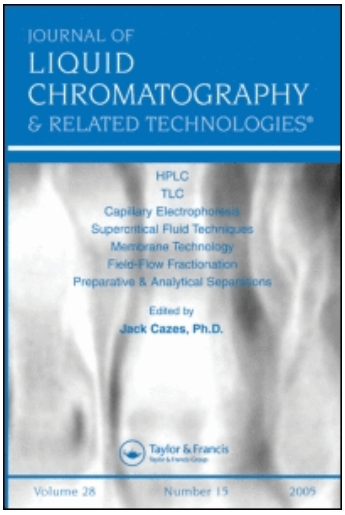

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SEPARATION AND DETERMINATION OF STEVIA SWEETENERS BY CAPILLARY ELECTROPHORESIS AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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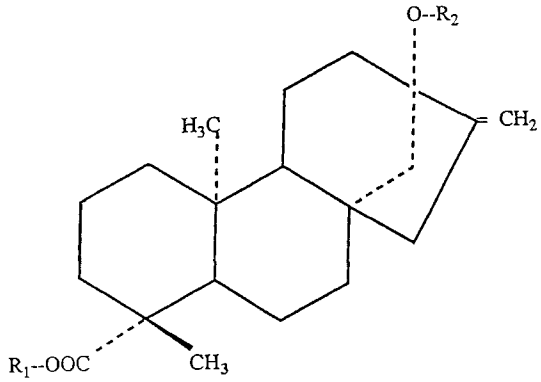
ABSTRACT

In this paper, the separation of steviol glycosides in stevia sweeteners including stevioside, rebaudioside A, rebaudioside C and dulcoside A by capillary electrophoresis and high performance liquid chromatography was investigated. A simple and efficient capillary electrophoretic method was developed. The results were compared with those obtained by HPLC. The individual steviol glycosides were obtained by HPLC fraction collection, and peaks in the electropherograms of the sweetener samples from Chinese refining factories were identified by comparing with those of individual steviol glycosides. The method was applied to the determination of real samples.

INTRODUCTION

Stevia sweeteners, extracted from the leaves of the plant Stevia rebaudiana, are commonly used as natural sweeteners in beverages, foods and medicines [1]. They contain eight steviol glycosides, in which stevioside (SS) has been shown



Name	R_1*	R_2*
1. Dulcoside A	glc	glc-rham
2. Stevioside	glc	glc-glc
3. Rebaudicoside C	glc	glc-rham \ glc
4. Rebaudicoside A	glc	glc-glc \glc

*glc, α-D-glucopyranosyl; rham, β-L-rhamnopyranosyl

Fig.1. Names and structures of steviol glycosides

to be the principal one, and rebaudioside A (RA), rebaudioside C (RC) and dulcoside A (DA) are the other main constituents. Their structures are shown in Figure 1 [2].

Various methods have been reported for the determination of stevia sweeteners, including gas liquid chromatography (GLC), thin layer chromatography (TLC), droplet countercurrent chromatography (DCCC), high performance liquid chromatography (HPLC), colorimetry and enzymatic determination [2]. Comparing with other methods, HPLC analytical methods are simple, rapid and accurate. However, in HPLC analysis of stevia sweeteners, a

special separation column must be used and the column is easily contaminated by the impurities of the real samples.

This paper described an efficient and simple method for determining stevia sweeteners by capillary electrophoresis (CE). It was evaluated by studying the effect of different separation conditions on migration time, selectivity and resolution. The method was applied in the separation and quantitation of real products. The results were compared with those obtained by HPLC.

MATERIALS AND METHODS

Reagents and materials

Stevioside standard was purchased from Wako (Osaka, Japan). Samples of stevia sweeteners were provided by Beijing Tianan Stevia Sweetener Products Company (Beijing, P.R.China). Pure stevioside was also obtained from the stevia sweeteners by methanol recrystallization, and other steviol glycosides (RA, RC and DA) were obtained from the stevia sweetener products by HPLC fraction collection and identified by HPLC.

Acetonitrile (HPLC grade) was purchased from BDH (Poole, England). Sodium tetraborate (analytical grade) was supplied by Fluka (Buchs, Switzerland). Water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare buffers for CE, mobile phase for HPLC and sample solutions.

Apparatus and conditions

CE was carried out on a commercial and a laboratory-built CE system. For

the laboratory-built CE system, a Spellman 30-kV power supply was used (Plainview, New York, USA). A fused-silica capillary tube of 50 cm effective length and 50 μm I.D. (Polymicro Technologies, Phoenix, AZ,USA) was used as the separation column. The peaks were detected by a Micro-UVis20 detector (Carlo Erba, Milan, Italy) with wavelength set at 210 nm. The window for the on-column detection cell was made by removing a small section of the polyimide coating on the fused silica capillary. Data processing was performed on a Shimadzu (Kyoto, Japan) Chromatopac C-R6A instrument. Samples were injected into the capillary by gravity feed with injection time of 20 seconds and injection height of 10 cm. The commercial system was a HP3D CE system (Hewlett Packard, Palo Alto, CA, USA), equipped with the same capillary, samples were injected into the capillary by pressure (30 mbar, 20 seconds). The buffer solution contained acetonitrile and sodium tetraborate solution. Other conditions were described where necessary in the next.

HPLC was carried out on a Shimadzu LC-9A system (Shimadzu Seisakusho, Kyoto, Japan), equipped with a normal phase LiChrospher NH_2 (5 μm) column (250 * 4 mm I.D.) (Merck, Darmstadt, Germany) and a Shimadzu SPD-M6A detector operated 210 nm. Data processing was performed through a computer by the SPD-M6A program. The HPLC mobile phase was prepared by mixing acetonitrile and Millipore water (80/20, V/V), and the flow rate was 0.8 ml/min.

Sample preparation

Samples of stevia sweeteners (100 mg) were dissolved in a 10 ml volumetric flask with Millipore water. The solution was diluted with the HPLC mobile phase to obtain the final concentration of 2 mg/ml. The solution were passed through a SPE C_8 cartridge (Whatman) before HPLC and CE analysis.

Solution for linearity response

Seven concentrations of stevioside, which ranged from 0.01 to 2.00 mg/ml, were prepared by dissolving and diluting in the HPLC mobile phase.

Isolation of individual glycosides from the stevia sweeteners

Stevia sweeteners (20 g) were dissolved in Millipore water (10 ml), and stevioside was recrystallized from the solution by adding methanol (60 ml). The solution was evaporated to dryness and the residue was redissolved in the HPLC mobile phase to get the final concentration of 5mg/ml. After treatment with a SPE C_8 cartridge, the solution was injected into HPLC system and the fractions of the steviol glycosides were collected. The collected fractions were evaporated to dryness and dissolved in HPLC mobile phase separately. Finally, the purity of the collected individual compounds was tested by HPLC.

RESULTS AND DISCUSSION

Fraction Collection by HPLC

Stevia sweeteners mainly contain four steviol glycosides, including SS, RA, RC and DA. Only stevioside (SS) standard could be obtained commercially. Other individual compounds were isolated from stevia sweeteners by HPLC fraction collection using a Lichrospher NH_2 column and the separation conditions given in ref. 3. The chromatograms of the mixture of stevia sweeteners and the individual steviol glycosides isolated from them are shown

in Fig. 2. It can be observed that the collected fractions of each of the individual compounds did not contain significant amounts of the other steviol glycosides present in the original mixture. These fractions were used in subsequent CE experiments for the purpose of peak identification.

Optimization of CE Conditions

The four steviol glycosides are similar in structure in that a steviol aglycone is connected at C-4 and C-13 to mono, di, trisaccharides consisting of glucose and/or rhamnose residues (see Figure 1). They are neutral compounds and usually cannot be separated electrophoretically. But in borate buffer systems, these compounds were transformed into negatively charged borate complexes [4], which could be partially separated in CE. Furthermore, by adding acetonitrile to the buffer solution, the separation was further improved.

The enhancement in selectivity could be attributed to differences in the stability of borate complexes, and in the solubilities of the steviol compounds in the acetonitrile/buffer mixture. The compounds which form more stable borate complexes and dissolved less well in the acetonitrile/buffer solution would be expected to exhibit a higher electrophoretic flow. Since the electrophoretic flow for negatively charged species would be in opposite direction to that of the electroosmotic flow, the compounds which form more stable complexes would be expected to have lower net migration rate and to migrate out later than those forming less stable complexes [4]. The difference in stability of the complexes therefore permitted the separation of the stevia sweeteners in electrophoresis. It is worth noting that differences in the stability of the borate complexes have been utilized previously in CE separation of carbohydrates [5-7] and catecholamines [8] to enhance selectivity.

Instead of acetonitrile, it was found that other organic modifiers, such as methanol and n-propanol also improved the resolution. However, these

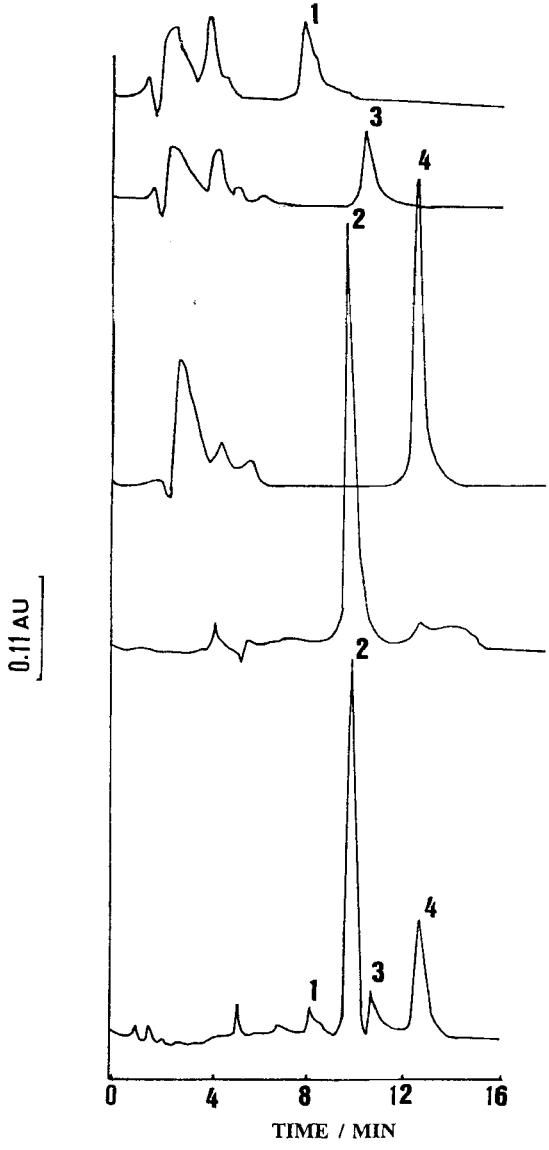


Fig.2. Liquid chromatograms of stevia sweeteners and the individual steviol glycosides obtained by fraction collection. Conditions: Column, Lichrospher NH_2 (5 μm) (250*4 mm I.D.); mobile phase, CH_3CN/H_2O (80/20, V/V); flow rate, 0.8 ml/min; UV (210 nm); att, 0.6; temperature, ambient. Peaks, 1 = DA; 2 = SS; 3 = RC; 4 = RA.

alternative modifiers reduced the electroosmotic flow significantly at the same time, and gave much longer analysis times than those for acetonitrile. Similar observations have been made previously in other CE separations utilizing organic modifiers as buffer additives [9]. Consequently, acetonitrile was used in all subsequent experiments as organic modifier.

In order to obtain the optimal conditions for CE separation, the effects of different experimental parameters on resolution were investigated. The experimental parameters which were studied included borate concentration, pH, acetonitrile concentration and voltage. The criteria used for selecting the optimal conditions were: 1. The resolution between any adjacent pair of peaks would be greater than unity, and 2. The capacity factors would be less than 0.7 (migration times less than 18 minutes).

The concentration of borate was varied from 10 to 60 mM. The migration times and resolution obtained are shown in Fig. 3 and Table 1, respectively. It was found that increasing borate concentration gave better separation but longer migration times for all of the stevia sweeteners. This result was expected owing to the increased concentrations of borate complexes and the higher electrophoretic mobilities at higher borate concentrations [4]. A concentration of 50 mM borate provided satisfactory separation ($R > 1$) within a relatively short analysis times (ca. 15 minutes) and hence was selected for further experiments.

The effect of pH on the migration times and resolution of the stevia sweeteners are shown in Fig. 4 and Table 2, respectively. It was note that increasing pH resulted in better separation, although the migration times would be significantly longer at higher pH. It was found that pH 9.30 provided satisfactory separation and relatively short migration times.

The effect of varying the concentration of acetonitrile is shown in Fig. 5 and Table 3. At higher concentrations of acetonitrile, the resolution improved albeit at the expense of longer analysis time. It was found that at an acetonitrile concentration of 45%, the migration times and the resolution satisfied the criteria stated above.

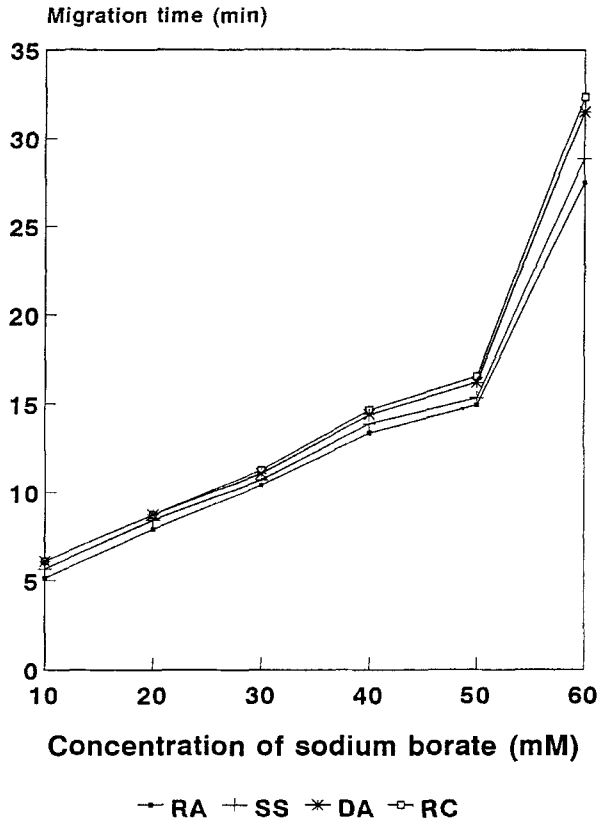


Fig.3. Influence of borate concentration on migration times of stevia sweeteners in CE system. Conditions: Buffer, sodium tetraborate (pH 9.3)/ acetonitrile (55/45, V/V), borate concentration ranging from 10 to 50 mM; UV detection at 210 nm; voltage,16.5 kV; temperature, ambient.

TABLE 1
Effect of Borate Concentration on Resolution

Concentration (mM)	10	20	30	40	50	60
Resolution (SS/RA)	*	0.64	0.88	1.01	1.04	2.12
Resolution (DA/SS)	0.32	0.51	1.06	1.29	2.24	2.78
Resolution (RC/DA)	*	*	0.73	0.79	1.13	2.20

* Peaks overlapping

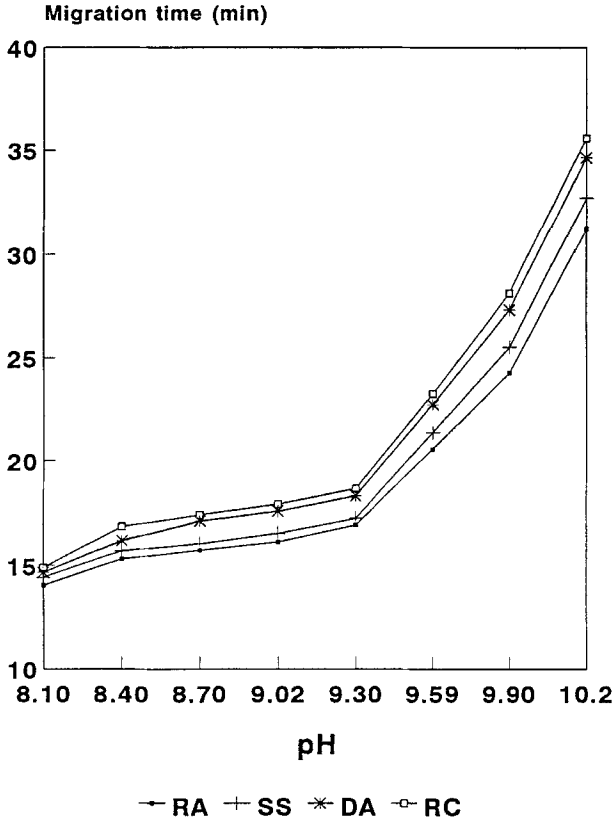


Fig.4. Influence of pH of buffer on migration times of stevia sweeteners in CE system. Voltage, 15 kV; buffer, 50 mM sodium tetraborate/acetonitrile, pH ranging from 8.1 to 10.2; other conditions as in Fig. 3.

TABLE 2
Effect of pH of Buffer on Resolution

pH	8.10	8.40	8.70	9.02	9.30	9.59	9.90	10.20
Resolution(SS/RA)	0.32	0.54	0.67	0.78	1.07	1.28	1.50	2.02
Resolution(DA/SS)	0.52	0.57	0.93	1.54	2.36	2.75	3.25	3.54
Resolution(RC/DA)	0.45	0.63	0.87	1.00	1.19	1.15	1.77	1.80

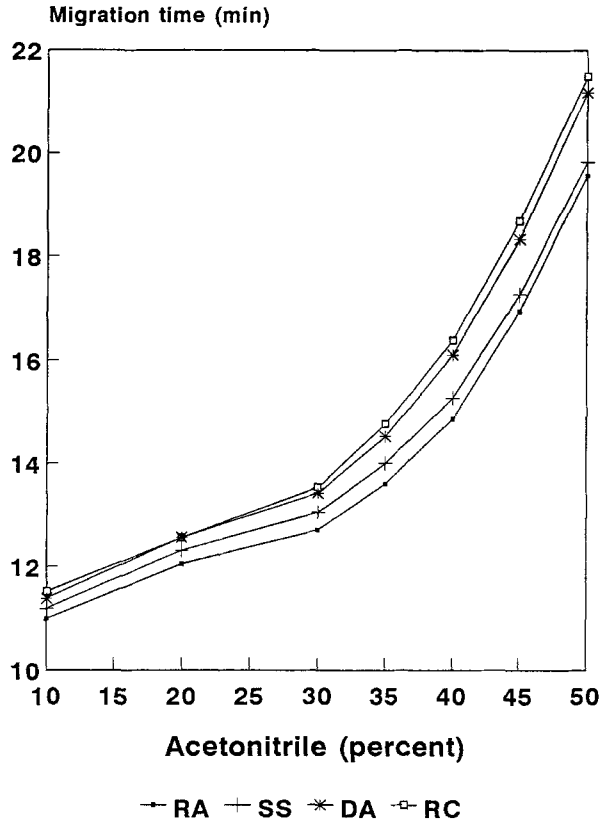


Fig.5. Influence of acetonitrile concentration on migration times of stevia sweeteners in CE system. Voltage, 15 kV; buffer, 50 mM sodium tetraborate/acetonitrile, acetonitrile concentration ranging from 10 % to 50 %; other conditions as in Fig. 3.

TABLE 3
Effect of Acetonitrile Concentration on Resolution

Concentration (%)	10	20	30	35	40	45	50
Resolution(SS/RA)	0.30	0.45	0.60	0.65	0.84	1.07	1.64
Resolution(DA/SS)	0.35	0.50	0.91	1.09	1.68	2.36	3.27
Resolution(RC/DA)	*	*	0.43	0.59	0.70	1.19	1.73

* Peaks overlapping

At higher applied voltages, the electroosmotic flow increased. Consequently shorter migration times would be obtained. The results of migration times and resolution for different voltages are shown in Fig. 6 and Table 4. It was noted from Fig.6 and Table 4 that although at higher voltages, shorter migration times were obtained, the resolutions between adjacent pairs of peaks were less than unity in some cases. Therefore, an applied voltage of 16.5 kV was chosen for subsequent experiments since it gave satisfactory separation and short migration times for all the peaks.

In summary, the optimum separation conditions based on the criteria chosen were determined as: applied voltage of 16.5 kV, a buffer solution containing 50mM sodium tetraborate (pH 9.3) and 55% acetonitrile (ratio of acetonitrile/buffer = 55/45, V/V). The electropherograms of stevia sweeteners obtained using these conditions are shown in Fig. 7. The peaks in the electropherograms were identified by comparing their capacity factors with those of the individual compounds, as shown in Table 5. It was noted that the migration order of stevia sweeteners in CE was very different from that in HPLC, as expected on the basis of different separation mechanisms in the two methods.

Determination of the purity of stevioside

Calibration graphs of HPLC and CE methods (peak-area ratio, y, vs concentration, x mg/ml) were constructed in the range 0.010-2.000 mg/ml for stevioside (The detection limit of the CE system was 0.010 mg/ml). The regression equations of the curves and their correlation coefficients were calculated as following:

HPLC: $y = 148.91x - 1.36$ $r = 0.9996$

CE: $y = 66795.68x - 2370.38$ $r = 0.9936$

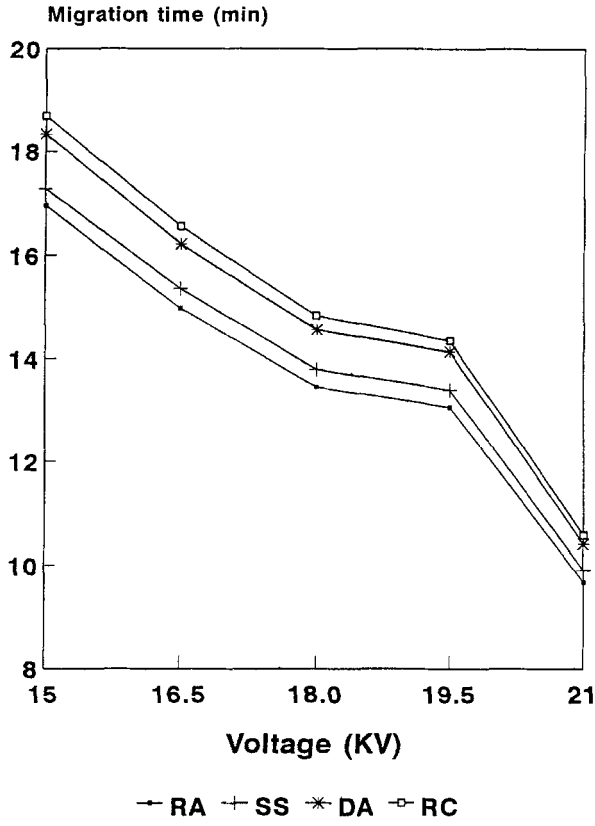


Fig.6. Influence of voltage on migration times of stevia sweeteners in CE system. Buffer, 50 mM sodium tetraborate/acetonitrile; voltage ranging from 15 to 21 kV; other conditions as in Fig. 3.

TABLE 4
Effect of Voltage on Resolution

Voltage (KV)	15	16.5	18	19.5	21
Resolution (SS/RA)	1.07	1.04	0.98	0.80	0.84
Resolution (DA/SS)	2.36	2.24	2.18	2.09	1.58
Resolution (RC/DA)	1.19	1.13	1.02	0.84	0.81

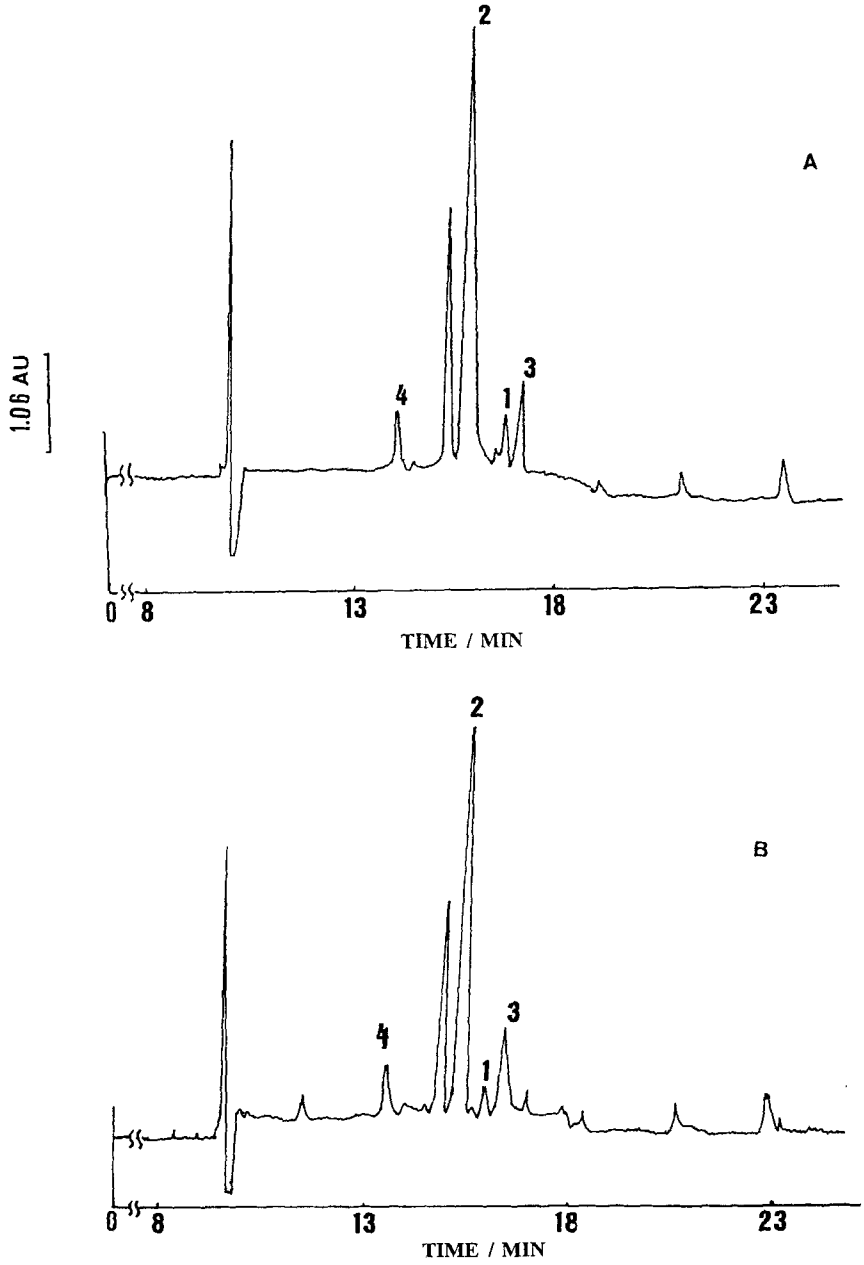


Fig.7. Capillary electropherograms of stevia sweeteners (A: sample 1, B: sample 2). Buffer, 50 mM sodium tetraborate/acetontrile (55/45, V/V); other conditions as in Fig. 3. Peak numbers as in Fig. 2.

TABLE 5
Capacity Factors of the Peaks in Capillary Electropherogram of Stevia Sweeteners (n=4)

	RA	SS	DA	RC
Individual glycosides	0.366±0.002	0.385±0.004	0.439±0.003	0.450±0.003
Sample 1	0.363±0.001	0.398±0.001	0.441±0.001	0.458±0.002
Sample 2	0.363±0.001	0.401±0.002	0.442±0.001	0.461±0.001

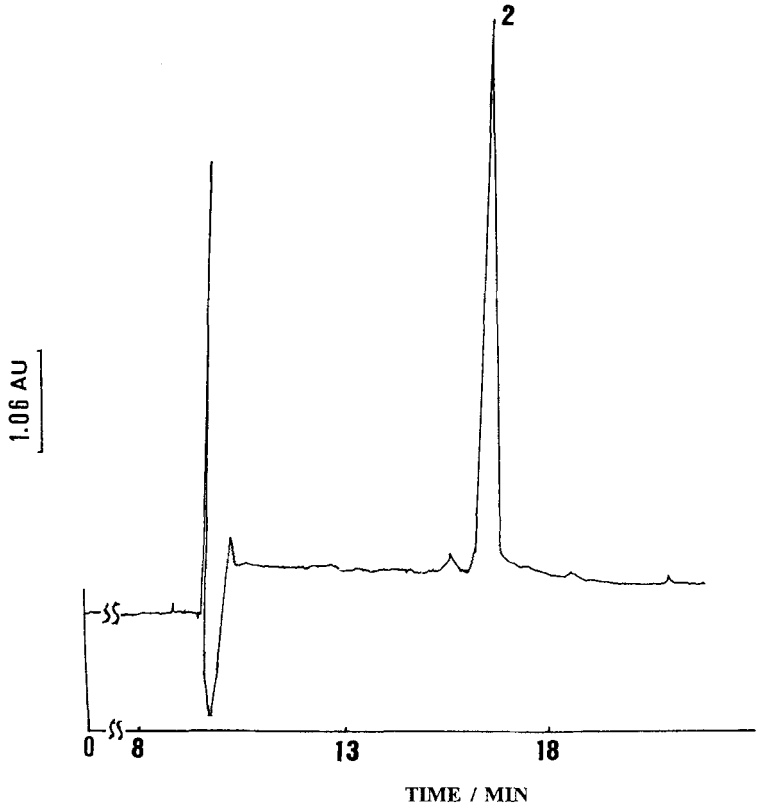


Fig.8. Capillary electropherogram of recrystallized stevioside. Conditions as in Fig. 7.

TABLE 6
Purity (%) of Stevioside in Real Samples (n=4)

	Sample 1	Sample 2	Recrystallized
HPLC	46.5±0.9	45.3±0.6	91.2±1.2
CE	46.2±1.3	45.8±1.8	91.8±1.8

The stevia sweeteners products and recrystallized stevioside were analyzed by capillary electrophoresis using the optimum conditions. The results are illustrated in Figure 7 and Figure 8. By substituting the area ratios of these peaks for y in the above equations, the content of stevioside in the samples were obtained as shown in Table 6. It was found that the results obtained by CE and HPLC were in very good agreement.

CONCLUSION

In this study, the separation and determination of stevia sweeteners by capillary electrophoresis was successfully demonstrated. Optimum conditions for the separation of the sweeteners were obtained. The results were consistent with those obtained using HPLC. Although the CE method has the advantage that it required only very small amounts of samples and little solvent usage, the HPLC method could be used successfully to obtain individual steviol glycosides by fraction collection. Both methods allowed simple, rapid and accurate analysis of the four steviol glycosides. Since the separation mechanisms are different in the two methods, the CE method can be used as an alternative analytical procedure to HPLC when the amount of sample available is small, or in an orthogonal manner to provide additional information.

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REFERENCES

1. K.C. Phillips in T.H.Grenby (Editor), Developments in Sweeteners-3, Elsevier Applied Science, London & New York, 1987, Ch.1, p.1.

2. B.Crammer and R.Ikan, in T.H.Grenby (Editor), Developments in Sweeteners-3, Elsevier Applied Science, London & New York, 1987, Ch.2, p.45.

3. Y. Kitada, M. Sasaki, Y. Yamazoe, J. Chromatogr., 474, 447-451, (1989)

4. S. Hoffstetter-Kuhn, A.Paulus, E.Gassmann, H.M.Widmer, Anal. Chem., 16, 1541-1547, (1991)

5. S. Honda, S.Iwasw, A. Makino, S. Fujiwara, Anal. Biochem., 176, 72, (1989)

6. P. Pietta, A. Bruno, J. Chromatogr., 593, 165-170, (1992)

7. C. Bjergegaard, S. Michaelsen, K. Mortensen & H. Sornsen, J. Chromatogr., 652, 477-485, (1993)

8. C.P. Ong, S.F. Pang, S.P. Low, H.K. Lee, S.F.Y. Li, J. Chromatogr., 559, 529-536, (1991)

9. S.F.Y.Li, Capillary Electropherosis: principles, practice and application, Elsevier, Amsterdam-London-New York-Tokyo, 1992, Ch.5, p.21

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