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Direct chiral resolution of lactic acid in food products by capillary electrophoresis

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Abstract

Chiral resolution of native DL-lactic acid was performed by capillary electrophoresis using 2-hydroxypropyl-β-cyclodextrin as a chiral selector. Various factors affecting chiral resolution, migration time, and peak area of lactic acid were studied. The running conditions for optimum separation of lactic acid were found to be 90 m*M* phosphate buffer (pH 6.0) containing 240 m*M* 2-hydroxypropyl-β-cyclodextrin with an effective voltage of −30 kV at 16°C, using direct detection at 200 nm. In order to enhance the sensitivity, sample injection was done under a pressure of 50 mbar for 200 s. On-line sample concentration was accomplished by sample stacking. With this system, D- and L-lactic acids in food products were analyzed successfully.

Keywords: Enantiomer separation; Food analysis; Lactic acid

1. Introduction

Lactic acid exists in two isomeric forms that can be present in various organisms as a metabolite of carbohydrate metabolism. It is well known that L- and D-lactates are oxidized to pyruvate by nicotinamide adenine dinucleotide (NAD) in the enzymatic reaction specifically catalyzed by L- and D-lactate dehydrogenases, respectively [1,2]. Thus, the catalytic action of L-lactate dehydrogenase permits measurement of L-lactate in terms of the generation of NADH, which can be spectrophotometrically measured at 340 nm. Similarly, the catalytic action of D-lactate dehydrogenase permits the measurement of D-lactate.

On the other hand, a number of investigations on the chiral resolution of lactic acid have been conducted by using chromatography. HPLC techniques using chiral ligand-exchange phases [3–7] or chiral fluorescent derivatization [8–10] are the most commonly used procedures, but thin-layer chromatographic [11] and capillary gas chromatographic [12,13] techniques have also been proposed. However, most of these chromatographic methods were not used to chiroptically separate lactic acid in food products.

Capillary electrophoresis (CE) is a recently developed powerful analytical technique with a wide range of applications. The availability of many chiral selectors make CE an important tool for chiral analysis. According to recent reviews [14–20], cyclodextrins (CDs) and their derivatives have been widely used in CE for the separation of enantiomers of many compounds. In the inclusion complexation

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mechanism, the hydrophobic interaction between the cavity of the native or derivatized CDs and the hydrophobic part of the compounds, such as an aromatic ring, plays an important role in the stereoselective interaction. Thus, most compounds described in these reviews have aromatic rings. It has been reported that monoterpenes such as pinenes, camphene, and limonene, which have no aromatic rings and are hydrophobic, are chiroptically separated by CE using CD [21]. Recently we reported the direct chiral resolution of pantothenic acid, a hydrophilic molecule having no aromatic rings, by using 2-hydroxypropyl-β-cyclodextrin (2HP-β-CD) [22]. Direct chiral resolution of the aliphatic α-hydroxy acids (lactic acid, 2-hydroxybutyric acid, 2-hydroxy-3-methylbutyric acid, 2-hydroxyisocaproic acid) were also possible by CE using 2HP-β-CD as previously reported [23]. Using spectrophotometry, it was shown that these α-hydroxy acids could be included in the cavity of 2HP-β-CD. However, sensitivity was insufficient for the measurements of D- and L-lactic acids in food products by using the above method. Sample stacking is well known as a method of sensitivity enhancement. Vinther and Soeberg [24] developed a theoretical model for the stacking phenomenon relating the mobilities of analytes to the conductivity in sample and buffer solutions. Burgi and Chien [25] reported the mathematical model for the optimization of peak variance in sample stacking. Sample stacking occurs when the conductivity of the injected sample is lower than that of the surrounding buffer. The electric field strength in the low-conductivity sample medium is higher than that in the high-conductivity running buffer, and ions rapidly migrate to the interface between the lower and higher conductivity zones. Upon reaching the interface, the analytes then slow (stack), resulting in the concentration of the sample zone. Sample stacking has been used extensively in many areas of CE [26–29].

The objective of the present study is to develop simple analyses of D- and L-lactic acids in food products without derivatization by CE using 2HP-β-CD. Various parameters influencing the resolution and the migration time of lactic acid by CE were investigated, and sample stacking was studied in order to enhance the sensitivity for food analysis. D- and L-lactic acid levels in several food products obtained by the CE method agreed well with those obtained by the enzymatic method.

2. Experimental

2.1. Chemicals

2-Hydroxypropyl-β-cyclodextrin (average degree of substitution, 7) and DL-lactic acid lithium salt were obtained from Sigma (St. Louis, MO, USA). L-Lactic acid lithium salt and other chemicals (guaranteed grade) were purchased from Wako (Osaka, Japan).

2.2. Apparatus for CE

Electrophoretic experiments were carried out using a HP^{3D} capillary electrophoresis system (Hewlett-Packard, Palo Alto, CA, USA). The injection of the samples was done by pressure (50 mbar, for 200 s). The separations were performed in a polyvinylalcohol (PVA)-coated bubble cell capillary of 50 μm I.D., 50-cm (Hewlett-Packard). The capillary was kept at 16°C. The analytes were detected at 200 nm. The power supply was operated in the constant-voltage mode, at −30 kV, and the substances migrated towards the positive pole.

2.3. Buffer and sample preparation

The background electrolyte (BGE) in the electrophoretic experiments, unless stated otherwise, was 90 m*M* phosphate buffer (pH 6.0) containing 240 m*M* 2HP-β-CD and was filtered with a 0.22-μm filter before use. Deionized water was prepared using a Yamato Auto Still Model WA-52G (Tokyo, Japan).

Stock solutions of 100 m*M* DL-lactic acid and 100 m*M* L-lactic acid were individually prepared in deionized water, stocked at 4°C and diluted to 1 m*M* (90 mg l^{-1}) and 0.5 m*M*, respectively, before use.

Three brands of yogurts, and four beverages (wine, sake, beer and a soft drink) were purchased from a local market. The yogurts were diluted 200-fold with deionized water and were centrifuged at 3000 rpm for 5 min. Then, the supernatants were filtered with 0.22-μm filters. Wine, sake, beer and soft drink were diluted 30-, 10-, 5- and 5-fold, respectively, and were filtered with 0.22-μm filters.

For recovery examination, racemic lactic acid was added to each diluted sample: 45 mg l^{-1} for yogurt, wine and sake, 22.5 mg l^{-1} for beer, 11.25 mg l^{-1} for soft drink.

2.4. Calculation of resolution

The resolution (R_S) of the enantiomer was calculated by using the following equation:

$$R_S = 2[(t_2 - t_1)/(w_2 + w_1)]$$

where t is the migration time, and w is the width of the peak at the baseline.

2.5. Enzymatic analysis of D- and L-lactic acids

A test combination (D-lactic acid/L-lactic acid, Boehringer Mannheim, Mannheim, Germany) was used for the enzymatic determination of D- and L-lactic acids.

3. Results and discussion

3.1. Factors affecting chiral separation

3.1.1. Cyclodextrin concentration

It was found that 2HP-β-CD was most effective for the chiral resolution of aliphatic α-hydroxy acids containing lactic acid using CE [23]. A PVA-coated capillary, in which the electroosmotic flow was almost completely suppressed, was used to avoid the longer migration time with a fused-silica capillary. Since the carboxyl group of lactic acid is dissociated in the BGE at pH 6, the analyte migrates electrophoretically to the anode. By the analysis of the mixture of DL- and L-lactic acids, it was found that the D-isomer moved slower than the L-isomer. This indicated that D-lactic acid formed a stronger diastereomer complex with 2HP-β-CD than did the L-isomer, because neutral 2HP-β-CD hardly migrates at all.

The effect of 2HP-β-CD concentration on the resolution and the migration time of lactic acid was studied (Fig. 1). The resolution and the migration time increased with increasing amounts of 2HP-β-CD. Wren and Rowe [30] developed a theoretical model relating mobility to the concentration of a CD selector and suggested that maximum resolution can be expected at the optimum CD concentration. We did not find a maximum resolution in the range of 2HP-β-CD concentrations that was used, suggesting that the 2HP-β-CD concentrations used were still below the optimum concentration. Also, it was considered that the increased migration time resulted both from complexation and from the increased viscosity of the buffer due to the high CD concentration.

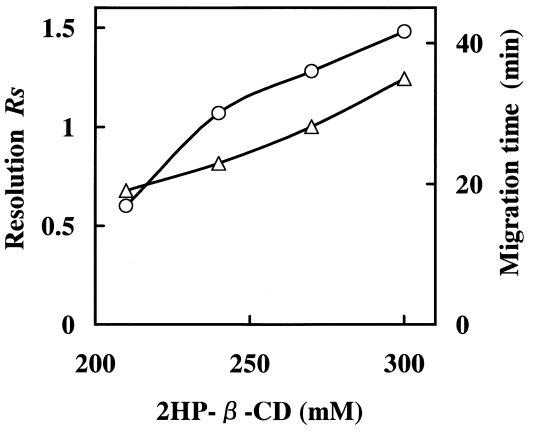

Fig. 1. Effect of 2HP-β-CD concentration on the enantiomeric resolution and migration time of lactic acid. Racemic lactic acid (1 m*M*) was analyzed by CE. The BGE was composed of various concentrations of 2HP-β-CD containing 90 m*M* phosphate buffer (pH 6.0). (○) resolution (R_S); (△) migration time.

3.1.2. Buffer conditions

The concentration of buffer can influence the CE separation. An increase in the concentration of phosphate buffer brought about an increase in the resolution (Fig. 2). This increase in the resolution correlated well with increase in the number of the theoretical plates of D- and L-lactate peaks. It is well known in sample stacking that a higher buffer concentration results in on-line concentration of analytes. Thus, we suggest that the increase in the resolution of D- and L-lactic acids with higher buffer concentration resulted from sample stacking. An increase in the concentration of phosphate buffer did not affect the migration time of lactic acid, but caused a decrease in the peak area of the acid. In general, when a fused-silica capillary is used, a high

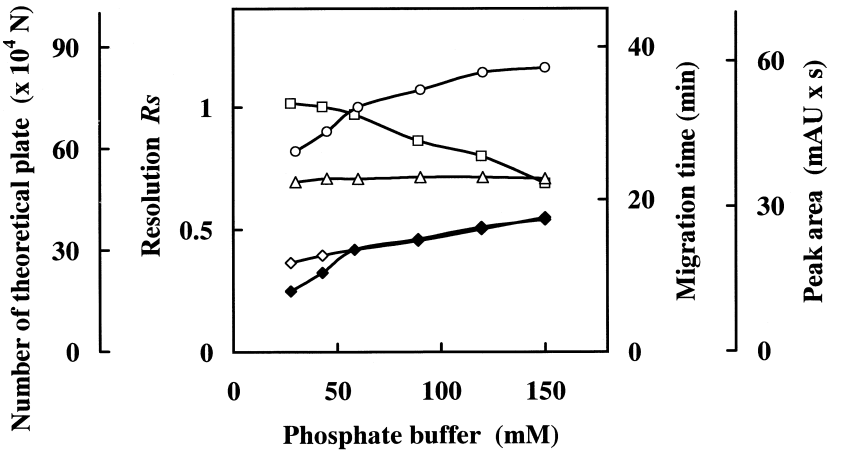

Fig. 2. Effect of phosphate-buffer concentration on the enantiomeric resolution, migration time, peak area, and number of theoretical plates of lactic acid. Racemic lactic acid (1 m*M*) was analyzed by CE. The BGE was composed of various concentrations of phosphate buffer (pH 6.0) containing 240 m*M* 2HP-β-CD. (○) resolution (R_S); (△) migration time; (□) peak area (◇) number of theoretical plate of L-lactate peak; and (♦) number of theoretical plate of D-lactate peak.

buffer concentration decreases the electroosmotic flow, resulting in a longer migration time. However, the migration time was unaffected by the buffer concentration, apparently because PVA-coated capillary that was used almost completely suppressed the electroosmotic flow.

Fig. 3 shows the effect of the pH of the BGE on the resolution and migration time of lactic acid. In the range of pH 5–8, the carboxyl group of the analyte is dissociated. The resolution showed a maximum at pH 6.0 for a racemic mixture of lactic acid. The larger decrease in resolution, which occurred both below pH 5.5 and above pH 6.5, was found to be due to peak tailing and/or leading of D- and/or L-isomer. Care needs to be taken in adjusting the pH to the optimum of 6.0, since even a minor shift to a lower or higher pH value can cause a significant decrease in the resolution of the enantiomers.

3.1.3. Temperature

The effect of capillary temperature on the resolution and the migration time of lactic acid was studied. It was found that a lower capillary temperature caused increases in both the resolution and the migration time of lactic acid. This result was the same as those observed with other chiral compounds [19,22,31–33]. According to Heuermann and Blaschke [33], the increase in the R_S value with a decrease in temperature might be explained by a decrease in rotational and/or vibrational energy, increasing the

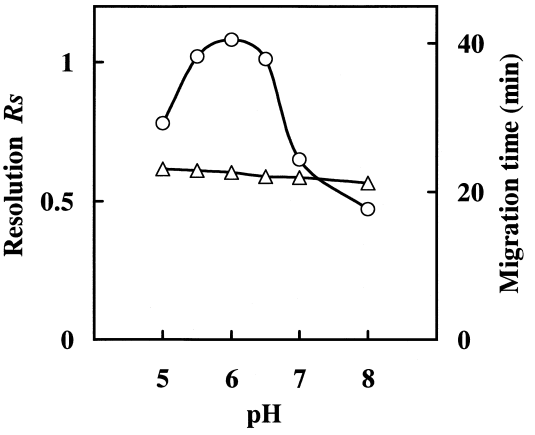

Fig. 3. Effect of pH of buffer solution on the enantiomeric resolution and migration time of lactic acid. Racemic lactic acid (1 m*M*) was analyzed by CE. The BGE was composed of 90 m*M* of phosphate buffer (pH 5.0–8.0) containing 240 m*M* 2HP-β-CD. (○) Resolution (R_S); (△) migration time.

fixation of the enantiomers inside or at the rim of CD and thus, increasing the enantioselectivity.

3.1.3.1. Sample stacking

In order to analyze with high sensitivity, sample stacking was investigated. That is, by increasing the injection time, ranging from 10 to 300 s with a pressure of 50 mbar, the influence of injection volume on the peak area, the resolution, and the migration time was studied (Fig. 4). Although increasing the injection time did not affect the migration time of lactic acid, it did increase the area of each peak of D- and L-lactic acid in a linear fashion ($r > 0.9999$). However, an increase in injection time gradually brought about a decrease in the resolution of the racemate. The sample injection time has been proven to be proportional to the volume of the sample introduced into the capillary. It was found that sample stacking enables an on-line concentration of analytes to yield analyte peaks large enough for quantitation.

Therefore, the optimum BGE conditions for both high resolution and short migration time were found to be 240 m*M* 2HP-β-CD in 90 m*M* phosphate buffer (pH 6.0) with an effective voltage of −30 kV at 16°C using sample injection by pressure with 50 mbar for 200 s.

3.2. Analysis of DL-lactic acids

Foods contain various compounds such as carbohydrates, free amino acids, organic acids, hydrophobic compounds, and so on. In the proposed CE method, only acidic substances migrated towards the positive pole and detector, because a PVA-coated capillary was used. We determined whether the migration times of 11 acidic substances, which are or might be included in food products, interfere with the chiral analysis of lactic acid. Fig. 5 shows the electropherograms of DL- and L-lactic acids analyzed in the CE system. The resolution (R_S) was 1.07. It was found that no racemation occurred in the CE method. It was also found that neither the D-lactic acid peak nor the L-lactic acid peak was affected by any of the 11 substances and that none of the chiral compounds in these 11 substances (DL-malic acid, DL-tartaric acid, DL-aspartic acid, DL-pyroglutamic acid and DL-glutamic acid) were chiroptically separated by the proposed method.

Racemic lactic acid (0.9–270 mg l^{-1}) was subjected to the CE method using the above optimum conditions. Linearity ($r^2 > 0.999$) was demonstrated in the range 0.45–135 mg l^{-1} (0.05–1.5 m*M*) by standard curves of each D- and L-lactic acid. The precision of five consecutive determinations was

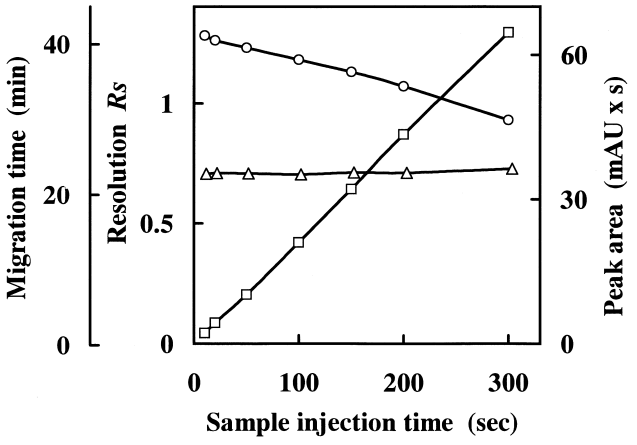

Fig. 4. Effect of sample injection time on the enantiomeric resolution, migration time and peak area of lactic acid. Racemic lactic acid (1 m*M*) was injected for 10–300 s with a pressure of 50 mbar. The BGE was composed of 90 m*M* of phosphate buffer (pH 6.0) containing 240 m*M* 2HP-β-CD. (○) Resolution (R_S); (△) migration time; (□) peak area.

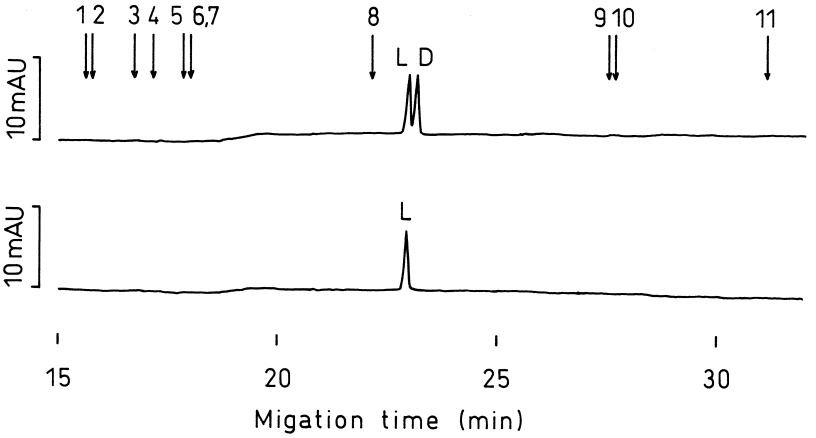

Fig. 5. Electropherograms of racemic and L-lactic acids: (upper) 90 mg l^{-1} racemic lactic acid; (lower) 45 mg l^{-1} L-lactic acid (L, L-lactic acid; D, D-lactic acid). (1–11) The migration times of substances as follows: (1) malonic acid; (2) fumaric acid; (3) DL-malic acid; (4) DL-tartaric acid; (5) acetic acid; (6) succinic acid; (7) pyruvic acid; (8) citric acid; (9) DL-pyroglutamic acid; (10) DL-aspartic acid; and (11) DL-glutamic acid.

evaluated at 90 mg l^{-1} (1 m*M*) for racemic lactic acid. High reproducibilities of peak areas (RSD< 0.7%) and migration times (RSD<0.5%) of both D- and L-isomers were obtained. Good recoveries (97–100%) of D- and L-lactic acids were obtained. L-Lactic acid and a racemic mixture of lactic acid were mixed at various ratios and the mixtures were analyzed by the CE method. It was found that the D-isomer could be as small as 3% in total lactic acid and still be detected.

Using the proposed CE method, D- and L-lactic acids in three brands of yogurts and four types of beverage (wine, sake, beer, and a soft drink) were determined (Table 1). Fig. 6 shows examples of electropherograms for yogurt and sake. The ratios between D- and L-lactic acids in the three yogurts obtained from different manufacturers varied. It seems that these different ratios result from the properties of the lactic bacteria that take part in lactic acid fermentation, because the three manufacturers use different types of lactic bacteria. Table 1 also compares the proposed CE method with the enzymatic method for quantitation of D- and L-lactic acids in food products. The regression equations for D- and L-lactic acids were $y=1.004x+6.165$ and $y=0.990x-6.021$ with correlations of 0.9988 and 0.9975, respectively. Therefore, this CE method was found to be simple, reproducible and useful for quantitative chiral analysis of lactic acid in food products.

Table 1
Comparison of the values determined for D- and L-lactic acids in food products using CE and enzymatic methods

Foods	Lactic acid	CE method (mg l^{-1})	Enzymatic method (mg l^{-1})
Yogurt (A)	L	6410	6270
	D	1890	1900
Yogurt (B)	L	5650	5510
	D	936	990
Yogurt (C)	L	6000	6170
	D	486	441
Wine	L	1520	1520
	D	333	387
Sake	L	495	468
	D	162	171
Beer	L	52	53
	D	47	44
Soft drink	L	28	32
	D	28	32

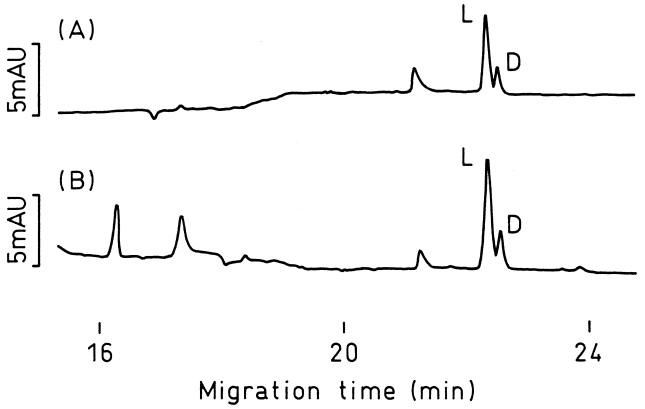

Fig. 6. Electropherograms of yogurt and sake. (A) Yogurt diluted 200-fold; (B) sake diluted 10-fold (L, L-lactic acid; D, D-lactic acid).

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