# 行政院國家科學委員會補助專題研究計畫成果報告

## 毛細管電泳法對抗壞血酸及異抗壞血酸之表異購物分離及蔬果中總抗壞血酸含量之定量

## Enantiomeric Separation of L-Ascorbic Acid and D-Isoascorbic Acid and Quantitative Analysis of Total Ascorbic Acid in Vegetables and Fruiots by Capillary Zone Electrophoresis

計畫類別：☒個別型計畫　　□整合型計畫
計畫編號：NSC 89-2313-B-002-090
執行期限：88 年 8 月 1 日至 89 年 7 月 31 日
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執行單位：台灣大學食品科技研究所

中　華　民　國　89　年　9　月　20　日

**Table 4. Parameters of Linear Regressions on Average Values ($n$ = 6), Obtained for Each Sample Analyzed by Standard Addition Method, and Comparison to the Mean of Six ELISA Determinations on Nonspiked Samples**

| | standard addition method | | | direct analysis |
|---|---|---|---|---|
| sample | slope | intercept (ng/mL) | correl coeff | amount determined[a] (ng/mL) |
| Dolcetto 2, 14% vol | 1.04 | 11.6 | 0.99 | 12.1 ± 0.5 |
| Barbera, 12.5% vol | 1.08 | 6.4 | 0.99 | 6.0 ± 0.6 |
| Nebbiolo, 12% vol | 1.01 | 3.4 | 0.99 | 3.6 ± 0.2 |
| Grignolino, 12% vol | 1.12 | 1.7 | 0.99 | 1.5 ± 0.3 |
| Brachetto, 11% vol | 1.02 | 4.5 | 0.99 | 4.5 ± 0.4 |
| Lambrusco, 10% vol | 0.89 | 0.01 | 0.99 | 0.4 ± 0.1 |

[a] Mean ± standard deviation.

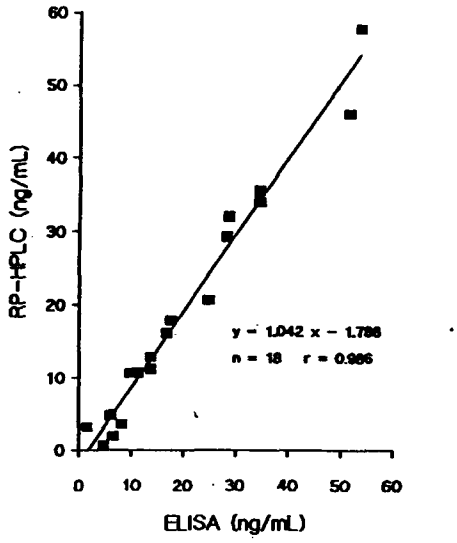


**Figure 3.** Correlation of RP-HPLC versus ELISA results for red wine samples spiked with benalaxyl.

benalaxyl concentration determined by the standard addition method and the benalaxyl concentration determined by ELISA direct analysis attests to the reliability of both the cleanup step and the ELISA developed for the determination of benalaxyl in red wine. It is important to emphasize that ELISA direct analysis is better than the standard addition method because it provides a more precise measurement.

Data obtained by recovery experiments on spiked and unspiked samples show that the coefficient of variation on a single sample varies from 4 to 10% in a range between ~3 and ~15 ng/mL and from 10 to 20% of the first considered range.

To confirm the reliability of ELISA analysis, some of the same spiked samples were analyzed also by RP-HPLC after the cleanup step. Correlation of RP-HPLC versus ELISA results is reported in Figure 3. It is evident that ELISA yields comparable values and reliable information about the degree of contamination in wine samples. The worst agreement between the two techniques, at a benalaxyl concentration <10 ng/mL, is attributable to the imprecision of HPLC determination at concentrations lower than the sensitivity of the chromatographic technique. The most remarkable aspect is the good detection of benalaxyl residues by ELISA at very low levels (0.5 ng/mL), whereas at this concentration no detection was achieved by RP-HPLC. Although ELISAs are generally less precise than chromatographic methods, they represent a feasible alternative to conventional analytical techniques for determination of agrochemical residues in food supplies, thanks to their higher sensitivity, rapidity, and lower expenses.

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Received for review February 4, 1999. Revised manuscript received September 21, 1999. Accepted October 13, 1999.

JF9901183

# Epimeric Separation of L-Ascorbic Acid and D-Isoascorbic Acid by Capillary Zone Electrophoresis

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Capillary zone electrophoresis (CZE) was used for separation of L-ascorbic acid (L-AA) and D-isoascorbic acid (D-IAA) in a model system. The effects of borate buffer concentration (0.05–0.25 M) and pH (pH 7.5–9.0) on migration time, resolution (Rs), and theoretical plates ($N$) were investigated. The migration times of L-AA and D-IAA increased with the increasing pH of carrier electrolyte (0.2 borate buffer), and the resolutions (Rs) of L-AA and D-IAA were calculated to be 12.98 at pH 9.0. Concentrations of borate buffer (pH 9.0) increased the Rs values of L-AA and D-IAA, and buffer concentrations >0.1 M were found to be effective for separation of L-AA and D-IAA. Methanol in the carrier electrolyte was also influential in improving the separation of L-AA and D-IAA, which increased with the increasing concentrations (0–10%) of methanol. The optimal separation conditions for L-AA and D-IAA were as follows: carrier electrolyte, 0.2 M borate buffer (pH 9.0); applied voltage, 25 kV, with an uncoated fused silica capillary, 75 μm (i.d.) × 57 cm.

**Keywords:** *Epimeric separation; L-ascorbic acid (L-AA); D-isoascorbic acid (D-IAA); capillary zone electrophoresis (CZE)*

## INTRODUCTION

Most vegetables and fruits contain abundant vitamin C; however, vitamin C is not stable and is liable to degradation when exposed to certain factors including light, temperature, heat, metal ions, oxygen, enzymes, etc. (Clegg, 1966; Clegg and Morton, 1965; Finholt et al., 1963). The fast degradation of vitamin C caused by the above factors results in the difficulty of precise quantitative determination of L-ascorbic acid (L-AA) with conventional methods. In addition, D-isoascorbic acid (D-IAA), the $C_5$ epimer of L-AA, displaying ~10% of the bioactivity of L-AA, is usually added to foods for nonvitamin purposes. However, the stereochemical structures and properties of L-AA and D-IAA are so close that the quantitative analysis of L-AA in food systems with D-IAA additives is difficult. On the basis of these two facts, rapid epimeric analysis of L-AA is of particular interest in the food industry. There are several highly sensitive high-performance liquid chromatography (HPLC) methods for the direct measurement of L-AA in foods and biological fluids (Pachla et al., 1985; Lloyd et al., 1988a). Some of these methods are also able to quantify D-IAA (Vanderslice and Higgs, 1988, 1990; Kutnink et al., 1985; Lloyd et al., 1988b).

Capillary electrophoresis has become a powerful and popular separation technique because of the fast separation and high resolution achieved. Compared with HPLC in the separation of chiral or epimeric compounds, capillary electrophoresis has the advantages of high separation efficiency, easy changes of carrier electrolyte media, and nanoliter levels of sample and media (Tsao and Salimi, 1982; Kutnink et al., 1985; Lloyd et al., 1988a; Bilic, 1991; Tsai et al., 1998). Capillary zone electrophoresis (CZE) is based on the difference of electrophoretic mobility that results from the varied charge numbers and particle sizes between electrolytes under applied voltage.

In the present research, L-AA and D-IAA were selected to be separated by CZE in a model system to investigate the effects of three factors—carrier electrolyte buffers, buffer concentration, and pH—on migration time, resolution (Rs) value, and electrophoretic mobility ($\mu$) of L-AA, D-IAA, and electroosmotic flow (EOF). In addition, the separation conditions were to be optimized. Furthermore, the relationships between organic modifiers such as methanol, or applied voltage, and the extent of separation are also discussed herein.

## MATERIALS AND METHODS

**Chemicals.** L-AA, D-IAA, boric acid, sodium borate, pyridine, and methanol were purchased from Sigma (St. Louis, MO).

Adequate amounts (100 μg/mL) of L-AA and D-IAA were dissolved in deionized water, which was obtained from a Mili-Q system (Millipore, Japan).

**Apparatus and Electrophoretic Conditions.** All of the experiments were carried out on a capillary electrophoresis instrument P/ACE system 5500 (Beckman, Palo Alto, CA), equipped with a diode array detector monitoring a wavelength of 254 nm (Chiari and Nesi, 1993; Koh et al., 1993; Marshall et al., 1995). An uncoated fused silica capillary (Beckman; total length = 57 cm, effective length = 50 cm, i.d. = 75 μm) was pretreated successively with 0.1 M hydrochloric acid and 0.1 M sodium hydroxide for 10 min each and then rinsed with deionized water and carrier electrolyte solution prior to use. The separation column was kept at a constant temperature of 25.0 ± 0.1 °C by means of a fluorocarbon liquid continuously

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10.1021/jf990399e CCC: $19.00 
Published on Web 12/14/1999

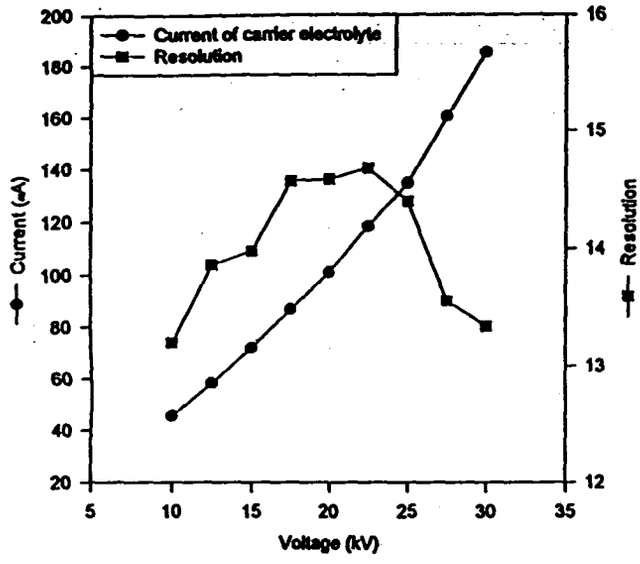


**Figure 1.** Effect of applied voltage on the current ($\mu$A) of carrier electrolyte and the Rs between L-AA and D-IAA. Separation conditions: carrier electrolyte, 0.2 M borate buffer (pH 9.0); fused silica capillary, 75 $\mu$m × 57 cm (50 cm to detector); separation temperature, 25.0 ± 0.1 °C; wavelength, 254 nm. $Rs = 1.18(t_{IAA} - t_{AA})/(w_{1/2(AA)} + w_{1/2(IAA)})$, where $t_{AA}$ and $t_{IAA}$ are the migration times of L-AA and D-IAA, respectively, and $w_{1/2(AA)}$ and $w_{1/2(IAA)}$ are the peak widths at half peak height of L-AA and D-IAA, respectively.

circulated through the cartridge, and the applied voltage was 25 kV. Sample introduction was performed using the pressure option for 3 s. Data collection was carried out with Gold Chromatography data system version 8.1.

The compositions of carrier electrolytes were 0.05−0.25 M borate buffer (pH 7.5−9.0). Buffers were filtered through a 0.45 $\mu$m membrane prior to use. Deionized water was obtained from a Mili-Q system (Millipore, Japan).

The pertinent parameters, electrophoretic mobility of electroosmotic flow ($\mu_{EOF}$), L-AA ($\mu_{AA}$), and D-IAA ($\mu_{IAA}$), resolution (Rs) of L-AA and D-IAA, and theoretical plates ($N$), were all calculated in the accepted manner (Kuhn and Hoffstetter-Kuhn, 1993; Baker, 1995). Rs value was used to expressed the separation results of the enantiomers (Wan et al., 1995). Pyridine was used as the EOF indicator to determine $\mu_{EOF}$.

## RESULTS AND DISCUSSION

**Applied Voltage.** Applied voltages were changed to conduct the epimeric separation of L-AA and D-IAA using 0.2 M borate buffer (pH 9.0) as carrier electrolyte. Initially, the increase of current (microamperes) produced was linearly proportional to the increase of applied voltage (kilovolts), ranging from 10 to 22.5 kV (Figure 1), which was consistent with Ohm's law. However, this linear relationship was not maintained and the current curve apparently moved upward when the applied voltage was >22.5 kV. The increase in current possibly resulted from heat accumulation caused at higher voltages, leading to the reduction of electric resistance in the carrier electrolyte. On the other hand, the Rs value reached maximum when the applied voltage was 22.5 kV, suggesting that higher voltage (within the voltage range of Ohm's law) was more effective in improving the resolution of analytes. This result could be due to the shortening effect of high voltage on peak width of analytes. Bjergegaard et al. (1992) and Akbay et al. (1997) have indicated that the migration times of analytes and EOF were shortened and the separation efficiency was increased at an increased voltage.

**Table 1. Effect of Electric Field on the Migration Time ($t$, min) and Mobility ($\mu$, × $10^4$ $cm^2$ $V^{-1}$ $S^{-1}$)[a] of EOF, L-AA, and D-IAA**

| voltage (kV) | $t_{EOF}$ | $t_{AA}$ | $t_{IAA}$ | $\mu_{EOF}$ | $\mu_{AA}$ | $\mu_{IAA}$ |
|---|---|---|---|---|---|---|
| 10.0 | 11.4 | 27.33 | 31.81 | 4.17 | −2.43 | −2.67 |
| 12.5 | 8.93 | 21.28 | 24.72 | 4.25 | −2.47 | −2.72 |
| 15.0 | 7.26 | 17.18 | 19.83 | 4.36 | −2.52 | −2.76 |
| 17.5 | 6.05 | 14.15 | 16.33 | 4.49 | −2.57 | −2.83 |
| 20.0 | 5.13 | 12.00 | 13.79 | 4.63 | −2.65 | −2.90 |
| 22.5 | 4.41 | 10.24 | 11.75 | 4.78 | −2.72 | −2.99 |
| 25.0 | 3.83 | 8.74 | 9.97 | 4.96 | −2.79 | −3.05 |
| 27.5 | 3.35 | 7.69 | 8.77 | 5.16 | −2.92 | −3.19 |
| 30.0 | 2.97 | 6.79 | 7.72 | 5.33 | −3.00 | −3.28 |

[a] $\mu_{EOF} = 1/t_{EOF} \times L/V$; $\mu_{AA} = [(1/t_{AA}) - (1/t_{EOF})] \times (L/V)$; $\mu_{IAA} = [(1/t_{IAA}) - (1/t_{EOF})] \times (L/V)$, where $l$ is the effective length of capillary, $L$ is the total length of capillary, and $V$ is the applied voltage.

Table 1 presents the effects of electric field on the migration time and mobility of EOF, L-AA, and D-IAA. It is clear that the increase in electric field enhanced the increase in $\mu_{EOF}$, $\mu_{AA}$, and $\mu_{IAA}$. However, the increase in $\mu_{EOF}$ (toward the cathode) was much larger than that in $\mu_{AA}$ and $\mu_{IAA}$ (toward the anode), thus apparently shortening the migration times of L-AA and D-IAA. For example, the migration times for L-AA were 27.33 and 6.79 min and for D-IAA were 31.81 and 7.72 min when the applied voltages were 10 and 30 kV, respectively.

**pH of Carrier Electrolyte.** The pH of the solution affects the ionization of carrier electrolyte, and the importance of pH for the separation results of L-AA and D-IAA needed clarification. At pH 7.5, the analytes' peaks almost overlapped and only two peaks were observed (Figure 2). At the increased pH, two analytes were completely separated at the baseline, but the peak shape of D-IAA was clearly not symmetrical at pH 8.0. The peak shape of D-IAA was apparently more symmetrical when epimeric separation was conducted at pH >8.5. On the other hand, the migration times for L-AA were 5.18 and 8.32 min and for D-IAA were 5.33 and 9.37 min when the pH values of the carrier electrolyte were 8.0 and 9.0, respectively. Most importantly, the Rs value for these two epimeric compounds increased from 2.29 to 12.98 when separation was conducted at the higher pH of 9.0. The ionization of L-AA and D-IAA ($pK_1 = 4.04$; $pK_2 = 11.4$) increased at the higher carrier electrolyte pH. $\mu_{AA}$, $\mu_{IAA}$, and separation efficiency increased (Table 2) at the higher pH, demonstrating the importance of the carrier electrolyte pH for the separation results. In the present study, 0.2 M borate buffers (at pH 8.0, 8.5, and 9.0) were used as carrier electrolytes and $\mu_{EOF}$ was investigated. It is noteworthy that $\mu_{EOF}$ was reduced when separation was conducted at the increased carrier electrolyte pH. Such results were considered to be due to the increase in the ionic strength in carrier electrolyte with an elevated pH, resulting from the stronger ionization of sodium borate. Similar results were also reported by Knox (1994) that the increase in the ionic strength reduced $\mu_{EOF}$.

**Concentration of Carrier Electrolyte.** Higher concentration of carrier electrolyte buffer solution has been shown to reduce the zeta potential, electrical double layer, and $\mu_{EOF}$ (Knox, 1994). In addition, the adsorption of electrolytes on capillary walls could be effectively prevented, thus increasing the separation results of analytes (Burgi and Chien, 1991). In the

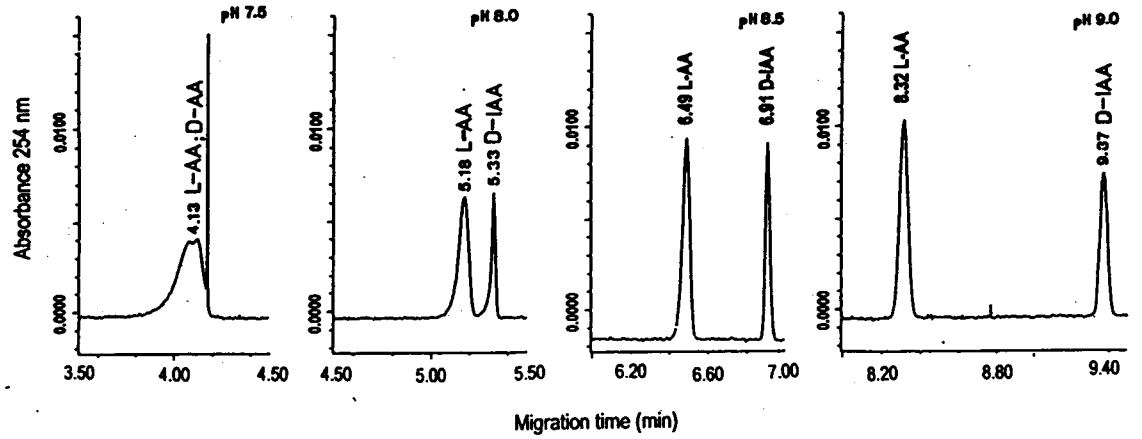


**Figure 2.** Influence of pH of carrier electrolyte on the epimeric separation of L-AA and D-IAA. Conditions: carrier electrolyte, 0.2 M borate buffer; applied voltage, 25 kV. The other experimental conditions were the same as in Figure 1.

**Table 2. Effects of the pH of 0.2 M Borate Buffer on Migration Time ($t$, min), Mobility ($\mu$, $\times 10^4$ cm$^2$ V$^{-1}$ S$^{-1}$),[a] Theoretical Plate ($N$),[b] and Resolution (Rs)[c] of EOF, L-AA, and D-IAA ($n$ = 3)**

| | | | | | | | $N$ | | |
|---|---|---|---|---|---|---|---|---|---|
| pH | $t_{EOF}$ | $t_{AA}$ | $t_{IAA}$ | $\mu_{EOF}$ | $\mu_{AA}$ | $\mu_{IAA}$ | L-AA | D-IAA | Rs |
| 8.0 | 3.12 | 5.18 | 5.33 | 6.10 | −2.43 | −2.54 | 46000 | 301000 | 2.29 |
| 8.5 | 3.46 | 6.49 | 6.91 | 5.49 | −2.56 | −2.74 | 129000 | 363000 | 7.08 |
| 9.0 | 3.75 | 8.32 | 9.37 | 5.07 | −2.78 | −3.04 | 162000 | 235000 | 12.98 |

[a] See footnote *a* of Table 1. [b] $N = 5.54(t_m/w_{1/2})^2$, where $w_{1/2}$ is the peak width at half peak height of peak L-AA or D-IAA; $t_m$ is the migration time of L-AA or D-IAA. [c] See Figure 1.

present research, when 0.05 M borate buffer (pH 9.0) was used as carrier electrolyte, peaks of L-AA and D-IAA did not achieve the baseline separation (Figure 3). The increased concentration of borate distinctly improved the separation results of L-AA and D-IAA. However, the migration times of both analytes were also increased when the separation was conducted at higher concentrations. When separation was conducted with 0.1 and 0.25 M borate buffer (pH 9.0), the migration times for L-AA were 5.31 and 9.83 min and for D-IAA were 5.65 and 11.42 min, respectively (Figure 3). The migration time of EOF, determined by using pyridine as the EOF indicator, also increased when epimeric separation was conducted at higher concentrations of borate buffer. Similar results were reported by Bruin et al. (1989) using $\beta$-naphthol as the EOF indicator and phosphate buffer as carrier electrolyte. However, the Rs value of L-AA and D-IAA increased from 0.85 to 18.53 in 0.05−0.25 M borate buffer (pH 9.0), indicating that the resolution of analytes is remarkably influenced by the concentration or ionic strength of carrier electrolyte. $\mu_{AA}$ and $\mu_{IAA}$ also increased with the higher concentrations of borate buffer (Table 3). This could be due to the possible complex formation between borate and the electrolyte. Hoffstetter-Kuhn et al. (1991) indicated that borate is liable to form complexes with polyols, especially with those possessing cis diol groups. The increase in $\mu_{AA}$ and $\mu_{IAA}$ (toward the cathode) at high concentrations of carrier electrolyte was less than that in $\mu_{EOF}$ (toward the anode). Thus, the migration times of L-AA and D-IAA all increased at higher concentrations of carrier electrolyte (Table 3).

**Concentration of Methanol.** Addition of organic modifiers has been shown to reduce the zeta potential of the electrical double layer in the capillary wall and to change the properties of enantiomers, such as their

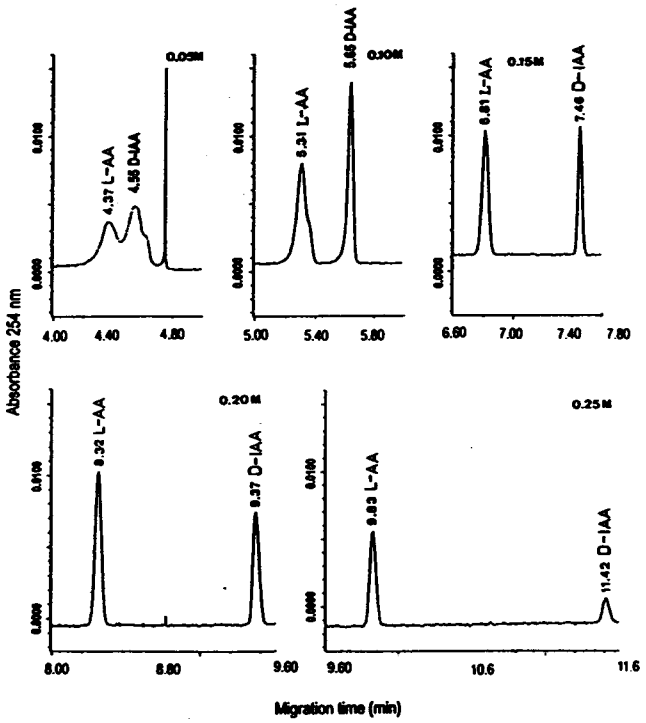


**Figure 3.** Influence of the concentration of carrier electrolyte on the epimeric separation of L-AA and D-IAA: (A) 0.05 M, (B) 0.1 M, (C) 0.15 M, (D) 0.2 M, and (E) 0.25 M borate buffer (pH 9.0) were used as carrier electrolyte. Applied voltage was 25 kV. The other experimental conditions were the same as in Figure 1.

hydrophobic and hydrophilic properties (Wang and Warner, 1995; Matchett et al., 1995), thus modifying the separation result of analytes. In addition, organic modifiers generally decrease the ionization of carrier electrolytes, resulting in the changes of pH value and ionic strength in the carrier electrolytes (Harrold et al., 1993). Figure 4 presents the separation results for the addition of 5 and 10% of methanol to the 0.1 M borate buffer (pH 8.5). It can be seen that the separation of L-AA and D-IAA was improved by the increased level of methanol and that the Rs value increased from 0.92 to 1.66 when epimeric separation was conducted using 10% methanol/0.1 M borate buffer (pH 8.5) as carrier electrolyte (Table 4). Moreover, the migration times for EOF, L-AA, and D-IAA were all prolonged with the higher concentrations of methanol, indicating that $\mu_{EOF}$, $\mu_{AA}$, and $\mu_{IAA}$ were reduced by the addition of methanol

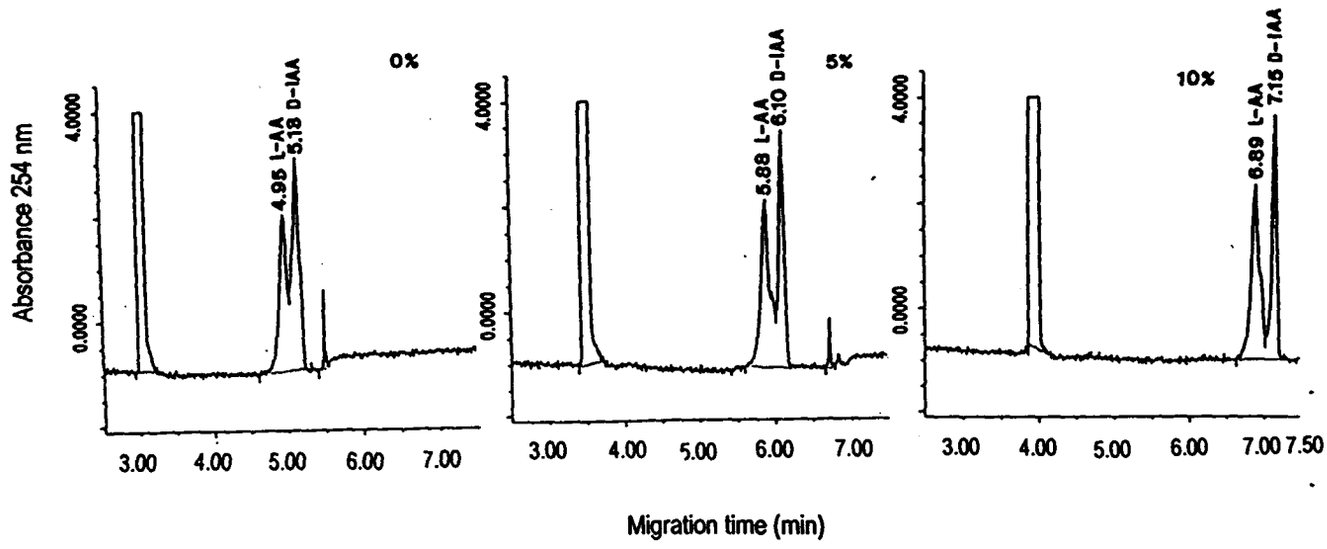


**Figure 4.** Effect of the concentration of methanol in carrier electrolyte on the epimeric separation of L-AA and D-IAA. Conditions: carrier electrolyte, 0.1 M borate buffer (pH 8.5); applied voltage, 25 kV.

**Table 3. Effects of Concentrations of Borate Buffer (pH 9.0) on Migration Time ($t$), Mobility ($\mu$),[a] Theoretical Plate ($N$),[b] and Resolution (Rs)[c] of EOF, L-AA, and D-IAA (Applied Voltage =25 kV; $n$ = 3)**

| concn (M) | $t_{EOF}$ | $t_{AA}$ | $t_{IAA}$ | $\mu_{EOF}$ | $\mu_{AA}$ | $\mu_{IAA}$ | $N$ L-AA | $N$ D-IAA | Rs |
|---|---|---|---|---|---|---|---|---|---|
| 0.05 | 2.80 | 4.37 | 4.55 | 6.78 | −2.43 | −2.60 | 10000 | 12000 | 0.85 |
| 0.10 | 3.10 | 5.31 | 5.65 | 6.14 | −2.56 | −2.77 | 43000 | 145000 | 3.69 |
| 0.15 | 3.48 | 6.81 | 7.46 | 5.46 | −2.67 | −2.91 | 114000 | 292000 | 9.59 |
| 0.20 | 3.75 | 8.32 | 9.37 | 5.07 | −2.78 | −3.04 | 162000 | 235000 | 12.98 |
| 0.25 | 3.96 | 9.83 | 11.42 | 4.79 | −2.86 | −3.13 | 222000 | 240000 | 18.53 |

[a] See footnote *a* in Table 1. [b] See footnote *b* in Table 2. [c] See Figure 1.

**Table 4. Effects of Concentrations of Methanol in Carrier Electrolyte on Migration Time ($t$), Mobility ($\mu$),[a] Theoretical Plate ($N$),[b] and Resolution (Rs)[c] of EOF, L-AA, and D-IAA [Carrier Electrolyte = 0.1 M Borate Buffer (pH 8.5); Applied Voltage = 25 kV; $n$ = 3]**

| methanol (%) | $t_{EOF}$ | $t_{AA}$ | $t_{IAA}$ | $\mu_{EOF}$ | $\mu_{AA}$ | $\mu_{IAA}$ | $N$ L-AA | $N$ D-IAA | Rs |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 3.03 | 4.95 | 5.13 | 6.28 | −2.44 | −2.58 | 10256 | 11016 | 0.92 |
| 5 | 3.48 | 5.88 | 6.10 | 5.47 | −2.34 | −2.35 | 17065 | 34780 | 1.41 |
| 10 | 3.92 | 6.89 | 7.15 | 4.85 | −2.09 | −2.19 | 20990 | 56199 | 1.66 |

[a] See footnote *a* in Table 1. [b] See footnote *b* in Table 2. [c] See Figure 1.

(Table 4). The increase in migration time is partly due to the reduction in the ratio of dielectric constant to viscosity, which results in reductions of both the zeta potential and the EOF (Harrold et al., 1993). However, addition of methanol in the carrier electrolyte did not appear to be beneficial in the separation of L-AA and D-IAA as a result of the much lower Rs value than that in Table 3.

**Conclusion.** Ascorbic acid is sensitive to light, oxygen, heat, metal ions, etc., and the conventional quantitative methods appear to be unable to obtain the precise value when ascorbic acid is exposed to the surrounding sensitive factors. D-IAA, an antioxidant for food use, is usually added to soft drinks and juices and could be a hindrance for ascorbic acid quantification. In the present study, CZE was used to develop a rapid method for the epimeric separation of L-AA and D-IAA, and the optimal separation conditions were determined in a model system. Applied voltage, pH, and concentration of carrier electrolyte were all found to be influential on the separation of L-AA and D-IAA. Under optimal conditions, L-AA and D-IAA could be separated in <10 min in a model system. The thus developed qualitative analysis for L-AA and D-IAA could contribute to the food industry.

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Received for review April 23, 1999. Accepted October 22, 1999. Financial support for this study from the National Science Council of the Republic of China under Grant NSC-88-2321-B-002-051 is greatly appreciated.

JF990399E