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Application Note

No. 61

Organic acid

Various Analysis Techniques for Organic Acids and Examples of Their Application

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Contents

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1. Introduction

Organic acids have traditionally been analyzed as tasty components of alcoholic beverages and fermented foods, counter-ions for target compounds of pharmaceutical products, and malodorous components in wastewater. In recent years, an increasing number of policies have aimed at realizing a sustainable society by ending dependence on fossil fuels, and active research is now underway on new energy technologies in the form of artificial photosynthesis and efficient production processes for bioethanol. Monitoring of the organic acids produced by these technologies is necessary in order to improve their efficiency. Moreover, until now, fossil fuels have been used as raw materials for plastic and film products, but efforts to replace organic acids produced in biomass fermentation processes, namely, succinic acid and propionic acid with raw materials for polymer products as intermediate materials are increasing. Monitoring of organic acids is also required in a diverse range of other situations, including research of intestinal bacteria that produce short-chain fatty acids as functional components and product development of high value-added foods and beverages by analysis of primary metabolites, such as organic acids.

Separation analysis has a long history of use in analysis of organic acids, but the respective techniques have distinctive features. For example, in GC and GC/MS, liquid samples are normally introduced into the column after vaporization. In another technique, only the gas phase (headspace) of a sample enclosed in a vial is injected into the column. In analysis of organic acids by HPLC, many different analytical techniques are available by combining various separation modes and detection methods, and in recent years, simultaneous analysis of organic acids and other components has become possible by use in combination with a mass spectrometer. This article introduces organic acid analysis techniques utilizing separation analysis, together with examples of their application by field. Table 1 summarizes the features of organic acid analysis by type of instrument.

Table 1 Classification of Analysis Techniques

Instrument	Main form of sample used in analysis	Derivatization used	Detection selectivity	Comprehensiveness
HPLC	• Water • Water-soluble solvent	No	CDD: Good UV: Poor	Poor
LC-MS			Excellent	Good
GC	• Solvent • Gas (*Used in combination with sample preparation system)	Yes	Poor	Average
GC-MS			Excellent	Excellent

2. Analysis of Organic Acids by HPLC

Organic acids are characterized by high polarity. Therefore, in analysis of organic acids by reversed-phase chromatography, which is a general-purpose separation mode, a mobile phase consisting of only aqueous solutions is selected. Ion exclusion chromatography and ion exchange chromatography are used in separation of organic acids that include multiple components, as these modes have high separation selectivity for ionic compounds.

Three detection methods are used with organic acids, absorbance (UV) detection, electroconductivity detection, and MS detection. However, it is difficult to obtain reliable analysis results by UV detection and electroconductivity detection in analyses of samples which have a complex matrix due to the background effects originating from co-eluted compounds or the mobile phase. Countermeasures for this problem include the post-column method and use of a suppressor cartridge or other removal treatment device.

Generally speaking, three methods may be mentioned: (1) Reversed-phase chromatography – UV detection method, (2) Ion exclusion chromatography using the post-column method (post-column electroconductivity detection method), and (3) Ion exchange chromatography – Electroconductivity detection method (ion chromatography: IC). This chapter introduces examples of organic acid analysis by these three methods using HPLC.

2-1. Reversed-Phase Chromatography: UV Detection Method

The analytical technique combining reversed-phase chromatography and the UV detection method makes it possible to analyze organic acids using a general-purpose HPLC. Since organic acids include a carboxyl group, they display absorption peaks in the short wavelength region near 210 nm. Fig. 1 shows the UV absorption spectrum of citric acid. Because many organic compounds have functional groups that possess UV absorbance, interference by co-eluted components peaks occurs easily in the short wavelength region with the UV detection method. However, this is a suitable detection method for analysis of samples with relatively few matrices.

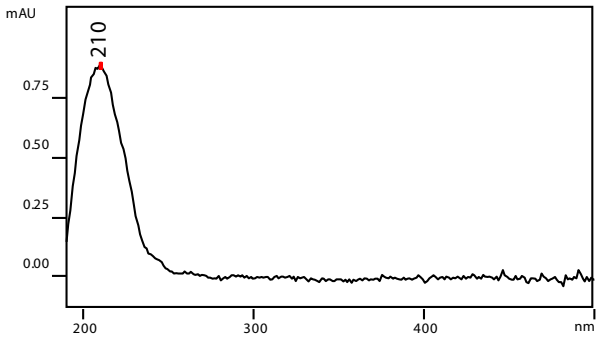


Fig. 1 UV Absorption Spectrum of Citric Acid

Reversed-phase chromatography is a mode in which compounds are separated by their differences in hydrophobic interaction, and is the most widely applicable separation method for HPLC analysis. Organic solvents are not used in analyses of highly hydrophilic compounds such as organic acids. Only aqueous solutions are used as the mobile phase. Among columns for reversed-phase chromatography, a large line-up of products for high speed analysis is available, enabling rapid analysis of organic acids. Fig. 2 shows an example of a high speed analysis of organic acids in a soft drink.

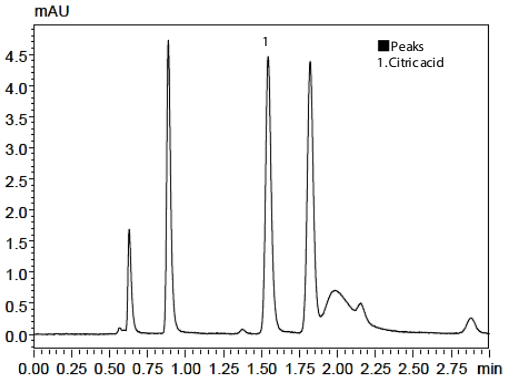


Fig. 2 High Speed Analysis of Organic Acids in Soft Drink

Although conventional columns for reversed-phase chromatography have the problems of retention time decreasing and poor repeatability due to the usage of the aqueous mobile phase for a long time, recently-developed columns realize stable retention over extended periods, even in use of this type of mobile phase. Fig. 3 shows a comparison of the performance of Shimadzu reversed-phase columns in the Shim-pack™ G series. In comparison with other ODS columns in the G series, the Shim-pack GIST C18-AQ (Fig. 4) has a high retention capacity and inertness. This column exhibits stable retention time repeatability when using an aqueous mobile phase and enables high speed, high resolution analysis of organic acids. Fig. 5 shows an example of a high speed analysis of organic acids in wine using a Shim-pack GIST C18-AQ.

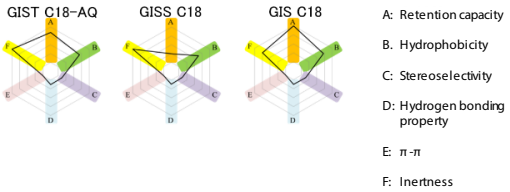


Fig. 3 Performance Comparison of Shim-Pack G series

Read here for the Shim-pack GIST series site.

Fig. 4 Shim-pack™ GIST C18-AQ

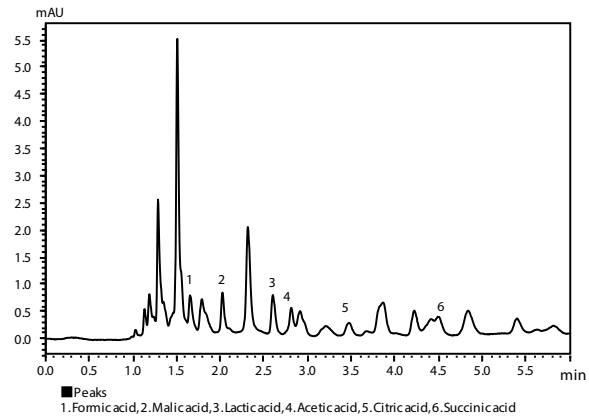


Fig. 5 Analysis of Organic Acids in Red Wine Using Shim-pack GIST C18-AQ

Analysis Conditions

Separation column	:	Shim-pack GIST C18-AQ (250 mm × 3.0 mm I.D., 3 µm)
Mobile phase	:	10 mmol/L (sodium) phosphate buffer solution (pH2.6)
Flow rate	:	1.0 mL/min
Column temp.	:	40 °C
Injection volume	:	4 µL
Detection	:	UV absorbance detector (210 nm)

Read here for Nexera series site.

Nexera
Ultra High Performance Liquid Chromatograph

Nexera™ series Ultra High Performance Liquid Chromatograph

2-2. Ion Exclusion Chromatography: Electroconductivity Detection Method

This method is provided as the LC organic acid analysis system, which is one part of Shimadzu line of LC application system products. Fig. 6 shows the principle of the Shimadzu LC organic acid analysis system. Organic acids in the sample are first separated by the ion exclusion column, and are then ionized by a continuously-added pH buffer solution and detected with high sensitivity by an electroconductivity detector. Because only the ionic components in the sample are detected by this method, highly selective analysis of organic acids is possible, virtually unaffected by interference by co-eluted peaks.

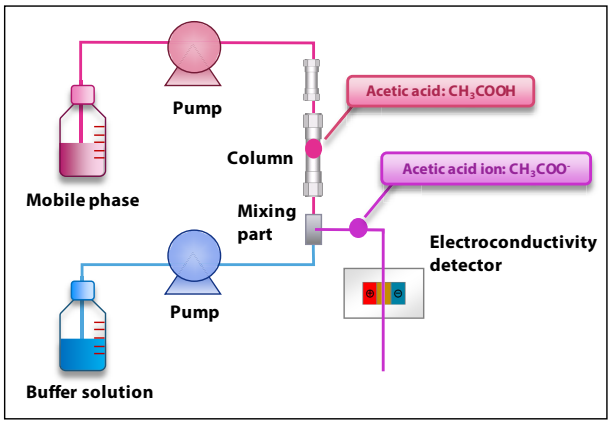


Fig. 6 Principle of Shimadzu LC Organic Acid Analysis System

In organic acid analysis by HPLC, ion exclusion chromatography is the first choice for separation. This separation mode has high selectivity for separation of weak acids such as organic acids. Fig. 7 shows the principle of ion exclusion chromatography. Strong acids are subject to strong electrostatic exclusion (ion repulsion) by the negative charge of the stationary phase (H-type ion exchanges groups), and pass through the column without access into the pores of the packing material. In the case of weak acids such as organic acids, the degree of access into the pores differs due to the degree of ion repulsion, depending on the degree of dissociation by pKa, and as a result, the elution time from the column differs depending on the acid. The columns for ion exclusion chromatography in the Shim-pack series product line are the Shim-pack SCR-102H, which is suitable for high resolution analysis, and the Shim-pack Fast-OA, which is suitable for high speed analysis.

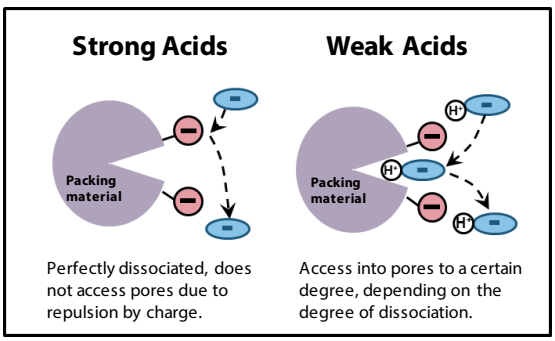


Fig. 7 Principle of Ion Exclusion Mode

The mobile phases used in organic acid analysis by ion exclusion chromatography are acidic aqueous solutions, namely, *p*-toluenesulfonic acid and perchloric acid. Under this kind of acidic mobile phase condition, weak acids such as organic acids exist in a state of suppressed ionization and high background electroconductivity originating from the mobile phase, which means adequate sensitivity cannot be obtained with an electroconductivity detector. Therefore, after the organic acids are separated by the column, a pH buffer solution is continuously added and mixed to adjust the pH of the mobile phase to near neutral, thereby promoting ionization of the organic acids and greatly improving the detection sensitivity of the electroconductivity detector.

Fig. 8 shows the relationship between the pH of the mobile phase and dissociation in the case of acetic acid, and Fig. 9 shows chromatograms comparing the results with and without pH buffering. It can be understood that the acetic acid is not almost completely dissociated at the pH of approximately 3 of the *p*-toluenesulfonic acid aqueous solution used as the mobile phase.

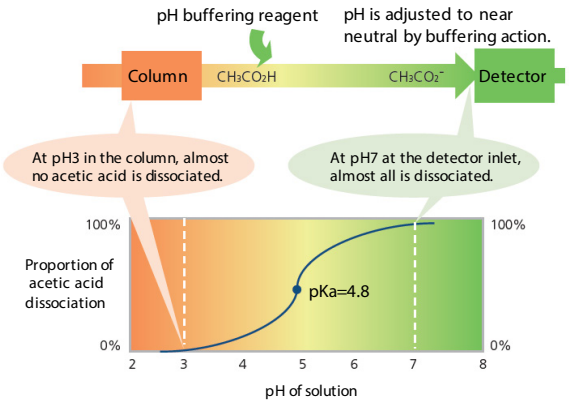


Fig. 8 Condition of Organic Acid in pH Buffering Method

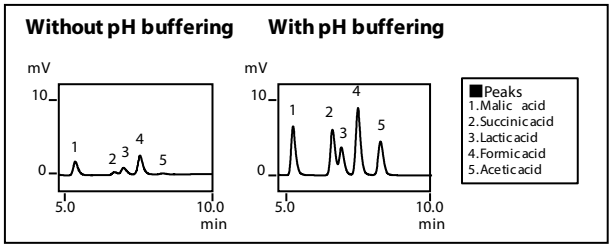
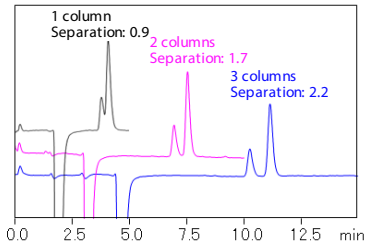


Fig. 9 Enhancement of Sensitivity by pH Buffering

Read here for Nexera organic acid analysis system site.

Nexera™ Organic Acid Analysis System

Because the ion exclusion mode is not suitable for gradient elution, separation capacity is improved by connecting multiple columns in series and controlling the column temperature appropriately. Fig. 10 shows the chromatograms when multiple columns were connected in series under the same condition. Separation is improved by coupling the columns. For details, please refer to Technical Report C190-0489 "High-Speed Analysis of Organic Acids by Shim-pack Fast-OA and pH-Buffered Electrical Conductivity Detection."



Read here to download Technical Report.

Fig. 10 Effect of Connecting Multiple Shim-pack Fast-OA Columns

Fig. 11 (left) shows an example of retention improvement by temperature control. Because the dissociation state of ionic compounds changes depending on the ambient temperature, the optimum separation for the target organic acid may be obtained by adjusting column temperature. In analyses where there are many target components and separation is inadequate even with multiple columns, further improvement of separation may be possible by individually controlling the temperatures of the connected columns. Fig. 11 (right) shows an example of organic acid separation when using different column temperatures. For details, please refer to Application News No. L442A "Effect of Column Temperature on Organic Acid Separation."

Read here to download Application News.

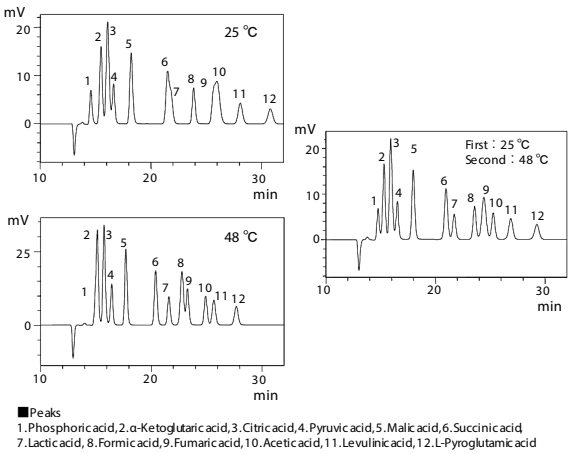


Fig. 11 Example of Retention Improvement by Column Temperature Control (When Using 2 Shim-Pack SCR-102H Columns)

2-3. Ion Chromatography

Ion chromatography is a specialized analytical technique for measurement of ionic compounds, and mainly uses a combination of ion exchange chromatography in separation and an electroconductivity detector in detection. Although ion chromatography is mostly used in analyses of inorganic ions, it is also possible to measure organic acids by ion chromatography, as organic acids are ionic compounds.

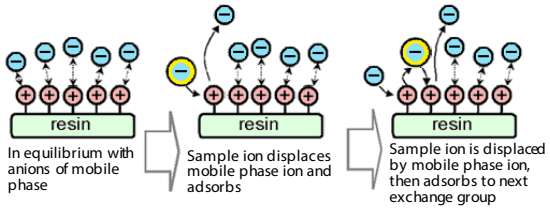


Fig. 12 Principle of Anion Exchange Chromatography

Fig. 12 shows the principle of separation by anion exchange chromatography. Retention is achieved as a result of the anion in the sample and the anion in the mobile phase being repeatedly exchanged on the cations in the stationary phase. An anionic mobile phase is used to desorb the anions from the stationary phase. In other words, in this mode, organic acids (anions) in the samples are separated by repeating adsorption and desorption at the cations of the stationary phase competitively. In organic acid analysis using this separation mode, the organic acid in the sample is dissociated in the mobile phase, and the organic acid in the anionic state is held in the stationary phase. An aqueous solution containing acid and salt is used as the mobile phase in separation analysis in ion exchange chromatography.

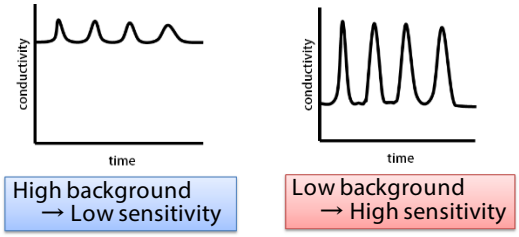


Fig. 13 Effect of Background when Using Electroconductivity Detector

As the principle of the electroconductivity detector, this type of detector uses an electric current, which depends on the existence of ions in an aqueous solution, and detects the change in eletroconductivity due to the difference in the ionic concentrations of the sample and the mobile phase flowing into the detector. Since the total ion content in the sample and mobile phase is detected as the sum of their electroconductivities, the observed conductivity response at the elution time of the target component of the chromatogram will be low if the background electroconductivity originating from the mobile phase is high (Fig. 13). Two analytical modes are used to solve this problem. The suppressor mode utilizes a device called a suppressor, which removes ions in the mobile phase, whereas the non-suppressor mode uses a low-background mobile phase. It may be noted that simultaneous detection of organic acids and inorganic ions is possible by ion chromatography.

2-3-1. Suppressor Mode

In order to separate organic acids in ion exchange chromatography, an eluent with a pH of 7 or higher is used. An aqueous solution of sodium carbonate and aqueous solution of potassium hydroxide are used as eluents. Because these eluents contain high conductivity ions such as the sodium ion and the potassium ion, a suppressor is used to remove these ions. Fig. 14 shows the principle of the ICDS™-40A suppressor of the HIC-ESP ion chromatograph.

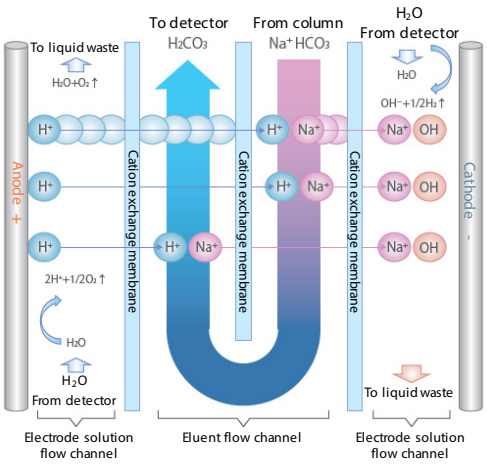


Fig. 14 Principle of ICDS-40A Suppressor

The background electroconductivity of the eluent is reduced after passing through the suppressor, as the suppressor removes dissolved sodium and other cations and replaces them with hydrogen ions. Since the organic acid is ionized, ions are detected with high sensitivity by the electroconductivity detector.

When using an electroconductivity detector, the optimum analytical technique corresponding to the purpose should be selected. That is, the organic acid analysis system should be used when the aim is simultaneous analysis of various organic acids, and suppressor mode ion chromatography should be selected when the priority is sensitivity.

Fig. 15 shows an example of separation of dicarboxylic acid using a Shim-pack IC-SA3 column for anionic analysis.

Analysis Conditions

Separation column	:	Shim-pack IC-SA3 (250 mm × 4.0 mm I.D., 5 µm)
Mobile phase	:	3.6 mmol/L sodium carbonate
Flow rate	:	0.8 mL/min
Column temp.	:	45 °C
Injection volume	:	50 µL
Detection	:	Electroconductivity detector (suppressor mode)

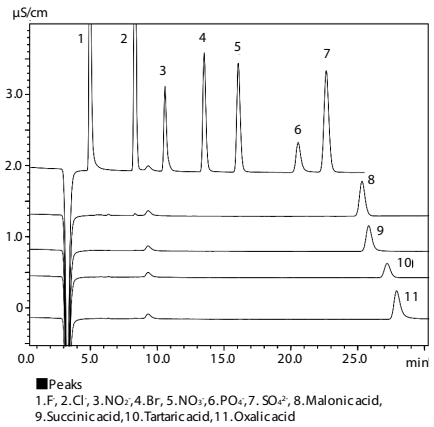


Fig. 15 Example of Separation of Dicarboxylic Acid (Suppressor Mode)

2-3-2. Non-Suppressor Mode

In separation of ionic compounds by ion exchange chromatography without a suppressor, it is common to use an acid with a low charge density for the mobile phase to minimize background electroconductivity.

Fig. 16 shows an example of separation of dicarboxylic acid using a Shim-pack IC-A3 column for anionic analysis. Because not only selectivity but the pH of eluent differs between the non-suppressor and suppressor, differences also appear in the separation patterns of organic acids.

Analysis Conditions

Separation column	:	Shim-pack IC-A3 (150 mm × 4.6 mm I.D., 5 µm)
Mobile phase	:	8.0 mmol/L *p*-hydroxybenzoic acid 3.2 mmol/L Bis-Tris, 50 mmol/L boric acid
Flow rate	:	1.2 mL/min
Column temp.	:	40 °C
Injection volume	:	50 µL
Detection	:	Electroconductivity detector (non-suppressor mode)

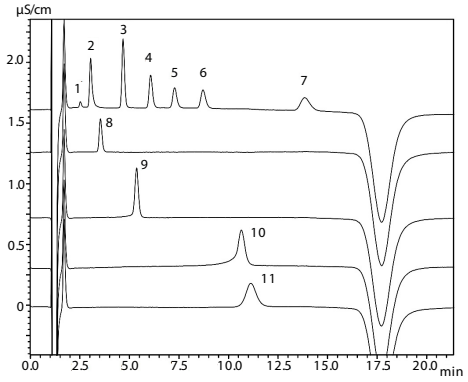


Fig. 16 Example of Separation of Dicarboxylic Acid (Non-Suppressor Mode)

Read here for HIC-ESP ion chromatograph site.

HIC-ESP Suppressor Mode Ion Chromatograph for Anion Analysis

3. Analysis of Organic Acids by LC-MS

The methods used in detection of organic acids by LC-MS are the direct detection method in the negative mode combined with separation by an acidic mobile phase, and the pre-column derivatization method (pre-label method) utilizing a nitrophenylhydrazine (NPH) reagent. LC-MS is used not only with organic acids, but also in simultaneous analysis of primary metabolites as well as amino acids, and in metabolomic analysis.

3-1. Direct Detection Method

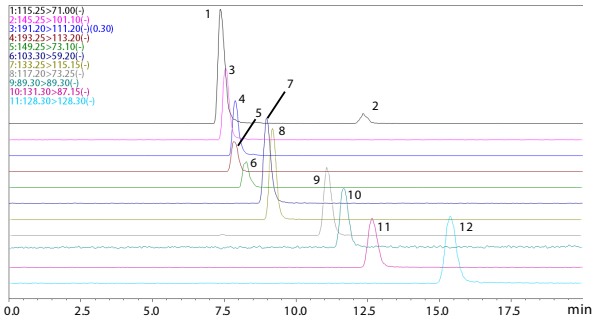
Fig. 17 shows an example of direct detection of organic acids by LC-MS/MS using a Shim-pack SPR-H with a sulfonate polystyrene resin stationary phase. Although the Shim-pack SPR-H uses the same separation mode as the Shim-pack SCR-102H introduced previously, an aqueous solution of formic acid is used in separation, as an aqueous solution of perchloric acid and aqueous solution of *p*-toluenesulfonic acid are not suitable mobile phases for LC-MS. Since formic acid and acetic acid are frequently added to the mobile phase in LC-MS analysis, this detection method is unsuitable for analyses which require highly sensitive determination for these acids.

Analysis Conditions (LC)

Separation column	: Shim-pack SPR-H (250 mm × 4.6 mm I.D., 8 µm)
Mobile phase	: 0.2 % formic acid aqueous solution
Mobile phase flow rate	: 0.2 mL/min
Post-column solvent	: Acetonitrile
Post-column solvent flow rate	: 0.2 mL/min
Column temp.	: 45 °C
Injection volume	: 10 µL

Analysis Conditions (MS)

Ionization method	: ESI (negative)
Nebulizer gas flow rate	: 1.5 L/min
Drying gas flow rate	: 10 L/min
DL temp.	: 250 °C
Heat block temp.	: 400 °C



■Peaks
1.Maleic acid, 2.Fumaric acid, 3.α-Ketoglutaric acid, 4.Citric acid, 5.Glucuronic acid, 6.Tartaric acid, 7.Malonic acid, 8.Malic acid, 9.Succinic acid, 10.Lactic acid, 11.Glutaric acid, 12.Pyroglutamic acid

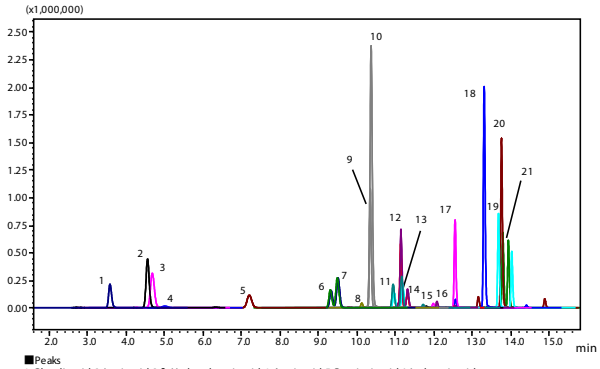
Fig. 17 Result of LC-MS/MS Analysis of Organic Acids Using Shim-pack SPR-H

3-2. Derivatization Method

Although direct detection of organic acids is possible by LC-MS, it is difficult to detect highly volatile organic acids with high sensitivity. However, detection of high volatility organic acids is possible by derivatization, that is, reacting the target component with a reagent in advance. Fig. 18 shows an example of simultaneous analysis of C2 to C5 short-chain fatty acids and pyruvic acid and other organic acids derivatized using a 3-NPH (nitrophenylhydrazine) reagent. Details of this analysis can be found in Application News No. C168 "Analysis of Short-Chain Fatty Acids/Organic Acids (3-NPH Derivatives) in Fecal Specimens from SPF and Antibiotic-Fed Mice."

Read here to download Application News.

Read here for LC/MS/MS method package for short-chain fatty acids site.



■Peaks
1.Glycolic acid, 2.Lactic acid, 3.β-Hydroxybutyric acid, 4.Acetic acid, 5.Propionic acid, 6.Isobutyric acid, 7.Butyric acid, 8.2-Hydroxyglutaric acid, 9.Malonic acid, 10.Succinic acid, 11.Isovaleric acid, 12.Fumaric acid 13.Valeric acid, 14.Maliec acid, 15.Isocitric acid, 16.Citric acid, 17.Glyoxylic acid, 18.Pyruvic acid, 19.Oxaloacetic acid, 20.Oxobutyric acid, 21.α-Ketoglutaric acid

Fig. 18 Simultaneous Analysis of Organic Acids and Short-Chain Fatty Acids by 3-NPH Derivatization

Read here for LCMS-8060 triple quadrupole high speed LC-MS/MS.

LCMS™-8060 High Speed Triple Quadrupole Liquid Chromatograph Mass Spectrometer

4. Analysis of Organic Acids by GC and GC-MS

In analyses of organic acids by GC and GC/MS, samples for analysis are derivatized, mainly with trimethylsilyl (TMS). Fig. 19 shows the MRM chromatograms from an analysis of organic acids using TMS derivatization. For details of this analysis, please refer to Application Data Sheet No. 59 "Analysis of Metabolites in Rat Urine Using MRM via GC-MS/MS."

Read here to download Application Data Sheet.

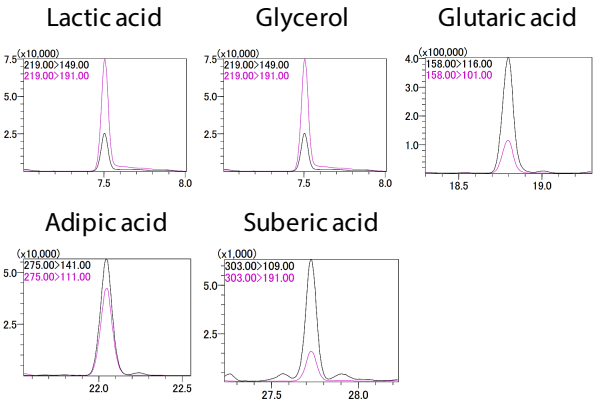


Fig. 19 MRM Chromatograms of Metabolites (TMS Derivatized) in Rat Urine using GC-MS/MS

Read here for GCMS-TQ8040 NX triple quadrupole GC-MS/MS site.

GCMS-TQ™8040 NX Triple Quadrupole Gas Chromatograph Mass Spectrometer

Short-chain fatty acids with carbon chains consisting of no more than 6 carbon atoms, such as acetic acid and propionic acid, are highly volatile, and many of these acids volatilize in the process of sample exsiccation during TMS derivatization. For this reason, TMS reagents are not suitable for derivatization of these compounds. It is known that a reagent which induces condensation reactions between carboxylic acid and amines in water or methanol, such as the reagent DMT-MM (4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmopholinium chloride), has been applied as a derivatization method for analyses targeting short-chain fatty acids with carbon chains of no more than 6 C atoms. Fig. 20 shows the mechanism of the condensation reaction between a short-chain fatty acid (formic acid) and n-octyl amine in the presence of the DMT-MM reagent, and Fig. 21 shows an example of analysis of short-chain fatty acids after derivatization with the DMT-MM reagent. Details of this analysis can be found in Application News No. M273, "Analysis of Short-Chain Fatty Acids in Biological Samples Using GC-MS."

Read here to download Application News.

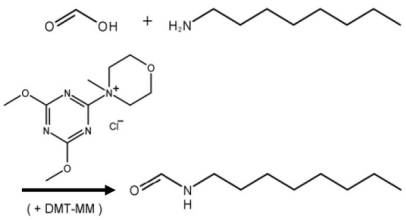


Fig. 20 Condensation Reaction Between Short-Chain Fatty Acid and n-Octyl Amine

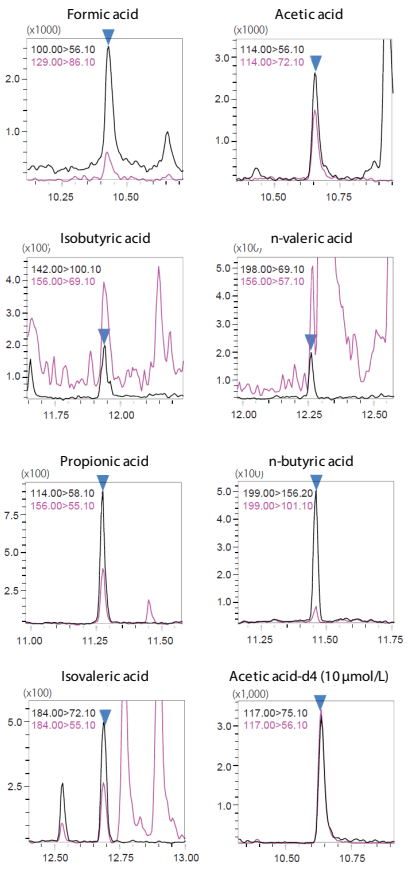


Fig. 21 MRM Chromatograms of Short-Chain Fatty Acids using GC-MS/MS

5. Examples of Analysis of Actual Samples

5-1. Examples of Organic Acid Analysis of Pharmaceutical Products

In drug development, ions called counter ions are selected for drug compounds. Selection of the optimum counter ion is critical for drug development because the properties of active pharmaceutical ingredients (API) vary depending on the formation of various salts in the drug product. Moreover, analysis of ionic contaminants is also necessary, as the residues of inorganic contaminants such as catalysts and ions which were used in the synthesis process may also affect the solubility and stability of the product. To eliminate contaminants from synthesized API, HPLC is commonly used for separation and preparative purification, and acetic acid, formic acid, and trifluoroacetic acid (TFA) are used in the mobile phase. TFA is also used in solid phase synthesis of peptides in order to isolate the synthesized peptides from the solid phase. Fig. 22 shows an example of an analysis of hydroxocobalamin acetate, and Fig. 23 shows an example of an analysis of angiotensin I TFA salt. For details of this analysis, please refer to Application News No. L457A, "Ion Analysis in Drugs (Part 4), Determination of Counterions (Anions) by Ion Chromatography."

Read here to download Application News.

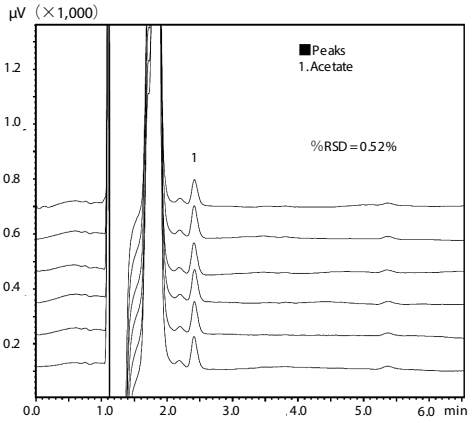


Fig. 22 Analysis of Hydroxocobalamin Acetate

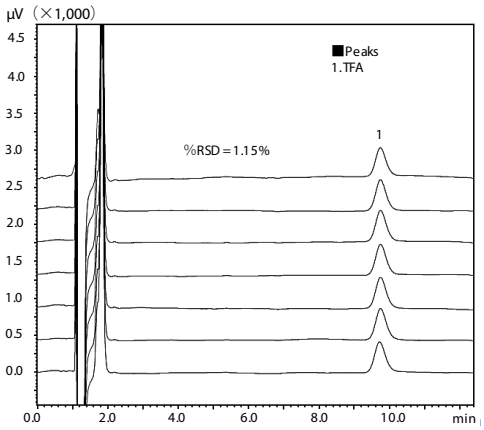


Fig. 23 Analysis of Angiotensin I TFA Salt

5-2. Examples of Life Science-Related Organic Acid Analysis

Various culture mediums are used in the production of antibody drugs and useful substances by fermentation and in industrialization of regenerative medicine, and optimization of the culture process and monitoring of process control are conducted. The composition of the culture medium used in culturing includes various compounds, such as organic acids, sugars, and amino acids, which are necessary for metabolism. It is necessary to monitor the state of metabolism of compounds when confirming/studying their bioprocess. A high-selectivity detection method must be used in determination of the organic acids in culture mediums that contain many matrices. Fig. 24 shows an example of an analysis of organic acids in a culture medium. For details of this analysis, please refer to Application News No. L490 "Organic Acids Analysis of Medium with Post-Column pH Buffering Organic Acid Analysis System."

Read here to download Application News.

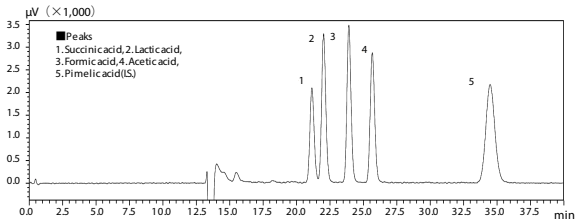


Fig. 24 Analysis of Organic Acids in Culture Medium

In recent years, it has become increasingly clear that intestinal flora contribute to maintaining and improving the health of their host. Intestinal flora produce short-chain fatty acids using dietary fiber and indigestible saccharides as energy sources. Since it is considered possible that these components are linked to autoimmune diseases, and to lifestyle-related diseases such as diabetes and obesity when absorbed in the body, quantitative analysis of these short-chain fatty acids is necessary in disease-related research. In many cases, LC-MS or GC-MS is used for comprehensive measurement of metabolites, but analysis is conducted by HPLC when the target is clear, as in determination of the short-chain fatty acids formed by microbial metabolism. Fig. 25 shows an example of an analysis of organic acids in mouse fecal extract using HPLC. For details of this analysis, please refer to Application News No. L516 "Applying an Organic Acid Analysis System to Intestinal Flora Research."

Read here to download Application News.

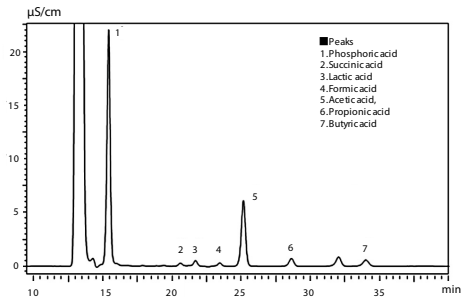


Fig. 25 Analysis of Mouse Fecal Extract using HPLC

Moreover, since short-chain fatty acids can be easily metabolized by the microorganisms in a fecal sample, it is important to store samples under conditions that prevent volatilization and metabolism and to shorten the time until measurement. Because the short-chain fatty acids in the fecal matter also contains butyric acid and valeric acid, which have long carbon chains, analysis time tends to be excessive under the separation conditions when using the Shim-pack SCR-102H. Therefore, Fig. 26 shows an example of measurement of the short-chain fatty acids in monkey feces in a short time by using a Shim-pack Fast-OA high speed analysis column for organic acids. Details of this analysis can be found in Application News No. L555 "Improvement of Productivity in Research on Intestinal Microbiota by Shim-pack Fast-OA High-Speed Organic Acid Analytical Column."

Read here for Shim-pack Fast-OA site.

Read here to download Application News.

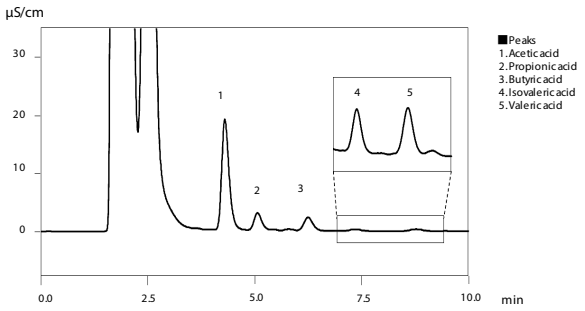


Fig. 26 Example of Analysis of Monkey Fecal Extract using Shim-pack Fast-OA

LC/MS or GC/MS is used to measure short-chain fatty acids produced by intestinal bacteria in samples with many components in a short time. In the case of analysis of many short-chain fatty acids using LC-MS, a derivatization method with an NPH-reagent is generally employed. An analysis of short-chain fatty acids and organic acids was conducted by this method using a sample of mouse feces containing physiological intestinal microbiota. Fig. 27 shows an example of the analysis results. For details of this analysis, please refer to Application News No. C168, "Analysis of Short-Chain Fatty Acids/Organic Acids (3-NPH Derivatives) in Fecal Specimens from SPF and Antibiotic-Fed Mice."

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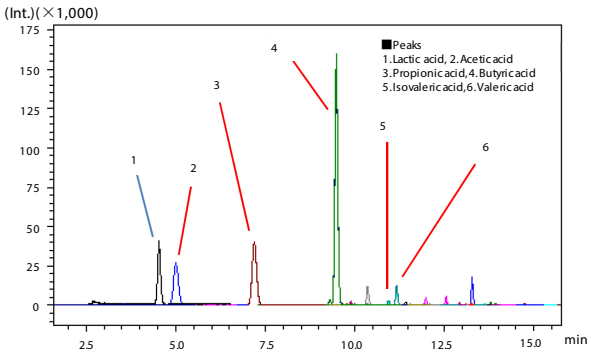

Fig. 27 Example of Analysis of Mouse Feces Sample

Metabolome analysis, which is a total analysis of organic acids, sugars, amino acids, and other primary metabolites, has attracted attention as one method for elucidating the influence of intestinal flora. As described above, simultaneous analysis of primary metabolites using LC-MS/MS is conducted under two analysis conditions, that is, using a mobile phase either with or without addition of an ion pair reagent, which are referred to as ion pairing or non-ion pairing LC-MS, respectively. Fig. 28 shows an example of measurements of mouse fecal extract by LC-MS/MS using these two conditions. Fig. 29 shows an example in which the same mouse fecal extract was derivatized and measured by GC-MS. For the specimen derivatization procedure, please refer to "Pretreatment Procedure Handbook for Metabolites Analysis" (C146-2181A). In the results of measurements of the same specimen by LC-MS/MS and GC-MS/MS, ion pairing LC-MS detected 17 components, which were mainly amino acids, while non-ion pairing LC-MS detected a total of 75 components, including amino acids, nucleotides, nucleosides, and organic acids. Measurement using GC-MS/MS detected 100 components, such as short-chain fatty acids, organic acids, and sugars. Thus, selective use of analytical methods corresponding to the purpose of the analysis is effective for total measurement of the metabolites in fecal samples. For details of analysis of the metabolites produced by intestinal flora, please refer to Application Note No. 48 "Comprehensive Measurement of Metabolites Using GC-MS/MS and LC-MS/MS – An Application to the Research of the Intestinal Environment –."

Read here to download Application Note.

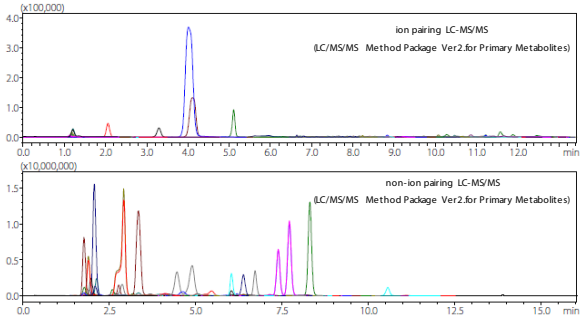

Fig. 28 Example of Analysis of Mouse Fecal Extract using LC-MS/MS

Read here to download Pretreatment Procedure Handbook for Metabolites Analysis.

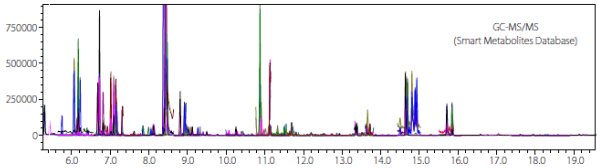

Fig. 29 Example of Analysis of Mouse Fecal Extract using GC-MS/MS

5-3. Examples of Energy-Related Organic Acid Analysis

Active research on next-generation renewable energy has begun in recent years with the aim of realizing a sustainable society. Artificial photosynthesis is one area of research on renewable energy, and the photochemical carbon dioxide reduction reaction by the photocatalyst such as titanium oxide is one important topic in this research. The reaction products of the carbon dioxide reduction reaction include not only carbon monoxide and lower hydrocarbons, but also organic acids such as formic acid. In HPLC analysis of formic acid dissolved in an organic solvent, the sample must be diluted with water or the mobile phase, analysis by GC or GC/MS sometimes can be appropriate. Here, Fig. 30 shows an example of direct analysis of formic acid with a concentration of ppm order without derivatization using a GC equipped with a barrier-discharge ionization detector (BID). Details of this analysis may be found in Application News No. G280C "High Sensitivity Analysis of Formic Acid Using GC-BID in Artificial Photosynthesis Research."

* Although derivatization is a general method in analysis of organic acids by GC, direct detection is also possible if the carboxyl group is one compound.

Read here for artificial photosynthesis analysis application site.

Read here to download Application News.

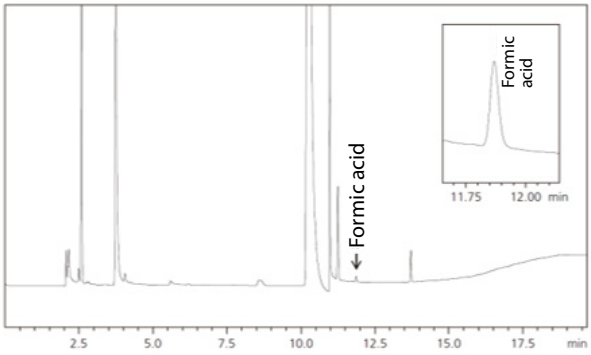

Fig. 30 Analysis of Formic Acid in Carbon Dioxide Reduction Reaction Aqueous Solution

Read here for Nexis GC-2030 gas chromatograph site.

Nexis™ GC-2030 Gas Chromatograph

Production of bioethanol and other biofuels from biomass resources as an alternative to fossil fuels has also attracted considerable interest. Biomass exists widely on earth and has a number of environmental and economic advantages, as it is a carbon-neutral resource and enables effective utilization of wood from forest thinning and waste materials. The biofuel production process by fermentation of biomass resources produces a variety of byproducts, including formic acid, acetic acid, and other organic acids and furan compounds such as furfural and hydroxymethylfurfural, but formation of these compounds must be monitored, as they inhibit fermentation. Fig. 31 shows an example of an analysis of the organic acids and furans in biomass. For details of this analysis, please refer to Application News No. L485 "Analysis of Biomass Using Organic Acid Analysis System."

Read here for biomass energy analysis application site.

Read here to download Application News.

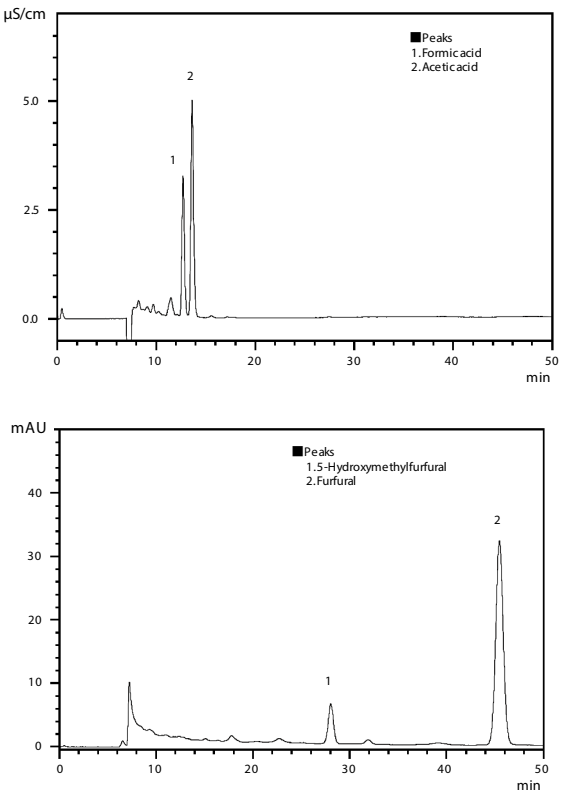

Fig. 31 Example of Analysis of (Top) Organic Acids and (Bottom) Furans in Biomass

Among the compounds in biofuels, formic acid, acetic acid, and propionic acid are thought to cause corrosion of metals. Thus, it is necessary to control the concentrations of these organic acids when biofuels are used in general vehicles. Fig. 32 shows an example of measurement of a biodiesel-diesel blended fuel. Details of this analysis can be found in Application News No. L352A "Analysis of Biofuel (Part 2) Determination of Formic Acid, Acetic Acid, and Propionic Acid in Bio Diesel Fuel Blended Oil."

Read here to download Application News.

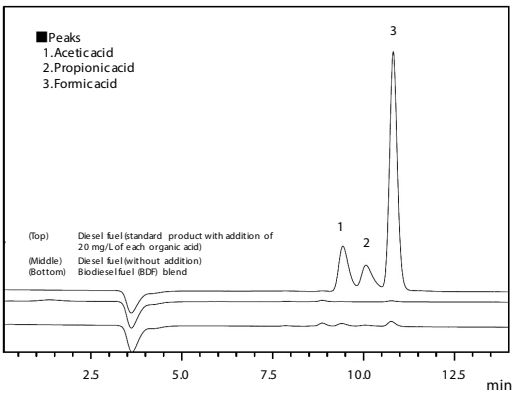

Fig. 32 Analysis of Biodiesel-Diesel Blend

In research on technologies for converting biomass resources to energy or chemical feedstocks, it is necessary to focus on conversion yield and the reaction process. Research has confirmed that high value-added organic acids such as hydroxymethylfurfural and lactic acid can be formed by a process in which cellulose, which makes up 60 to 70 % of woody biomass, is converted to glucose, and the target organic acids are then formed from glucose by a further catalytic reaction. It is necessary to identify the volatile components when analyzing this process. Fig. 33 shows an example of measurement of the aqueous glucose solution after the catalytic reaction. For details of this analysis, please refer to Application Data Sheet No. 94 "GC-MS Analysis of Catalytic Reaction Products of Glucose in Biomass Research."

Read here to download Application Data Sheet.

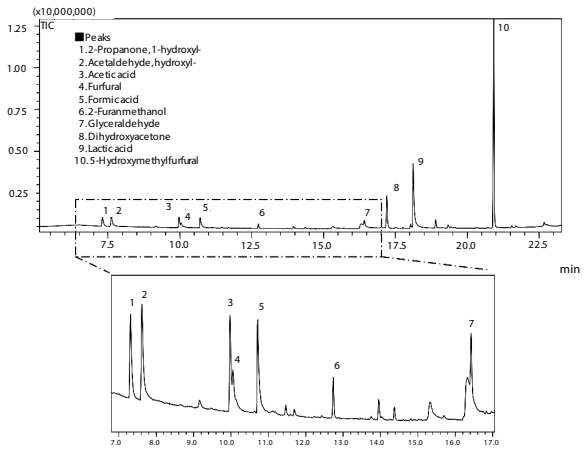

Fig. 33 Analysis of Aqueous Glucose Solution After Catalytic Reaction

5-4. Examples of Environment-Related Organic Acid Analysis

Many factories conduct appropriate treatment of the water used in production processes before release into the environment. Although the components in industrial wastewater differ depending on the product being produced, wastewater may contain organic acids such as butyric acid and valeric acid which cause offensive odors. Fig. 34 shows an example of measurement of the organic acids in wastewater.

Analysis Conditions

Separation column	:	Shim-pack SCR-102H (300 mm × 8.0 mm I.D., 7 µm)
Guard column	:	Guard column SCR-102H (50 mm × 6.0 mm I.D.)
Mobile phase	:	5 mmol/L *p*-toluenesulfonic acid (aq)
Mobile phase flow rate	:	0.8 mL/min
pH buffer	:	5 mmol/L *p*-toluenesulfonic acid (aq) containing 0.1 mmol/L EDTA and 20 mmol/L Bis-Tris
pH buffer flow rate	:	0.8 mL/min
Column temp.	:	40 °C
Injection volume	:	10 µL
Detection	:	Electroconductivity detector

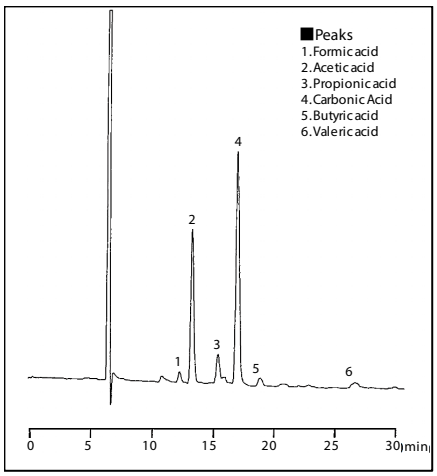

Fig. 34 Analysis of Organic Acids in Wastewater

5-5. Examples of Analysis of Organic Acids in Foods

Various foods and beverages are produced through fermentation processes. Not only organic acids, but also sugars, amino acids, and other primary metabolites are complexly involved in those products, and are factors that give the products their distinctive features. Wines contain organic acids which are produced by the brewing process and organic acids originating from grapes, and these organic acids in particular are thought to affect the flavor of wine. Fig. 35 shows an example of an analysis of the organic acids in wine by HPLC. In general, UV absorbance detectors are frequently used, but highly selective analysis unaffected by sample matrices is possible by using an electroconductivity detector.

Analysis Conditions

Separation column	:	Shim-pack SCR-102H (300 mm × 8.0 mm I.D., 7 µm) × 2
Guard column	:	Guard column SCR-102H (50 mm × 6.0 mm I.D.)
Mobile phase	:	5.0 mmol/L perchloric acid (aq)
Mobile phase flow rate	:	0.8 mL/min
pH buffer	:	5 mmol/L perchloric acid (aq) containing 0.1 mmol/L EDTA and 20 mmol/L Bis-Tris
pH buffer flow rate	:	0.8 mL/min
Column temp.	:	35 °C
Injection volume	:	10 µL
Detection 1	:	Electroconductivity detector
Detection 2	:	UV absorbance detector: 210 nm

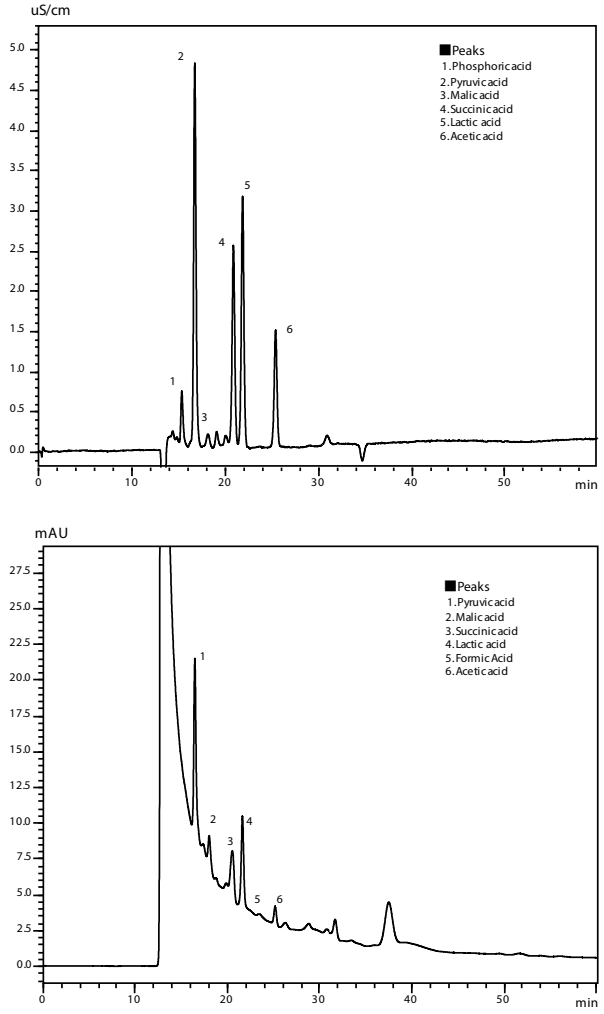

Fig. 35 Analysis of Organic Acids in Wine using HPLC (Top: Electroconductivity Detection, Bottom: UV Absorbance Detector)

Fig. 36 shows an example of an analysis of wine using an ion chromatograph. In addition to the organic acids in the wine, it is also possible to analyze the sulphate ion originating from the sulfite antioxidant and other inorganic ions.

Analysis Conditions

Separation column	:	Shim-pack IC-A3 (150 mm × 4.6 mm I.D., 5 µm)
Mobile phase	:	8.0 mmol/L *p*-hydroxybenzoic acid 3.2 mmol/L Bis-Tris, 50 mmol/L boric acid (aq)
Flow rate	:	1.2 mL/min
Column temp.	:	40 °C
Injection volume	:	50 µL
Detection	:	Electroconductivity detector (non-suppressor mode)

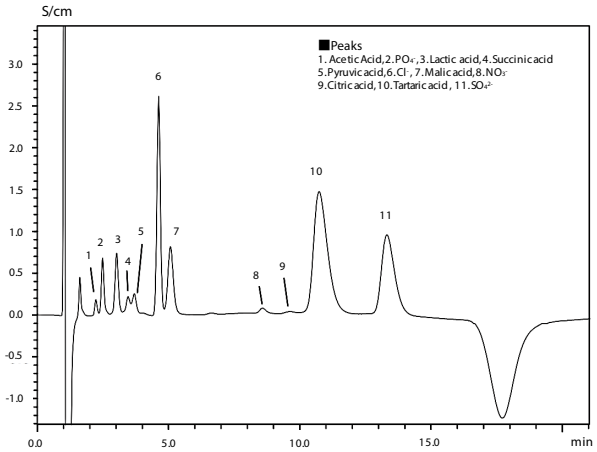

Fig. 36 Analysis of Wine using Ion Chromatograph

Foods and beverages contain various organic acids that contribute to flavor and smell. Coffee contains many organic acid components. Malic acid and citric acid are contained in coffee of the raw bean state, and formic acid and acetic acid form when coffee beans are roasted. These organic acids components have a large influence on the acidity of coffee. Fig. 37 shows an example of an analysis of the organic acids in coffee.

Analysis Conditions

Separation column	:	Shim-pack SCR-102H (300 mm × 8.0 mm I.D., 7 µm) × 2
Guard column	:	Guard column SCR-102H (50 mm × 6.0 mm I.D.)
Mobile phase	:	5 mmol/L *p*-toluenesulfonic acid (aq)
Mobile phase flow rate	:	0.8 mL/min
pH buffer	:	5 mmol/L *p*-toluenesulfonic acid (aq) containing 0.1 mmol/L EDTA and 20 mmol/L Bis-Tris
pH buffer flow rate	:	0.8 mL/min
Column temp.	:	40 °C
Injection volume	:	10 µL
Detection	:	Electroconductivity detector

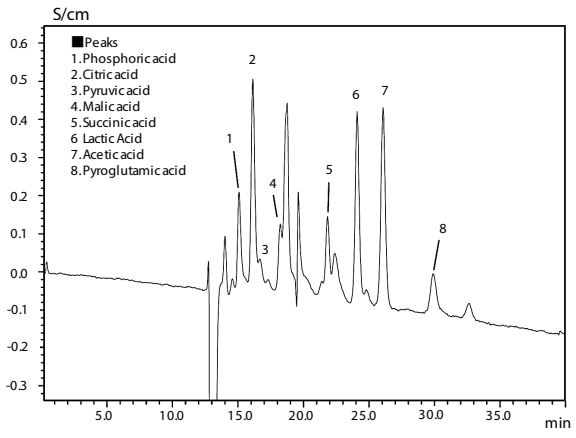

Fig. 37 Analysis of Organic Acids in Coffee

When developing fermented products, multiple compounds such as organic acids, sugars, and amino acids are monitored in order to optimize the fermentation conditions. In analysis of these compounds by HPLC, separate instruments are required for each compound group because the most suitable separation mode and detection method differ depending on the group. The new dual injection function of autosamplers for the Nexera series enables injection of samples into two independent flow channels, and consequently two types of analysis under different conditions can be performed simultaneously. Fig. 38 shows the flow channel diagram for simultaneous high speed analysis of organic acids and analysis of saccharides with a single system, and Fig. 39 shows the analysis results. Details of this analysis may be found in Application News No. L548 "Fermentation Processes Monitoring Using a Nexera Dual Injection System."

Read here for materials concerning the dual injection function.

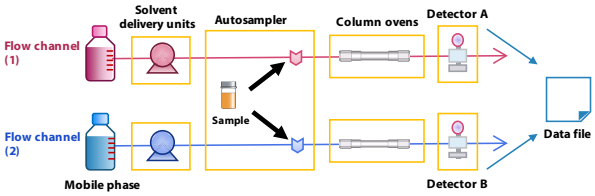

Fig. 38 Flow Channel Diagram of Dual Injection System for Simultaneous Analysis of Sugars and Organic Acids

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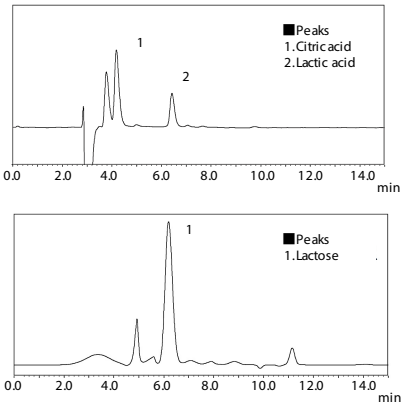

Fig. 39 Simultaneous Analysis of Organic Acids and Sugars in Yogurt using HPLC (Top: Organic Acids, Bottom: Sugar)

The components contained in foods are interrelated to some extent, and contribute to the taste of food products. Examples of efforts to establish a correspondence between quantitative information on primary metabolites such as organic acids and the results of sensory evaluation tests can be found in the food industry. The following introduces an example concerning coffee.

A sensory test was conducted with eight types of coffee, which were prepared under the same conditions (Table 2). The eight types of coffee were also analyzed using GC-MS, and 192 primary metabolites were detected. A statistical analysis was carried out for the results of the sensory test and the results of the metabolites detected on the GC-MS analysis. Fig. 40 shows the results of a comparison of the sensory test results and 4-hydroxybenzoic acid, which was one of the compound groups that showed a high correspondence in Table 3. These results suggested that a correspondence can be established between the results of sensory tests and the results of metabolite analysis. For the details of this analysis, please refer to Application News No. M274 "Construction of a Regression Model for a Coffee Sensory Evaluation Through the Comprehensive Analysis of Metabolites."

Read here for the food metabolomics site.

Read here to download Application News.

Table 2 Results of Sensory Evaluation

	A	B	C	D	E	F	G	H
Bitterness score	34	28	25	19	20	22	25	22

Table 3 Compound Groups with High Correspondence to Sensory Evaluation Results

Compounds with positive correspondence	Compounds with negative correspondence
Glycine-3TMS	4-Hydroxybenzoic acid-2TMS
Arabitol-5TMS	Glyceraldehyde-meto-2TMS
Mannitol-6TMS	Erythrulose-meto-3TMS
Glucose-meto-5TMS	Gluconic acid-6TMS
3-Phenyllactic acid-2TMS	Coniferyl aldehyde-meto-TMS
Glucuronic acid-meto-5TMS	5-Oxoproline-2TMS
Octanoic acid-TMS	Niacinamide-TMS
2-Aminoethanol-3TMS	Dihydroxyacetone-meto-2TMS
	Tryptamine-2TMS

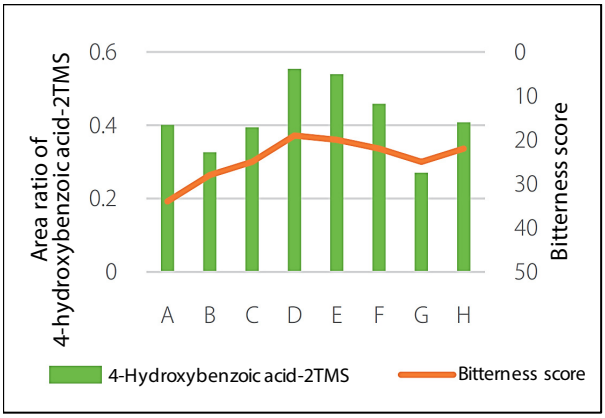

Fig. 40 Comparison of Sensory Evaluation Results and Analysis Results of 4-Hydroxybenzoic Acid

The number of complaints related to offensive odor from products, such as foods, has tended to increase in recent years. Measurement by GC/MS is used to determine the substances that cause offensive odors. Although offensive odors can be classified as moldy odor, disinfection odor, and putrefaction odor, the substances that cause putrefaction odor are said to be mainly organic acids and lower fatty acids. Because complaints related to offensive odors require an urgent response, sample preparation and analysis must be carried out quickly. Fig. 41 shows an example in which a product which was the subject of a offensive odor complaint (defective product) and a normal product were sliced thinly, and the samples were then heated to a high temperature (thermal desorption (TD) method) and the evolved compounds were measured with a GC-MS. Details of this analysis may be found in Application Data Sheet No. 136 "Off - Flavor Analysis in Chemical Material Using a Thermal Desorption Method."

Read here for GC/MS off-flavor analyzer system site.

Read here to download Technical Report on off-flavor analyzer.

Read here to download Application Data Sheet.

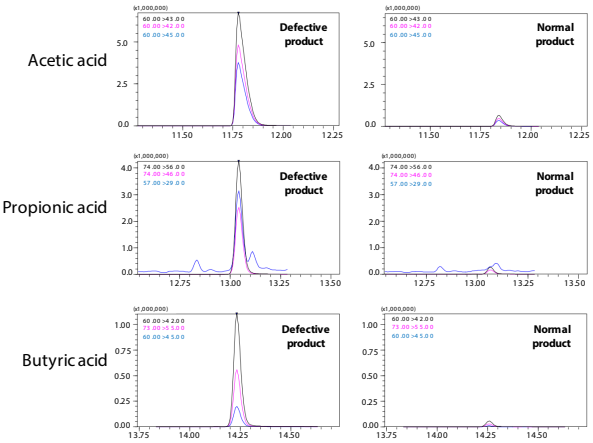

Fig. 41 Example of Analysis of Odor-Complaint Product and Normal Product

Read here for thermal desorption GCMS site.

Thermal Desorption (TD) GCMS™ System

5-6. Examples of Comprehensive Metabolomic Analysis

Metabolomics is a technique for comprehensive detection and analysis of various metabolites formed in-vivo as a result of biological activities and comprehensive investigation of the phenomena of life in living organisms. Different instruments are used in "quantitative metabolomics," which measures the total amount of metabolites, that is, the states of fluctuation or transition of metabolites in a sample as a whole, and "imaging metabolomics," which measures the distribution of metabolites. The following explains mainly quantitative metabolomics.

In quantitative metabolomics, GC/MS and LC/MS are used in measurements of the total amount of metabolites in a sample. Fig. 42 shows the target compounds of measurements by each technique, and Table 4 shows the features of the techniques. It is necessary to use the instruments appropriately, depending on the target compounds and the purpose of the analysis.

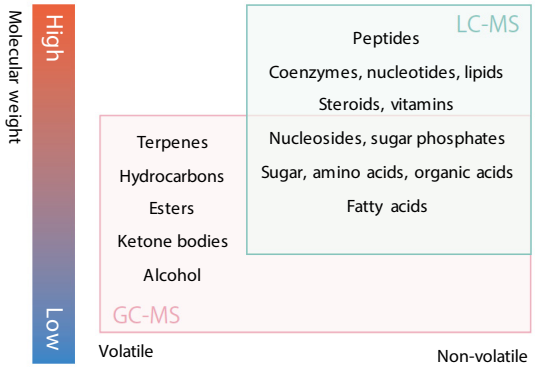

Fig. 42 Target Compounds of Metabolomics by GC/MS and LC/MS

Table 4 Features of Measurement by GC/MS and LC/MS

GC/MS	LC/MS
Permits comprehensive measurement of several hundred components in a single measurement.	Simple measurement of specific metabolites (up to 100 components).
Standard measurement methods with excellent robustness.	Quick measurement, including pretreatment.
Low installation cost.	Measurement of high-molecular weight non-volatile metabolites is possible.
First choice for comprehensive measurement.	Ideal for efficient routine measurement of specific components.

The objects of metabolomics are mainly sugars, amino acids, and organic acids as primary metabolites. With LC-MS, it is possible to conduct simultaneous analysis of primary metabolites under two different separation conditions. One is a combination of a mobile phase containing an ion pairing reagent and ODS column, and the other is a combination of a non-ion pairing mobile phase and PFPP column. In particular, simultaneous analysis using a non-ion pairing mobile phase enables analysis of a large number of organic acids, including components, such as citric acid and fumaric acid, which are produced by the TCA cycle. Fig. 43 shows an example of a simultaneous analysis of primary metabolites by LC-MS/MS. Details may be found at the product site "LC/MS/MS Method Package for Primary Metabolites Ver. 2."

Read here for LC/MS/MS Method Package for Primary Metabolites Ver. 2 site.

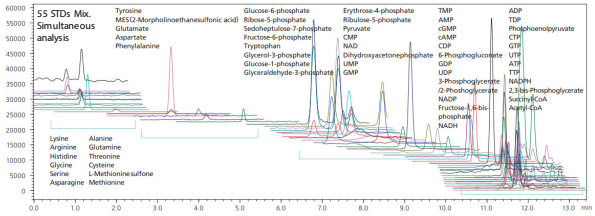

Fig. 43 Example of Simultaneous Analysis of Primary Metabolites using LC-MS/MS

As described above, derivatization is necessary when measuring organic acids by GC/MS, but simultaneous analysis of 475 metabolites is possible in one analysis. Fig. 44 shows an example of a simultaneous analysis of the metabolites in mature tomato leaves using the analysis conditions in the Smart Metabolites Database™, and Fig. 45 shows an example of the mass chromatograms of sugars, organic acids, amino acids, and nucleic acids which were detected. It was possible to detect 170 types of metabolites from the sample. For details of this analysis, please refer to Technical Report C146-0356 "Application for Plant Metabolome Analysis Using the GC/MS/MS Smart Metabolites Database."

Read here for Smart Metabolites Database site.

Read here to download Technical Report.

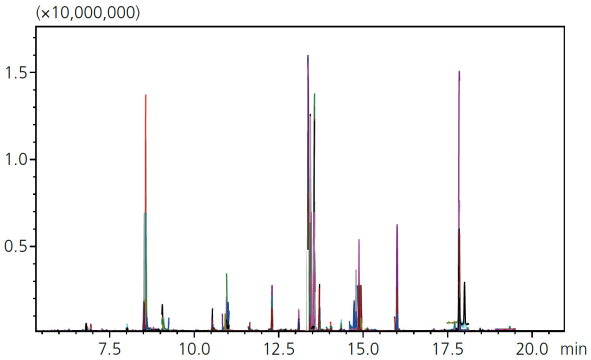

Fig. 44 TIC of Metabolite Compounds in Mature Tomato Leaves

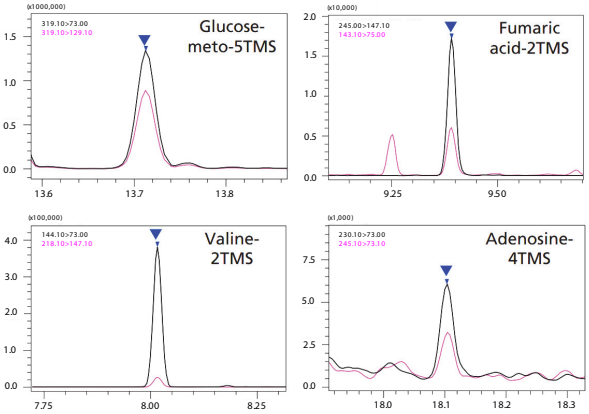

Fig. 45 Mass Chromatograms of Sugars, Organic Acids, Amino Acids, and Nucleic Acids

6. Conclusion

Various analytical techniques are used in analyses of organic acids in respective fields. As could be understood from this Application Note, the optimum analytical instrument for use will differ depending on the sample, target compound, and purpose of the analysis. Pretreatment such as dewatering or extraction may be required in some cases, depending on the sample solvent, but on the other hand, there are methods in which higher sensitivity and enhanced selectivity are achieved by derivatization. Moreover, even when using methods that enable comprehensive analysis of components other than organic acids, such as in metabolomic analysis, it is necessary to select the optimum analytical technique for the purpose. LC/MS is recommended when the purpose is routine analysis, and GC/MS is recommended for simultaneous analysis of several hundred components.

First Edition: Jul. 2020

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