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Principles and Applications of the Prominence Organic Acid Analysis System

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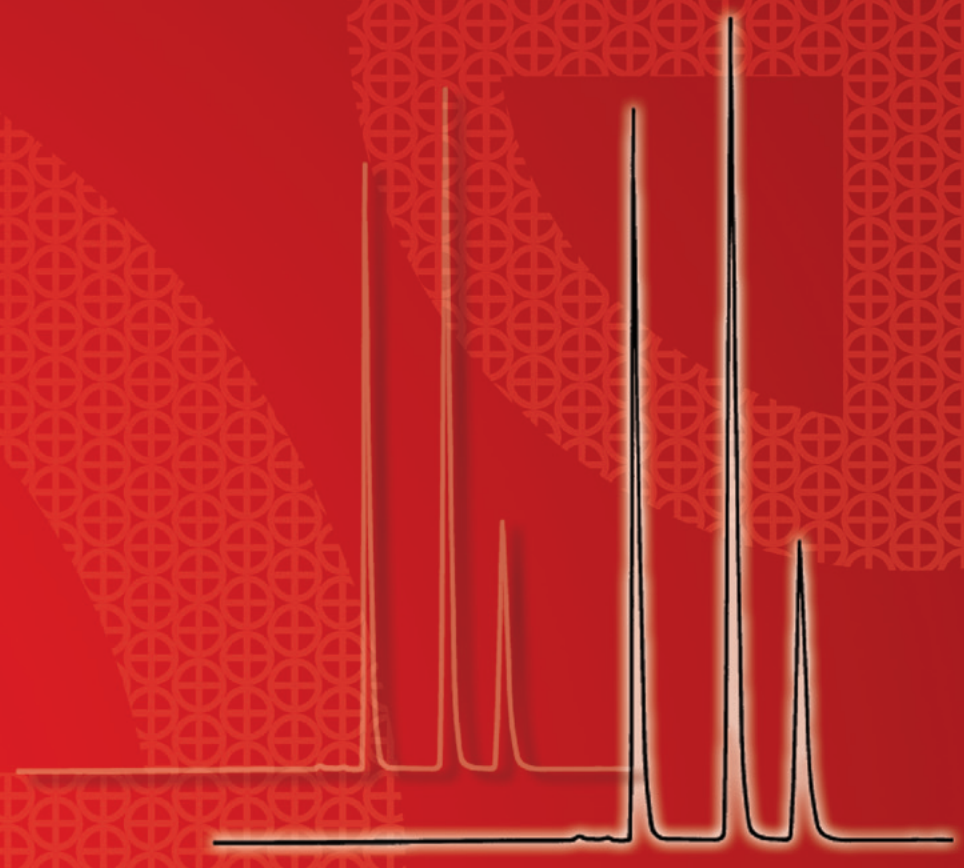

Principles and Applications of the Prominence Organic Acid Analysis System

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1. Introduction

Organic acids*, along with amino acids and sugars, are important substances in biochemistry. They are major analytical target components in various fields such as the environment and food products. They are particularly important components in determining the fragrance and flavor of food products and are commonly analyzed by high-performance liquid chromatography (HPLC).

Conventionally, spectrophotometric detection has been used for HPLC organic acid analysis. However, as the detection wavelength is near 205 nm, equivalent to the absorption maximum of the carboxyl group, detection is subject to the effects of impurity components and the analysis of some samples is difficult. Consequently, methods were developed to improve the detection selectivity of organic acids, including a method to measure the pH changes that accompany organic ion elution as changes in absorption[1] by applying pH indicators (titration indicators) to the post-column detection method[1] and a post-column derivatization method[2] using condensing agents. While these methods achieved some improvements in selectivity, extremely high sensitivity cannot be expected. In addition, problems remain with these methods: the linear range of the calibration curve is narrow for the method using pH indicators, and the post-column derivatization method requires a complex equipment configuration.

On the other hand, as the electroconductivity method can selectively detect ionic substances at high sensitivity, it can be applied to analyzing organic acids, which are ionic substances.[3] However, as acidic aqueous solutions are used as the mobile phase for the ion exclusion chromatography[4,5] method that is widely used for organic acid separation, the background electroconductivity increases and the ionization of the target organic acids is suppressed at a low pH. Consequently, great sensitivity can not be expected by simply using the electroconductivity detection method. Therefore, Shimadzu developed the "post-column pH-buffered electroconductivity method" that achieves outstanding selectivity and sensitivity by promoting the ionization of organic acids through the addition of a buffer solution to the column eluate to achieve an approximately neutral pH. This method was combined with ion exclusion chromatography to create the Organic Acid Analysis System in the Shimadzu HPLC series.[6]

This report explains the basic principles of the Prominence Series Organic Acid Analysis System that is based on this post-column pH-buffered electroconductive detection method, and introduces some application examples in the food products field.

* "Organic acids," the subjects for analysis with this analytical method, mainly refer to lower fatty acids.

2. What is Ion Exclusion Chromatography?

2.1 Ion Exclusion and Ion Exchange

Ion exclusion chromatography can be thought of simply as exclusion chromatography that exploits the "Coulomb repulsion force" between the ions. On the other hand, the similarly named "ion exchange chromatography" is chromatography based on interactions due to the "Coulomb attraction" between the ions. In ion exchange mode, if the analysis targets are anionic substances, for example, the packing material (stationary phase) has an anion exchange group (that is, a cationic residue) and separation occurs by ion exchange with the anions in the mobile phase. The situation is different for ion exclusion chromatography. If the analysis targets are anionic substances, such as organic acids, the packing material (stationary phase) is a cation exchange resin (that is, anionic residue) and acidic aqueous solutions are normally used as the mobile phase. (The mobile phase must mostly contain the counterions identical to the cations that are the counterions for the anionic residue in the packing material.)

2.2 Principles of Ion Exclusion Chromatography

H^+ type cation exchange resin is used as the packing material in ion-exclusion-mode organic acid analysis, and the organic acids are separated according to the magnitude of the Donnan exclusion between the mobile phase and the H^+ type ion exchange residue on the stationary phase surface. A schematic diagram is shown in Fig. 1. Strong acids in the sample (Cl^-, SO_4^{2-}, etc) are strongly excluded by static electricity due to the negative charge on the packing material surface and cannot permeate into the pores of the packing material. Weak acids such as organic acids, on the other hand, are less affected by this electrostatic exclusion. Consequently, the strength of the acid (pKa value) determines the degree of permeation into the pores. This results in differences in the elution time of each organic acid during separation. Theoretically, organic acids elute sequentially from low pKa and all organic acids will elute before the elution position of a neutral substance (corresponding to the permeation limit in exclusion chromatography). However, in practice, in addition to permeation into the pores, hydrophobic interaction also affects the elution of organic acids.

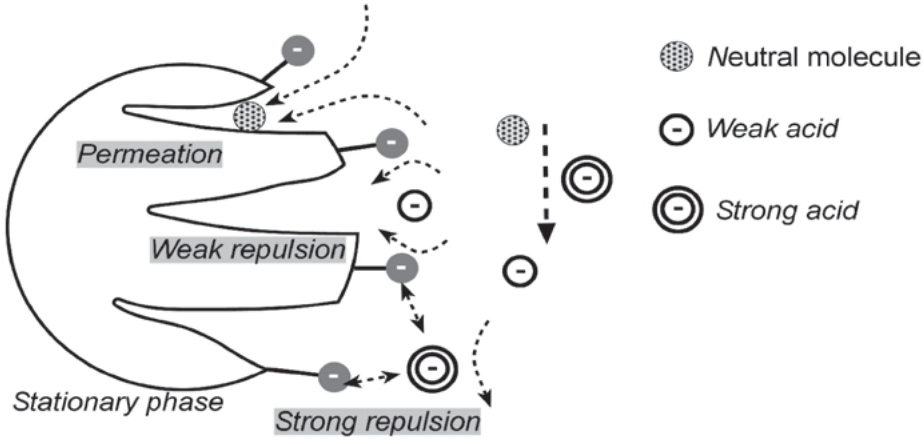

Fig. 1 Interaction on the Surface of the Ion Exclusion Stationary Phase

As H^+ type cation exchange resin is used as the packing material for this separation method, cation components other than hydrogen (H^+) ions in the sample will exchange with hydrogen ions, such that the counterions in the column packing material may gradually be replaced by hydrogen ions. Consequently, a guard column must always be used with this separation method to prevent the flow of cation components into the analysis column. The guard column is also essential to prevent the adsorption of non-ionic substances in the sample onto the analysis column by hydrophobic interaction.

3. What is the Post-column pH-buffered Electroconductivity Detection Method?

3.1 Electroconductivity Detection and the Ion Exclusion Mode

Ionic components can be selectively detected by electroconductivity detection. Changes in the ion content are strongly related to the response of this detection method and application of the method is expected not only for inorganic ions, which are typical ionic components, but also for organic acid ions. However, as a strongly acidic aqueous solution must be used as the mobile phase when separating organic acids by ion exclusion chromatography, the dissociation (ionization) of many organic acids is suppressed. Consequently, it is not possible to obtain adequate sensitivity and a wide calibration curve linearity range with electroconductivity detection. The dissociation efficiency can be improved by neutralization. However, simple neutralization causes changes in the concentrations of hydrogen ions and hydroxide ions, sacrificing the calibration curve linearity. Consequently, buffering of pH and other factors is required.

3.2 Principle of the Post-column pH-buffered Electroconductivity Detection Method

Consider an example of ion exclusion chromatography using a completely dissociated strong acid (HA) aqueous solution as the mobile phase, where a buffering solution, including a base (B), is added to the column eluate to buffer the pH. The responsivity (S) of the detector for the target organic acids (HO) is given by the following expression:

$$S = [O^-]\,\lambda_O + \Delta[A^-]\,\lambda_A + \Delta[BH^+]\,\lambda_B + \Delta[H^+]\,\lambda_H + \Delta[OH^-]\,\lambda_{OH} \qquad (1)$$

λ_O, λ_A, λ_B, λ_H and λ_{OH}, are the ionic equivalent electroconductivity of O^-, A^-, BH^+, H^+ and OH^-, respectively. Of these, $\Delta[A^-]$ can be ignored for ion exclusion chromatography. If the pH of the mixed solution is sufficiently larger than the pKa of HO, it can be considered that HO is completely dissociated. Therefore, under the conditions where the buffering capacity of B is at a maximum, that is, the conditions where the mixed solution is buffered at a pH equal to the pKa of B (at this point, the concentration of B is double that of HA), the changes in concentration of each ion are expressed as follows.

$$\left.\begin{aligned} [O^-] &= C_O \\ \Delta[BH^+] &= C_O \\ \Delta[H^+] &= \frac{(C_A + C_O)}{(C_A - C_O)} K_B - K_B = \frac{2C_O}{(C_A - C_O)} K_B \\ \Delta[OH^-] &= \frac{(C_A - C_O)}{(C_A + C_O)} \cdot \frac{K_W}{K_B} - \frac{K_W}{K_B} = -\frac{2C_O}{(C_A + C_O)} \frac{K_W}{K_B} \end{aligned}\right\} \qquad (2)$$

Where, C_O and C_A are the HO and HA concentrations, respectively; K_B is the acid dissociation factor for B; and K_W is the ionic product of water. By substituting these expressions into expression (1), the responsivity of the electroconductivity detector can be rewritten as follows:

$$S = C_O(\lambda_O + \lambda_B) + \frac{2C_O}{(C_A^2 + C_O^2)}\left[(C_A + C_O)\cdot K_B\cdot\lambda_H - (C_A - C_O)\cdot\frac{K_W}{K_B}\cdot\lambda_{OH}\right] \quad (3)$$

When K_B satisfies the following condition:

$$pK_B = \frac{1}{2}(pK_W + \log\lambda_H - \log\lambda_{OH}) \quad (4)$$

The value of the second term of expression (3) becomes minimal and expression (3) can be rewritten as follows:

$$S = C_O\cdot(\lambda_O + \lambda_B) + \frac{4C_O^2K_B}{(C_A^2 - C_O^2)}\cdot\lambda_H \quad (5)$$

If $C_O \ll C_A$, the second term of expression (5), can be ignored, so that when the HO concentration is sufficiently small, the responsivity can be considered a linear function of the organic acid concentration, and linearity is achieved, as follows:

$$S = C_O\cdot(\lambda_O + \lambda_B) \quad (6)$$

As λ_H, λ_{OH} and pKw are 349.8 S/(cm^2•eq), 198.3 S/(cm^2•eq)[7] and 14 (25 °C), respectively, when these are substituted into expression (4), the solution of pK_B = 7.12 (25 °C) is obtained. Therefore, we can conclude that detection sensitivity and linearity are improved by using a pH-buffered method where a base with an acid dissociation factor close to pKa = 7.12 is added to the mobile phase at a concentration double that of the acid in the mobile phase.

The Prominence Series Organic Acid Analysis System is a unique system that combines ion exclusion chromatography and post-column pH-buffered electroconductivity detection. p-toluenesulfonic acid (*p*-TSA), a strong acidic aqueous solution with a comparatively small equivalent conductivity, is used as the mobile phase. Bis-Tris [Bis (2-hydroxyethyl) iminotris (hydroxymethyl) methane] at pKa = 6.45 (25 °C), which also has a low ionic equivalent electroconductivity, is used as the base. In addition, to improve the mixing efficiency and to minimize the effects on background noise, various measures were adopted, such as adding *p*-TSA to the reaction solution and adding ethylenediaminetetraacetic acid (EDTA-4H) to prevent destruction of the peak shapes of coordinated organic acids such as citric acid.

4. The Prominence Organic Acid Analysis System

4.1 Equipment Configuration

The Shimadzu High-Performance Liquid Chromatograph Prominence Series Organic Acid Analysis System comprises two LC-20AD solvent delivery units (one for mobile phase and the other for reaction reagent), SIL-20AC autosampler, CTO-20AC column oven, CDD-10A$_{VP}$ electroconductivity detector, SCL-10A$_{VP}$ system controller, DGU-20A$_3$ degasser, and a reaction kit (pipe parts J). In addition, an LCsolution workstation is used for data processing and equipment control. Fig. 2 shows the flow diagram for this system.

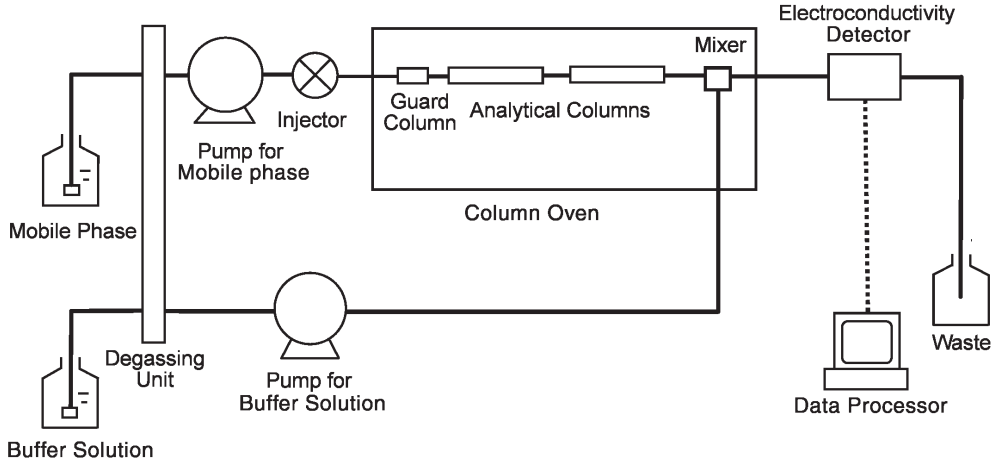

Fig. 2 Flow Diagram of the Prominence Organic Acid Analysis System

4.2 Reagents and Analysis Conditions

p-TSA (for automatic amino acid analysis), Bis-Tris and EDTA-4H are used to prepare the mobile phase and the buffer solution. The organic acid standard samples are adjusted to the required concentration; acetic acid, formic acid, and pyruvic acid are prepared by adjusting the concentration of their sodium salts and lactic acid is prepared by adjusting the concentration of its lithium salts and other free acids.

Two Shim-pack SCR-102H ion-exclusion chromatography columns (8 mm ID x 300 mm long) (only one column for some samples) with an SCR-102H guard column are used for the separation of organic acids. As differences in the net electric charge, molecular weight, and hydrophobic interaction of organic acids contribute to separation in the ion exclusion mode, the lower the pH of the mobile phase (that is, the higher the strong acid concentration in the mobile phase), normally the slower the elution and the easier the separation of each organic acid. With this system, the standard concentration of *p*-TSA in the mobile phase is set to 5 mmol/L to optimize the separation of major organic acids. As column temperature also affects separation (see section 6.2), the standard column temperature is set to 40 °C to achieve efficient separation and stable temperature control that is unaffected by the installation environment. The buffer solution added to the column eluate for detection consists of 5 mmol/L *p*-TSA, 20 mmol/L Bis-Tris and 100 μmol/L EDTA-4H, which are the same concentrations as the mobile phase. The detector cell temperature is set to 43 °C. Table 1 shows the standard analytical conditions for this system.

Samples containing a comparatively small number of organic acid components can be analyzed with one column to shorten the analysis time. In this case, the column temperature is set to 45 °C to improve separation (especially

for succinic and lactic acids) and the cell temperature is set to 48 °C. (These conditions are referred to hereafter as “single-column conditions.”)

Table 1 Standard Analytical Conditions

<Separation>	
Columns	: Two Shim-pack SCR-102H（300mmL. x 8.0mmi.d., 7μm） and Guard Column SCR-102H（50mmL. x 6.0mmi.d.）in series
Mobile Phase	: 5 mmol/L p-TSA
Flow Rate	: 0.8 mL/min
Column Temp.	: 40°C
<Detection>	
Reagent	: 5 mmol/L *p*-TSA, 20 mmol/L Bis-Tris, 100μmol/L EDTA-4H
Flow Rate	: 0.8 mL/min
Cell Temp.	: 43°C
Detection	: Electroconductivity

5. Analysis Using Standard Analytical Conditions

5.1 Simultaneous Analysis of Organic Acids

Fig. 3 shows the chromatogram of a standard solution containing 11 organic acids and phosphoric acid (each concentration 10^{-3} equivalent*/L, 10 μL injected volume) obtained using the standard analytical conditions. These organic acids are generally the most common subjects of analysis and the standard conditions can be widely applied to the analysis of organic acids in food-product samples, such as soy sauce, sake and wine. However, other organic acids may not be sufficiently separated under these conditions. The negative peak at about 12 minutes is a system peak due to *p*-TSA. The region around this position is the exclusion limit for the SCR-102H column used in this system.

Fig. 4 shows the chromatogram of a standard solution containing nine organic acids and phosphoric acid (each concentration 10^{-3} equivalent*/L, 10 μL injected volume) obtained using the single-column conditions. The separation of α-ketogultaric acid from phosphoric acid and the separation of fumaric acid from formic acid and acetic acid are more difficult than in the chromatogram with two columns in Fig. 3.

* Equivalent: The number of moles for each organic acid multiplied by their ion valence.

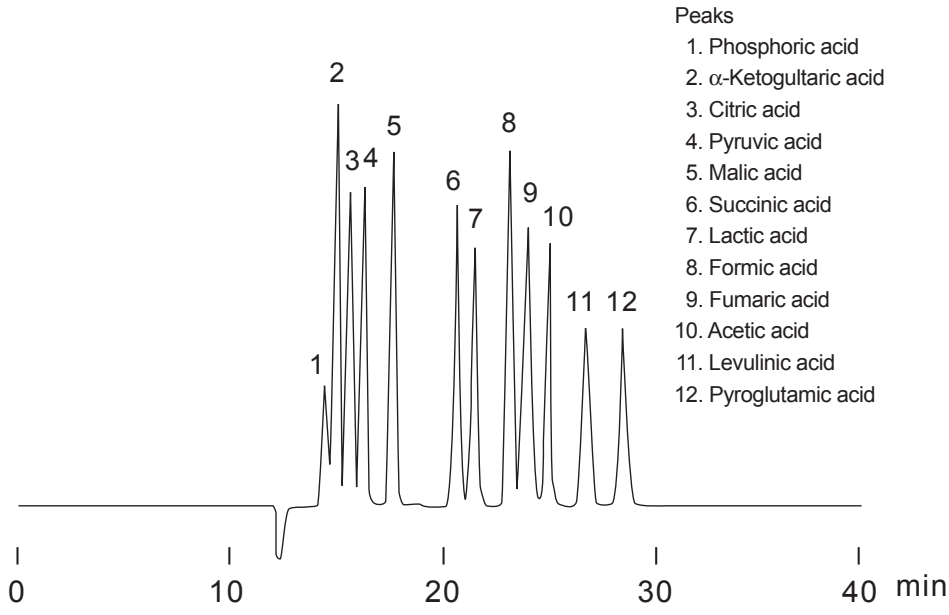

Fig. 3 Chromatogram of a Standard Mixture of Organic Acids and Phosphoric Acid
(Standard Analytical Conditions:Two SCR-102H Columns in Series)

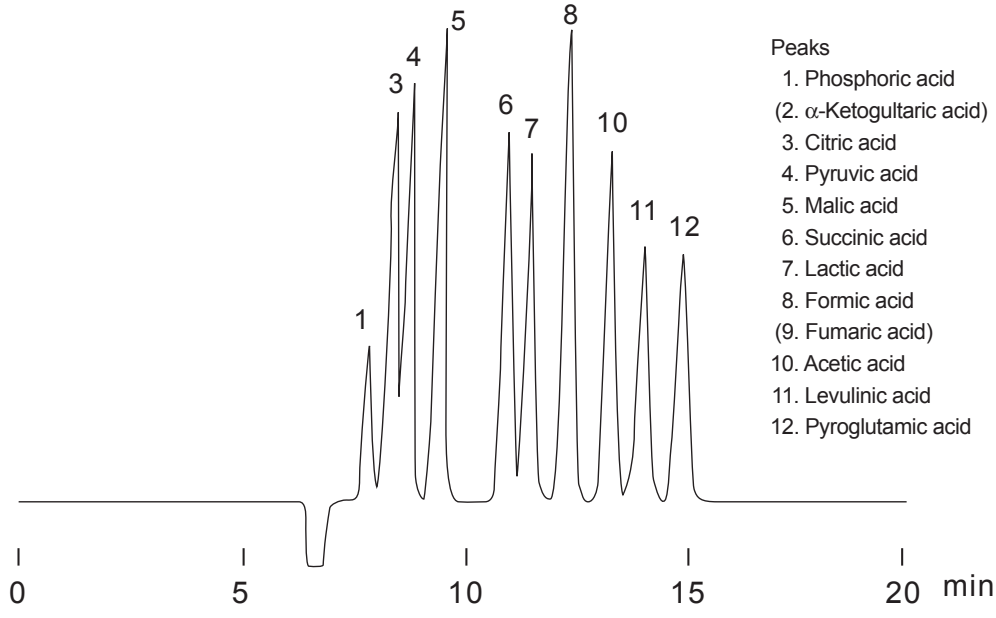

Fig. 4 Chromatogram of a Standard Mixture of Organic Acids and Phosphoric Acid
(One SCR-102H Column, Column Temp.: 45 °C, Cell Temp.: 48 °C)

5.2 Linearity and Repeatability

Table 2 shows the relationship between the injected volume of each organic acid and the detector responsivity. It indicates a good linear relationship that passes through the origin in a range up to an equivalent of 1 x 10^{-6} for each organic acid. Although the lower limit of detection tends to rise as the molecular weight decreases and the elution rate increases, it remained in the range from 3 x 10^{-11} to 5 x 10^{-11} equivalent (S/N = 2) for all the components. The maximum concentration in the cell when an amount of 1 x 10^{-6} equivalent reached the detector cell is 2 mmol/L or less for each component, under the analytical conditions shown in Table 1. This concentration adequately satisfies the C_O « C_A condition used to establish the expression (6).

Table 2 Relationship between Detector Response and the Amount of Organic Acids

Organic acids	Response(10^{-6} S/cm)						
	1 x 10^{-9}	1 x 10^{-8}	1 x 10^{-7}	1 x 10^{-6}(equiv.)	a(10^{-7})	b	r
Citric acid	0.284	2.80	28.2	276.2	2.09	276	1.000
Pyruvic acid	0.294	3.02	29.5	294.1	0.61	294	1.000
Malic acid	0.330	3.32	33.0	329.1	0.43	329	1.000
Succinic acid	0.285	2.78	26.5	259.3	2.69	259	1.000
Lactic acid	0.258	2.45	24.5	241.2	1.49	241	1.000
Formic acid	0.332	3.35	33.1	329.1	0.87	329	1.000
Acetic acid	0.241	2.44	24.3	241.1	0.75	241	1.000
Levulinic aicd	0.176	1.78	17.6	174.1	0.79	174	1.000
Pyroglutamic aicd	0.170	1.72	16.9	167.1	1.04	167	1.000

a and b: from correlation equation, y=a + bx.

x: the amount of the individual organic acids, y: the detector response.

r: coefficient of correlation.

Table 3 shows the retention time repeatability (n = 5) when injecting 10 μL of a standard sample containing the nine organic acids at a concentration of 1 x 10^{-3} equivalent/L each. Good results were obtained, with 0.8% maximum relative standard deviation (RSD%) for each organic acid.

Table 3 Repeatability of Retention Time

Oraganic acids (1 x 10^{-8} equiv.)	RSD% (n=5)
Citric acid	0.78
Pyruvic acid	0.64
Malic acid	0.52
Succinic acid	0.57
Lactic acid	0.62
Formic acid	0.44
Acetic acid	0.32
Levulinic acid	0.41
Pyroglutamic acid	0.33

5.3 Comparison with the Direct Electroconductivity Detection Method

In order to compare the post-column pH-buffered electroconductivity detection method used in this system with the direct electroconductivity detection method, we detected the column eluate with a direct electroconductivity detector. Fig. 5 shows the chromatogram of a standard solution containing nine organic acids and phosphoric acid (each concentration 1 x 10^{-2} equivalent/L, 10 μL injected volume) obtained under single-column conditions. Based on these results and the results in Fig. 4, we compared the sensitivities of the post-column pH-buffered electroconductivity detection method and the direct electroconductivity detection method. The comparison results are summarized in Table 4.

Since the post-column pH-buffered electroconductivity method is not associated with changes in the concentration of hydrogen ions, which have an extremely large ionic equivalent electroconductivity, sensitivity is lower than the direct detection method for organic acids with small pKa values, such as pyruvic acid and pyroglutamic acid. However, sensitivity is significantly improved for other organic acids, despite dilution of the column eluate, due to the addition of the buffer solution. In particular, for acetic acid and levulic acid, which have pKa values of 4 or more, the buffering method can achieve sensitivities at least 19 times greater than the direct detection method.

The concentration of strong acid in the mobile phase significantly affects the sensitivity of the direct detection method: the higher the concentration of strong acid (that is, the lower the pH of the mobile phase), the lower the sensitivity for organic acids. On the other hand, with the post-column pH-buffered electroconductivity method, a certain degree of sensitivity can be achieved by adjusting the buffer concentration according to the strong acid concentration. This means that with this system, the mobile phase conditions can be changed as necessary.

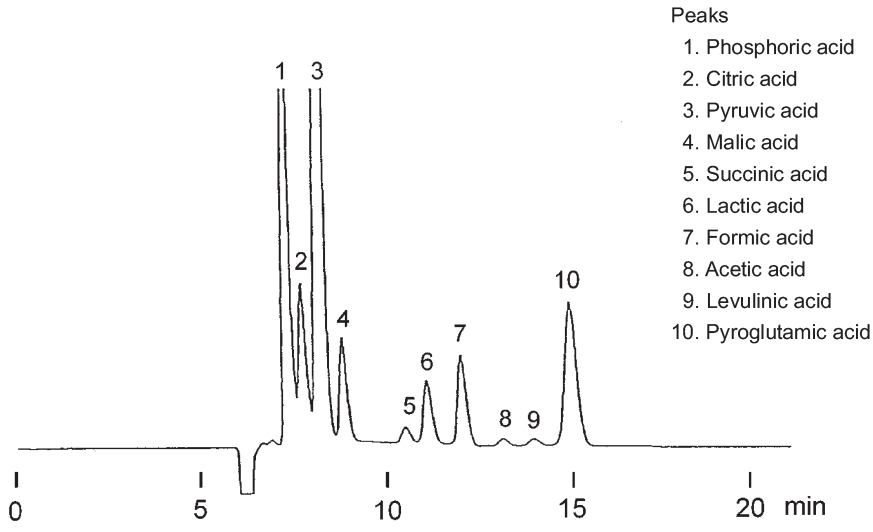

Fig. 5 Chromatogram of a Standard Mixture of Organic Acids and Phosphoric Acid Obtained by Direct Detection Using Electroconductivity Detector

Table 4 Comparison of the sensitivity between the pH-buffered Method and the Direct Detection Method

Organic acid (1×10^{-7} equiv.)	(A) pH-buffered method (10^{-6} S/cm)	(B) Direct detection (10^{-6} S/cm)	Ratio A / B
Citric acid	23.26	15.16	1.53
Pyruvic acid	23.05	121.43	0.19
Malic acid	26.74	10.11	2.64
Succinic acid	21.79	1.62	13.45
Lactic acid	19.01	6.17	3.08
Formic acid	25.05	9.05	2.77
Acetic acid	18.53	0.70	26.47
Levulic acid	13.43	0.69	19.46
Pyroglutamic acid	13.26	14.15	0.94

6. Analytical Conditions and Elution Behavior of Organic Acids

In ion exclusion chromatography, the net electric charge, molecular weight and hydrophobic interaction of organic acids contribute to separation. However, the degree of contribution differs somewhat for each of the organic acids, causing differences in their separation patterns. However, this also means that analytical conditions can be optimized for individual organic acid components. As a reference for optimization, we introduce examples of how the mobile phase concentration and column temperature affect the elution positions of organic acids. (The following data was obtained under single-column conditions.)

6.1 Effects of *p*-TSA Concentration in the Mobile Phase

Fig. 6 shows the relationship between the *p*-TSA concentration in the mobile phase (1 mmol/L, 5 mmol/L, 10 mmol/L) and the retention times of major organic acids. As a general tendency, the lower the acid concentration in the mobile phase, the faster the elution of the organic acid. This tendency is more evident for pyruvic, fumaric and pyroglutamic acids than for other organic acids, indicating a high relative-elution position selectivity due to the acid concentration in the mobile phase.

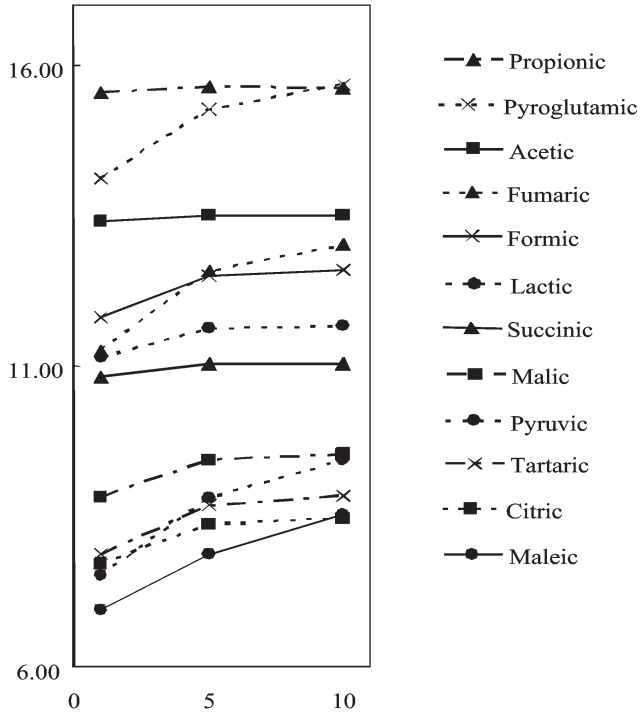

Fig. 6 Relationship between the Retention Time of Organic Acids and the Concentration of *p*-TSA in the Mobile Phase

6.2 Effects of Column Temperature

Fig. 7 shows the relationship between column temperature and the retention times of major organic acids. In this case, we may conclude that changes in the organic acid pKa, due to temperature, contribute to changes in the elution positions. Generally, raising the temperature improves the degree of separation. However, with the exception of a few components, the changes in the elution position are not large.

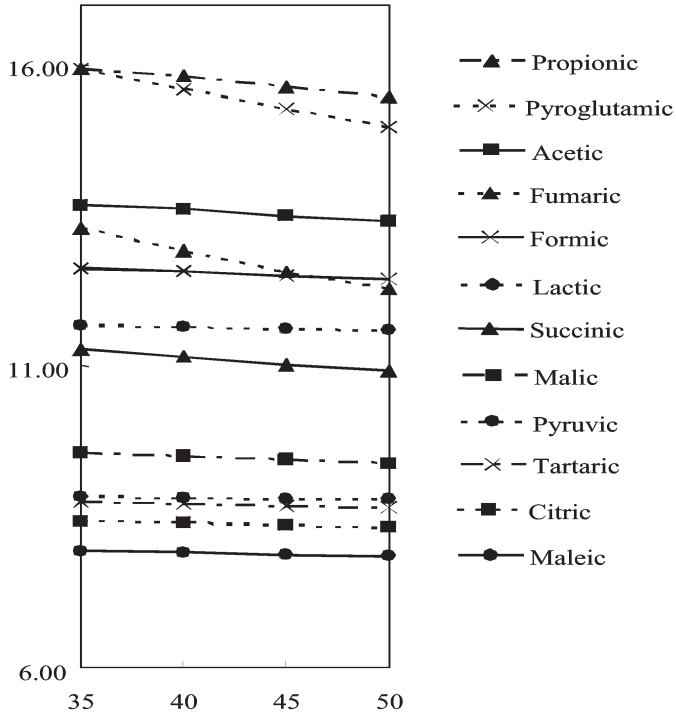

Fig. 7 Relationship between the Retention Time of Organic Acids and the Column Temperature

7. Applications in the Field of Food and Beverage

The Prominence Series Organic Acid Analysis System that permits high-sensitivity selective analysis of organic acids is widely used in various fields. Here, we introduce some typical application examples in the field of food and beverage.

7.1 Analysis of Beer

Fig. 8 shows an example of the analysis of beer using single-column conditions. After shaking the beer to remove the carbon dioxide, it was filtered through a membrane filter (0.45 μm pore diameter) and 10 μL of it was injected. Among the peaks observed in the chromatogram, peaks 1 to 9 match the elution positions of the eight organic acid components and phosphoric acid. Peak 10 and the negative peak immediately following it are assumed to be carbonic acid and ethanol, respectively. This analysis example indicates that these amino acids can be analyzed without being affected by impurities, even without any special sample pretreatment.

By means of comparison, Fig. 9 shows the results of analyzing the same beer sample using the absorption detection method (210 nm). The sample was pretreated in the same way as the analysis in Fig. 8. The analytical conditions are shown in Table 5. As *p*-TSA has strong UV absorption, perchloric acid was used as the mobile phase for the absorption detection method. (Elution positions differ slightly in Fig. 8 and Fig. 9 due to the different acidic solutions.) With absorption detection, a large number of impurity peaks, which hinder the identification of the organic acids, appear.

As shown in this example, the Prominence Series Organic Acid Analysis System greatly contributes to improving the analysis reliability, particularly for samples originating from natural products.

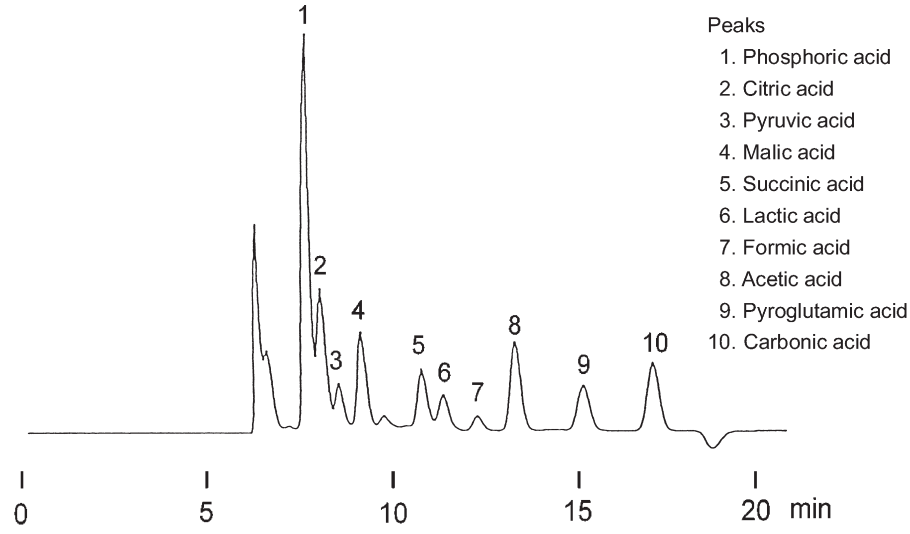

Fig. 8 Chromatogram of a Beer Sample

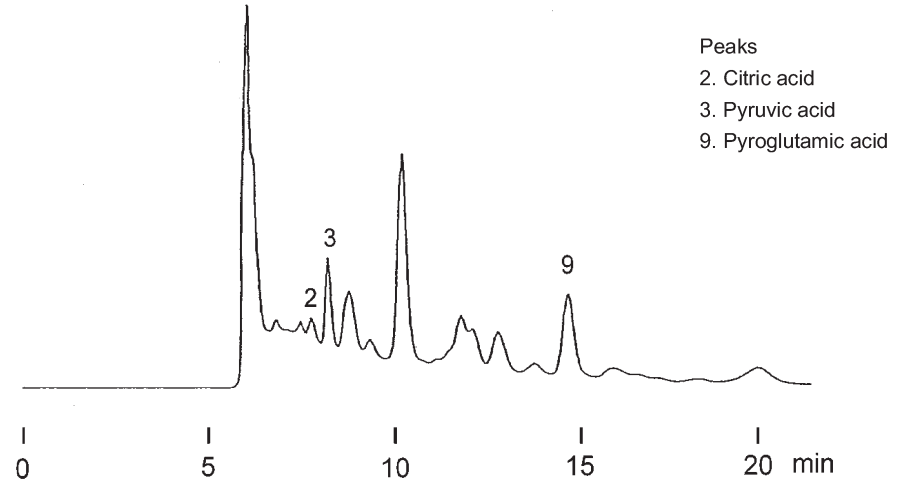

Fig. 9 Chromatogram of a Beer Sample Obtained by Absorption Detector

Table 5 Analytical Conditions (Absorption Detection)

Columns	: Shim-pack SCR-102H (300mmL. x 8.0mmi.d., 7μm) and Guard Column SCR-102H (50mmL. x 6.0mmi.d.) in series
Mobile Phase	: 5 mmol/L Perchloric acid aqueous solution
Flow Rate	: 0.8 mL/min
Column Temp.	: 50°C
Detection	: UV absorption at 210nm

7.2 Analysis of Japanese Sake and Wine

Fig. 10 shows an example of the analysis of Japanese sake and Fig. 11 the analysis of wine. The sake sample was analyzed without dilution, and the wine was diluted with water ten times. Both samples were filtered through a membrane filter (0.45 μm pore diameter) and 10 μL of each were injected. In both cases, negative peaks appeared due to the ethanol contained in the sample, similar to the beer sample.

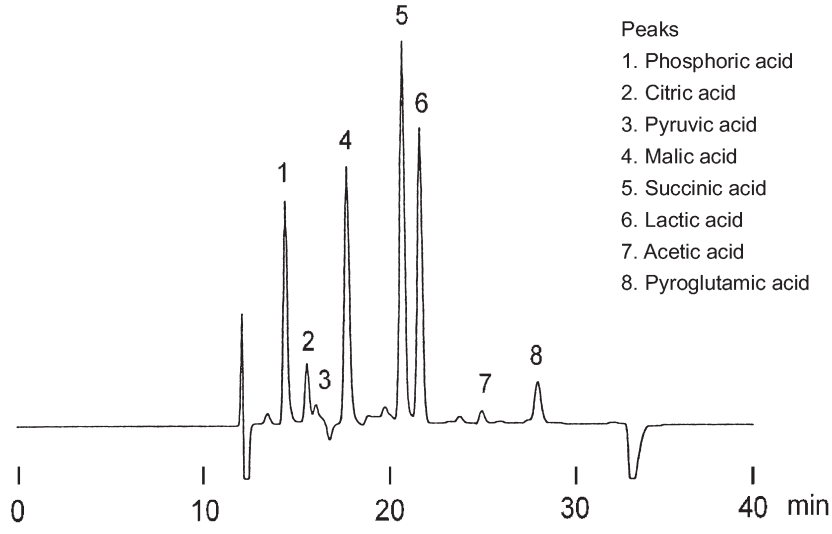

Fig. 10 Chromatogram of a Japanese Sake Sample

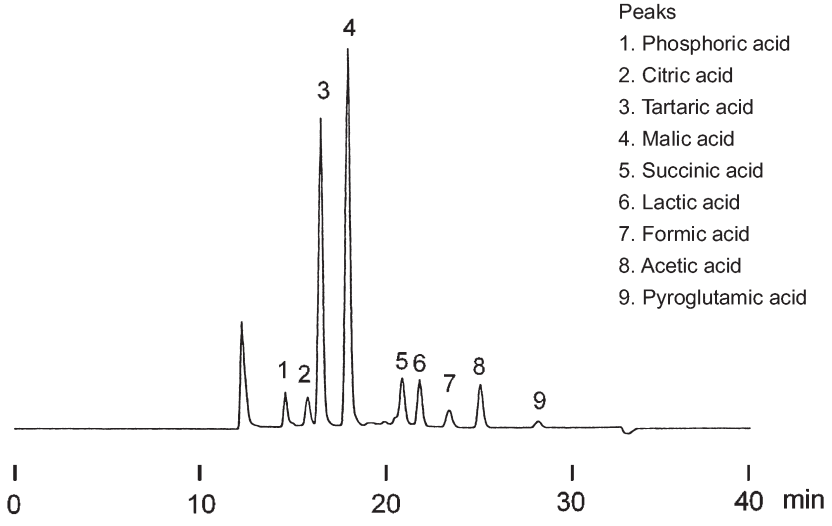

Fig. 11 Chromatogram of a Wine Sample

7.3 Analysis of Soy Sauce

Fig. 12 shows an example of the analysis of soy sauce. After diluting the sample ten times with water, it was filtered through a membrane filter (0.45 μm pore diameter) and 10 μL of it was injected.

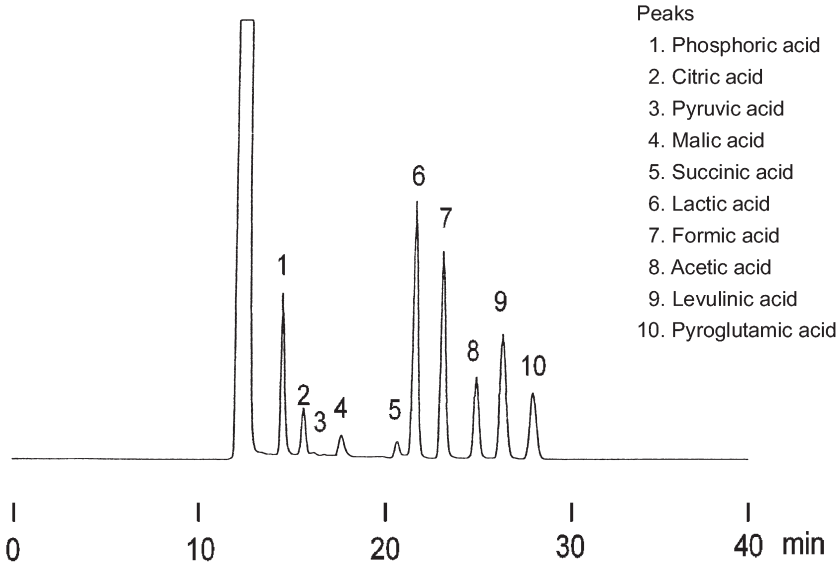

Fig. 12 Chromatogram of a Soy Source Sample

7.4 Analysis of Tomato Juice

Fig. 13 shows an example of the analysis of tomato juice. After grinding up a tomato and obtaining the juice, the juice was diluted ten times with purified water, filtered through a membrane filter (0.45 μm pore diameter) and 10 μL of it was injected.

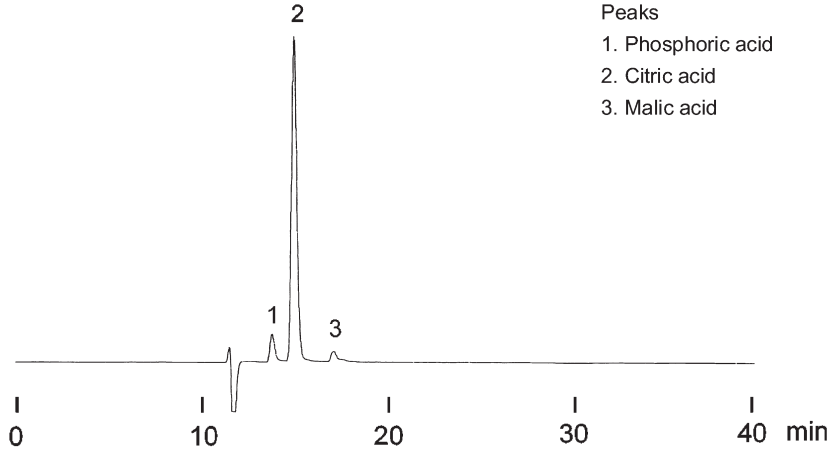

Fig. 13 Chromatogram of a Tomato Juice Sample

8. Conclusions

Electroconductivity detection, which has selectivity for ionic substances, is a suitable detection method for the analysis of organic acids in complex samples such as food products. However, due to its incompatibility with ion exclusion chromatography used for the separation of organic acids, there were few cases where electroconductivity detection was directly applied to the analysis of food products. The post-column pH-buffered electroconductivity detection method used by the Prominence Series Organic Acid Analysis System has outstanding detection sensitivity, selectivity and linearity, and is especially suitable for samples with large numbers of impurities, such as food products. This analysis system provides valuable information for samples that cannot be efficiently analyzed by conventional methods using absorption detection.

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