Post-column pH Buffered Electric Conductivity Detection of Organic Acids with Ultra High Performance Liquid Chromatography

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Introduction

For the analysis of organic acids by HPLC, UV detection at around 210 nm has been conventionally used as a convenient and simple method. The UV detection method, however, is often lack of sensitivity and/or selectivity when analyzing organic acids in food samples which include many kinds of UV absorbing co-existing compounds.

For highly sensitive and selective determination of organic acids, "Post-column pH-buffered electric conductivity detection" was developed by Shimadzu Corporation[1), 2)]. After separating organic acids by ion-exclusion column, pH-buffered solution is added to the column eluent constantly in order to utterly ionize the organic acids which are weak anions (Fig. 1). This technique greatly improves the selectivity of organic acids in complicated samples (Fig. 2).

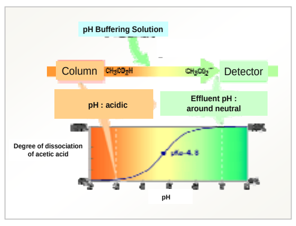
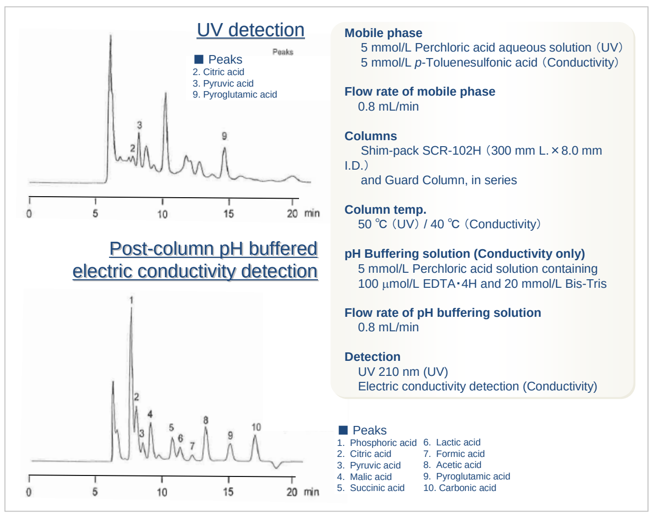


Fig. 1 Principle of the post-column pH-buffered electric conductivity detection



Mobile phase
5 mmol/L Perchloric acid aqueous solution（UV）
5 mmol/L *p*-Toluenesulfonic acid（Conductivity）

Flow rate of mobile phase
0.8 mL/min

Columns
Shim-pack SCR-102H（300 mm L.×8.0 mm I.D.）
and Guard Column, in series

Column temp.
50 ℃（UV）/ 40 ℃（Conductivity）

pH Buffering solution (Conductivity only)
5 mmol/L Perchloric acid solution containing
100 µmol/L EDTA･4H and 20 mmol/L Bis-Tris

Flow rate of pH buffering solution
0.8 mL/min

Detection
UV 210 nm (UV)
Electric conductivity detection (Conductivity)

■ Peaks

1. Phosphoric acid	6. Lactic acid
2. Citric acid	7. Formic acid
3. Pyruvic acid	8. Acetic acid
4. Malic acid	9. Pyroglutamic acid
5. Succinic acid	10. Carbonic acid

Fig. 2 Comparison of UV and conductivity detection in food analysis (with ion-exclusion columns)

In this study, we applied the post-column pH buffered electric conductivity detection to reversed phase ultra high-performance liquid chromatography (UHPLC) for the high speed analysis of organic acids. Analytical conditions for separation and detection were optimized. Under optimized conditions, organic acids were separated within a few minutes, and good linearity and repeatability were obtained. Based on these results, this method is one of the promising ways for the rapid determination of organic acids in food, which contains a small amount of co-existing compounds or a large amount of co-existing compounds.

1) M. Hayashi:Shimadzu Review, 49, 59 (1992)
2) M. Hayashi:GIT Spezial Chromatographie, 1, 64 (1995)

Experimental

■ Instruments

For HPLC system, *"Nexera"* (Shimadzu) was used. To optimize the conditions for separation, UV-VIS detector "SPD-20AV" was used. And the electric conductivity detector "CDD-10A*VP*" was used for optimization of detection conditions and actual food sample analysis with post-column system (Fig. 3).

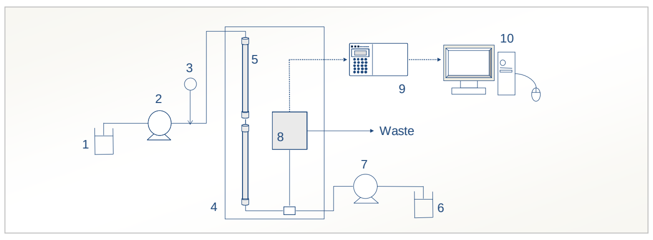


Fig. 3 Flow diagram of post-column pH buffered electric conductivity detection with "*Nexera*"

1; Mobile Phase, 2; Pump for Mobile Phase (LC-30AD), 3; Autosampler (SIL-30AC), 4; Column Oven (CTO-20AC), 5; Column(s), 6; pH Buffering Solution, 7; Pump for pH Buffering Solution (LC-30AD), 8; Cell of Conductivity Detector, 9; Conductivity Detector (CDD-10A*VP*), 10; Data Processor (LabSolutions LC)

■ Analytical Conditions

Analytical conditions are shown in Table 1. Under the conditions, we optimized 1) separating conditions (for rapid separation), 2) concentration of Bis-Tris and 3) concentration of EDTA-4H in buffering solution (for good sensitivity and linearity without losing peak resolution).

Table 1 Analytical Conditions

Column	:	Phenomenex Synergi Hydro-RP （100 mm L.×3.0 mm I.D., 2.5 µm）
Mobile phase	:	Perchloric acid solution（5～100 mmol/L）
Flow rate of mobile phase	:	0.6 mL/min
Column temp.	:	35 ℃ / 40 ℃
pH buffering solution	:	5 mmol/L Perchloric acid solution containing **EDTA･4H（0～200 µmol/L）** **Bis-Tris（0～30 mmol/L）**
Flow rate of buffer solution	:	0.6 mL/min
Detector	:	UV detector / Electric conductivity detector

Results and Discussion

■ 1) Optimization of separation conditions of organic acids

We tried several kinds of columns and mobile phases (both acidic aq. solution and buffer). In the results, we adopted "Synergi Hydro-RP column" (100 mm L.×3.0 mm I.D., 2.5 µm, Phenomenex) as a column and perchloric acid solution as a mobile phase, so that the resolution of each organic acid was good, and background level of electric conductivity detector was low.

The relationship between the concentrations of perchloric acid in mobile phase and retention factors (*k*) were shown in Fig. 4. These results indicated that the concentration of perchloric acid hardly affected the retention of organic acids. Considering the background level of conductivity detector, low concentration acid would be better. We adopted the low concentration of perchloric acid (5 mmol/L) for mobile phase.

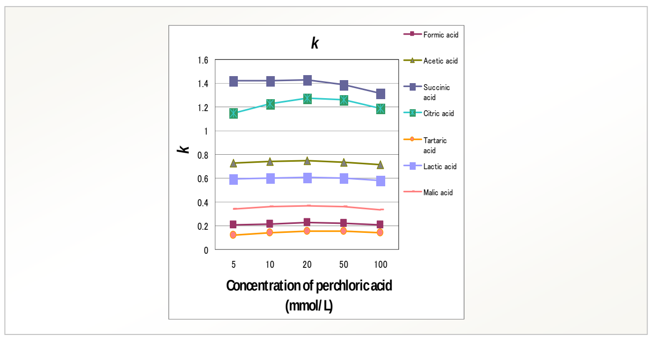
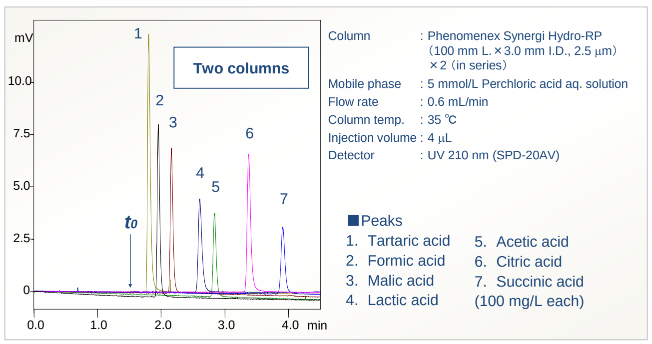


Fig. 4 Relationship between the concentrations of perchloric acid and retention factors (*k*) of organic acids

To improve a resolution of organic acids, we connected two "Synergi Hydro-RP " columns in series. Coupling two columns resulted in improved resolution of tartaric and formic acid (Fig. 5) .



Column	:	Phenomenex Synergi Hydro-RP （100 mm L.×3.0 mm I.D., 2.5 µm） ×2（in series）
Mobile phase	:	5 mmol/L Perchloric acid aq. solution
Flow rate	:	0.6 mL/min
Column temp.	:	35 ℃
Injection volume	:	4 µL
Detector	:	UV 210 nm (SPD-20AV)

■Peaks

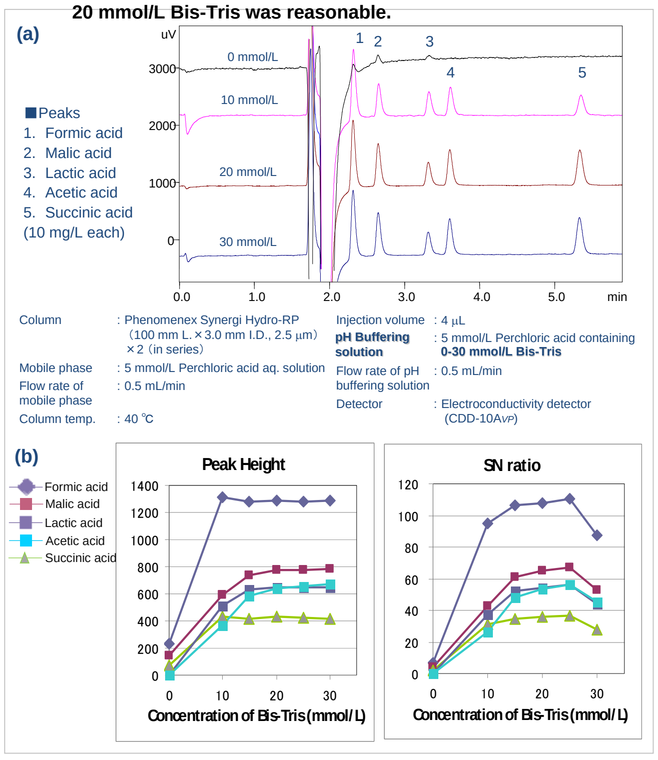
1. Tartaric acid	5. Acetic acid
2. Formic acid	6. Citric acid
3. Malic acid	7. Succinic acid
4. Lactic acid	(100 mg/L each)

Fig. 5 Improvement in resolution of organic acids by coupling two columns

■ 2) Optimization of detection conditions of organic acid - concentration of Bis-Tris

In the case of reversed phase chromatography, the acidic solution is commonly used as a mobile phase. Our post-column detection method could provide improved sensitivity and selectivity coupled with reversed phase separation.

We examined the concentrations of Bis-Tris, which is as a base in the pH buffering solution. Fig. 6 (a) shows chromatograms obtained under different concentrations of Bis-Tris, and (b) shows peak heights and S/N ratios of organic acids in Fig. 6 (a). In the results, 20 mmol/L Bis-Tris was reasonable.



■Peaks
1. Formic acid
2. Malic acid
3. Lactic acid
4. Acetic acid
5. Succinic acid
(10 mg/L each)

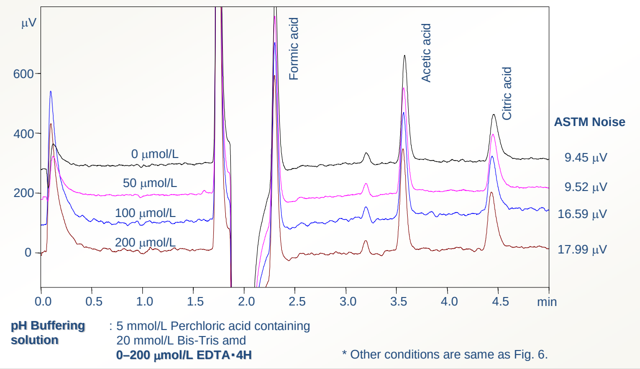
Column	:	Phenomenex Synergi Hydro-RP （100 mm L.×3.0 mm I.D., 2.5 µm） ×2（in series）
Mobile phase	:	5 mmol/L Perchloric acid aq. solution
Flow rate of mobile phase	:	0.5 mL/min
Column temp.	:	40 ℃
Injection volume	:	4 µL
pH Buffering solution	:	5 mmol/L Perchloric acid containing **0-30 mmol/L Bis-Tris**
Flow rate of pH buffering solution	:	0.5 mL/min
Detector	:	Electroconductivity detector (CDD-10A*VP*)

Fig. 6 (a) Chromatograms of organic acids under the different concentrations of Bis-Tris in the pH buffering solution; (b) Peak heights and S/N ratios of organic acids depending on the concentrations of Bis-Tris in pH buffering solution

■ 3) Optimization of detection conditions of organic acid - concentration of EDTA-4H

Some organic acids (for example, citric acid) form chelate compounds with metal ion existing in the column and/or mobile phase. Consequently sensitivity and/or linearity might get worse. To prevent this phenomenon, EDTA-4H is quite effective.

Fig. 7 shows chromatograms with 20 mmol/ L Bis-Tris pH buffering solution, which contains different concentrations of EDTA-4H. The result indicated that 50 µmol/L EDTA-4H was the best, because the background noise was as small as non-EDTA buffer. Linearity of citric acid was quite good in this condition (we describe this later).



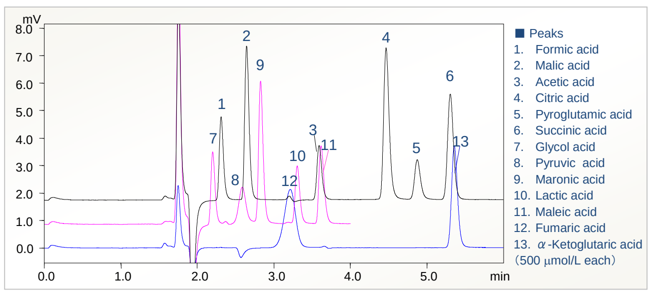
pH Buffering solution	:	5 mmol/L Perchloric acid containing 20 mmol/L Bis-Tris amd **0–200 µmol/L EDTA･4H**

* Other conditions are same as Fig. 6.

Fig. 7 Chromatograms of organic acids under the different concentrations of EDTA-4H in pH buffering solution

■ Analysis of organic acid standard

Chromatograms of 13 organic acids are presented in Fig. 8. The analytical conditions which have been optimized are shown in Table 2. Under the conditions, popular organic acids were separated in 5 min.



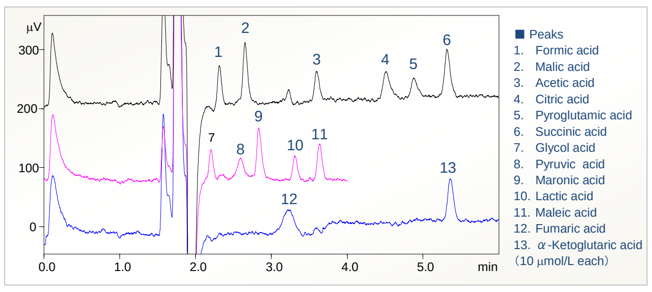
■ Peaks
1. Formic acid
2. Malic acid
3. Acetic acid
4. Citric acid
5. Pyroglutamic acid
6. Succinic acid
7. Glycol acid
8. Pyruvic acid
9. Maronic acid
10. Lactic acid
11. Maleic acid
12. Fumaric acid
13. α-Ketoglutaric acid
（500 µmol/L each）

Fig. 8 Analysis of organic acids by the optimized analytical conditions

Table 2 Optimized analytical conditions

Column	:	Phenomenex Synergi Hydro-RP （100 mm L.×3.0 mm I.D., 2.5 µm） ×2（in series）	pH Buffering solution	:	5 mmol/L Perchloric acid containing 20 mmol/L Bis-Tris amd 50 µmol/L EDTA･4H
Mobile phase	:	5 mmol/L Perchloric acid aq. solution	Flow rate of pH buffering solution	:	0.5 mL/min
Flow rate of mobile phase	:	0.5 mL/min	Detector	:	Electroconductivity detector (CDD-10A*VP*)
Column temp.	:	40 ℃			
Injection volume	:	4 µL			

Fig. 9 shows chromatograms of standard solution of organic acids at high sensitivity. Each concentration of organic acid was 10 µmol/L (4 µL injected). LOD (S/N ratio = 3.3) and repeatability of peak area (n=6) of formic, acetic, citric acid are given in Table 3.



■ Peaks
1. Formic acid
2. Malic acid
3. Acetic acid
4. Citric acid
5. Pyroglutamic acid
6. Succinic acid
7. Glycol acid
8. Pyruvic acid
9. Maronic acid
10. Lactic acid
11. Maleic acid
12. Fumaric acid
13. α-Ketoglutaric acid
（10 µmol/L each）

Fig. 9 Highly sensitive analysis of organic acid standard

Table 3 LOD and repeatability of peak area (from Fig. 9)

	SN ratio	LOD (pmol)	Repeatability of Peak Area (%RSD, n=6)
Formic acid	7.9	16.8	1.7
Acetic acid	5.3	25.1	2.1
Succinic acid	8.6	15.3	1.0

■ Linearity of calibration curves

Calibration curves of formic, acetic, citric, succinic and lactic acid are given in Fig. 10 (10 µmol/L - 2 mmol/L, 4 µL injected). We obtained good linearity for respective compounds.

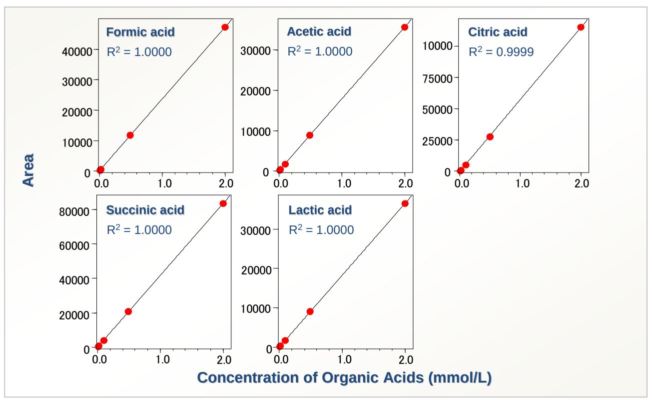
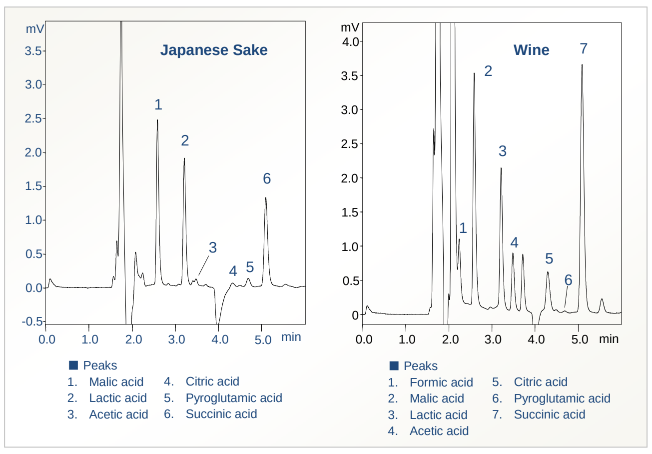


Fig. 10 Calibration curves

■ Analysis of brewed food

We applied this method to the brewed food. Fig. 11 shows chromatograms of Japanese "Sake" and wine, and the results indicated that this method might be quite useful for analysis of samples which contain a slight amount of organic acids and a large amount of co-existing compounds.



■ Peaks

1. Malic acid	4. Citric acid
2. Lactic acid	5. Pyroglutamic acid
3. Acetic acid	6. Succinic acid

■ Peaks

1. Formic acid	5. Citric acid
2. Malic acid	6. Pyroglutamic acid
3. Lactic acid	7. Succinic acid
4. Acetic acid	

Fig. 11 Analysis of organic acids in brewed food

Conclusions

To apply "post-column pH buffering electric conductivity detection" to UHPLC, we optimized analytical conditions. Under the optimized conditions, major organic acids could be separated in about 5 min, with good resolution. LOD of formic acid was 16.8 pmol. Linearity and repeatability of peak area were quite good. We believe that this method is extremely useful for the analysis of organic acids in complicated samples such as food, which contains a large amount of co-existing compounds.