cheese.

FSCE with phosphate–urea–ACN run buffer separated caseins and large peptides, but problems with capillary performance and ability to detect small molecules in cheese extracts limit its utility in studies of cheese maturation. MECC with borate–SDS run buffer simultaneously and rapidly separated $\alpha$- and $\beta$-caseins, an oligopeptide, four free amino acids, and three small aromatic molecules in a standard solution. The ability to detect small molecules as well as large proteins and peptides may allow investigators to monitor the degradation (disappearance) of large proteins and the appearance of smaller compounds in a single analytical run. MECC electropherograms from $M_r$ 30 000 filtrates and whole extracts were far more complex than those obtained from similar cheese fractions with FSCE (Figs. 3, 4, and 6 versus 1b,c). MECC elution patterns are complex, and it may be difficult to identify peaks. Several peaks in the cheese extracts co-eluted with compounds in the standard mixture (data not shown), and work is now in progress to identify these and other peaks in cheese extracts. The MECC method has good repeatability (Table 1) and ongoing work shows that the method is very robust with cheese extracts. Because of its speed, sensitivity, and flexibility with respect to different compounds, the MECC method described in this report may be very effective in characterizing the biochemical changes associated with cheese maturation.

## Acknowledgments

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