Determination of Organic Acids by High-Performance Liquid Chromatography with Electrochemical Detection during Wine Brewing

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Voltammetric determination of acids by means of the electrochemical reduction of quinone was applied to high-performance liquid chromatography (HPLC) with electrochemical detection (ED) for determining organic acids in fruit wines. A two-channel HPLC−ED system was fabricated by use of an ion-exclusion column and an electrochemical detector with a glassy carbon working electrode. Aqueous solution of 0.1 mM $HClO_4$ and ethanol containing 2-methyl-1,4-naphthoquinone served as a mobile phase and reagent solution, respectively. Determination of acetic, citric, lactic, malic, succinic, and tartaric acids was made by measuring the peak areas of the flow signals due to the reduction current of quinone caused by the eluted acids. The peak area was found to be linearly related to the acid amount ranging from 0.1 to 40 nmol per 20 μL injection. The present method was characterized by reproducibility with the simple and rapid procedure without derivatization of analytes. The method was shown as an effective means for following acid contents in fruit juices during fermentation with wine yeast.

KEYWORDS: Electrochemical detection; HPLC; acid determination; wines, fruit juices

INTRODUCTION

Various kinds of organic acids, such as tartaric acid, citric acid, malic acid, and lactic acid, are contained in beverages such as fruit juices and wines and serve as a source of taste and aroma. Each acid content differs depending on the particular origin and growth conditions of the original fruits and changes during the fermentation process. The acid determination of fruit juices and wines is thus essential for their quality and process controls.

Although acidity measurements (*1*, *2*) and photometric, chromatographic (*3*), and enzymatic methods (*4*) have been commonly employed for determining organic acids in fruit juices and wines, the sensitivity and selectivity of the responses in these methods are not always sufficient for practical uses. In HPLC, because of the weak light-absorptive property of carboxylic acids, derivatization with an appropriate labeling reagent is often required before or after the column separation for sensitive detection of acids; however, the operating procedures for the field tests are sometimes troublesome and time-consuming. A simpler, more sensitive and rapid assay that requires no derivatization procedure is thus highly desirable.

Since most carboxylic acids are less active electrochemically, a few papers have so far been concerned with the use of electrochemical detection methods in HPLC. Recently, a pulsed electrochemical detection was employed for the determination

of several aliphatic acids in fruit juices and wines by HPLC (*5*). We previously developed a new method for determining acids by means of the voltammetric reduction of quinone (*6*, *7*). Since the method was shown to be quite sensitive and selective for acid components, the method was successfully applied to the determination of the free fatty acid content of fats and oils (*8−11*) and the total acid content of fruit juices (*12*, *13*), wines (*12*, *13*), and coffee (*14*, *15*). On the basis of this method, electrochemical detection in HPLC was recently examined to determine lactic acid in yogurt (*16*).

In the present study, an HPLC system with electrochemical detection (HPLC−ED) was fabricated for determining organic acids in fruit wines, and an assessment of the HPLC−ED method was made as an effective means for monitoring the acid content of wine brewing through the fermentation of fruit juices with wine yeast.

MATERIALS AND METHODS

Reagents. All the chemicals used were of reagent grade. 2-Methyl-1,4-naphthoquinone (vitamin K_3, VK_3, >98.5%), $LiClO_4$ (>98%), $HClO_4$ (60−62%), ethanol (99.5%, JIS K 8101), citric acid (anhydrous, >98.0%), tartaric acid (>99.8%), malic acid (>98.0%), lactic acid (analytical value 91.7%), succinic acid (>99.5%), acetic acid (>99.7%), and 3-methylglutaric acid (99%) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Dried cultured wine yeast was obtained from Auvelcraft Co. (Aichi, Japan).

HPLC System and Its Operation Conditions. The HPLC-ED system is shown in **Figure 1**. It consists of a degasser (model DG-

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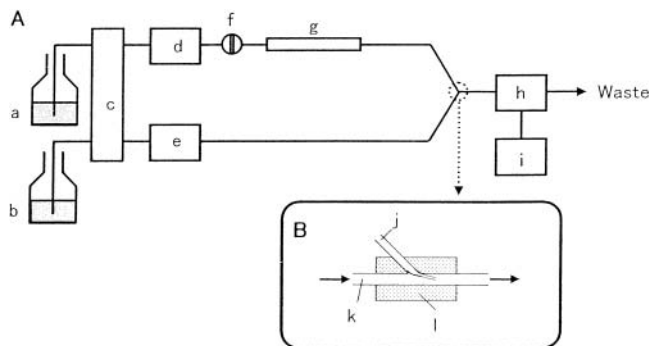
Figure 1. Block diagram of the HPLC-ED system (A) and mixing junction (B). (a) Mobile phase, 0.1 mM HClO$_4$; (b) quinone solution, 6 mM VK_3 + 76 mM LiClO$_4$ in ethanol; (c) degasser; (d and e) pump; (f) sample injector; (g) separation column; (h) electrochemical detector, electrochemical cell, and potentiostat; (i) recorder; (j) PTFE tube (i.d. 0.5 mm); (k) PTFE tube (i.d. 1.0 mm); (l) epoxy resin.

980-50, Jasco, Tokyo, Japan), two pumps (model PU 980, Jasco), a sample injector (model 7125, Rheodyne, Cotati, CA), an ion-exclusion column (RSpak KC-811, 300 mm × 8 mm i.d., Shodex, Tokyo, Japan) with a guard column (RSpak KC-LG, 50 mm × 8 mm i.d., Shodex) and an electrochemical cell (model EC-840, Jasco), a potentiostat (model 331B, Huso Electrochemical Systems, Kawasaki, Japan), and a recorder (model 807-IT, Jasco). The electrochemical cell (cell volume 0.7 μL) was constructed from a glassy carbon working electrode, a saturated calomel reference electrode (SCE), and a stainless steel auxiliary electrode.

An aqueous solution of 0.1 mM HClO$_4$ and an ethanol solution containing 6 mM VK_3 and 76 mM LiClO$_4$ served as the mobile-phase solution and the quinone solution, respectively. Each solution was deaerated by the degasser and made to flow at the rate of 1.0 mL/min in each flow line. Then 20 μL of the test solution was injected into the ion-exclusion column maintained at 45 °C by use of a column oven. An eluate from the column was allowed to merge with the quinone solution at the junction (B) of the system, and the mixture was permitted to move to the electrochemical cell, in which the working electrode potential for detecting acids was set at -0.7 V vs SCE. The detection potential was decided from the measurement of the hydrodynamic voltammogram of each standard acid so as to obtain the maximum current. Each acid in the mixture was monitored by measuring the current height of a flow signal.

Test Solution Preparation. Standard acid mixture was prepared by dissolving citric acid, tartaric acid, malic acid, lactic acid, succinic acid, 3-methylglutaric acid, and acetic acid in 0.1 mM HClO$_4$. 3-Methylglutaric acid was used as an internal standard.

The wine brewing experiment was carried out with 700 mL of commercially available grape or orange juice (100%). Following the addition of dried cultured wine yeast (1 g) and sucrose (16.2 g/dL), the mixture was incubated at 20 °C in a wine-brewing bottle (Auvelcraft Co.). The bottle was kept covered with a special stopper in which water was placed to exclude undesirable bacteria from the air. During fermentation, 50-μL aliquots of the mixture were withdrawn from the wine-brewing bottle and mixed with 1-mL aliquots of 0.1 mM HClO$_4$ containing 3-methylglutaric acid and 3% phenol. The mixture was filtered through a 0.45 μm membrane filter, and 20 μL of the supernatant served as the test solution for the HPLC measurements.

RESULTS AND DISCUSSION

Acid Assay by Means of Voltammetric Reduction of Quinone. In previous studies on the electrochemical reduction of VK_3 in a nonbuffered ethanol solution, the addition of small amounts of acid to the solution were found to give rise to a new peak (termed the prepeak) at a more positive potential than the original reduction peak (*6, 7*). The occurrence of the prepeak is ascribed to the increased proton availability of the added acid compared to the solvent molecules, leading to the reduction potential shift to a more positive direction depending on the
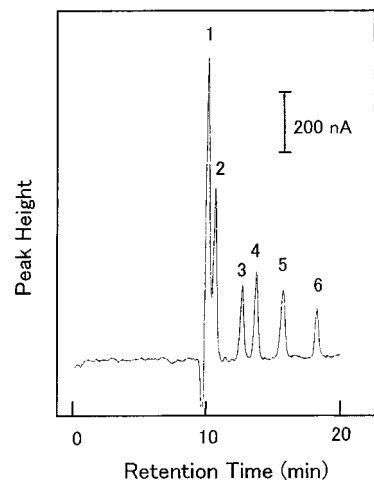

Figure 2. Chromatogram of standard acid mixture. Each acid amount injected: 20 nmol. Peaks: (1) tartaric acid and citric acid; (2) malic acid; (3) lactic acid; (4) succinic acid; (5) 3-methylglutaric acid (internal standard); (6) acetic acid.

pK_a of the added acid. The prepeak height was proportional to the acid concentration ranging from 8 μM to 3 mM; the half-peak potential of the prepeak shifted to a more positive value accompanied by a decrease in pK_a of the acid (*6, 7*).

On the basis of these findings, acid in the eluate from the separation column in the HPLC system was detected by measuring the current height of a flow signal at the fixed potential. The choice of VK_3 as a quinone reagent was favorable for our purposes, as it facilitated this determination owing to its solubility and stability in ethanol (*7*).

Determination of Organic Acids by the HPLC–ED Method. A typical chromatogram for a standard acid mixture containing tartaric, citric, malic, lactic, succinic, 3-methylglutaric, and acetic acids at 20 nmol each is shown in **Figure 2**. Although the separation of citric acid from tartaric acid, whose resolution (Rs) was less than 0.359, was difficult, other acids were well separated within 20 min. The peak area of each acid was found to be linearly related to the acid amount injected, ranging from 0.1 to 40 nmol: 15.0 ng–6.0 μg of tartaric acid, 21.0 ng–8.4 μg of citric acid, 13.4 ng–5.4 μg of malic acid, 9.0 ng–3.6 μg of lactic acid, 11.8 ng–4.7 μg of succinic acid, and 6.0 ng–2.4 μg of acetic acid (*r* > 0.998). The standard solution at 2.0 nmol was determined 10 times with a relative standard deviation (RSD) of less than 2.5%. Acid detection limits (at the signal-to-noise ratio of 3) for a single injection of the present method for tartaric, citric, malic, lactic, succinic, and acetic acids were 15.0, 21.0, 13.4, 9.0, 11.8, and 6.0 ng, respectively. Acid determination was made on the basis of comparison of the signal peak area with that of 3-methylglutaric acid as the internal standard.

In the method, the separation of tartaric acid and citric acid was poor. Although the level of tartaric acid is nothing to that of citric acid in oranges, both acids are present in grapes. Therefore, by increasing the HClO$_4$ concentration in the mobile phase, the separation of tartaric and citric acids in ion-exclusion chromatography was achieved. When 2 mM HClO$_4$ and 2-propanol containing 10 mM VK_3 and 76 mM LiClO$_4$ were used as mobile phase and quinone solution, respectively, the peaks of tartaric and citric acids were separated with Rs of 1.01. However, the background current became very high and the detection limits for a single injection for tartaric and citric acids were 1.8 μg and 2.1 μg, respectively. Thus 0.1 mM HClO$_4$ is recommended as the most suitable mobile phase for monitoring fermentation of fruit juices except that of grape juice.
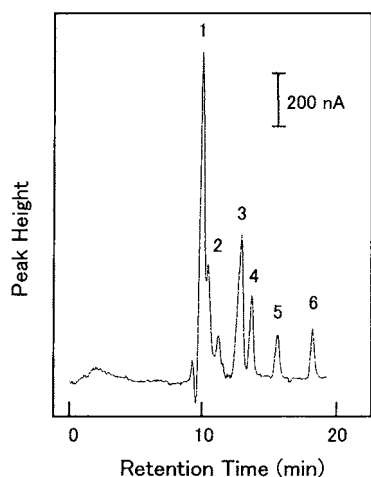
Figure 3. Chromatogram of organic acids in red wine. Sample wine was diluted 50-fold with 0.1 mM HClO$_4$. Peaks: (1) tartaric acid and citric acid; (2) malic acid; (3) lactic acid; (4) succinic acid; (5) 3-methylglutaric acid; (6) acetic acid.

Table 1. Analytical Results of Acid Content of Commercial Wines

	wine color (country of origin)	acid concentrations (g/L)				
		tartaric + citric[a]	malic	lactic	succinic	acetic
A	white (Chile)	2.46	1.21	0.30	0.32	0.46
B	red (France)	3.02	0.84	1.85	0.67	0.38
C	red (France)	3.80	0.82	2.24	0.63	0.42
D	rosé (France)	3.03	0.98	1.57	0.59	0.27
E	rosé (U.S.A.)	2.58	1.84	0.70	0.40	0.21

[a] The sum concentrations of tartaric and citric acids are presented as concentrations of tartaric acid.

Determination of Organic Acids in Wines. The method was first applied to acid determination in commercially available wines. Test solution was prepared by 50-fold diluting a sample wine with 0.1 mM HClO$_4$ containing 3-methylglutaric acid. An example of the chromatogram obtained for a red wine (made in France) is shown in **Figure 3**. Although the peaks of tartaric and citric acids are not well separated, malic, lactic, succinic, and acetic acids exhibit very well separated peaks within 20 min. Analytical results for several commercial white, red, and rosé wines are listed in **Table 1**.

Sulfites and sorbic acid are often added in wines as preservatives. The retention times of both additives obtained under the present HPLC conditions were 8.2 and 88.3 min, respectively, indicating no interference from the additives on the analytical results obtained by the present method.

Monitoring of Acid Content during Wine Brewing. Change of each acid content during the fermentation of fruit juices with wine yeast was followed by the present method. Panels A and B of **Figure 4** show the chromatograms obtained for grape juice before and after fermentation for 4 days, respectively, in which tartaric and malic acids tended to decrease, while lactic, succinic, and acetic acids increased following fermentation for 4 days.

Analytical results obtained for grape and orange juices after fermentation for 4 days are listed in **Table 2**. Each acid content was determined with RSD less than 3.4%. Recovery tests of each acid were made with each standard acid spiked in the sample juices, and the results were 91−101%, indicating that the matrix components in the samples gave practically no influence on the present method.

In panels **A** and **B** of **Figure 5**, the content values for the six acids are plotted against the incubation time during the course of the fermentation of grape and orange juices, respectively.
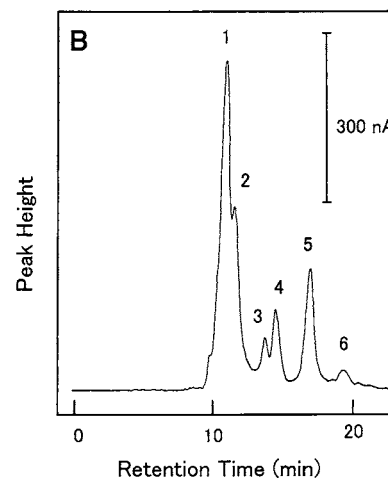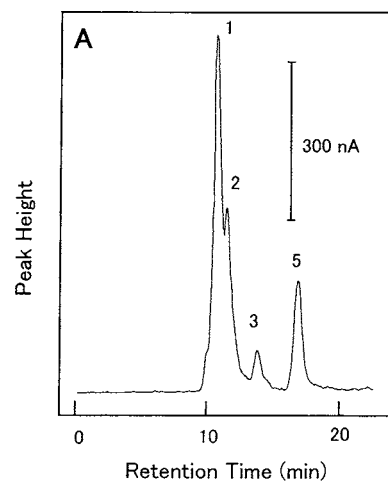

Figure 4. Chromatograms of grape juice obtained before (**A**) and after (**B**) fermentation with wine yeast for 4 days. Peaks: (1) tartaric acid and citric acid; (2) malic acid; (3) lactic acid; (4) succinic acid; (5) 3-methylglutaric acid; (6) acetic acid.

Table 2. Analytical Results Obtained for Grape and Orange Juices after Fermentation with Wine Yeast for 4 Days

organic acids	content (n = 5)		recovery (n = 5)		
	concn (g/L)	RSD (%)	added concn (g/L)	recovery (%)	RSD (%)
	Grape Juice				
tartaric and citric	2.80[a]	2.6	3.00[a]	101	2.2
malic	1.49	3.2	1.34	97	2.7
lactic	0.55	2.4	0.45	95	2.8
succinic	1.52	2.0	1.18	99	2.6
acetic	0.33	2.9	0.30	92	3.3
	Orange Juice				
tartaric and citric	7.10[b]	3.0	6.30[b]	98	2.3
malic	2.56	2.8	2.68	93	1.6
lactic	0.37	2.1	4.50	91	2.5
succinic	2.33	2.7	2.36	92	2.5
acetic	0.14	3.4	0.12	95	3.1

[a] The sum concentration of tartaric and citric acids is presented as concentration of tartaric acid. [b] The sum concentration of tartaric and citric acids is presented as concentration of citric acid.

The features of the content changes for these acids are consistent with the findings so far reported (*17−20*). Increase of lactic and succinic acids following fermentation has been known to provide flavor to the wines.

To clarify how acid content differs depending on the kinds of fruit and fermentation conditions, a further experiment was
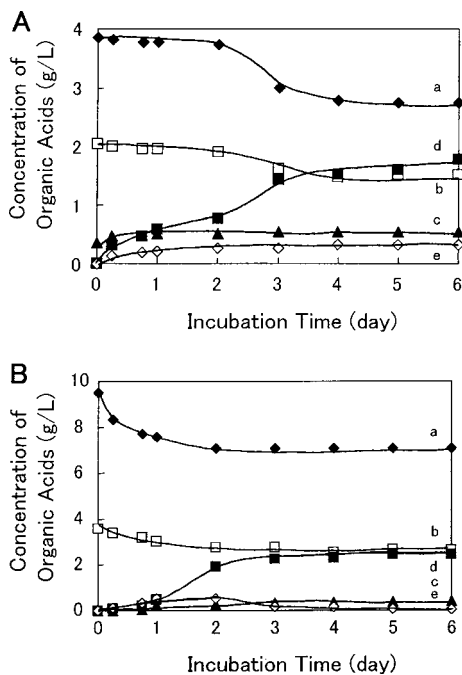
Figure 5. Acid contents during the fermentation of grape (**A**) and orange (**B**) juices with wine yeast: (a) tartaric acid and citric acid (◆); (b) malic acid (□); (c) lactic acid (▲); (d) succinic acid (■); (e) acetic acid (◇). (**A**) Sum concentrations of tartaric and citric acids are presented as concentrations of tartaric acid. (**B**) Sum concentrations of tartaric and citric acids are presented as concentrations of citric acid.

carried out under diluted conditions. When the brewing was started with grape or apple juice diluted with water (1:6.7, total volume 700 mL) with the addition of a smaller amount of sucrose (2.1 g/dL), the acid content changes demonstrated somewhat different patterns (**Figure 6**) compared to the cases as shown above. As shown in **Figure 6**, even though decreases in tartaric, citric, and malic acids are not clearly shown, lactic, succinic, and acetic acids tended to increase following fermentation for several days.

Advantages of the Present HPLC-ED Method. To realize the advantages of the HPLC–ED over the HPLC–UV method, the chromatogram obtained by a UV (210 nm) detection method for the same test solution of grape juice as that used in **Figure 4B** is shown in **Figure 7**. All the acid peaks in **Figure 7** are ill-defined compared to those obtained by the ED method. UV detection at 210 nm was sensitive for other compounds present in the test solution, as well as the acids, although the ED method was selective for the acids. In the UV method, there are many intermediate peaks, and the peak resolutions seem to be poorer than the ED method. To detect only acids by UV method, further purification of the test solution would be necessary.

Comparison of the analytical results obtained by both detection methods was made in **Table 3**. By the ED method, acid content was determined with smaller RSD values and lower detection limits as compared with the UV detection method in all cases. The results in **Table 3** demonstrate that the HPLC-ED method is characterized by several tens times higher sensitivity and higher reproducibility than the HPLC–UV method.

An ion-exchange chromatographic method with conductometric detection has also been used for determining organic acids in wine (*21*). The method requires extraction of organic acids from wine samples by use of solid-phase extraction cartridges. The detection limits of organic acids are about 3 times larger than the present ED method.
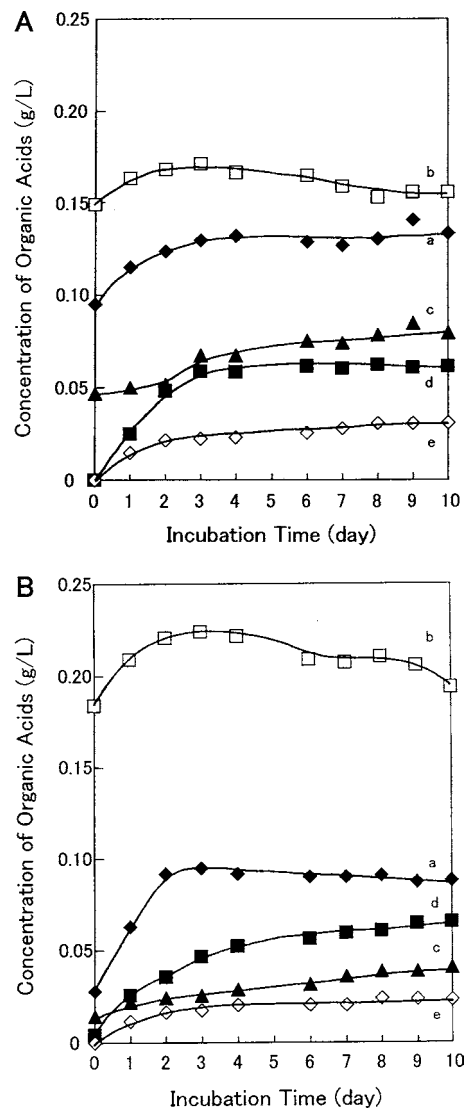

Figure 6. Acid contents during the fermentation of grape (A) and apple (B) juices with wine yeast under diluted conditions: (a) tartaric acid and citric acid (◆); (b) malic acid (□); (c) lactic acid (▲); (d) succinic acid (■); (e) acetic acid (◇). The sum concentrations of tartaric and citric acids are presented as concentrations of tartaric acid.

Recently, pulsed amperometric detection was proposed for the determination of several aliphatic acids in foods and beverages by ion-exclusion HPLC, based on the formation/inhibition of a platinum oxide layer on the platinum working electrode surface (*5*). In this method, the electrode potential steps periodically between the positive (oxidation) and negative (reduction) potentials. Application of positive potential at the platinum working electrode in a flowing stream containing chloride ion seems liable to cause undesirable problems, such as baseline instability with subsequent poor precision and high detection limits. When chloride (also bromide or iodide) ions are present in wine samples (*22, 23*) or mobile phase, anodic dissolution of platinum will occur through the formation of chloroplatinum complexes (*24, 25*) for the formation of platinum oxide layer. These factors lead to less reliability of acid determination. Contrary to this, in the present study, glassy carbon was used as the working electrode. Glassy carbon electrode is stable over the wide potential range even in the presence of halide ions. In this study, the applied potential for monitoring carboxylic acids was negative enough not to cause electrode surface oxidation. Consequently, the working electrode
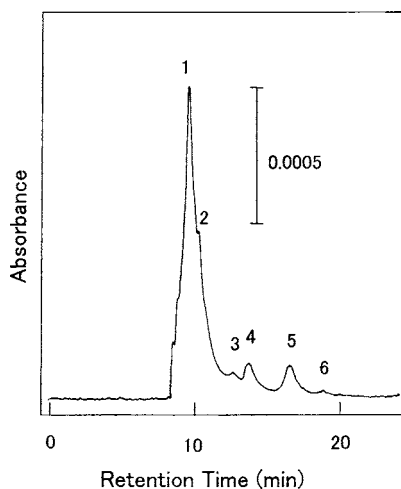
Figure 7. Chromatogram of grape juice obtained by HPLC–UV method. Test solution was the same as used in **Figure 4B**. Acid detection was made at 210 nm. Peaks: (1) tartaric acid and citric acid; (2) malic acid; (3) lactic acid; (4) succinic acid; (5) 3-methylglutaric acid; (6) acetic acid.

Table 3. Comparison of the Analytical Results Obtained by the HPLC–ED and HPLC–UV Methods[a]

organic acids	HPLC-ED			HPLC-UV		
	concn (g/L)	RSD (%, $n = 5$)	detection limit (ng)	concn (g/L)	RSD (%, $n = 5$)	detection limit (ng)
lactic	0.43	2.6	9.0	0.45	11.2	450
succinic	1.01	2.7	11.8	0.98	18.0	282
acetic	0.96	2.8	6.0	0.10	12.5	360

[a] Test solution was the same as used in Figure 4B.

surface was found to remain stable for periods of more than 4 months for analysis of more than 10 samples a day with the HPLC system.

By the present HPLC–ED method, several organic acids in fruit wines were determined with satisfactory results. The present ED method is selective for acids and required small sample amounts, only 50 μL. Therefore, the merits of the present method in saving sample amounts and time for pretreatment are favorable for monitoring wine fermentation. Besides fruit wines, the method was also useful for determining acids in fermented dairy products such as yogurt, cheese, fermented seasonings, and pickled vegetables. Because of the simple and rapid procedure without the derivatization of analytes, the present method was thus shown to be effective for monitoring acid content changes during the bacterial fermentation of food.

The present method requires small sample amounts, and accordingly, freshly prepared specimens can be analyzed at frequent time intervals. The present method is thus promising for the kinetic studies of bacterial fermentation processes of foods.

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