Rapid Analysis of Essential and Branched-Chain Amino Acids in Nutraceutical Products by Micellar Electrokinetic Capillary Chromatography

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A rapid method for the analysis of dansylated essential and branched-chain amino acids (BCAAs) by micellar electrokinetic capillary chromatography (MECC) is reported. Optimization of analytical conditions has been carried out, evaluating the influence on the performance of several parameters such as sodium dodecyl sulfate (SDS) concentration in the running electrolyte, temperature, and voltage. The effect of the addition of small amounts of isobutanol to the electrolyte has also been investigated. The best separation in the shortest time with a 37 cm capillary was obtained employing a 20 mM Borax buffer (pH 9.1) + 70 mM SDS at 25 °C and 20 kV. Under these conditions a mixture of nine essential amino acids was analyzed in 7 min, while separation of BCAAs occurred in less than 4 min. Using a shorter capillary (20 cm to the detector), the BCAA separation was performed in only 2.5 min. The method was applied to the quantitative analysis of amino acids in three commercial nutraceutical preparations. Assessment of analytical performance in terms of precision, linearity, and limit of detection has also been reported.

Keywords: *Essential amino acids; branched-chain amino acids (BCAAs); nutraceuticals; dansylated amino acids; micellar electrokinetic capillary chromatography (MECC)*

INTRODUCTION

Capillary electrophoresis (CE) and micellar electrokinetic capillary chromatography (MECC) are found to offer a good alternative to conventional HPLC methods in different fields of analytical chemistry. Analysis of amino acids by CE has been reported in several papers and extensively reviewed (Smith, 1997; Issaq and Chan, 1995). Derivatization with dansyl chloride is a common method to allow the UV detection. Most authors have employed micellar electrokinetic capillary chromatographic methods to separate dansyl derivatives of amino acids. In MECC, a surfactant such as sodium dodecyl sulfate (SDS) is added to the running electrolyte in an amount above its critical micelle concentration to provide a two-phase chromatographic separation medium: an aqueous phase and a micellar pseudophase. The separation principle is based on the combination of electrokinetic migration in CE and the partitioning mechanism of solute between the bulk solution and the micelles (Terabe et al., 1984). Furthermore, organic modifiers can be used in MECC to improve the separation, extending the migration-time window, improving the resolution, and modifying the migration order of analytes due to their effect on electrokinetic migrations and the capacity factor of homologous compounds (Liu et al., 1998).

Some applications in food chemistry have involved the use of MECC for the quantitative analysis of dansyl derivatives of amino acids in samples from different sources, including corn seed flour (Skocir and Prosek, 1995), fish (Skocir et al., 1997a), maize seed and seaweed (Skocir et al., 1997b), and honey (Corradini et al., 1997).

The aim of this work is to provide a rapid method to separate essential and branched-chain amino acids (BCAAs) by MECC, carrying out the quantitative analysis of BCAAs in nutraceutical preparations. Data about this subject were not found in the literature, although these products seem to have importance in commercial markets.

Essential amino acids are not directly synthesized by humans, and it is necessary to introduce them by diet or nutritional supplement. Valine, isoleucine, and leucine are three essential amino acids of the branched-chain type. It has been demonstrated that BCAA administration in rats (Calders et al., 1997) as in humans (Bolmstrand et al., 1997; Bigard et al., 1996) enhances muscular performance during exercise. For this reason they are widely employed by superathletes and body builders.

MATERIAL AND METHODS

Instrumentation. The samples were analyzed by MECC employing a Beckman P/ACE System 2050 (Beckman Instruments Inc., Fullerton, CA) capillary electrophoresis unit

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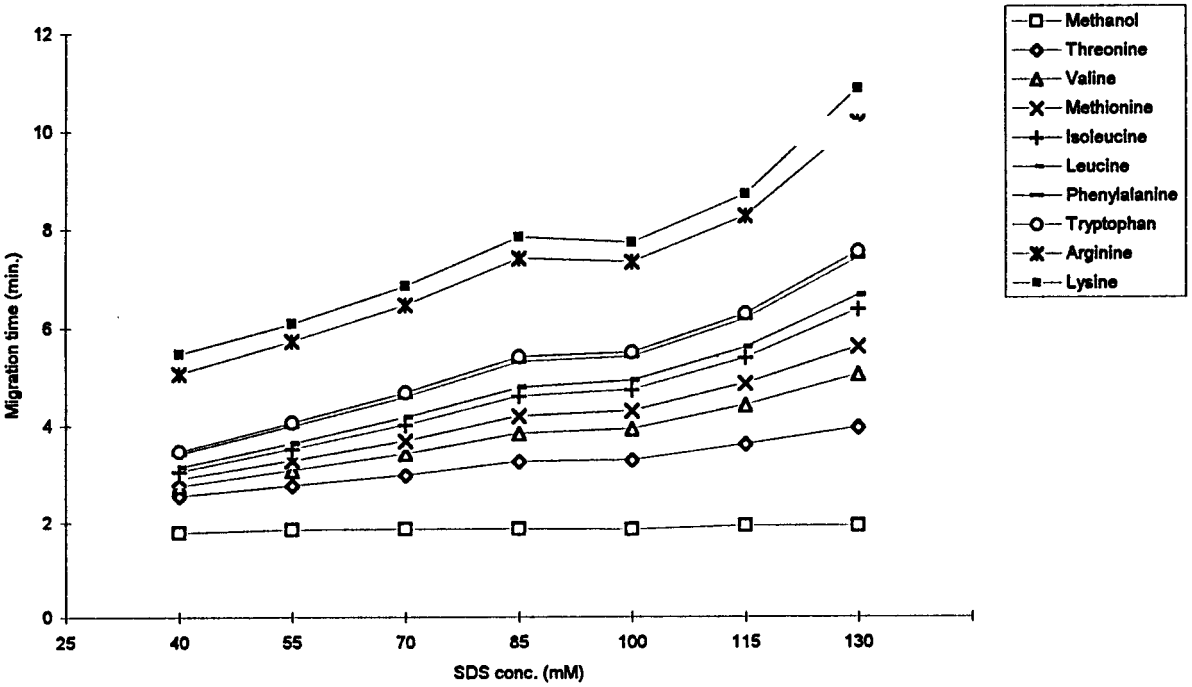

Figure 1. Dependence of the migration time (min) of dns-amino acids on the concentration of SDS in the 20 mM borate buffer (pH 9.1). Experimental conditions: capillary, bare fused silica, 50 μm i.d. × 37 cm total length (30 cm to the detector); applied voltage, 20 kV; temperature, 25 °C; detection wavelength, 214 nm at the cathodic end.

equipped with a UV detector functioning at $\lambda = 214$ nm. Data processing was performed using the Beckman System Gold 7.11 software.

Two fused silica capillaries (Quadrex Corp., New Haven, CT) of 37 and 27 cm (30 and 20 cm to the detector) in length, respectively, and 50 μm i.d. were used for the experiments. The temperature of the capillary cartridge was kept constant by circulation of a fluorocarbon liquid. Samples were injected by applying nitrogen pressure over 4 s. The volume introduced in the capillary was calculated by CE-inject software (Beckman Instruments Inc.) and was found to be, at the temperature of 25 °C, approximately 8.0 nL.

Materials. Borax, standard amino acids, dansylated amino acids (dns-amino acids), dansyl chloride, and sodium bicarbonate were purchased from Sigma Aldrich (Milan, Italy), SDS was purchased from BioRad (Milan, Italy), and methanol, acetonitrile, isobutanol, and water (HPLC grade) were purchased from Carlo Erba (Milan, Italy). The samples of nutraceutical preparations were commercially available.

Methods. *Derivatization of the Standard Mixtures and Samples.* Standards of leucine, isoleucine, valine, alanine, and norleucine and samples of nutraceutical preparations were derivatized by modifying the method proposed by Taphui et al. (1981). Solutions of amino acids were prepared at a concentration of 1 mg/mL. Nutraceutical samples were prepared by dissolving 0.5 g of product in 50 mL of water. Volumes of 0.5 mL of 500 mM sodium bicarbonate and 0.5 mL of a solution of 10 mg/mL dansyl chloride in acetonitrile were added to a vial containing 0.5 mL of the sample. The mixture was put in an ultrasonic bath (at 20 kHz, 25 °C) for 10 min and then placed in an oven at 60 °C for 1 h. After derivatization, the solvent was evaporated and the residue was dissolved in 1 mL of water/methanol (80:20) (Michaelsen et al., 1994). Solutions were filtered through a 25 mm filter unit (0.2 μm) (Lida Manufacturing Corp., Kenosha, WI) and stored in a dark place.

To perform a quantitative determination, it is important to take into account that dansylation is not 100% complete for all amino acids. Therefore, it was necessary to derivatize the standard mixture used for the calibration graphs following the same procedure employed for the samples. Calibration graphs were thus prepared by diluting a solution of the standards after derivatization, at five different concentrations.

Resolution and Efficiency Calculations. Analogous to chromatography, resolution was calculated by applying the following equation:

$$R_s = 2(t_2 - t_1)/(W_2 + W_1)$$

where t_2 and t_1 are the migration times of two adjacent peaks and W_1 and W_2 are the peak widths measured at the peak base.

Peak efficiency was calculated using the following equation:

$$N = 5.54(t_M/t_{W_{1/2}})^2$$

where N = theoretical plate number and t_M and $t_{W_{1/2}}$ are the migration time of the peak and the width at the half peak height, respectively. Efficiency is expressed as the number of theoretical plates per meter (N/m).

RESULTS AND DISCUSSION

Optimization of the Electrophoretic Conditions. The first step of our work was to develop a CE method which allowed the separation of the essential amino acids in a short time. The study was conducted on a mixture of nine essential dns-amino acids. The electrolyte employed was a 20 mM Borax buffer (pH 9.1) to which different amounts of SDS were added. The effect of SDS concentration on the separation of different mixtures of dns-amino acids with 20 or 100 mM borate buffer is reported in the literature. Several ranges, including 30−50 mM (Ong et al., 1991), 90−102.5 mM (Skocir at al., 1994), and 100−180 mM (Michaelsen et al., 1994), of SDS concentration were investigated. We studied the behavior of nine essential dns-amino acids in the range from 40 to 130 mM SDS. Figure 1 shows a considerable influence on migration time of analytes, recorded by keeping the temperature constant at 25 °C and the applied voltage at 20 kV. As observed, migration times became longer as SDS concentration increases. This behavior depends on the higher amount of micelles in the background electrolyte, which enhances the

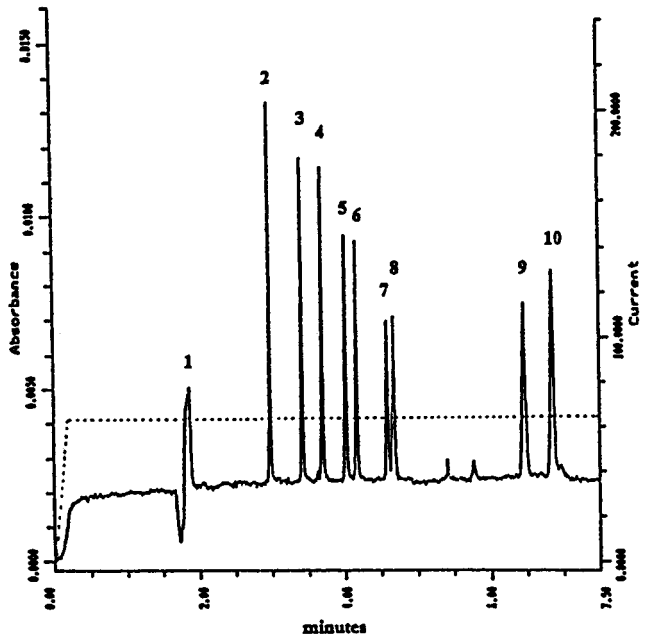

Figure 2. Electropherogram of a mixture of essential dns-amino acids. Electrophoretic conditions: running electrolyte, 20 mM Borax (pH 9.1) + 70 mM SDS. Other conditions as in Figure 1. Peak identification: (1) methanol (EOF marker), (2) dns-threonine, (3) dns-valine, (4) dns-methionine, (5) dns-isoleucine, (6) dns-leucine, (7) dns-phenylalanine, (8) dns-tryptophan, (9) dns-arginine, (10) didns-lysine. Dotted line = current value.

interaction of the analyte with a sort of stationary phase formed by the surfactant. The electroosmotic flow (EOF) is represented in the graph by methanol that was used as a marker due its noninteraction with the micelles. From the graph, it is evident that EOF did not change significantly over the whole SDS concentration range, which was varied from 40 to 130 mM, and it was reproducible between runs under the specific conditions used in this work.

As expected, the resolution of several pairs of dns-amino acids changed with variation of the surfactant concentration. For instance, the R_s value for the pair isoleucine−leucine changed from 0.21 at 40 mM SDS to 3.37 at 130 mM. A value of 70 mM was chosen to obtain a good resolution in the shortest time for all the peaks detected. Studies on resolution were also carried out by varying other parameters. Increasing the temperature (in the range 25−45 °C) or the applied voltage (from 20 to 30 kV) resulted in a loss in resolution as the time of analysis became shorter and the peaks eluted closely. Therefore, the best conditions which allowed a good resolution of all peaks in the minimum analysis time were 70 mM SDS, 20 kV, and 25 °C. Under these analytical conditions, the separation of a mixture of the nine essential amino acids was performed. All peaks were well resolved in 7 min as presented in Figure 2.

The effect of the addition of small amounts of an organic solvent to the running electrolyte was also investigated. The employment of a cosurfactant not only affects the polarity of the mobile phase, but also changes the EOF and the partition coefficient of the solutes in the micelles. This effect was previously studied on four didansyl derivatives by Skocir et al. (1997a). Our investigation was focused on the influence of isobutanol on the behavior of nine essential amino acids. The analytical conditions were 85 mM SDS in 20 mM Borax buffer, a voltage of 10 kV, and a temperature of 25 °C. These conditions, although longer in time, permitted us to monitor in an unequivocal way the variations in migration times and resolution for all the peaks. The addition of isobutanol in the buffer, in the range 1−8% v/v, was found to decrease the EOF, consequently increasing the migration times of all analytes, and influenced the resolution. As reported in Figure 3, with 2% isobutanol in the running electrolyte, phenylalanine and tryptophan coeluted at around 12 min. With an increase in isobutanol from 2% to 4%, the two amino acids were resolved in reverse order to that observed without the addition of isobutanol. However, retention times lengthened, and phenylalanine and tryptophan were retained for 14.5 and 14.0 min, respectively. Therefore, the addition of an organic modifier to the running electrolyte

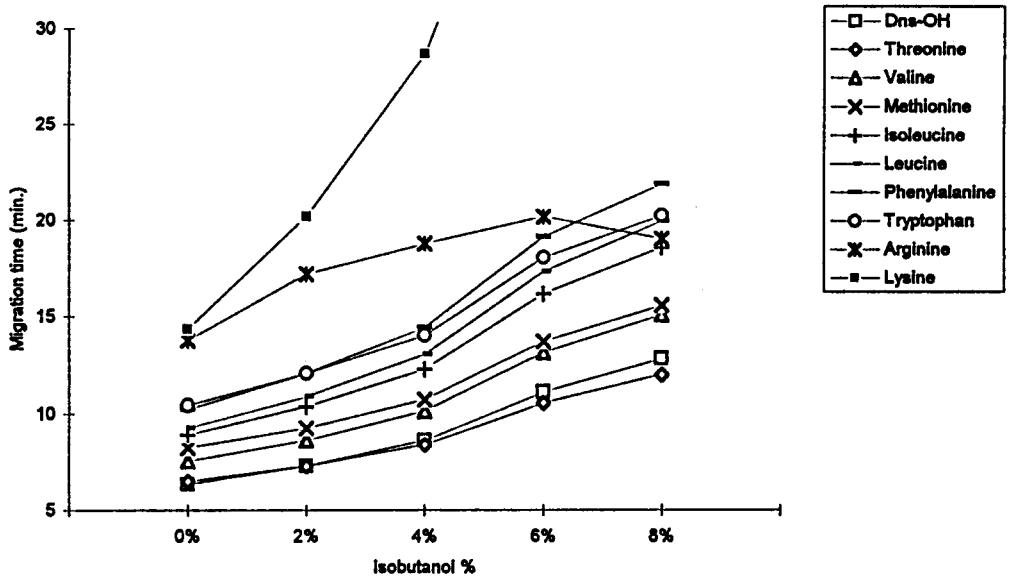

Figure 3. Effect of isobutanol percentage on the migration times (min) of dns-amino acids. Other conditions: 20 mM borate buffer (pH 9.1) + 85 mM SDS; temperature, 25 °C; applied voltage, 10 kV.

Table 1. Peak Efficiency (N/m[a]) and Resolution (R_s) of the Selected Dansylated Amino Acids

analyte	N/m	R_s	analyte	N/m	R_s
alanine	420.000		leucine	288.000	
valine	340.000		(isoleucine−leucine)[b]		1.95
(alanine−valine)[b]		6.2	norleucine	315.000	
isoleucine	325.000		(leucine−norleucine)[b]		3.77

[a] N/m = plate count per meter. [b] Adjacent peaks.

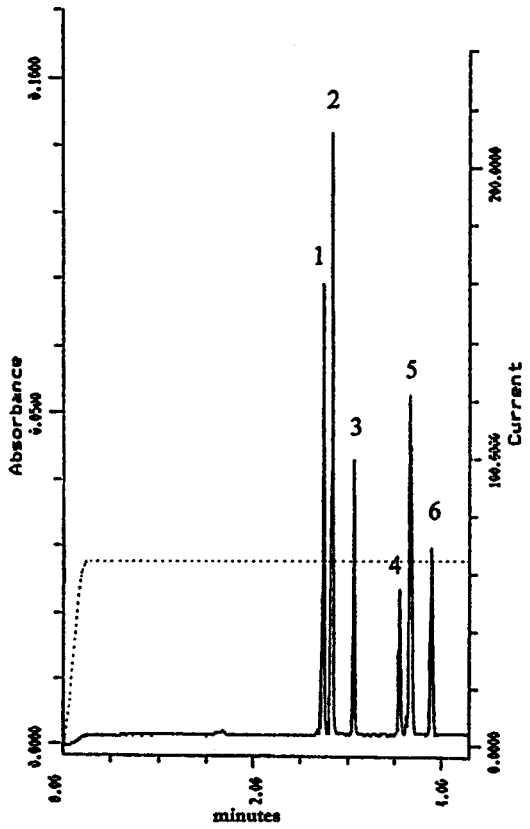

Figure 4. Electropherogram of a mixture of derivatives of amino acids employed for the calibration graphs. Conditions of analysis as in Figure 2. Peak identification: (1) dns-OH, (2) dns-alanine, (3) dns-valine, (4) dns-isoleucine, (5) dns-leucine, (6) dns-norleucine.

was found not to be useful, and the research was carried out using the above-mentioned conditions.

Assessment of Analytical Performance. The optimum conditions for rapid routine analysis of the amino acids contained in the nutraceuticals investigated and the internal standard norleucine, all as dansylated derivatives, were found by employing a running electrolyte consisting of 70 mM SDS added in 20 mM borate buffer (pH 9.1). The applied voltage was 20 KV and the capillary temperature 25 °C. The average current corresponding to these conditions was found to be 65 μA. The resulting electropherogram (Figure 4) shows the best separation for the dansylated amino acids of interest with high peak efficiency and R_s values in the range 1.95−6.2, as reported in Table 1.

The validation criteria commonly employed in the evaluation of CE methods are similar to those tested for HPLC (Jenke, 1996a; Jenke, 1996b).

Repeatability. The repeatability of the migration time for the proposed analytes is expressed in terms of relative standard deviation [RSD = (standard deviation/mean) × 100]. A mixture of five dansylated amino acids in water/methanol was injected using the above optimized conditions. Eight injections of standards were done sequentially. Results for within-day analyses are summarized in Table 2, and the migration time repeatability was excellent, with RSD values ranging from 0.33% to 0.36% for all the amino acids investigated. This operation was repeated over 5 days, and the between-day RSD for all analytes was better than 2%. This indicates a good performance of the method for qualitative analysis. Furthermore, within-day and between-day repeatability data for peak areas, normalized peak areas, and normalized peak area ratio of the dansylated amino acid are reported in Table 3. Peak area reproducibility for the studied analytes was in the region of 0.95−4.00%, and better results were obtained with the normalized peak areas and normalized peak area ratio. This performance suggests that the method is also entirely suitable for quantitative determination of these compounds.

Table 2. Repeatability of Migration Times Calculated from Within-Day Analyses, and Limit of Detection (LOD)

	mean (n = 8)	SD	RSD (%)	LOD (fmol)
alanine	2.847	0.009	0.33	5.1
valine	3.080	0.010	0.35	10.5
isoleucine	3.568	0.011	0.33	13.4
leucine	3.681	0.013	0.36	8.8
norleucine	3.914	0.012	0.33	6.7

Table 3. Within-Day and Between-Day Peak Area, Normalized Peak Area, and Normalized Peak Area Ratio Reproducibility of Dansylated Amino Acid[a]

	within-day RSD (%)[b]			between-day RSD (%)[b]		
	A	AL/t_M	$(A/t_M)/(A_{IS}/t_M)$	A	AL/t_M	$(A/t_M)/(A_{IS}/t_M)$
alanine	0.95	0.98	0.75	2.41	2.12	1.31
valine	1.15	1.08	0.89	2.55	2.17	1.41
isoleucine	1.28	1.22	1.13	2.60	2.44	1.47
leucine	2.25	2.16	1.75	3.72	3.27	1.75
norleucine	2.15	2.06		3.97	3.61	

[a] A = peak area. AL/t_M = normalized peak area (L = capillary effective length; t_M = retention time). $(A/t_M)/(A_{IS}/t_M)$ = (normalized peak area/normalized peak area of the internal standard). [b] n = 10.

Linearity and Limit of Detection (LOD). Detector response measured for all amino acids was linearly correlated with the sample concentration injected over a range of 0.1−160 μg/mL. Higher concentrations were not assayed because we considered that the range was wide enough for the proposed applications. The linearity was estimated from repeated injection at five different concentrations of each amino acid. The linearity of the present method for all analytes was good, with correlation coefficient higher than 0.998. The linearity was achieved without an internal standard, which would have increased the correlation coefficient, as demonstrated in the application of BCAAs and alanine quantitation.

The limit of detection was determined by finding the concentration of amino acids at which the peak area had a signal-to-noise (S/N) ratio of 3. The detectable amount was found to be between 0.20 and 0.60 μg/mL for all

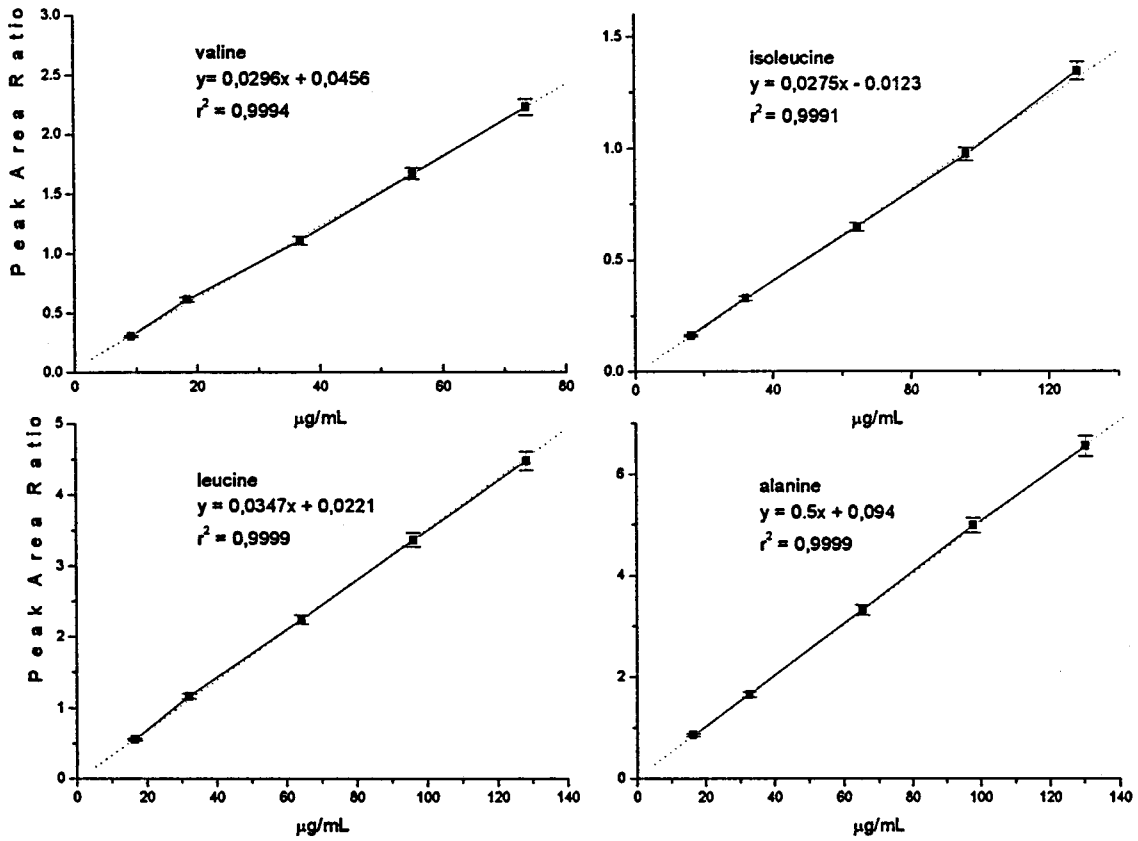

Figure 5. Calibration graphs for dansylated amino acids. Error bars are calculated at an RSD of 3%. Dotted line = linear fit. Concentration range: valine, 9.2−73.6 μg/mL; isoleucine, 6.1−48.8 μg/mL; leucine, 16.1−128.4 μg/mL; alanine, 16.3−130.4 μg/mL. Electrophoretic conditions as in Figure 2.

Table 4. Amount of Amino Acids Found in Three Different Nutraceuticals Containing BCAAs[a]

	sample A		sample B		sample C	
amino acid	found	labeled[b]	found	labeled[b]	found	labeled[b]
alanine	7.78 ± 0.16	8.0				
valine	4.87 ± 0.07	5.0	5.79 ± 0.08	6.0	7.55 ± 0.16	7.8
isoleucine	1.04 ± 0.02	1.0	5.82 ± 0.10	6.0	5.58 ± 0.11	5.8
leucine	7.86 ± 0.15	8.0	11.68 ± 0.17	12.0	13.97 ± 0.22	14.5

[a] Mean values ± SD (n = 4). [b] Reported on the label of the commercial product.

compounds when analyzed under the above-mentioned conditions. The LODs for alanine and BCAAs expressed in femtomol were calculated as reported in the experimental part and are reported in Table 2. Similar values were also recorded for the other essential amino acids investigated.

Applications. The optimized conditions described in this paper were applied to carry on the quantitative determination of amino acids in three different nutraceutical preparations, which are essentially a mixture of leucine, isoleucine, and valine, the three essential amino acids of the branched-chain type. In one of the samples the amino acid alanine was also included. The quantitation calculation of the dansylated amino acids was based on the internal standard method. The peak areas were normalized to the migration time. The amino acid norleucine was selected as the internal standard because it is not present in the analyzed nutraceuticals, it is completely resolved in the electropherogram from the other amino acids, and it migrated close to the peaks of interest.

The calibration graphs for the three BCAAs and alanine obtained by the peak area ratio method showed excellent linearity over the concentration range reported for each amino acid, with correlation coefficients better than 0.999, and nearly passed through the origin. Results obtained from five repeated measurements are graphically reported in Figure 5.

A typical electropherogram of alanine, valine, isoleucine, and leucine with the internal standard norleucine is reported in Figure 4. A good separation of all peaks of interest was achieved in less than 4 min, and the electrophoretic profiles recorded under the same conditions for two of the nutraceutical samples are depicted in Figure 6 (samples A and B). Sample C, a nutraceutical having only amino acids of the branched-chain type, showed an electropherogram similar to that of sample B. Other components of the analyzed samples either were not detectable or displayed migration properties that did not interfere with the separation. Data from quantitative investigations of samples A, B, and C are reported in Table 4. Results are in good agreement with those declared on the label of the products.

Furthermore, the use of a shorter capillary (20 cm to the detector) allowed the separation of the branched-chain amino acids in 2.5 min. This achievement im-

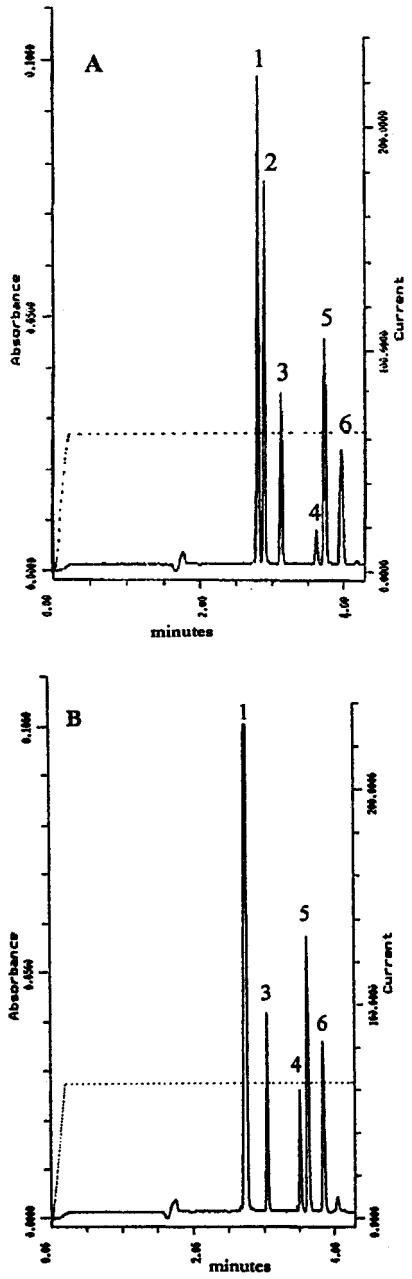

Figure 6. (A) Dansylated branched-chain amino acids and dns-alanine of sample A. (B) Dansylated branched-chain amino acids of sample B. Electrophoretic conditions and peak identification as in Figure 4.

proves the advantages of the proposed method for routine analyses.

Results of this work show that MECC is an effective separation method for the rapid determination of essential and branched-chain amino acids in nutraceuticals. This technique provides highly efficient separation with high precision, short analysis time, and very low reagent consumption. Furthermore, MECC did not show any decrease in performance during at least 100 analyses, and no replacement of the low-cost separation capillary was necessary.

LITERATURE CITED

Bigard, A. X.; Lavier, P.; Ullmann, L.; Legrand, H.; Douce, P.; Guezennec, C. Y. Branched-chain amino acid supplementation during repeated prolonged skiing exercises at altitude. *Int. J. Sport Nutr.* **1996**, *6*, 295−306.

Bolmstrand, E.; Hassmen, P.; Ek, S.; Ekblom, B.; Newsholme, E. A. Influence of ingesting a solution of branched-chain amino acids on perceived exertion during exercise. *Acta Physiol. Scand.* **1997**, *159*, 41−49.

Calders, P.; Pannier, J.-L.; Matthys, D. M.; Lacroix, E. M. Pre-exercise branched-chain amino acid administration increases endurance performance in rats. *Med. Sci. Sports Exercise* **1997**, *29*, 1182−1186.

Corradini C.; Canali, G.; Cavazza, A.; Cogliandro, E.; Nicoletti, I. Determination of honey authenticity and its botanical origin by micellar electrokinetic chromatography (MEKC) and HPLC. *Proceedings of Euro Food Chem IX*, Amadó, R., Battaglia, R., Eds.; Druckerei Sailer: Switzerland, 1997; Vol. 3, pp 664−670.

Issaq, H. J.; Chan, K. C. Separation and detection of amino acids and their enantiomers by capillary electrophoresis: a review. *Electrophoresis* **1995**, *16*, 467−480.

Jenke, D. R. Chromatographic method validation: a review of current practices and procedures. I. General concepts and guidelines. *J. Liq. Chromatogr. Relat. Technol.* **1996a**, *19*, 719−736.

Jenke, D. R. Chromatographic method validation: a review of current practices and procedures. II. Guidelines for primary validation parameters. *J. Liq. Chromatogr. Relat. Technol.* **1996b**, *19*, 737−757.

Liu, Z.; Zou, H.; Zhang, Y. Roles of organic modifiers in micellar electrokinetic capillary chromatography. *J. High Resolut. Chromatogr.* **1998**, *21*, 234−240.

Michaelsen, S.; Moller, P.; Sorensen, H. Analysis of dansyl amino acids in feedstuffs and skin by micellar electrokinetic capillary chromatography. *J. Chromatogr.* **1994**, *680*, 299−310.

Ong, C. P.; Ng, C. L.; Lee, H. K.; Li, S. F. Y. Separation of Dns-amino acids and vitamins by micellar electrokinetic chromatography. *J. Chromatogr.* **1991**, *559*, 537−545.

Skocir, E.; Prosek, M.; Oskomic, M. Separation of di-dansyl amino acids by MEKC with a cosurfactant. *Chromatographia* **1997a**, *44*, 267−273.

Skocir, E.; Prosek, M.; Strazisar, M.; Golob, T.; Plestenjak, A.-M. Quantitative determination of some amino acids in maize seed and seaweed by micellar electrokinetic chromatography. *J. Microcolumn Sep.* **1997b**, *9*, 451−457.

Skocir, E.; Prosek, M. Determination of amino acid ratios in natural products by micellar electrokinetic chromatography. *Chromatographia* **1995**, *41*, 638−644.

Skocir, E.; Vindevogel, J.; Sandra, P. Separation of 23 dansylated amino acids by micellar electrokinetic chromatography at low temperature. *Chromatographia* **1994**, *39*, 7−10.

Smith, J. T. Review: Developments in amino acids analysis using capillary electrophoresis. *Electrophoresis* **1997**, *18*, 2377−2392.

Tapuhi, Y.; Schmidt, D. E.; Lindner, W.; Karger B. L. Dansylation of amino acids for high-performance liquid chromatography analysis. *Anal. Biochem.* **1981**, *115*, 123−129.

Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchiya, A.; Ando, T. Electrokinetic separations with micellar solutions and open-tubular capillaries. *Anal. Chem.* **1984**, *56*, 111−113.

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