ELSEVIER

Journal of Chromatography A, 964 (2002) 243–253

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Use of capillary electrophoresis and laser-induced fluorescence for attomole detection of amino acids

M. Ummadi[1], B.C. Weimer*

Western Dairy Center, Center for Microbe Detection and Physiology, Department of Nutrition and Food Sciences, Utah State University, Logan, UT 84322-8700, USA

Received 1 June 2001; received in revised form 16 May 2002; accepted 17 May 2002

Abstract

A capillary electrophoresis and laser-induced fluorescence (CE–LIF) method was developed to identify and quantitate at amol (10^{-18}) concentration. Amino acids were derivatized with 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde prior to CE–LIF analysis. The assay was developed by varying the sodium borate concentration, buffer pH, operating voltage, and operating temperature. A run buffer system containing 6.25 m*M* borate, 150 m*M* sodium dodecyl sulfate, and 10 m*M* tetrahydrofuran (pH 9.66) at 25 °C, and 24 kV provided analysis conditions for a high-resolution, sensitive, and repeatable assay of amino acids. The rate of derivatization, stability of the labeled amino acids, and amino acid quantitation varied for each amino acid. Amino acids were detected with greater efficiency by this method than automated HPLC amino acid analysis. The repeatability of the assay ranged from 0.3 to 0.9% within a day and 0.7 to 1.5% between analysis days. Bacterial amino acid utilization in a chemically defined medium was successfully monitored using this method. This work defines a sensitive and repeatable method for the detection of amino acids during bacterial metabolism.

Keywords: Derivatization, electrophoresis; Bacteria; Amino acids; Carboxybenzoylquinolinecarboxaldehyde

1. Introduction

Amino acid analysis is an area of interest and challenge for biological samples. There is a long-standing need for a high-resolution separation technique for amino acids (AA) combined with ultra-sensitive detection [1]. Methods to identify amino acids, proteins, and peptides help clarify the nature of proteins. Another need for this analysis is in growth media to study metabolic pathways [2].

Amino acids comprise a mixture of acidic, basic, and neutrally charged molecules, thereby posing a major challenge for optimal separation of each type. For example, changing the pH of the run buffer can improve the resolution of one group of amino acids, but it produces another overlap among other amino acids [3]. High-performance liquid chromatography (HPLC) procedures are utilized extensively in the separation of amino acids with adequate results to resolve the amino acids, but lack the sensitivity needed for some applications, bacterial metabolism specifically.

*Corresponding author. Tel.: +1-435-797-2126; fax: +1-435-797-2379.

E-mail address: milkbugs@cc.usu.edu (B.C. Weimer).

[1]Present address: Chr. Hansen, Inc., 9015 W. Maple Street, Milwaukee, WI 53214, USA.

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00692-1

Capillary electrophoresis (CE) has gained popularity as an analytical tool because of short analysis times, analysis of small molecules, and additional resolution capability as compared to other electrophoresis methods. The main advantage of using CE over HPLC is its speed of analysis and elimination of the need for gradient elution [2]. Other advantages include robustness of CE methods since they rely on mobility differences between solutes or the analyte interaction with additives, and the ability to analyze complex samples such as serum plasma and glycoproteins [3].

Micellar electrokinetic capillary chromatography (MEKC) is a technique that involves the use of charged micelles to separate uncharged and charged molecules by means of a pseudo-micellar phase that is created through hydrophobic interactions between solute molecules and detergent [3]. This approach offers advantages for resolution of compounds that are similar in structure over other analytical methods.

Because many amino acids are not readily detectable in their native state by analytical techniques such as HPLC or CE (with the exception of aromatic amino acids), a pre- or post-column derivatization with a chromogenic or fluorescent compound is needed. A variety of derivatizing agents (chromophores or fluorophores) such as dansyl chloride (DNS) [4,5], *o*-phthalaldehyde (OPA) [6,7] naphthalene-2,3-dicarboxaldehyde (NDA) [4], fluorescein isothiocyanate (FITC) [8,9], fluorescamine [10], and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [11] are used to label the amino acids [1]. Although use of chromogenic labels is common, the additional sensitivity of fluorophores makes them particularly useful in CE applications [2].

Increasing knowledge of CE and laser-induced fluorescence (LIF) has greatly improved the ability to efficiently separate and quantitatively detect even low concentrations of amino acids, peptides, and proteins [11,12]. Nickerson and Jorgenson [13] compared the relative sensitivities of OPA, FITC, and NDA derivatization procedures on amino acids using CE–LIF detection. An extremely rapid and efficient separation (75 s) of eight NDA-labeled amino acids ($<$0.5 s peak width) was reported using a high field (30 kV) in short capillaries (35 cm$\times$10 μm I.D.) with a detection limit of 0.4 a*M*. Nishi et al. [14] separated 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)-derivatized DL amino acids using MEKC with different levels of sodium dodecyl sulfate (SDS). At 0.2 *M* SDS, 10 amino acids were resolved in neutral and alkaline conditions within 40 min. Mank and Yeung [15] developed a sensitive method using diode-LIF and synthesized diacarbocyanine fluorophore to separate 18 amino acids and tyramine. Using a neutral (pH 7.0) run buffer with CBQCA-derivatized amino acids qualitatively separated 17 amino acids within 30 min [10,12]. These studies suggest a large number of derivatization agents and run conditions can be coupled in different combinations to separate a mixture of amino acids. However, the selection of a suitable combination largely depends upon the specific requirements of the sample. Such an approach must also consider factors such as cost, time, simplicity of method, and overall feasibility and utility.

To further optimize the resolution of the amino acids, the run buffer concentration or use of buffer additives is common. An example of this concept is the alteration of the borate concentration and buffer pH. Modification of operating temperature and voltage are also used to optimize separation, but these can bring about only subtle changes that complement the optimization achieved with a suitable buffer condition [1]. The concentration of borate in the buffer greatly influences the separation of mixtures of compounds by its ability to alter the electroosmotic flow (EOF) [1,16]. For these reasons, the work described in this paper optimized the run buffer concentration first, followed by the buffer pH, operating temperature, and CE voltage.

The application of current techniques to detect, separate, and quantitate amino acids for this study proved inadequate in preliminary studies. Therefore, the focus of this work was to develop a quantitative and sensitive method to monitor the metabolism of amino acids during bacterial growth in a chemically defined medium (CDM). In addition to run condition optimization, the rate of derivatization, stability of the labeled amino acids, and amino acid quantitation were also investigated. These investigations defined the CE–LIF conditions needed to achieve an optimal, sensitive, and repeatable separation using precolumn amine derivatization with CBQCA followed by MEKC using a borate run buffer with SDS and

tetrahydrofuran (THF) additives. Amino acids in CDM, a bacterial growth medium were compared by this method with a conventional automated amino acid analysis to demonstrate the utility of this method.

2. Experimental

2.1. Chemicals

LIF performance test mixture (fluorescein), capillary performance run buffer A, and capillary regeneration solution A were purchased from Beckman Instruments (Fullerton, CA, USA). All amino acids, standard solution of amino acids, and nor-leucine were obtained from Sigma (St. Louis, MO, USA). Analytical-grade SDS, THF, 2-[*n*-tris(hydroxymethyl)methyl}amino]ethanesulfonic acid (TES), β-cyclodextrin, 1,5-diaminopentane (DAP), sodium hydroxide, methanol (HPLC grade), and potassium cyanide were also purchased from Sigma. The ATTO-TAG CBQCA derivatization kit was obtained from Molecular Probes (Eugene, OR, USA).

2.2. CE separation and LIF detection

Run buffers of different pH and sodium borate concentrations were used. All buffers were freshly prepared by dissolving appropriate amounts of sodium borate (6.25, 12.5, 25, and 50 m*M*), and 150 m*M* SDS in distilled deionized water, and THF was added to a final concentration of 10 m*M*. The pH was adjusted using 1 *M* NaOH (pH 9.05, 9.3, 9.6, and 9.66). Operating voltage (22.5 and 24 kV) and temperature (22, 25, 27, and 30 °C) of electrophoresis were also varied.

The CE/MEKC method was used as described by Strickland et al. [17] was used as an initial run condition. A P/ACE 2100 automated CE system fitted with the LIF detector, the laser module 488, and Beckman System Gold chromatography software (Beckman Instruments) were used. Optical filters of 488 and 560 nm were used for excitation and emission, respectively. Sample electrophoresis was performed using the appropriate run buffer, operating voltage and temperature with a 1 s pressure injection (~6 nl) into a 107 cm×75 μm I.D. untreated silica capillary. 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 [17].

Individual amino acids and the internal standard nor-leucine (Nle) were derivatized with CBQCA as described in Section 2.3 before determining the detection limit and dynamic range in a standard curve format. The limit of detection for each amino acid was evaluated using an amino acid mixture with known concentrations that were serially diluted for a signal-to-noise ratio (S/N) less than 5. To determine the within-run variability, five replicates of each amino acid in mixture were done in five consecutive runs on the same day. To determine the between-run reproducibility, five replicate samples were tested on 5 different days during a 6-month period. During CE analysis, care was taken to keep the capillary well rinsed (as described above) and regenerated to minimize the variability.

2.3. Derivatization

A 10 m*M* solution of ATTO-TAG CBQCA in methanol was prepared in a sterile 15-ml centrifuge tube by sonication until a clear solution was obtained (~1–5 min). A working solution of 420 μ*M* of CBQCA was prepared by dilution in methanol. All CBQCA stocks were stored at −20 °C in a capped vial protected from light. A 420 μ*M* working solution of potassium cyanide (KCN), and 1 m*M* Nle (pH of both solutions adjusted to 11.0 with 1 *M* NaOH) were prepared with fresh distilled deionized water and stored at 4 °C. At least a sixfold molar excess of CBQCA and a fivefold excess of KCN were used for derivatization of amino acids. Amino acid solutions and the internal standard were adjusted to pH 9.0 using 1 *M* NaOH. A typical reaction mixture contained 2 μl (10 μ*M*) of amino acid solution, 10 μl (120 μ*M*) of KCN, 6 μl (30%) of methanol, 11 μl (70%) of water, and 5 μl of CBQCA (60 μ*M*). Prior to addition of the derivatization agent, 0.5 μl (8.163 μ*M*/sample) of internal standard was added to all the reaction mixtures. After gentle mixing, the reaction mixture was incubated in the dark at 25 °C for 24 h. Before analysis the samples were placed in the temperature-controlled vial housings of the CE instrument (10–

15 °C) to reduce solvent evaporation during analysis. Fresh and spent CDM samples (1, 3, 11, 14, 18 days) were also derivatized as above.

To measure the rate of derivatization, the amino acids in a CDM [18] were labeled with CBQCA as above and samples were analyzed at hourly intervals for 64 h. The time of maximal labeling and stability of the labeled amino acid was determined.

To compare the detection efficiency of the CBQCA-derivatized CDM with a conventional amino acid analyzer, an aliquot of freshly prepared sterile CDM sample was analyzed by the CE–LIF system and by SynPep (Dublin, CA, USA) using an a 6300 amino acid analyzer (Beckman Instruments).

2.4. Bacterial growth medium

An amino acid growth medium, CDM lacking carbohydrate (pH 6.5), was used to grow the bacteria [18]. The bacteria were grown in three different media: CDM, CDM without aromatic amino acids and in CDM containing all aromatic amino acids. Individual amino acids concentrations in the spent medium were calculated by comparing the specific amino acid peak area to a standard curve for that amino acid after the peak location was compared to the Nle internal standard to verify the consistency of the location and time.

The standard curves were done using a five-point concentration series. The concentration ranged from 0.1 to ~3000 μ*M*, the exact upper limit depended on the concentration in the sterile medium and the concentration series included the upper limit in the medium. Each curve was linear and the correlation coefficient (R^2) for the regression equations ranged from 0.997 to 0.999.

2.5. Bacterial strain

Brevibacterium linens strain BL2 (Utah State University culture collection) was used in this study. Stock cultures were frozen in tryptic soy broth (TSB) containing 30% glycerol and kept at −70 °C. Before each use, stocks were incubated in TSB at 25 °C with shaking at 220 rpm, and subcultured twice prior to inoculation into 100 ml of CDM.

3. Results and discussion

3.1. Preliminary investigation of electrophoresis run buffers

Use of previously reported amino acid analysis methods with CBQCA derivatization [11] and CE–LIF was unsuccessful in resolving mixtures of amino acids in CDM (data not shown). Consequently, MEKC was used to examine various buffers and run conditions to define an acceptable assay. Initial experiments using buffer pH, β-cyclodextrin, DAP, and SDS as additives were unsuccessful (data not shown) in separating individual amino acids adequately. Consequently, the need arose for an alternative buffer system for adequate separation and identification of an amino acid mixture. Therefore, a combination of sodium borate, an anionic surfactant (SDS), and an organic solvent (THF) were investigated to decrease the EOF, which resulted in an improved separation (data not shown for all combinations).

3.2. Optimization of run condition and run buffer composition

3.2.1. Borate concentration

Increasing the ionic strength of the run buffer causes longer migration times and increases zone focusing [1], so we chose to optimize the buffer concentration, thereby balancing the increasing run time with an improved separation, before evaluating the buffer pH and additives to further optimize the buffer mixture. The effect of four borate buffer concentrations (6.25, 12.5, 25, 50 m*M*) were examined at an operating voltage of 22.5 kV, operating temperature of 25 °C, and run buffer, pH 9.66. Each condition allowed identification of various groups and individual amino acids; however, use of 6.25 m*M* borate yielded the best separation for each amino acid (Fig. 1, line D).

As the concentration of borate in the run buffer increased, amino acids changed their migration order and co-migrated. For example, at 12.5 m*M* borate concentration, amino acids proline (10) and cysteine (11), phenylalanine (13) and leucine (14), and arginine (17) and aspartic acid (18) co-migrated (Fig. 1, line C). At 25 m*M* borate levels histidine (9)

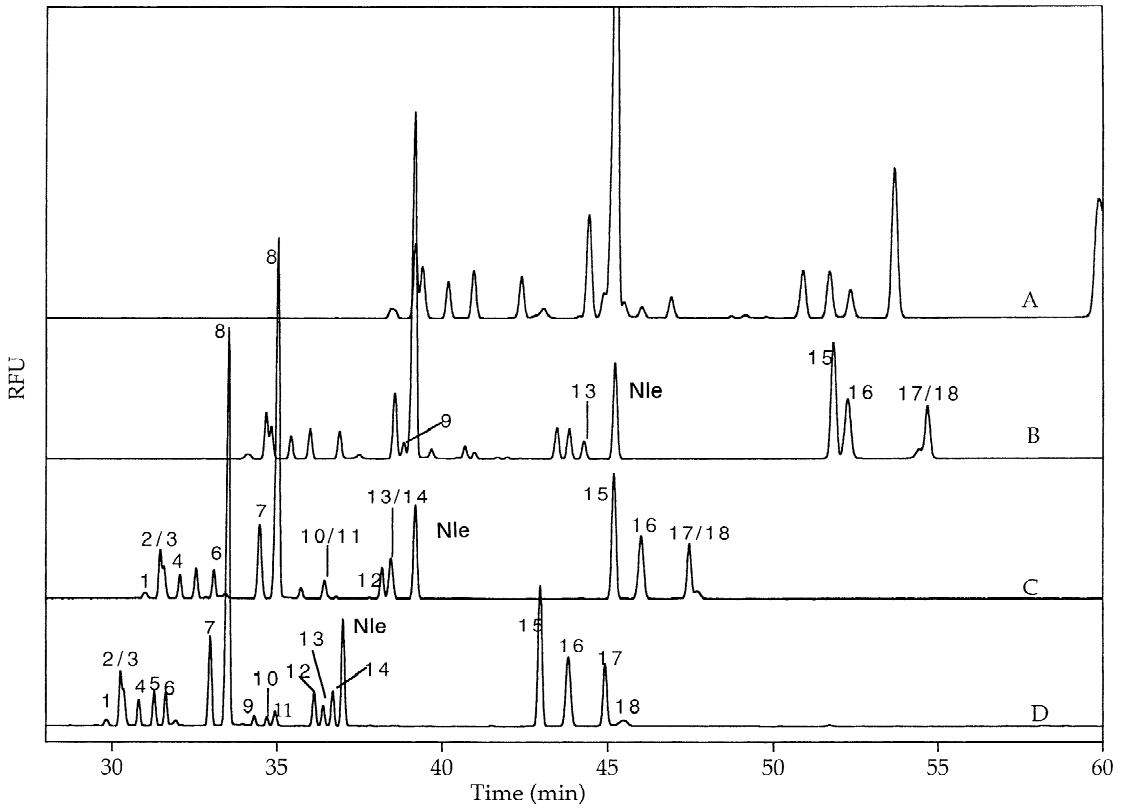

Fig. 1. An example of the influence of sodium borate concentration on the separation of amino acids in a chemically defined bacterial (CDM) growth medium [18] after the buffer mixture was optimized for the pH and additive concentrations. MEKC was done using 150 m*M* SDS, 10 m*M* THF, 22.5 kV, pH 9.66, and 25 °C with varying borate concentrations: (A) 50 m*M*; (B) 25 m*M*; (C) 12.5 m*M*; (D) 6.25 m*M* sodium borate. Sterile CDM was used as a standard amino acid mix as defined in Table 2. The amino acids are labeled as 1=Ser, 2=Gln, 3=Met, 4=Asn, 5=Thr, 6=Tyr, 7=Ala, 8=Gly/Val, 9=His, 10=Pro, 11=Cys, 12=Ile, 13=Phe, 14=Leu, norleucine (Nle, internal standard), 15=Glu, 16=unknown, 17=Arg, 18=Asp.

migrated between alanine (7) and glycine (8), and phenylalanine (13) with leucine (14) (Fig. 1, line B). The change in the order of migration with changing concentration of borate can be explained by the similarity of the nature of side chains among amino acids in each of the respective groups. Additionally, the run time increased with increasing borate concentrations (65 min at 50 m*M*) (Fig. 1, line A). The separation was optimized for individual amino acid resolution, but balanced for run time with a run buffer containing 6.25 m*M* borate, 150 m*M* SDS, 10 m*M* THF with a pH of 9.66 at 24 kV and a temperature of 25 °C (Fig. 2).

Three migration groups were observed at 6.25 m*M* borate concentration. All amino acids with uncharged polar side chains (threonine, tyrosine, asparagine, glutamine, and serine), except cysteine, were included in the first group of amino acids that migrated between 25 and 27 min. The second group migrated between 27 and 31 min and included all amino acids with non-polar side chains (alanine, glycine, valine, isolucine, phenylalanine, leucine, and proline). The last group, consisted of all the amino acids with charged side chains (aspartate, arginine, and glutamate) except histidine, which migrated between 35 and 38 min. This method did not identify tryptophan and lysine. The lack of tryptophan detection is in contrast with the work of Liu et al. [11],

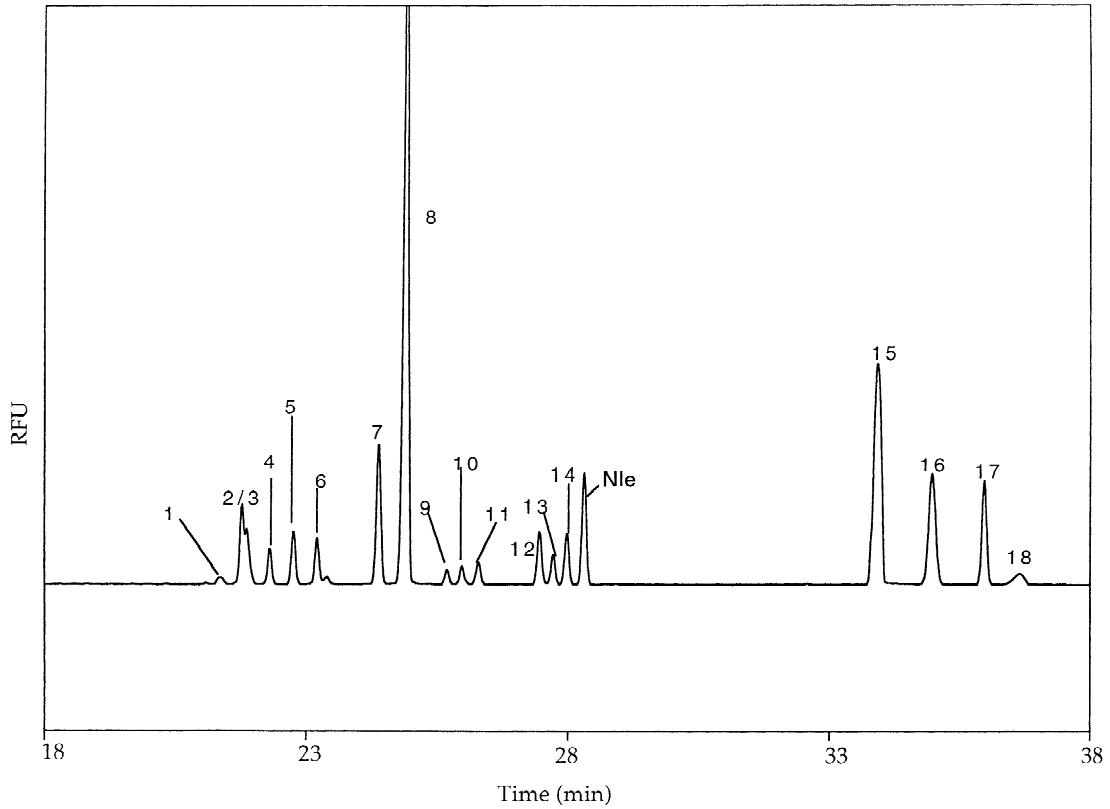

Fig. 2. Optimum conditions for separation of an amino acid standard solution using 6.25 m*M* sodium borate, 150 m*M* SDS, 10 m*M* THF (pH 9.66) buffer and run conditions of 24 kV, 25 °C. 1=Ser, 2=Gln, 3=Met, 4=Asn, 5=Thr, 6=Tyr, 7=Ala, 8=Gly/Val, 9=His, 10=Pro, 11=Cys, 12=Ile, 13=Phe, 14=Leu, Nle, 15=Glu, 16=unknown, 17=Arg, 18=Asp. Amino acid concentrations are indicated in Table 2.

but the inability to detect lysine is consistent with their method and occurs due to fluorescent quenching [11].

The basis for the amino acid migration order may be explained by the mechanism of MEKC separation, which relies on partitioning of the solute molecules between the micelles and the solvent phase [19]. The ionic micelle generally migrates slower than the bulk solution in the capillary. Consequently, the amino acid that is associated with the micelle migrates slower than the amino acid in the solvent phase or bulk solution [20–22]. Neutral molecules separate because of differential partitioning into micelles. Highly polar, uncharged groups do not interact with the micelles, so they migrate at the velocity of the EOF. Accordingly, hydrophilic amino acids migrated faster than hydrophobic amino acids.

3.2.2. Operating voltage

Increase in operating voltage increases EOF and decreases the time of migration, but may cause excessive Joule heating that ruins the resolution and repeatability. Joule heating is an important negative effect of electrophoretic separation of molecules that caused peak broadening. To select a suitable voltage that effectively reduced retention time and minimized Joule heating, an Ohm's plot [16] for dissipation of heat was used (data not shown). Operating voltages of 22.5 and 24 kV were used in a run buffer (pH 9.66, 6.25 m*M* borate), at an operating tempera-

ture of 25 °C. In this run buffer, the operating voltages resulted in acceptable Joule heating that did not influence the peak shape. However, the higher run voltage (24 kV) reduced the run time of migration while maintaining resolution of each amino acid (Fig. 2).

3.2.3. Buffer pH

Changing the buffer pH alters the mobility of analytes by changing the charge of analyte molecules and the capillary wall [1]. High buffer pH is correlated to an increase in EOF and longer migration of amino acids [1]. To identify the optimal pH for separation of a mixture of CBQCA-labeled amino acids, buffer pH was examined in a run buffer containing 6.25 m*M* borate at an operating voltage of 24 kV and an operating temperature of 25 °C (Fig. 2). Migration times of different amino acids and the pattern of their migration varied with pH. Run buffer pH was an important factor in obtaining optimal separation of amino acids. At pH 9.66, 17 amino acids were optimally separated (Fig. 2).

3.2.4. Operating temperature

The buffer temperature during operation is an important parameter that influences the migration of amino acids with MEKC. Operating temperature was investigated with the aim of reducing run time and optimizing the separation. MEKC was done at 22, 25, 26, and 27 °C with the same buffer (6.25 m*M* borate, pH 9.66) and voltage conditions (24 kV). The amino acid migration time decreased with increasing operating temperatures (data not shown) and was shortest at 27 °C, as expected. However, the optimal resolution of 17 amino acids was achieved at 25 °C (Fig. 2), not at the highest temperature.

Based on the influence of the sodium borate concentration, run buffer pH, operating temperature, and operating voltage the baseline separation of 17 CBQCA-labeled amino acids was attained within 38 min in a run buffer containing 6.25 m*M* sodium borate, 150 m*M* SDS, and 10 m*M* THF (pH 9.66) at 24 kV and 25 °C (Fig. 2). The separation defined in Fig. 2 was reproducible (see Section 3.5 for assessment of this parameter) during the development experiments and was used as a basis for further assay development for quantitation and investigation of amino acid metabolism by *B. linens*.

3.3. Amino acid quantitation

3.3.1. Derivatization rate, stability and sensitivity

Initial use of Molecular Probes methods for ATTO-TAG CBQCA amine derivatization did not label amino acids maximally with 1 h of incubation as noted in the instructions. The need for a longer period of incubation to achieve maximal derivatization was reported earlier [12]. Thus, the time for complete CBQCA derivatization and the stability of each labeled amino acid was examined in this work. Maximal derivatization time for the amino acids in CDM varied from 6 to 20 h, while 14 of the 18 amino acids required ~12 h (Table 1). This resulted in a calculated derivatization rate that ranged from 8.2 to 226.8 μ*M*/h (Table 1). Once labeled, the amino acids remained stable for an average of 34 h (Table 1). These data indicate that at least 6 h is needed to derivatize the amino acids in CDM and that the resulting labeled amino acids were stable and acceptable for analysis up to 24 h–34 h.

3.4. Commercial analysis vs. CE–LIF CBQCA detection efficiency

The ability to quantitate amino acids in sterile CDM with this method was compared to conventional automated HPLC amino acid analysis. The CE method for amino acid quantitation was linear (R^2 = 0.992 to 0.997) for each amino acid over the concentration ranges used in the bacterial growth medium (data not shown). In CDM the lowest detectable concentration for CBQCA-labeled amino acids was 0.1–2.4 amol for a 1 s injection (~6 nl injection volume), which is consistent with the range of detection limits reported by Liu et al. [11] and at least three orders of magnitude better than Bergquist et al. [12] (Table 1). Surprisingly, proline was detected with this method—presumably as a hydroxyproline derivative [23]. HPLC analysis efficiency of detection for individual amino acids ranged from 0 to 127%, while CE–LIF analysis ranged from 0 to 182% (Table 2). Automated HPLC analysis did not detect 6/19 amino acids, while

Table 1
CBQCA derivatization of amino acids in CDM [18]

Amino acid	Time for complete derivatization (h)	Stability time (h)	Rate of derivatization (μM/h)	Lowest detectable concentration (aM)[a]
1. Ser	12	34	79	0.8
2. Gln	12	34	57	0.6
3. Met	6	24	22	0.1
4. Asn	12	34	63	0.6
5. Thr	14	34	60	0.7
6. Tyr	12	28	23	0.2
7. Ala	12	34	94	0.9
8. Gly/Val	12	24	182	1.1
9. His	6	34	54	0.3
10. Pro	12	34	145	1.5
11. Cys	20	34	8	0.1
12. Ile	12	34	13	0.1
13. Phe	12	34	10	0.1
14. Leu	12	36	13	0.1
Nle	6	34	24	ND
15. Glu	12	28	227	2.3
16. Unknown	12	34	–	ND
17. Arg	12	34	24	0.3
18. Asp	12	34	63	0.6

[a] This is the lowest detection limit of $S/N \geq 5$.

Table 2
Comparison of efficiency of amino acid analysis using conventional HPLC and CE–LIF with CBQCA derivatization

Amino acid	Amino acid added to CDM (μM)	Concentration by automated HPLC analysis (μM)	Automated HPLC analysis detection efficiency[a] (%)	Amino acid concentration by CE–LIF (μM)	CE–LIF detection efficiency[a] (%)
Ser	952.3	603.2	63.3	1124.4	118.1
Gln	684.9	0.0	0.0	571.6	83.5
Met	134.2	0.0	0.0	106.2	79.1
Asn	757.5	0.0	0.0	777.9	102.7
Thr	840.3	701.1	83.4	855.2	101.8
Tyr	276.2	221.7	80.3	273.9	99.2
Ala	1123.5	926.8	82.5	1311.1	116.7
Gly/Val	2188.0	1553.1	71.0	2188.0	100.0
His	322.5	261.8	81.2	331.8	102.9
Pro	1739.1	1518.2	87.3	1879.8	108.1
Cys	165.2	0.0	0.0	112.4	68.0
Ile	152.6	194.0	127.1	155.1	101.6
Leu	152.6	0.0	0.0	277.6	181.9
Phe	121.2	125.4	103.5	65.6	54.1
Glu	2721.0	1836.8	67.5	4403.5	161.8
Arg	287.3	222.1	77.3	292.8	101.9
Asp	751.8	522.8	69.5	631.4	84.0
Trp	122.5	0.0	0.0	0.0	0.0

[a] Percent detection efficiency=[estimated amino acid concentration (μM)/added amino acid concentration (μM)]·100.

CE–LIF missed Lys and Trp. These data indicate that this CE–LIF method was useful in detecting and quantitating 18/19, suggesting it is a useful method in determining amino acid metabolism during bacterial growth.

3.5. Repeatability

The repeatability of this method was assessed by examining replicates of a standard amino acid mixture for the migration time. Within a day of analyses for within-day variation had migration RSD values that ranged from 0.33 to 0.99% for individual amino acids (Table 3), and had an average RSD value over all amino acids of 0.44%. Tests done to estimate the variability between days, resulted in RSD values that ranged from 0.72 to 1.51%. These results provide evidence that this test has adequate variability to provide reliable migration times, identification, and quantitation of amino acids in unknown samples.

Table 3
Variability of amino acid identification and detection

Peak	Within-day RSD (%)[a]	Between-day RSD (%)[b]
Ser	0.3	0.7
Gln	0.3	0.7
Met	0.3	0.7
Asn	0.8	0.7
Thr	0.3	0.8
Tyr	0.4	1.1
Ala	0.3	0.8
Gly/Val	0.3	0.8
His	0.4	0.8
Pro	0.4	0.8
Cys	0.4	0.9
Ile	0.4	0.9
Phe	0.4	1.1
Leu	0.4	0.9
Nle	0.4	0.9
Glu	1.0	1.5
Arg	0.5	1.0
Asp	0.4	1.1
Average RSD (%)	0.4	0.9

[a] This number determined with five repeated injections within a day.
[b] This number was determined with five replicates between 5 days over 6 months.

3.6. Usefulness of the method

Selection of a suitable method, one should consider factors such as cost effectiveness, time, simplicity of method, and overall feasibility for the sample under analysis. The primary interest of this work was to develop a method to monitor the metabolism of all amino acids during bacterial growth at very low concentrations. The developed method was simple, sensitive, cost-effective, identified 17 amino acids and quantitated 18 amino acids within 17 min (with a total run time of 46 min), compared to a run time of a similar method of 70 min [23]. Though the total test time was slightly longer than other methods [11], a satisfactory separation, quantitation, and repeatability of all the amino acids was obtained. This CE–LIF method was judged acceptable for use in a complex, unknown mixture—such as a bacterial growth medium. Therefore, it was selected as the method to examine bacterial amino acid utilization.

3.7. Bacterial utilization of amino acids

Brevibacterium linens BL2 was grown in CDM to determine the pattern of amino acid utilization and demonstrate the application of the method in a bacterial growth medium. This method detected changes in the amino acid concentration due to the metabolism of *B. linens* BL2 during incubation in CDM (Table 4). During the initial 24 h of growth, all the amino acids decreased as expected. However, it is interesting to note that this organism produced amino acids in some cases (for example Ser), while in other cases the amino acid was depleted (for example Ile) during subsequent incubation days. These observations are consistent with known functions of this organism and use of this genus to produce amino acids for industrial applications. These data demonstrate the need for an assay with a very sensitive detection limit and highlight the utility of this method in following amino acid utilization in bacteria.

4. Summary and conclusions

An MEKC method to identify, separate, and quantitate amino acids in CDM using CBQCA with

Table 4
Concentration of amino acids during growth of *B. linens* BL2 in CDM[a]

Amino acid	Sterile CDM (μM)	Day of growth[b]				
		1	3	11	14	18
Ser	952.3	54.4	66.8	111.9	119.2	122.4
Gln	684.9	39.1	10.7	2.1	1.6	0.4
Met	134.2	7.7	1.9	5.7	0.0	0.3
Asn	757.5	43.3	39.2	10.4	3.3	0.4
Thr	840.3	48.0	46.7	36.4	0.7	5.4
Tyr	276.2	15.8	15.2	12.8	0.2	0.3
Ala	1123.5	64.2	21.0	67.8	2.6	2.0
Gly/Val	2188.0	125.0	94.2	100.9	2.8	1.9
His	322.5	18.4	14.4	12.9	3.1	2.0
Pro	1739.1	99.4	105.7	132.9	20.6	31.5
Cys	165.2	9.4	9.5	10.0	0.1	0.3
Ile	152.6	8.7	8.2	7.3	0.1	0.1
Phe	152.6	8.7	8.8	4.7	1.3	1.3
Leu	121.2	6.9	6.8	6.6	1.3	0.9
Glu	2721.0	155.5	157.8	129.3	128.8	137.1
Arg	287.3	16.4	17.7	28.0	2.0	3.6
Asp	751.8	43.0	16.2	30.9	6.3	3.2

[a] Amino acid concentrations in the spent medium were calculated by comparing the specific amino acid peak area to a standard curve (the R^2 ranged from 0.997 to 0.999) for that amino acid after the peak location was compared to the Nle internal standard to verify the consistency of the location and time.
[b] The data in these columns are amino acid concentrations (μM).

pre-column derivatization and CE–LIF detection was developed. CE–LIF with a run buffer containing 6.25 m*M* borate, 150 m*M* SDS, and 10 m*M* THF (pH 9.66) at 25 °C and 24 kV was used to identify 18 CBQCA-derivatized amino acids. Seventeen of the above 18 amino acids were quantitated at amol concentration. The derivatization rate was amino acid dependent and varied between 6 and 20 h, while the labeled amino acids remained stable up to 34 h. Comparison with automated HPLC analysis showed that the CE–LIF method had greater detection efficiency for 14 of the amino acids and minimal variability to suggest that this method may be used for unknown samples. This method was useful to determine amino acid concentration changes during bacterial growth of *Brevibacterium linens* BL2.

Acknowledgements

We would like to acknowledge the Utah Agricultural Experiment Station (approved as journal paper 7105), Dairy Management, Inc., and the Western Dairy Center for providing partial funding for this project. We would also like to acknowledge Dr. Marie Strickland for technical assistance.

References

[1] H.E. Schwartz, R.H. Palmieri, R. Brown, in: P. Camilleri (Ed.), Capillary Electrophoresis: Theory and Practice, CRC Press, Boca Raton, FL, 1993, p. 204.
[2] H.J. Issaq, K.C. Chan, Electrophoresis 16 (1995) 467.
[3] M.G. Khaledi, in: R.P. Oda, J.P. Landers (Eds.), Handbook of Capillary Electrophoresis, CRC Press, Boca Raton, FL, 1994, p. 46.
[4] K. Hayakawa, N. Imaizumi, O.S. Wong, M. Miyazaki, J. Chromatogr. 464 (1989) 343.
[5] I.Z. Atamna, C.J. Metral, G.M. Muschik, H.J. Issaq, J. Liq. Chromatogr. 13 (1990) 3201.
[6] H. Koning, H. Wolf, K. Venema, J. Korf, J. Chromatogr. 533 (1990) 171.
[7] M. Albin, R. Weinberger, E. Sapp, S. Moring, Anal. Chem. 63 (1991) 417.
[8] N.J. Dovichi, Y. F Cheng, Am. Biotechnol. Lab. 7 (1989) 10.
[9] G. Nouadje, M. Nertz, Ph. Verdeguer, F. Couderc, J. Chromatogr. A 717 (1995) 335.
[10] R.A. Wallingford, A.G. Ewing, Anal. Chem. 59 (1987) 1762.
[11] J. Liu, Y.-Z. Hsieh, D. Wiesler, M. Novotny, Anal. Chem. 63 (1991) 408.

[12] J. Bergquist, M.J. Vona, C.-O. Stiller, W.T. O'Connor, T. Falkenberg, R. J Elkman, J. Neurosci. Methods 65 (1996) 33.
[13] B. Nickerson, J.W. Jorgenson, J. Chromatogr. 480 (1989) 157.
[14] H. Nishi, T. Fukuyama, M. Matsuo, J. Microcol. Sep. 2 (1990) 234.
[15] A.J.G. Mank, E.S. Yeung, J. Chromatogr. A 708 (1995) 309.
[16] R. Weinberger, Am. Lab. 6 (1997) 17X.
[17] M. Strickland, B.C. Weimer, J.R. Broadbent, J. Chromatogr. A 731 (1996) 305.
[18] P.R. Jensen, K. Hammer, Appl. Environ. Microbiol. 59 (1993) 4363.
[19] S. Terabe, M. Shibata, Y. Miyashita, J. Chromatogr. 480 (1989) 403.
[20] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.
[21] S. Terabe, K. Otsuka, T. Ando, Anal. Chem. 57 (1985) 834.
[22] S. Miyashita, S. Terabe, Application Data No. DS-767, Beckman Instruments, Fullerton, CA, 1990.
[23] E. Arriaga, Y. Zhang, N.J. Dovichi, Anal. Chim. Acta 299 (1995) 319.