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# Determination of 2-keto-L-gulonic, 2-keto-D-gluconic and 2,5-diketo-D-gluconic acids by capillary zone electrophoresis

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**Abstract**

During the biosynthetic processing of 2-keto-L-gulonic acid (2-KLG) from 2,5-diketo-D-gluconic acid (2,5-DKG) by *Corynebacterium* sp., 2-keto-D-gluconic acid (2-KDG) is produced as a byproduct. These organic acids have been analyzed by high-performance liquid chromatography (HPLC) on an Aminex HPX-87H column, but the resolution was not good enough for quantitative analysis. We investigated the quantitation of 2-KLG, 2-KDG and 2,5-DKG using capillary electrophoresis (CE) and the results were compared with those of HPLC. With CE, in contrast to HPLC, good resolution, efficiency and rapid analysis were demonstrated as well as low consumption of solvent and samples. The CE system was applied at 15 kV with UV detection at 195 nm using 100 m*M* sodium borate (pH 8.4) as an electrolyte. The results were shown within 5 min with efficiency approaching 100 000 theoretical plates. The relative standard deviations of migration time and peak area were less than 0.9% and 1.6%, respectively. The detection limits for quantitative determination were 0.5–1.3 $\mu M$ level. The above compounds, in fermentation broth, were analyzed under the optimum conditions. Considering the results of our study, the CE method should be highly suitable for the separation of 2-KLG, 2-KDG and 2,5-DKG in the fermentation broth.

*Keywords:* Fermentation broth; Ketogulonic acid; Organic acids; Ketogluconic acids

## 1. Introduction

The ascorbic acid (vitamin C) has received considerable attention over many years due to its commercial value for human health as a vitamin [1]. Only 2-keto-L-gulonic acid (2-KLG) is a key intermediate in vitamin C synthesis. There are two methods to produce this organic acid, a stable compound, which can be readily and efficiently converted into vitamin C by a simple chemical procedure.

One is a modified route of the Reichstein-Grussner synthesis that involves the chemical reduction of D-glucose to D-sorbitol, followed by a microbial oxidation to L-sorbose, which is then protected by acetonization, chemically oxidized at $C_1$, and deprotected to give 2-KLG [2]. At present most vitamin C is produced by this almost chemical method. Over the past three decades many efforts have been made to find other efficient and economical routes using microbial methods for preparing 2-KLG. An important advance in this method was the discovery of microorganisms, *Corynebacterium* sp., that were capable of carrying out the stereospecific reduction of 2,5-diketo-D-gluconic acid (2,5-DKG) to 2-KLG [3,4].

During the course of this process 2-keto-D-gluconic acid (2-KDG), the stereoisomer of 2-KLG,

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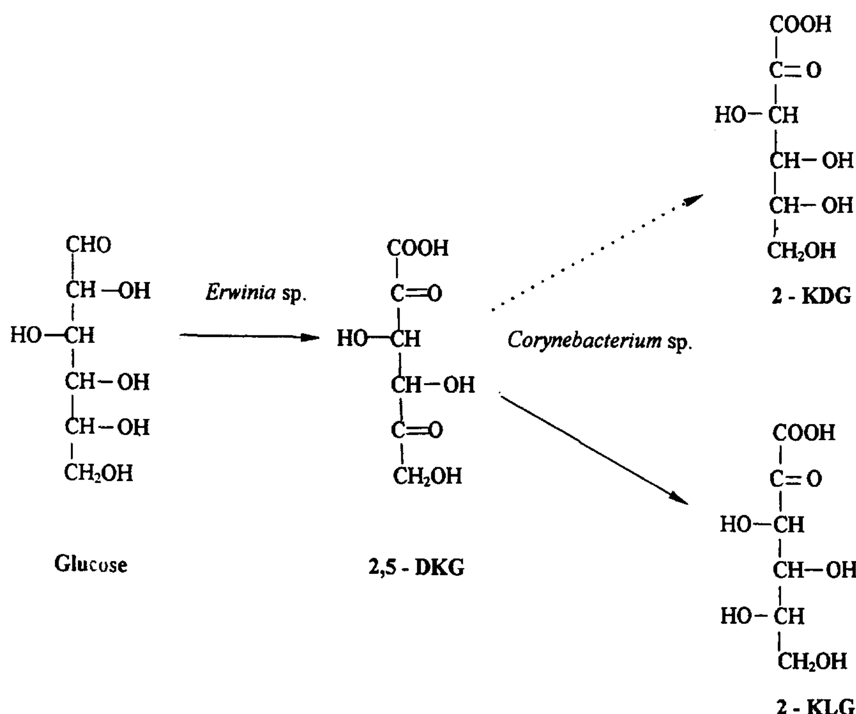


Fig. 1. Simple scheme indicating the transformation of glucose to 2-keto-L-gulonic acid.

might be accumulated simultaneously with 2-KLG from other organic acids such as 2-KDG and 2,5-DKG in the fermentation broth (Fig. 1). We have applied high-performance liquid chromatography (HPLC) with a column packed with sulfonated polystyrene–divinylbenzene copolymer resin to the analysis of these organic acids [5]. This method often delivers only 1000–2000 theoretical plates yielding relatively overlapping peaks, long run time and poor resolution.

Capillary electrophoresis (CE) exhibits several unique characteristics that make it an excellent technique for the analysis of complicated biological samples, and these have led to its rapid development over the past 15 years [6–9]. It is a powerful micro-analytical separation technique that combines the advantages of HPLC and conventional electrophoresis. Also, its high resolving power, simplicity of operation and versatility make it an important analytical tool in pharmaceutical, clinical and food analysis [10–14].

In this paper, we present the procedure of CE which separated stereoisomeric organic acids that are formed in the fermentation process for synthesis of ascorbic acid and compare it with an HPLC method.

## 2. Experimental

### 2.1. Reagents and the sample preparation

The 2-KLG was prepared from the monohydrates of diacetone gulonic acid by acid hydrolysis [15]. Calcium 2-KDG was purchased from Sigma (St. Louis, MO, USA). Calcium 2,5-DKG was obtained from D-glucose as the oxidation product of *Erwinia* sp. (ATCC 31626) by the method of Sonoyama et al. [16]. Calcium 2-KLG fermentation broth was prepared by incubating the strain MWS 43821, a mutant derived from *Corynebacterium* sp. (ATCC 31090), on 2,5-DKG culture broth. Sodium borate and sodium hydroxide were obtained from Beckman (Fullerton, CA, USA). Phosphate was purchased from Fluka (Buchs, Switzerland).

### 2.2. Apparatus and method

A CE system equipped with a P/ACE 5500 diode array detector, automatic injector, a fluid cooled column cartridge and a system Gold data station (Beckman) was used in this study. All runs were carried out at 20°C. The electrolyte was passed through 0.2 μm nylon filers and degassed prior to use. The capillary inlet and outlet vial were replenished after every run. Injections were made using the pressure mode for 5 s at $3.45 \cdot 10^{-3}$ MPa. Detection was performed at a wavelength of UV 195 nm. A 47 cm length of a 75 μm I.D. fused-silica capillary was rinsed with water and filled with electrolyte for 2 min prior to sample injection.

HPLC was performed using a Model HP 1050 series dual pump (Hewlett-Packard, Basel, Switzerland) and a Model Waters 484 detector (Millipore, Milford, MA, USA). The column (300 mm×7.8 mm I.D.) was Aminex HPX-87H (Bio-Rad, Richmond, CA, USA) packed with strong cation-exchange resin. The mobile phase consisted of 0.006 $M$ $H_2SO_4$. The flow-rate was 0.6 ml/min at 65°C.

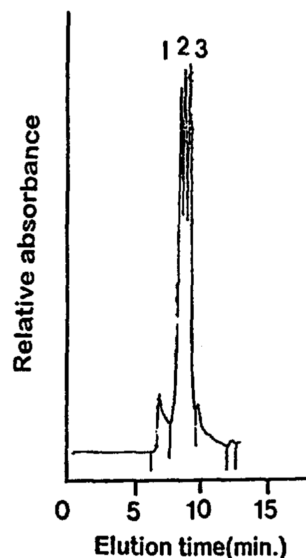


Fig. 2. HPLC chromatogram of organic acids. Peaks: (1) 2,5-diketo-D-gluconic acid; (2) 2-keto-L-gulonic acid and (3) 2-keto-D-gluconic acid. Mobile phase: 0.006 $M$ $H_2SO_4$. Flow-rate: 0.6 ml/min. Column: 300 mm×7.8 mm I.D. Aminex HPX-87H. Temperature: 65°C. Detection wavelength: 210 nm. Injection: 50 μl.

## 3. Results and discussion

Currently, the general instrumental method for the determination of these stereoisomer components is HPLC with a cation-exchange column. Prior to the application of capillary zone electrophoresis (CZE) to the organic acids in the fermentation broth, we tried to separate a standard acid mixture containing 2-KLG, 2-KDG and 2,5-DKG using HPLC on Aminex HPX-87H (Fig. 2). The retention times of 2,5-DKG, 2-KLG and 2-KDG were 8.6 min, 9.0 min and 9.4 min, respectively. This resolution was not good enough for quantitative analysis.

### 3.1. Optimization of CZE

For the determination of these acids with CE, the factors for the separation of organic acids were examined. The pH effect on migration time was examined with 100 m$M$ phosphate electrolyte and 100 m$M$ borate, over the pH range 6.0–8.0 and 8.0–9.0. The migration times of 2-KLG, 2-KDG and 2,5-DKG remained almost constant and the electroosmotic velocity also. The resolution in CZE could, in principle, be improved either by increasing the difference in electrophoretic mobility of the separated zones or by reducing the electroosmotic flow of the running buffer [17]. Fig. 3 shows the change in

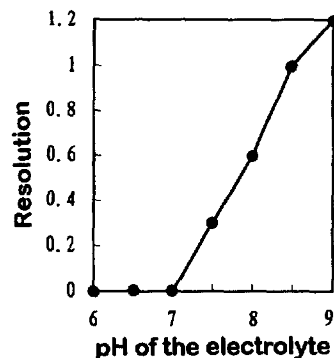


Fig. 3. Effect of pH of electrolyte on the resolution of 2-KLG and 2-KDG. Electrolyte: 100 m$M$ phosphate (pH 6–8) and 100 m$M$ borate (pH 8–9). Applied voltage: 15 kV. Capillary: 47 cm×75 μm fused-silica. Detection wavelength: 195 nm. Injection: pressure 5 s.

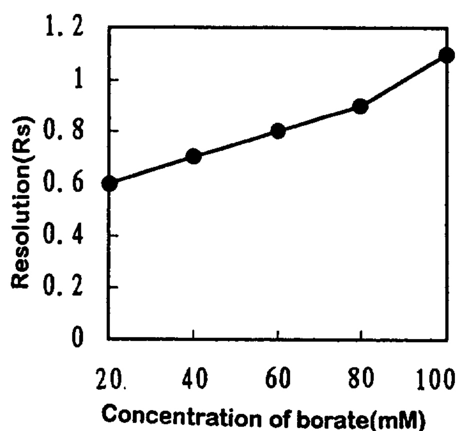


Fig. 4. Effect of ionic strength on the resolution of 2-KLG and 2-KDG. Other conditions are as in Fig. 3.

the resolution of 2-KLG and 2-KDG with the pH of the electrolyte. Although within range of pH 6.0 these acids were not separated completely, but above pH 8.0 the resolution was improved. At pH 8.4 analytes were well separated as shown in Fig. 6 (below) and the peaks were almost symmetric and sharp.

The ionic strength of the background electrolyte plays a role in the variation of the electroosmotic flow and efficiency. The electrolytes can also lead to significantly increased Joule heating, which may result in low resolution and concomitant analyte ability. Fig. 4 shows the migration time obtained by varying the borate concentration from 20 m*M* to 100 m*M* at pH 8.4. It is evident that the 2-KDG begins to comigrate with 2-KLG at low ionic strength. The analytes were completely separated with 100 m*M* borate concentration. The lower the applied field strength, the larger the peak areas owing to decreasing velocities of the analytic bands.

Proper voltage was obtained at the point where the Ohm's plot just deviates from linearity [18]. Therefore, regression analysis of the average of the data points was performed to achieve greater accuracy for the current values and determination of the linearity. Attempts were made to optimize the separation conditions by using different applied voltages ranging 5–30 kV. The effect of voltage on efficiency, resolution and mobility is depicted in Fig. 5. The values of theoretical plate number were then plotted against voltage for 2-KDG. The results of this experiment clearly indicate that as the applied voltage increases up to 15 kV, the theoretical plate number increases, but decreases beyond 15 kV. Voltage ranging 15–30 kV appears to generate pronounced Joule heating resulting in lower theoretical plate number and resolution.

### 3.2. Reproducibility and accuracy

The precision of the peak is affected by variation in the migration time, so stable and reproducible separation conditions must be established prior to the quantitative analysis. We carried out precision tests for migration time and peak area under the optimum conditions based on the above investigations. The amount of each component in the mixture injected

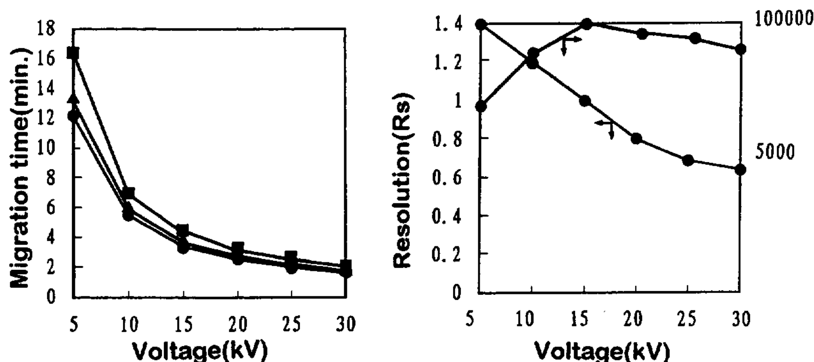


Fig. 5. Effect of the applied voltage on the mobilities (●, 2-KLG; ▲, 2-KDG; ■, 2,5-DKG), efficiency (2-KLG) and resolution (2-KLG and 2-KDG). Electrolyte: 100 m*M* borate (pH 8.4). Other conditions are as in Fig. 4.

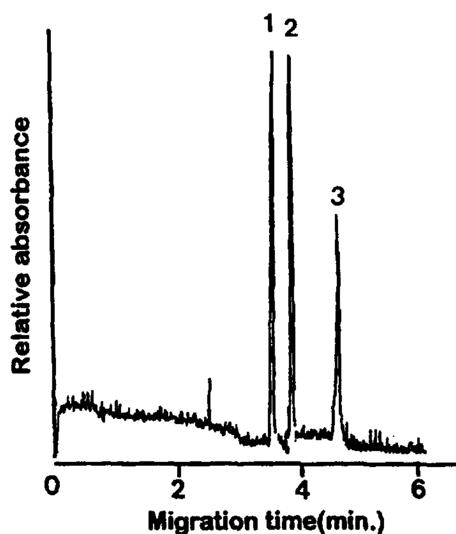


Fig. 6. Typical electropherogram of stereoisomeric organic acids by CE. Peaks: (1) 2-KLG; (2) 2-KDG; (3) 2,5-DKG. Electrolyte: 100 m*M* borate (pH 8.4). Other conditions are as in Fig. 3.

was 5–10 $\mu M$. The applied voltage between two ends of the 47 cm capillary was 15 kV. All the compounds were completely resolved within 5 min and the minimum detectable amount was 0.5–1.3 $\mu M$. The electrolyte was 100 m*M* borate (pH 8.4) and the CE instrument was equipped with a 47 cm×75 $\mu$m I.D. fused-silica capillary column. Fig. 6 shows the electropherogram for a standard mixture of organic acids obtained under the optimum condition. In order to confirm the reproducibility of the migration time and peak area of the three standard acids, 2-KLG, 2-KDG and 2,5-DKG the standard mixture was injected repeatedly three times per day for three consecutive days. Day-to-day variation was almost not observed, the relative standard deviations (R.S.D.) of the migration time were less than 0.89% (Table 1).

### 3.3. Linearity and sample analysis by CZE

For the quantitative analysis, the correlation between the peak area and the sample concentrations in the range of 1.0–100.5 $\mu M$ was studied. The linear regression equations for 2-KLG, 2-KDG and 2,5-DKG were $y=10.1937x+0.4140$ ($r=0.998$), $y=9.1391x+0.4517$ ($r=0.997$) and $y=13.0620x+0.1211$ ($r=0.987$), respectively. This linearity guaranteed the determination of 100.5 $\mu M$ of 2-KLG in the fermentation broth with the detection limit of 0.5 $\mu M$.

Under the above optimum conditions, we separated organic acids in the fermentation broth of MWS 43821, a mutant of *Corynebacterium* sp. The samples were monitored during fermentation at 22 h, 48 h and 72 h, respectively (Fig. 7). The accumulation of 2-KLG began immediately after 2,5-DKG broth was fed and in the final broth 2,5-DKG did not remain unconverted. 2-KDG was not detected by means of CE or by HPLC in the fermentation broth of this mutant which differed from that of the wild strain, ATCC 31090.

Compared to HPLC, the present method is superior in at least four respects: improved separation efficiency, shorter run time, better selectivity and improved sensitivity. For example the number of theoretical plates obtained per meter was 100 000 for 2-KLG, however the highest plate counts attainable by HPLC are smaller than 2000. Separation of the standard by CZE is accomplished within 5 min, whereas HPLC separations of identical mixtures take 8–15 min on average. Injection volumes in CE are

Table 1
Reproducibility of migration time and peak area

| | Migration time (min) | | | Peak area | | |
|---|---|---|---|---|---|---|
| | Mean | S.D.[a] | R.S.D.[b] (%) | Mean | S.D. | R.S.D. (%) |
| 2-Keto-L-gulonic acid | 3.45 | 0.02 | 0.58 | 19 883 | 188.5 | 0.95 |
| 2-Keto-D-gluconic acid | 3.72 | 0.02 | 0.53 | 23 914 | 231.0 | 0.97 |
| 2,5-Diketogluconic acid | 4.48 | 0.04 | 0.89 | 94 716 | 150.1 | 1.59 |

[a] S.D.: Standard deviation.
[b] R.S.D.: Relative standard deviation ($n=9$).

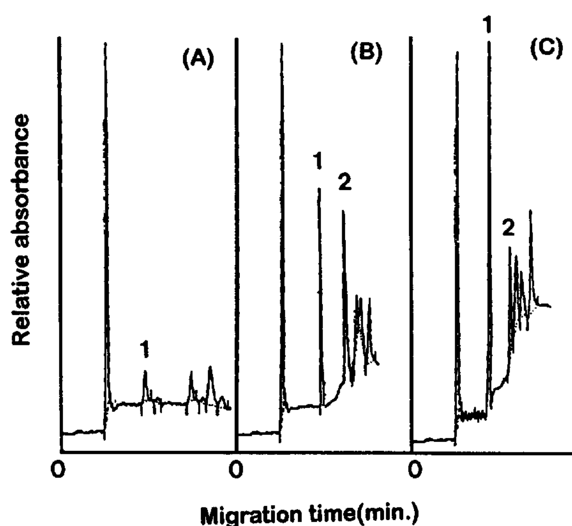


Fig. 7. Electropherogram of fermentation broth by *Corynebacterium* sp.. Cultivation time: (A) 22 h; (B) 48 h; (C) 72 h. Peaks: (1) 2-KLG; (2) 2,5-DKG. The conditions are as in Fig. 6.

less than about 20 nl, compared to 50–100 $\mu$l for HPLC.

## 4. Conclusion

CE is applicable to the analysis of stereoisomeric organic acids produced during the biosynthetic processing of ascorbic acid. CE shows an excellent run-to-run reproducibility and a good linearity in the plot of concentration vs. area under the curve. CE, in comparison with HPLC, has the potential to be used as a simple, fast and economical analytical tool in the screening of 2-KLG, 2-KDG and 2,5-DKG in fermentation broths. The results described here show that the role of CE could be extended to the quality control and the monitoring of fermentation broth in biological and biosynthetic laboratories.

## References

[1] H.J. Rehm and G. Reed, Biotechnology, VCH, New York, 1984.
[2] T. Reichstein and A. Grussner, Helv. Chem. Acta, 17 (1934) 311.
[3] T. Sonoyama, H. Tani, H. Kyotani and K. Mitsushima, Appl. Environ. Microbiol., 43 (1982) 1064.
[4] T. Sonoyama, B. Kageyama and T. Honjo, US Pat., 3 922 194 (1975).
[5] R. Eero, J. Chromatogr., 218 (1981) 695.
[6] F.E.P. Mikkers and F.M. Verheggen, J. Chromatogr., 169 (1979) 11.
[7] J.W. Jorgenson and K.D. Lukacs, Anal. Chem., 53 (1981) 1298.
[8] W.G. Kuhr and C.A. Monnig, Anal. Chem., 64 (1992) 389R.
[9] R. Weinberger, Practical Capillary Electrophoresis, Academic Press, San Diego, 1993.
[10] Y. Xu, Anal. Chem., 65 (1999) 425.
[11] K.D. Altria, J. Chromatogr., 646 (1993) 245.
[12] M. Korman, J. Vindervogel and P. Sandra, J. Microcol. Sep., 5 (1993) 525.
[13] J. Wolfisberg, A. Schmutx, R. Stotzer and W. Thormann, J. Chromatogr. A, 652 (1993) 407.
[14] A. Pluym, W. Vanel and M. Desmet, Trends Anal. Chem., 11 (1992) 27.
[15] T. Reichstein, US Pat., 2 301 811 (1942).
[16] T. Sonoyama, S. Yagi, B. Kageyama and M. Taminoto, Eur. Pat., 46 284 (1982).
[17] R. A. Wallingford and A.G. Ewing, J. Chromatogr., 441 (1988) 299.
[18] J.L. Beckers and F.M. Everaerts, J. Chromatogr., 508 (1990) 19.