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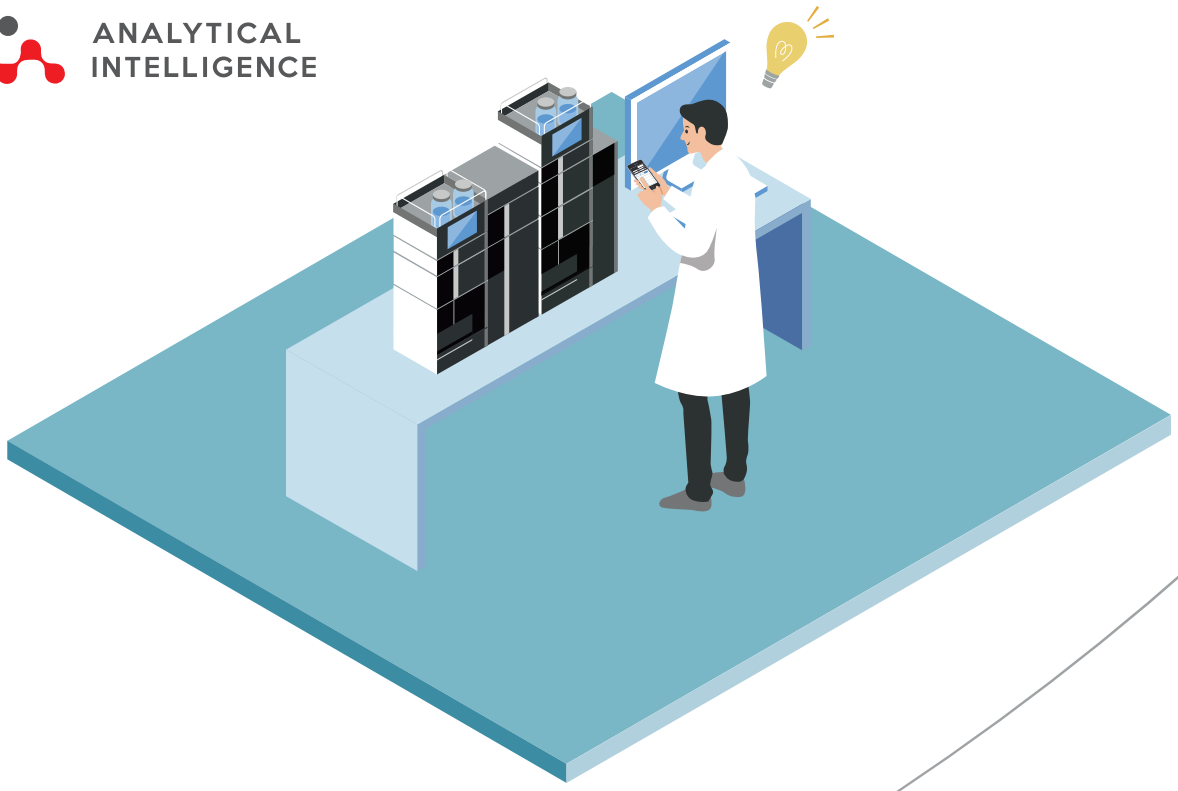
Excellence in Science

Ultra High Performance Liquid Chromatograph

Nexera series
Technical Reports / Applications

ANALYTICAL
INTELLIGENCE



EXPERIENCE NEW BENCHMARKS

The Nexera Series:
Reinvent Your Workflow

Just as the internet and smartphones have changed our lifestyles, and robots with AI capabilities continue to make our lives more convenient, so the new Nexera series is designed to reinvent daily LC analysis for a smoother, more efficient workflow.

ANALYTICAL INTELLIGENCE

Automated support functions utilizing digital technology, such as M2M, IoT, and Artificial Intelligence (AI), that enable higher productivity and maximum reliability.

Nexera series: Introducing Key Features

A new day of LC analysis using the Nexera™ series begins.

Start analysis right away with optimal conditions

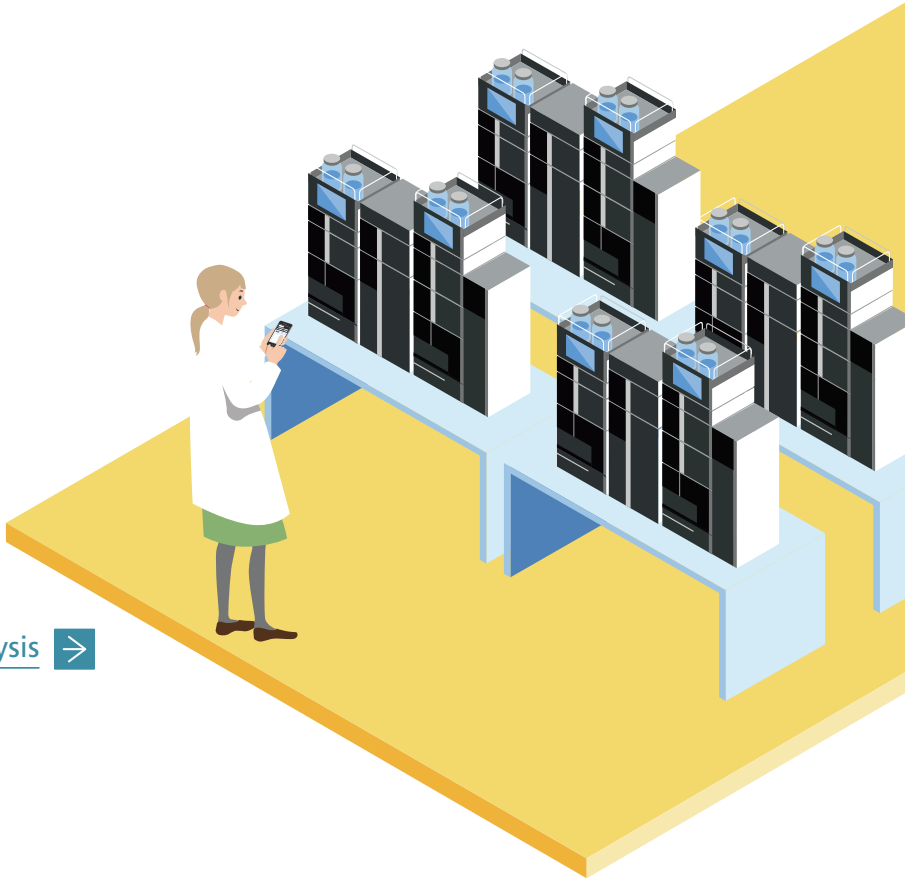
The Nexera series makes it easy to automate analysis preparation workflows so that your system is always in optimal condition. The instrument is ready for analysis as soon as you arrive in the lab.

Automate skilled manual work: FlowPilot

Mobile phase levels monitored in real time

Mobile phase management is an important daily task. The Nexera series provides real-time monitoring of remaining mobile phase from a smart device to prevent analysis failures.

Mobile phase monitoring to avoid running out of mobile phase during analysis



Problems in analysis are automatically detected and resolved

Stable analysis sessions are ensured.

The Nexera series allows the system to detect instrumental abnormalities during analysis, and restore normal operating status if they occur. This function reduces downtime and ensures reliable output.

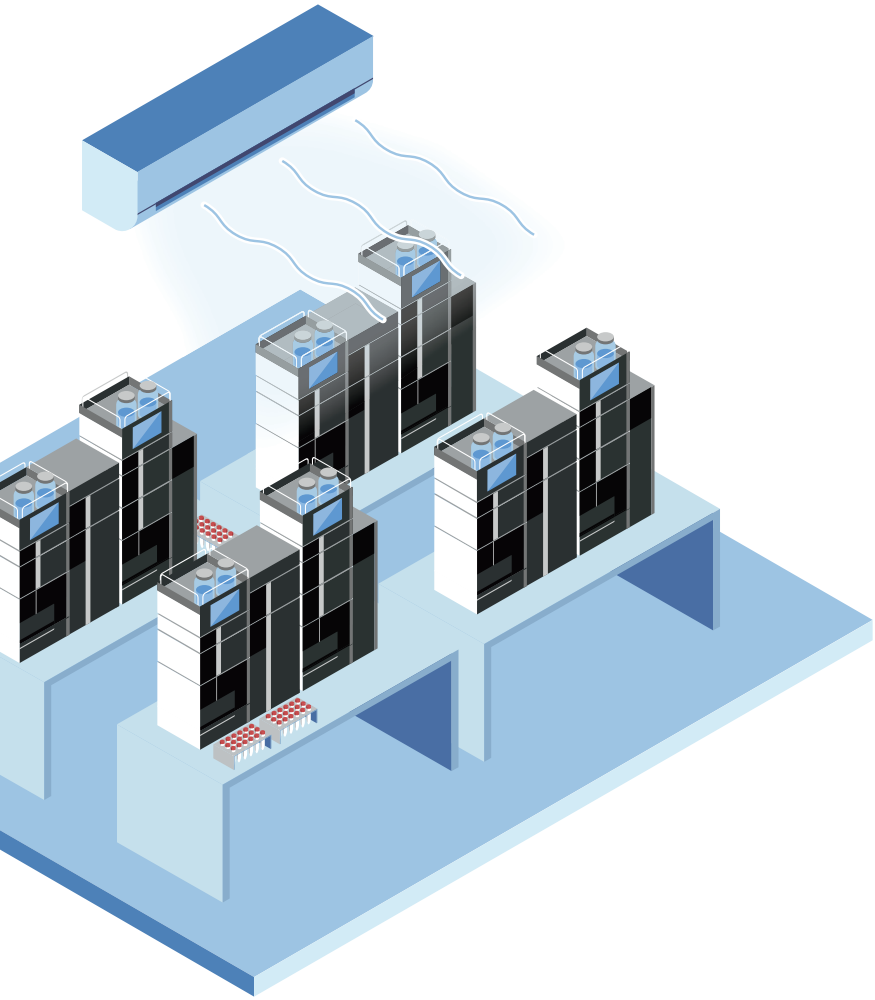
Auto-diagnostics and auto-recovery

View instrument status any time, anywhere

You don't have to stay in front of the instrument.

The Nexera series enables you to work efficiently by using smart devices to check the analysis status from outside the lab, for example in the office.

Monitor laboratory status any time, anywhere



Results unaffected by temperature changes

No need to worry about changes in the ambient temperature during analysis

The Nexera series not only controls the temperature of column ovens and autosamplers, but also detectors, ensuring stable analysis results.

Stable baseline independent of temperature changes

IoT technology for managing consumables and maintenance timing

Keep the instrument in its best condition

The Nexera series allows consumables usage to be managed in the cloud, enabling service engineers to remotely diagnose problems and optimize part replacement times.

Centralize part management, optimize maintenance planning and minimize downtime

For the next day's analysis

Smart shutdown

The Nexera series supports unattended shutdown as well as start-up. Set the time for the next day's analysis to begin, and the instrument will be ready when you are.

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Fully Automated Workflow for HPLC Analysis Using Automatic Startup with FlowPilot Function

- Analytical Intelligence Part 3 -

Takayuki Kihara[1], Davide Vecchietti[1]

Abstract:

An appropriate start-up procedure, a warm-up of the LC system and a specific System Suitability Test (SST) are critical steps before any analytical LC session in order to ensure high data quality in terms of reproducibility, accuracy, etc., and to reduce maintenance costs (e.g. by prolonging the lifetime of analytical columns). These procedures are often time-consuming for operators, and, if not performed properly, can lead to the loss of data and the waste of time and resources due to the need for re-analysis. In this report, we explain the ways in which we have improved and completely automated system startup and SST through a combination of different technologies.

Keywords: Intelligent start-up, Intelligent shut-down, System Suitability Test, FlowPilot

1. Automation of Entire Analytical Procedures

The Nexera LC system is equipped with various technologies that allow enhanced automation of all routine operations within the analytical workflow.

Intelligent start-up includes both the FlowPilot function (See section 2). It can be coupled with the warm-up function and scheduled depending on the user requirements. The system can also be evaluated automatically using the automatic SST function (See section 4). Scheduled shutdown automatically turns off the system and switches it to power-saving mode when all analytical operations are complete.

The combination of these functions allows the user to fully automate an entire analytical cycle: Shutdown -> Start-up -> SST -> Analysis -> Results report -> Shutdown (Fig. 1).

2. Intelligent Start-up with FlowPilot

It is well known that pressure shock can affect column performance by reducing column lifetime and leading to channeling, which results in peak-splitting in the corresponding chromatogram.

In order to avoid this issue, operators usually need to start up the system by slowly increasing flow rate, waiting for column pressure to stabilize and finally setting the flow rate for analysis. Nexera solvent delivery units use the FlowPilot function to fully automate all these steps by synchronizing flow ramping and oven temperature stabilization, (Fig. 2):

1. Flow rate is set to 50% of the flow rate for analysis and the oven is turned on.
2. Flow rate remains constant until the column oven reaches the set temperature.
3. Flow rate starts increasing toward the set value once the column oven reaches the set temperature.

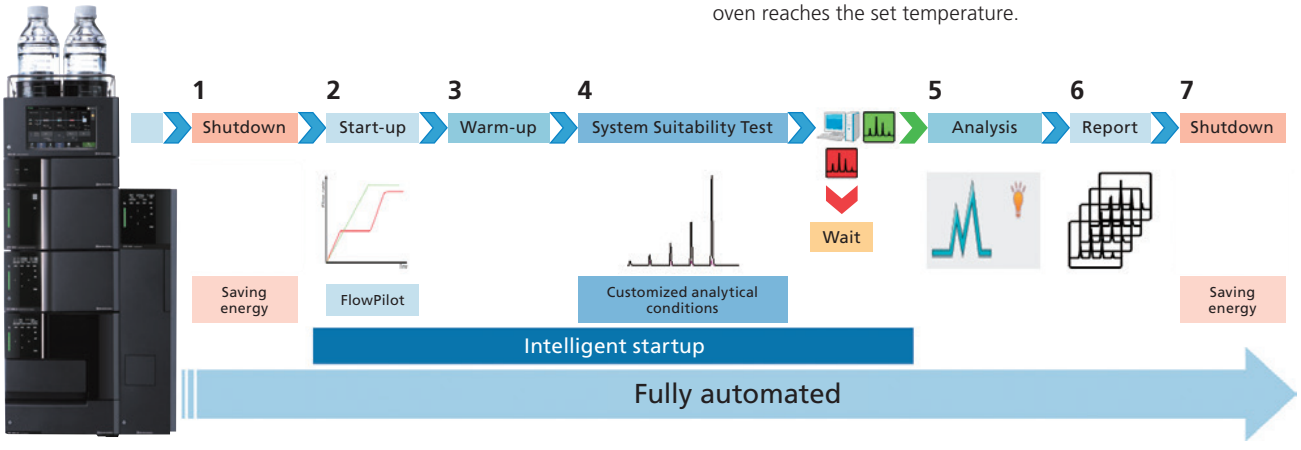

Fig. 1 Workflow diagram showing the fully-automated operation achievable with Nexera LC systems

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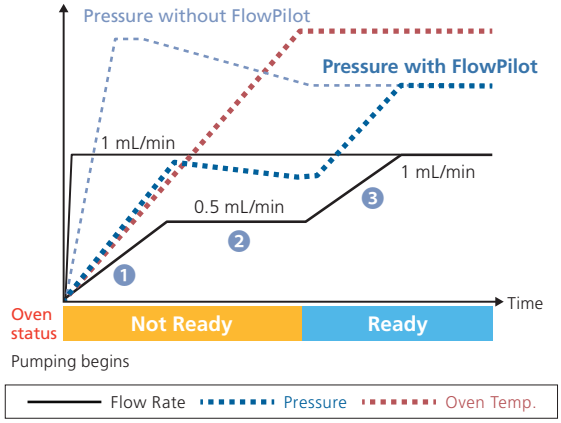

Fig. 2 Diagram of system pressure profile during start-up with the FlowPilot function

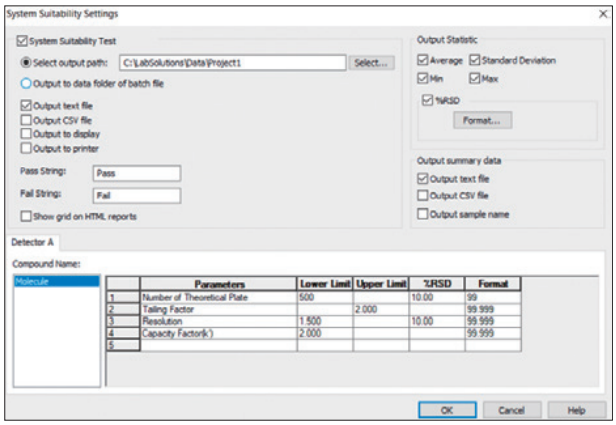

Fig. 3 System Suitability Test - example of 4 user-selected "pass/fail" criteria

3. System Suitability Test

SST are used to verify that the chromatography system is adequate for the intended analysis. The tests are based on the concept that the equipment, electronics, analytical operations, and samples analyzed constitute an integrated system that can be evaluated as such.

SST is mandatory in USP, FDA, and EP standards to check and ensure the ongoing performance of analytical systems. Nevertheless, several different parameters can be evaluated depending on the system and the analytical conditions. For this reason, there is a growing demand for a degree of flexibility in the set-up of SST parameters and possibilities for their customization in modern LC systems.

4. Fully-automated SST

SST parameters are embedded in the analytical method file. This means that users can easily create an SST with specific analytical conditions, in which selected parameters are evaluated (e.g. number of theoretical plates, tailing factor, resolution, capacity factor *k* ; see Fig. 3). After creating the SST, it is possible to choose when to run the SST during a batch analysis (at the beginning, after analysis of some samples or at the end of the batch).

Once the SST is complete, a "pass" or "fail" result is issued depending on the previously-selected criteria, and this result will then trigger specific actions based on user preferences (see Fig. 4).

Fig. 4 shows an example where the user has selected a batch composed of 4 samples for calibration and 7 unknown samples. By customizing SST parameters, it is possible to inject the SST sample after warm-up; in the case of a "pass" result, the analysis of the batch will continue with subsequent samples (both calibration and unknown samples).

In the case of a "fail" result, a blank is injected and the SST is repeated. A second "fail" will trigger the suspension of the batch processing and the instrument will be automatically put into standby mode. If the user has selected automatic shutdown, the instrument will be put into power-saving mode at the end of the batch.

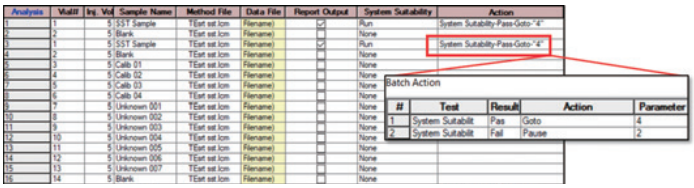

Fig. 4 System Suitability Test during batch analysis creation

5. Conclusions

- The Intelligent Start-up and FlowPilot functions can be used to automate routine procedures related to system start-up and warm-up prior to analysis.
- The user can select SST parameters for a method to fully automate the validation of a batch session, saving time and ensuring high reliability of analytical results.
- By using the shutdown function after sample analysis has been completed, a series of analysis workflows can be fully automated.

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Technical Report

Maximizing Analytical Efficiency with Real-time Measurement of Mobile Phase Consumption

- Analytical Intelligence Part 2 -

Yoshino Saki[1], Davide Vecchietti[1]

Abstract:

Management of the mobile phase is of great importance in high-performance liquid chromatograph analysis. Depletion of the mobile phase during analysis not only results in failure of the analysis, but also risks damaging expensive analytical columns. To prevent these problems, it is necessary to estimate the amount of mobile phase required for analysis before starting, but such an estimate is laborious to calculate, especially with regards to gradient elution. There is also the possibility that the mobile phase may nonetheless be depleted during analysis due to a calculation error. While, it is time-consuming for the user to visit the laboratory frequently to check the quantity of mobile phase remaining. In this report we describe the effectiveness of the Mobile Phase Monitor Module (MPM-40) for real-time measurement of mobile phase quantity and automated estimate of consumption of mobile phase with LabSolutions™.

Keywords: Mobile phase monitoring, Real-time gravimetric measurement

1. The MPM-40 Module

The MPM-40 is a Shimadzu module that includes sensors to accurately weight mobile phase. This technology enables real-time display of the actual amount of mobile phase remaining, not the amount calculated indirectly from forecast consumption.

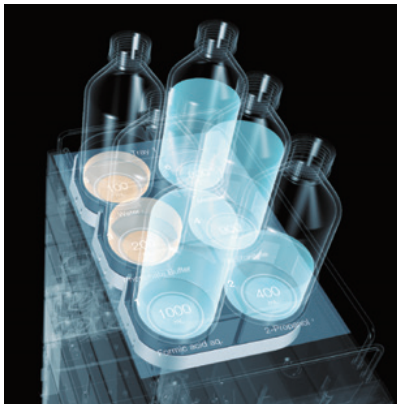

Fig. 1 A Nexera™ system equipped with an MPM-40 module

The module continuously records the weight of mobile phase and exchanges real time data with connected workstations, and smart devices. And, before starting the analysis, the quantity of mobile phase required for the series of analysis is automatically calculated and compared with the current quantity of mobile phase. A warning is displayed if there is insufficient mobile phase for the analysis. The mobile phase volume can be checked on a PC or smart device through a dedicated application. It is possible to receive warnings about the lack of mobile phase and perform automatic actions such as stopping analysis. This prevents data loss due to mobile phase depletion during continuous analysis and enables more efficient overall analysis.

The MPM-40 can also be used with LC systems other than the Nexera series. (If LabSolutions is not used, some functions such as predicting and confirming the consumption of mobile phase will not be available.)

2. Improvement of Analytical Efficiency

Monitoring functions can always be accessed directly through LabSolutions, or remotely through a dedicated mobile application on a smart device.

Up to 12 solvents can be simultaneously monitored through an MPM-40 module connected to one LC system. Mobile phase monitoring modules are connected to workstations via a LAN network. It is possible to use the dedicated software MPMChecker™ for monitoring and customizing parameters (calibration, warning levels, alarm levels).

In addition, by linking this mobile phase monitoring technology with Shimadzu LabTotal™ Smart Service Net Asset Agent, it is possible to track the operation status of devices from their mobile phase consumption. This supports the efficient allocation of resources in the lab.

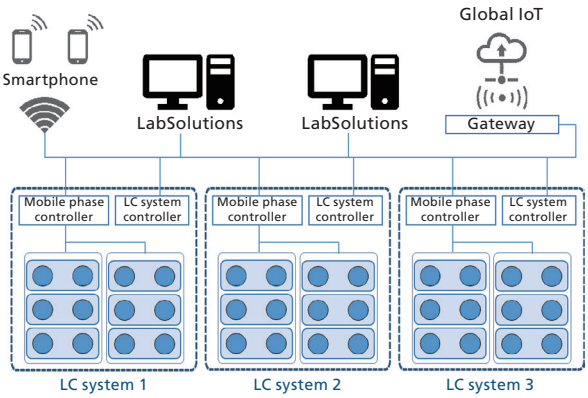

Fig. 2 Mobile phase monitoring technology integrated into laboratory workflow (representative system configuration)

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3. Using the Mobile Phase Monitor

Operators can easily calibrate the sensors by following the step-by-step procedure included in the software. Calibration consists of measuring the weight of the empty bottle and then that of the filled bottle (Fig. 3).

During this phase it is also possible to customize information about the composition of the mobile phases, warning levels and line connections (Fig. 4).

The Mobile Phase Monitor is automatically turned on, and warning levels and alarm levels will be activated according to user settings. Each time an analysis is started (either single or multiple analysis), the volume of mobile phase needed is estimated and a warning will appear if the current volume of mobile phase is insufficient (Fig. 5).

Since all bottles are connected to the system, the total amount of mobile phase consumption is estimated, including all rinse steps, giving an accurate consumption prediction regardless of the differences in analytical methods used.

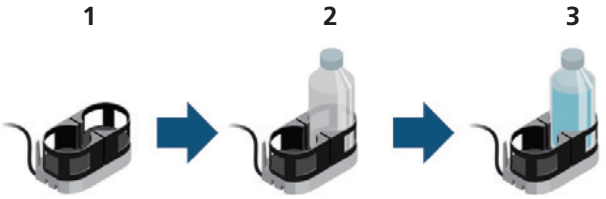

Fig. 3 Sensor calibration procedure

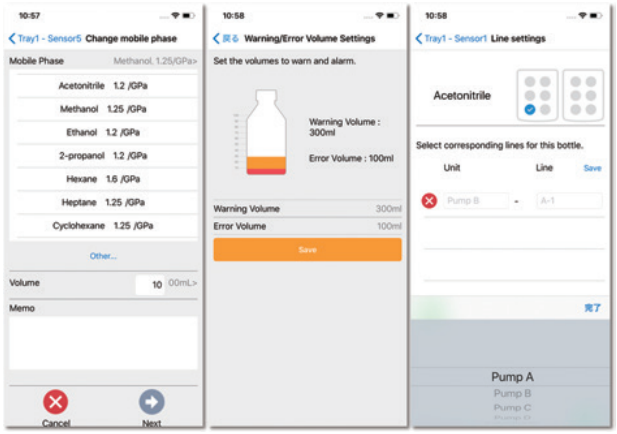

Fig. 4 Customizable settings for mobile phase monitoring

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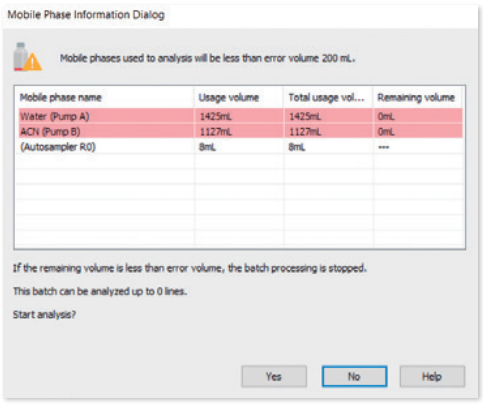

Fig. 5 Warning dialog before analysis start

4. Real Time Monitoring Possibilities

Even while the analysis is in progress, it is always possible to check the mobile phase consumption using smart devices or LabSolutions software. In particular, with a smart device, it is possible to check the remaining mobile phase even from outside the lab by configuring the Wi-Fi setting (Fig. 6). A notification will also be sent when the mobile phase becomes insufficient. So there is no need for the user to go to the lab to check the amount of mobile phase remaining.

Fig. 6 Software interface for MPM

5. Conclusions

- The Mobile Phase Monitor MPM-40, together with its dedicated software and application MPMChecker, weighs the mobile phase in order to display the amount of mobile phase on a PC or smart device, updated in real time. Moreover It is possible to receive warnings about the lack of mobile phase and perform automatic actions such as stopping analysis thanks to the integration of the Mobile Phase Monitor with LabSolutions.
- The MPM-40 can be used with any LC system. If LabSolutions is not used, some functions such as predicting and confirming the consumption of mobile phase will not be available.
- By eliminating the risk of mobile phase depletion by mobile phase monitoring, the interruption of analysis is prevented, and overall laboratory productivity is improved.

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Technical Report

Watch the product video

Use of Solvent Delivery Unit Equipped with Auto-diagnostics and Auto-recovery Functions to Enhance Lab Productivity

- Analytical Intelligence Part 1 -

Tomohiro Gomi[1], Davide Vecchietti[1]

Abstract:

In order to improve productivity in modern analysis laboratories, it is essential to reduce analysis time and maximize throughput through regular maintenance. One issue to be resolved is the interruption of analysis due to unforeseeable problems. An example of this is air bubbles in the flow line, which can cause shifts in retention times, pulsating baselines, unexpected changes in peak shapes. In this report, we describe the effectiveness of auto-diagnostics and auto-recovery functions in detecting and resolving this problem automatically. These functions minimize system downtime due to air bubbles and contribute to the optimization of laboratory productivity.

Keywords: Auto-diagnostics, Auto-recovery, Nexera™ solvent delivery unit

1. Bubble Formation in Flow Lines

The amount of gas that a liquid can absorb depends on several factors, such as the pressure and temperature gradients, and the nature and type of the liquid and gas (see reference).

Gas bubbles are produced in a liquid when the amount of dissolved gas in a solution exceeds the saturated solubility (supersaturation). Usually, the bubbles are removed through the degassing unit. However, in rare cases, they can appear in the flow line of an HPLC / UHPLC and reach the pump. These bubbles can cause shifts in retention times, pulsating baselines, unexpected changes in peak areas, irregular peak shapes.

This can dramatically affect the analytical results due to inaccuracies, poor precision, or inability to distinguish between trace amounts of analytes and the baseline. It also prevents the identification of analytes that are close to their detection limits.

2. Auto-diagnostics and Auto-recovery

Air bubbles can appear in HPLC/UHPLC systems when air has not been removed from the mobile phase, when room temperature varies dramatically or surfactants are added to mobile phase.

When air bubbles are encountered, they require the presence of an operator to be dealt with. The operator will usually remove bubbles by stopping the analysis in progress and purging the flow lines.

When the instrument is running unattended (e.g. at night), undetected air bubbles within flowlines can affect a large number of analysis samples, resulting in data loss and time-consuming re-runs.

Auto-diagnostics and auto-recovery functions prevent data loss and waste of samples by automatically detecting abnormal pressure variations triggered by air bubbles within the system and performing corrective actions such as flow line purging until the system regains normal operational status (Fig. 2).

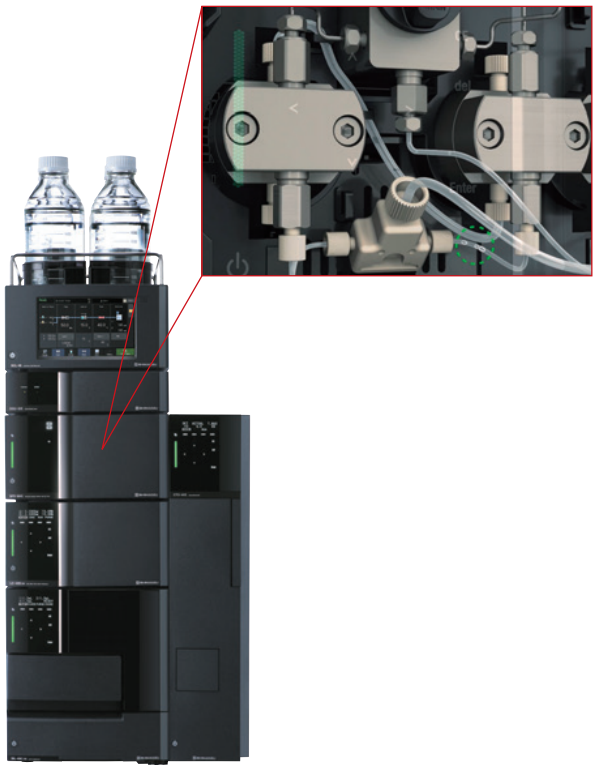

Fig. 1 Diagram of the Nexera™ solvent delivery unit flow lines

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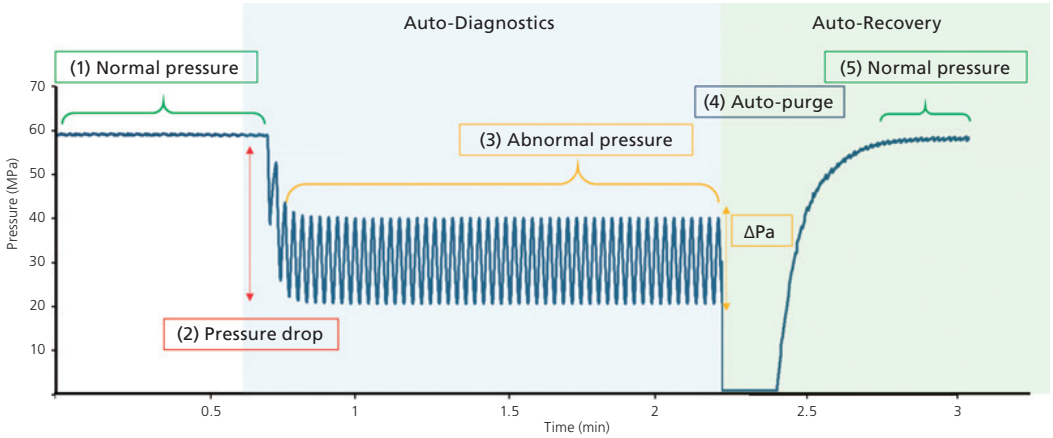

Fig. 2 Pressure changes during auto-diagnostics and auto-recovery. The time spent in each phase can vary depending on analytical conditions and user-defined settings.

3. Fully-automatic Recovery

Auto-diagnostic and auto-recovery functions are based on a specific algorithm providing the following capabilities. When air bubbles appear in the system, the pressure will drop (Fig. 2, stage 2), and this abnormal pressure will continue (Fig. 2, stage 3). If the new pressure variability ΔPa is abnormal compared to the reference value, the auto-recovery function will be triggered.

In this case, all the subsequent analyses are temporarily suspended. An auto-purge is performed in order to remove any air bubbles from the flow lines (Fig. 2, stage 4) and a column rinse is performed.

After the auto-recovery process, the pressure profile is checked and compared to the reference values. If pressure variability is normal, the system will return automatically to analysis mode and resume all analyses in the queue.

After auto-recovery, the user can choose to start the interrupted analysis again or to skip this and start from the next line of the batch.

4. User Settings

The settings for the auto-diagnostics and auto-recovery functions can be easily changed with LabSolutions™. First, if the system detects that the pressure is abnormal, select the operation to be performed as follows.

Enter auto-recovery mode: Auto-purge is performed.
Ignore: No action is performed.
Stop batch processing: Analysis stops and the system goes to standby.

In addition, it is possible to customize the purge time at the time of auto-recovery, the number of attempts at recovery, and the steps after recovery (Fig. 3).

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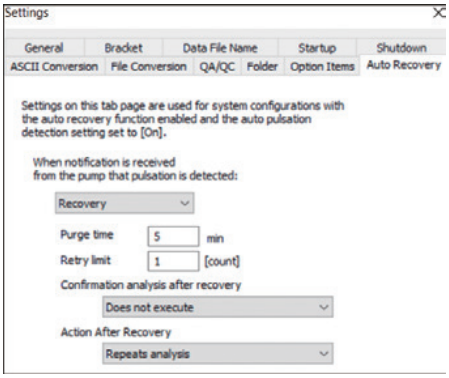

Fig. 3 LabSolutions auto-recovery settings window

5. Conclusions

- Auto-diagnostics and auto-recovery functions are available using all Nexera solvent delivery units being controlled via LabSolutions software.
- Both functions are fully automatic and do not require any human intervention, resulting in increased overall analytical efficiency.

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Technical Report

Increased Analysis Throughput by Overlapped Injection Using the SIL-40 series Autosampler

- Analytical Intelligence Part 6 -

Hidetoshi Terada[1], Katsuaki Koterasawa[1], Takayuki Kihara[1]

Abstract:

The use of high-speed analysis methods is now widespread because of improvements in separation efficiency due to the use of ultra-small particles in column packing materials and surface porous particles, and the associated higher pressure resistance and lower size of the instruments. However, to improve the overall analysis throughput, it is necessary to effectively utilize the sample injection time and the times when peaks are not eluted, periods outside the time for the chromatogram to become visible. The minimum injection time of the SIL-40 series autosampler (SIL-40) is 7 seconds or less, which is extremely effective for improving the throughput of the overall analysis. In addition, by performing a simple setting and injecting the sample for the next analysis during the current analysis (overlapped injection), the whole analysis cycle time be can be shortened and throughput increased. Here, we describe how to increase efficiency in size exclusion mode, where improving throughput is normally difficult, by utilizing overlapped injection.

Keywords: automatic pretreatment, overlapped injection, SIL-40 series, GPC

1. Overlapped Injection

The Nexera Series SIL-40 has not only a standard sample injection operation but also an automatic pretreatment function that is capable of a variety of operations, including overlapped injection.

Overlapped injection is a method of injecting the next sample while the immediately previous sample is being analyzed so that contaminant and solvent peaks do not overlap during the period that the target components are being eluted. This enables the analysis time for a continuous series of analyses to be shortened (Fig. 1). The longer the overlap time, the more efficiently the analysis can be performed.

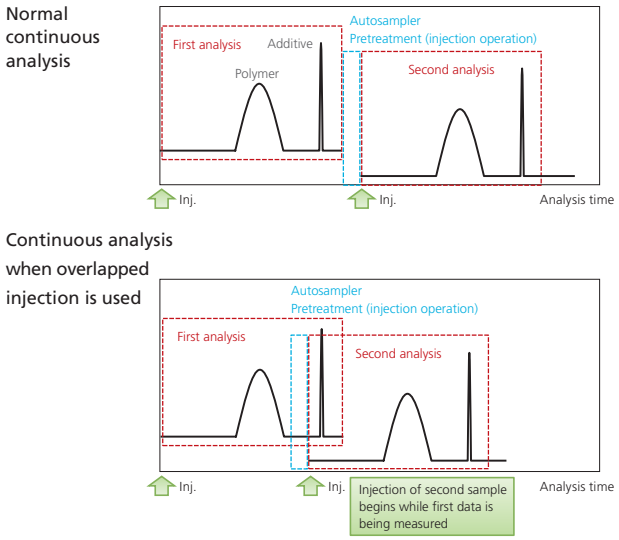

Fig. 1 Comparison of Normal Continuous Analysis and Analysis when Overlapped Injection is Used in Size Exclusion Mode

Overlapped injection achieves improved continuous analysis efficiency in modes such as size exclusion, ligand exchange, and ion exclusion, where adjustment of elution separation is almost impossible and high speed is difficult. However, because the next sample is injected while the previously injected sample is being analyzed, special attention must be paid to the following points when setting up the method.

- The separation mode must be isocratic
- The injection timing must be set up so that the target peaks to be detected in the next analysis do not cover any of the peaks in the previous analysis

2. Overlapped Injection Settings

Overlapped injection is set as shown in Fig. 2. Using the following chromatogram examples, the concepts for overlapping injection setting are introduced.

0 to 10 minutes: period when no components are eluted . . . (1)

10 to 30 minutes: period when components are eluted . . . (2)

Around 20 minutes: period when target components in (2) are not eluted . . . (3)

Note: It is assumed that all components are eluted within 30 minutes.

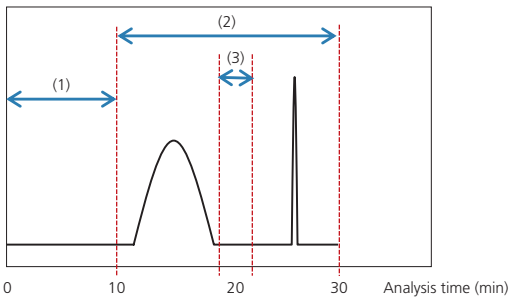

Fig. 2 GPC Chromatogram Example

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In Fig. 2, because no components are being eluted in period (1), there is no need to record any data, and this time is overlapped with the latter half of (2) of the immediately previous analysis.

To avoid any influence by the injection operation on the chromatogram, the injection operation for the second analysis is started at around 20 minutes (Fig. 2 (3))

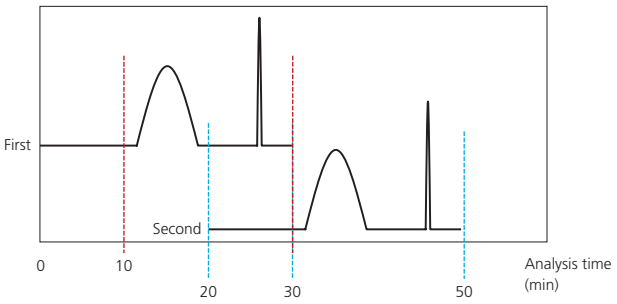

Fig. 3 Example of Successful Overlapped Injection

If overlapped injection is performed with this setting, then in the first analysis data is acquired in the 10 to 30 minute period. In the second analysis, no data is acquired in the period 20 to 30 minutes from the start of the first analysis, but data is acquired in the 30 to 50 minute period (Fig. 3). (The overlap time changes slightly due to the injection operation and data processing.)

On the other hand, if for example the second injection operation starts 15 minutes after the analysis begins, then the elution time of the polymer component in the second analysis and the time of the peak of the additive in the first analysis will overlap, so this setting cannot be used (Fig. 4).

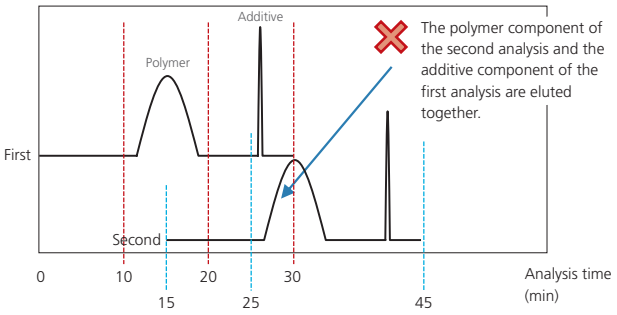

Fig. 4 Example of Unsuccessful Overlapped Injection

3. LabSolutions Setting Method

Overlapped injection can be set up easily from the LabSolutions analysis data system. The times determined in the procedure in Section 2 are entered into a method file as analysis conditions. The Fig. 2 (2) time is entered as [LC End Time] (Fig. 5).

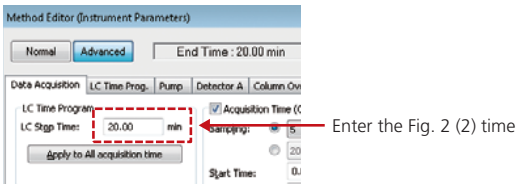

Fig. 5 Data Acquisition Time Setting

Next, select [Overlap] mode in the autosampler's pretreatment program setting window

Set the [Data processing time] to 0.5 min. (initial value), and enter the value of the difference between the time when data is not being acquired (Fig. 2 (1)) and the [Data processing time] (9.5 minutes in this example) as the [Overlap time].

For the [Pretreatment overlap time], enter the same value as the [Overlap time].

If the [Pretreatment overlap time] is too large, the first analysis data acquisition and the second analysis peak elution will overlap.

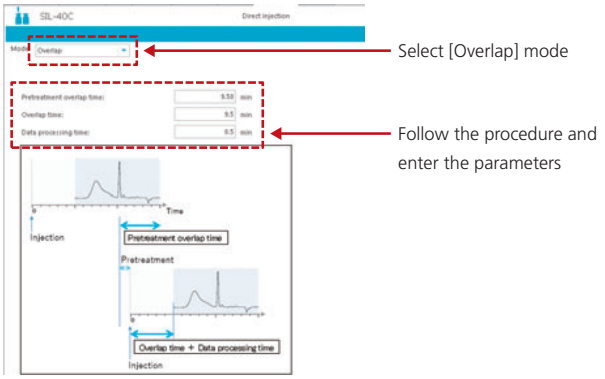

Fig. 6 Overlap Time Setting Input Window

4. Conclusion

- By using overlapped injection, analysis can be performed more efficiently even in analysis separation modes where throughput improvements are normally difficult, such as size exclusion, ligand exchange, and ion exclusion modes.
- The settings for overlapped injection can be easily set up from the LabSolutions control software.

References

Application News No. L537 (Increased Throughput with Nexera™ GPC system: Overlapped Injection and Simultaneous Determination of Polymer Additives)

First Edition: June, 2019

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Technical Report

Heightened Analytical Efficiency and Integrated Management of Multifaceted Data Using the Dual Injection Function in the SIL-40 Series Autosampler

Katsuaki Koterasawa[1], Kenichi Yasunaga[1], Hidetoshi Terada[1], and Keiko Matsumoto[1]

Abstract:

The unique dual injection function is optionally available for the Nexera series. At analysis sites, there are cases in which analysis must be performed twice under different analytical conditions because multiple components from a single sample cannot be separated at the same time, or two completely different compounds must be quantified. With the dual injection system, two independent flow lines are built into a single platform. Chromatograms are acquired simultaneously under two different conditions, and the results obtained are integrated into a single data file. Examples of such analyses include fermentation monitoring, which involves the simultaneous measurement of organic acids and amino acids, the metabolites of microorganisms, as well as the test of complex impurity property in the drug that are hard to separate at the same time under a single set of conditions. In these analyses, this function not only significantly improves analytical efficiency, but also integrates the two chromatograms into a single data file, ensuring traceability with respect to the sample. Additionally, not only the data files but also the method files and batch files are each integrated respectively into a single file, simplifying data management, and ensuring data integrity. In this article, we introduce the actual process starting with how to use the dual injection system, as well as the sequence of steps up to integrated data management, and the heightening of efficiency.

Keywords: Dual injection function

1. Dual Injection Function

The dual injection function is an optional function for the SIL-40 series. It enables a sample to be injected into two analysis flow lines.*

With a dual injection system using this function, two flow lines are incorporated into a single system, enabling two analyses to be performed simultaneously (Fig. 1).

The two sets of analysis data obtained by using this system are integrated into a single data file. This ensures traceability of the data with respect to the sample and eliminates the risk of incorrect associations between pairs of data files originated from different samples.

Additionally, the method files, batch files, and data files from the two analyses are each integrated into a single file. This simplifies data management and ensures data integrity.

Furthermore, analyses that conventionally used multiple systems, as well as analyses implemented by switching the conditions for each target component can now be performed simultaneously. This results in shorter analysis times (Fig. 2), smaller installation space requirements, and lower initial costs (Fig. 3).

* An optional kit is required to use the dual injection function.

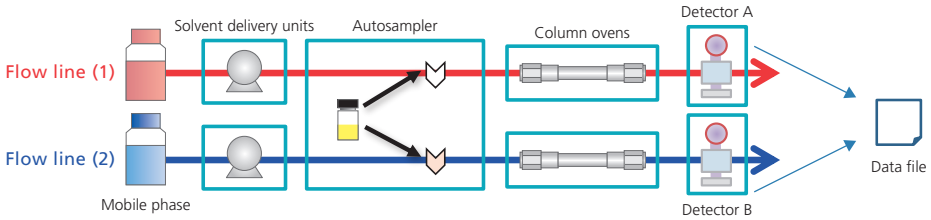

Fig. 1 Flow Lines in the Nexera Dual Injection System

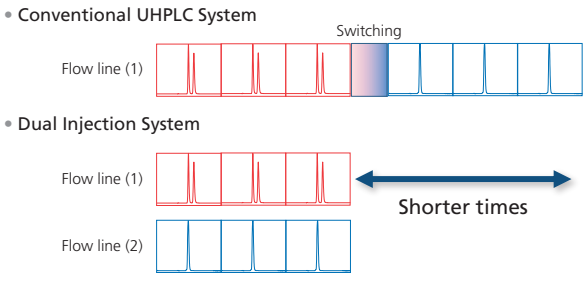

Fig. 2 Dual Injection System Contributes to Shorter Analysis Times

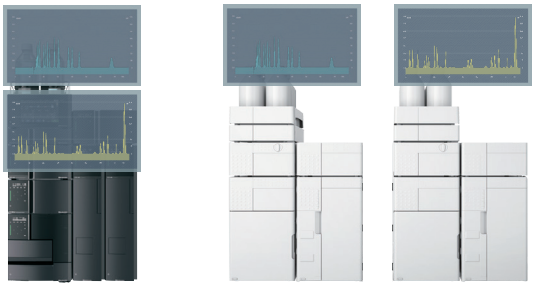

Fig. 3 Dual Injection System Contributes to Installation Spaces Savings

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2. Operation of the Dual Injection System

When using the dual injection function, the injection ports in the autosampler and the valves in the neighboring column ovens are increased by one each respectively. The respective injection ports are connected to separate valves and sample loops, resulting in a configuration in which they are connected with the sample loops and flow lines via valve switching (Fig.4, 5).

The loop injection method is used for both flow lines. It is possible to select the sample loop with the optimal capacity and change the injection volume to suit the intended analysis.

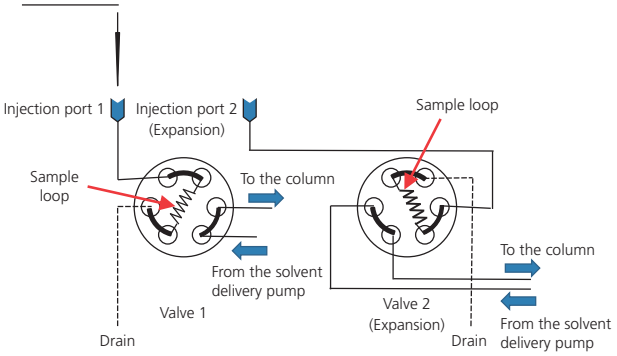

Fig. 4 Flow Line when the Sample is Loaded into the Sample Loops

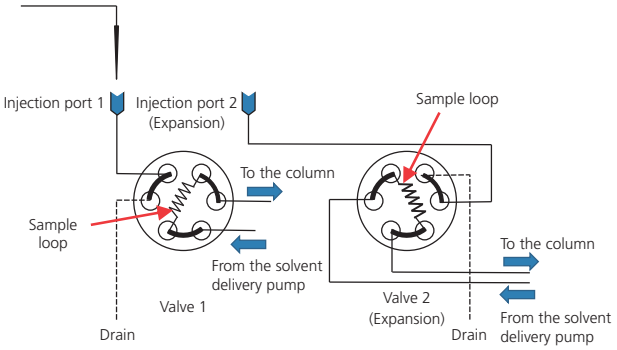

Fig. 5 Flow Line when the Sample is Injected into the Columns

An overview of the autosampler injection process is shown below (Fig. 6).

After the valve is switched to select a flow line during sample loading, the sample is loaded into the sample loop (pretreatment). After loading the sample into each flow line, the valves are switched to flow line (1) and flow line (2) simultaneously. The sample in the flow lines during injection is then introduced to the columns. Afterwards, the autosampler rinses the flow lines used for injection (post treatment).

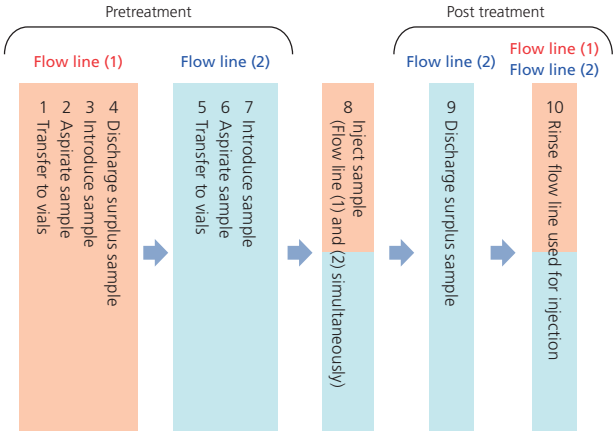

Fig. 6 Autosampler Injection Process

3. Settings and Data Analysis in LabSolutions

The dual injection system is configured using LabSolutions. The analytical conditions and analysis sequence for the two flow lines are configured in a single method file and batch file (Fig. 7 to 9). Conventionally, for two analyses, it was necessary to create different files. However, using the dual injection function, the respective information is integrated in a single file, simplifying data management and ensuring data integrity.

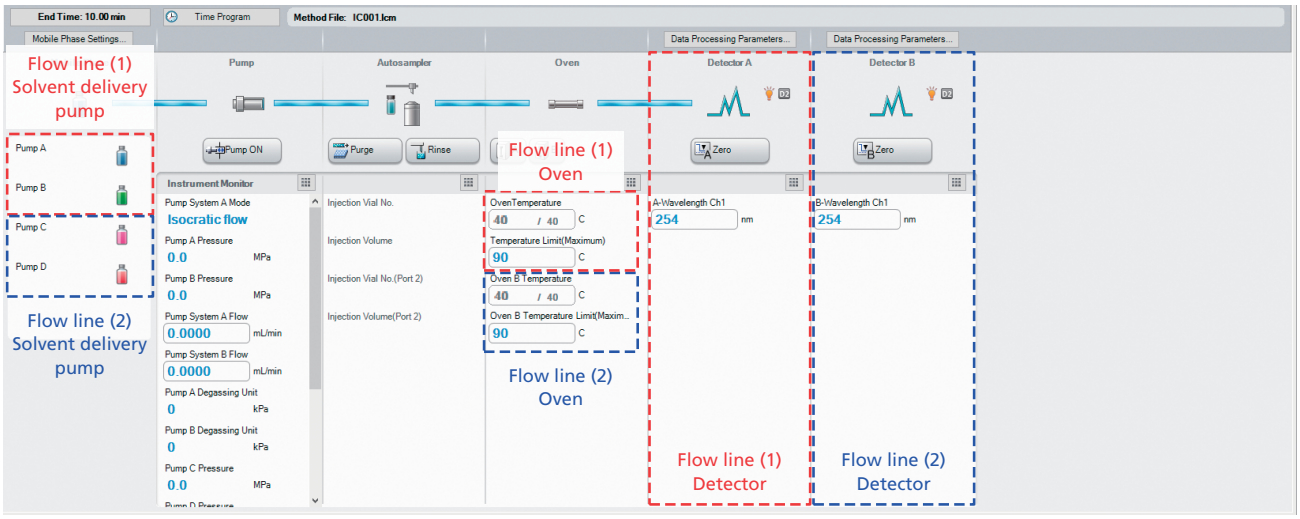

Fig. 7 Example of the Analytical Condition Settings (Solvent Delivery Pump, Oven, and Detector)

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Additionally, use of the dual injection function is enabled by selecting [Simultaneous (Port 1, 2)] for the ports used in the settings for the autosampler in the method file (Fig. 8). Also, it is possible to perform an analysis using only one of the flow lines by specifying the port to use.

In the settings for consecutive analysis, the vial number and injection volume for the sample injected into each flow line are entered on one line. Either the same vial numbers or different vial numbers can be specified (Fig. 9).

For example, after injecting the standard samples used to create a calibration curve into each of the flow lines from the different vials, the actual sample can be injected into both flow lines from a single vial.

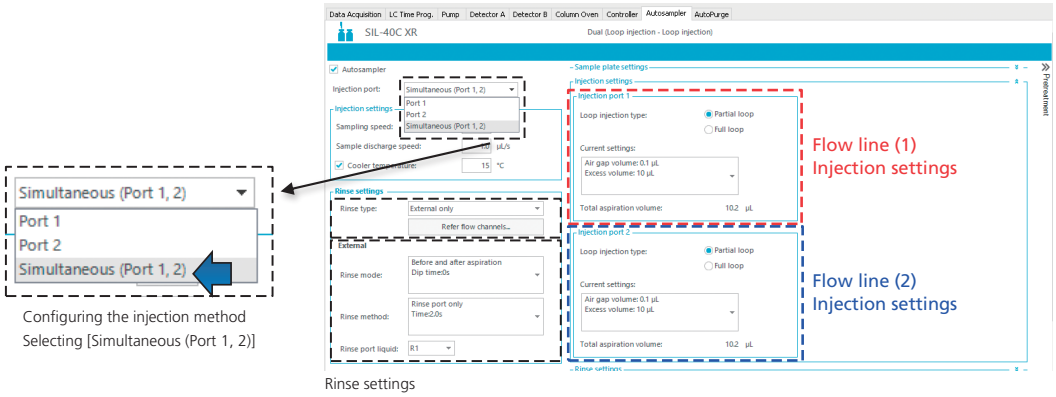

Fig. 8 Example of the Analytical Condition Settings (Autosampler)

Analysis	Tray Name	Vial#	Inj. Volume	Tray Name (Port 2)	Vial# (Port 2)	Inj. Volume (Port 2)
1	1	13	0.1	1	13	0.1
2	1	14	0.1	1	13	0.1

Flow line (1)
Tray, vial No., injection volume

Flow line (2)
Tray, vial No., injection volume

Fig. 9 Example of the Vial Number Settings

With the dual injection system, the two sets of data for the same sample are managed in an integrated way by automatically combining them into a single data file. This eliminates the risk of incorrect associations of sample and data file pairs, ensuring data traceability.

Additionally, using the LabSolutions data browser function and quantitative browser function, the data can be displayed in an easy to understand manner, simplifying the data analysis process (Fig. 10 and 11).

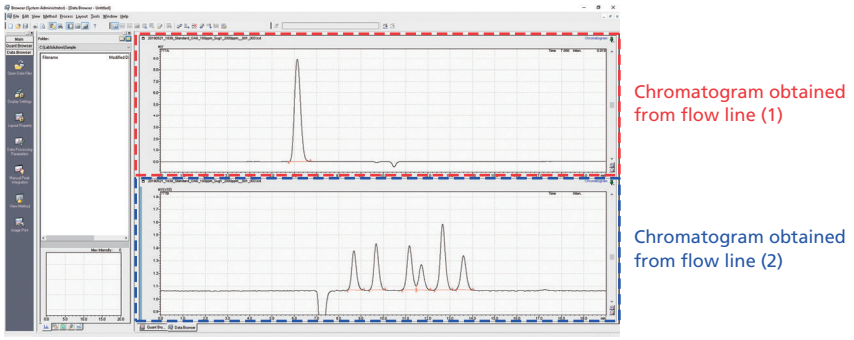

Fig. 10 Example of the Batch Display of Two Chromatograms Using the Data Browser Function

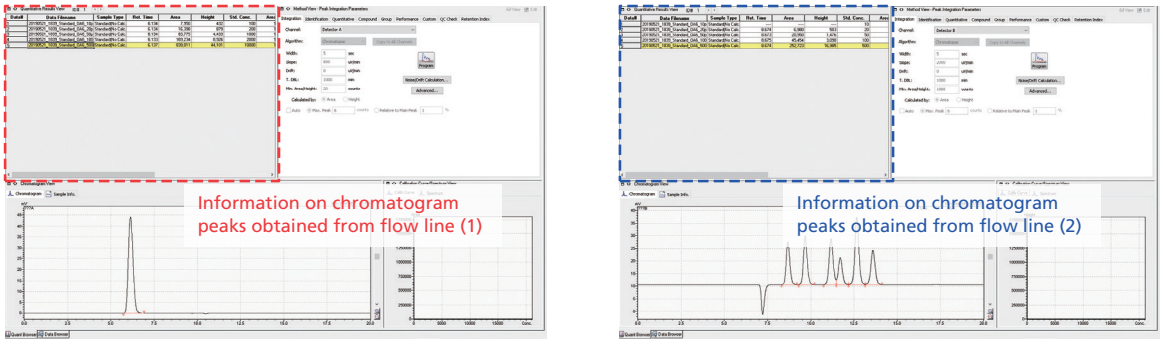

Fig. 11 Example of the Display of Quantitative Results with Two Chromatograms Using the Quantitative Browser Function

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4. Application Example

To quantify the organic acids and sugars in yogurt, a simultaneous analysis was performed under two different conditions using the dual injection system. The column temperature conditions differ for each analysis method, but with this system, the temperatures can be controlled separately using two column ovens (Fig. 12).

With these analytical conditions, the citric acid, lactic acid, and lactose in yogurt were analyzed in 15 minutes.

These two chromatograms were integrated into a single data file, simplifying the association and management of the data sets obtained from one vial.

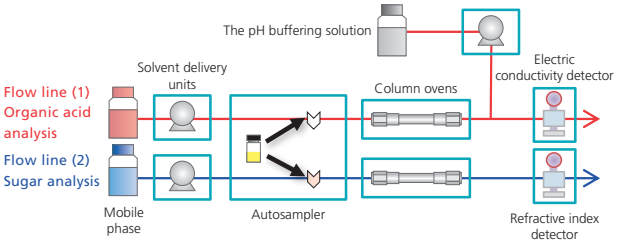

Fig. 12 Flow Line for the Batch Analysis of Organic Acids and Sugars

Table 1 Analytical Conditions of Organic Acids

Column	: Shim-pack SCR-102H (10 µm, 7.9 x 300 mm)
Mobile Phase	: 5.0 mmol/L aqueous *p*-toluenesulfonic acid solution
pH Buffer Solution	: 5.0 mmol/L *p*-toluenesulfonic acid 20 mmol/L Bis-Tris, 0.1 mmol/L EDTA mixed aqueous solution
Column Temperature	: 40 °C
Detector	: Electric conductivity detector (Post column pH buffering method)
Injection Volume	: 10 µL

Table 2 Analytical Conditions of Sugars

Column	: Shim-pack SCR-101C (10 µm, 7.9 x 300 mm)
Mobile Phase	: Water
Column Temperature	: 80 °C
Detector	: Refractive index detector
Injection Volume	: 10 µL

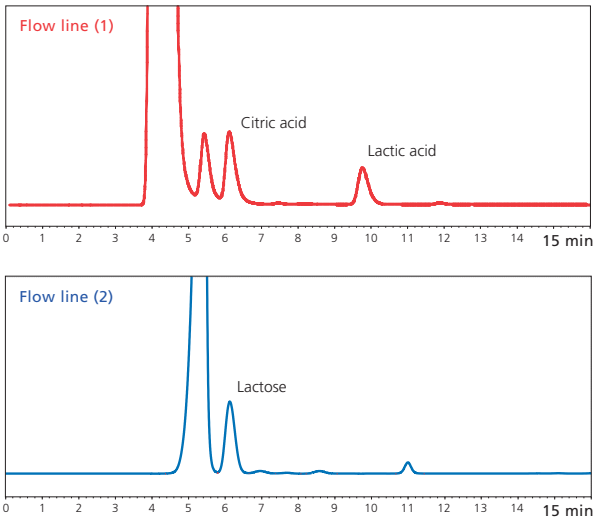

Fig. 13 Chromatograms of Yogurt
(Top: Organic Acid Analysis; Bottom: Sugar Analysis)

5. Conclusions

- The SIL-40 series features the dual injection function, which enables the injection of a single sample into two analysis flow lines.
- The dual injection system simplifies the data analysis process by automatically integrating the two data sets with respect to the sample into a single data file. Additionally, this ensures traceability with respect to the same sample.
- The two analysis method files, batch files, and data files are each integrated into a single file, simplifying data management and ensuring data integrity.
- Analyses that used multiple systems, as well as analyses implemented by switching the conditions for each target component can now be performed simultaneously using the same system. This shortens analysis times, reduces the installation space requirements, and lowers initial costs.

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Technical Report

Improving Peak Shape Using the Automatic Pretreatment Function (Co-Injection) in the SIL-40 Series Autosamplers

Satoshi Akita[1], Takato Uchikata[1], Kenichi Yasunaga[1], Hidetoshi Terada[1], Takanari Hattori[1], Keiko Matsumoto[1]

Abstract:

If a sample solvent selected in accordance with the solubility of a compound has a higher elution strength than the mobile phase, this can have a negative impact on the chromatogram. For example, if the ratio of organic solvent in a sample solvent is heightened in order to dissolve a compound with low polarity, this can have a detrimental effect on peak shape for quickly eluted compounds in reverse-phase chromatography, and even compromise the reliability of quantitative results. This article illustrates the use of the co-injection function, one aspect of the automatic pretreatment functionality included as standard in autosamplers in the Nexera™ series (SIL-40 series), to improve peak shape by injecting a solvent with a weak elution strength simultaneously with the sample in order to reduce the effect of the sample solvent. The co-injection function can be specified easily via the LabSolutions workstation software.

Keywords: Automated pretreatment functionality, co-injection

1. Co-Injection Function

In addition to regular injection procedures, autosamplers in the Nexera series (SIL-40 series) are equipped as standard with a variety of automatic pretreatment functions for diluting samples or adding reagents for example. One such function, the co-injection function, can be used to take a specified volume of reagent or solvent from a specified vial, and inject it together with the sample, or to mix it with the sample inside the needle.

Using this function, a specified volume of reagent (or solvent) from a specified vial can be injected together with the sample (Fig. 1).

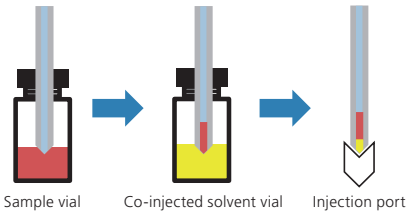

Fig. 1 Illustration of the Injection Process

2. Settings for the Co-Injection Function

Settings for co-injection and other standard automatic pretreatment functions can be configured easily using a LabSolutions template, and saved in a method file (Fig. 2).

The applicable parameter settings are indicated below.

(1) Tray and vial number for the reagent to be co-injected
(2) Injection volume of reagent to be co-injected
(3) Injection timing (before, after, or before and after the sample)
(4) Mixing count and volume (number of agitation cycles within the needle, and the volume)
(5) Wait time (wait time after mixing before injection)
(6) Air gap volume inserted before and after aspirating the sample and the co-injected reagent

It is also possible to successively aspirate user-defined volumes from multiple vials for co-injection, as shown in Fig. 3.

In addition to co-injection, programmed pretreatment actions not specified in a template can also be changed and executed.

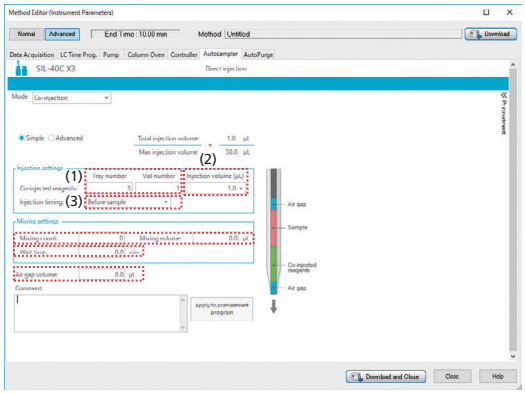

Fig. 2 Settings for Co-Injection Function

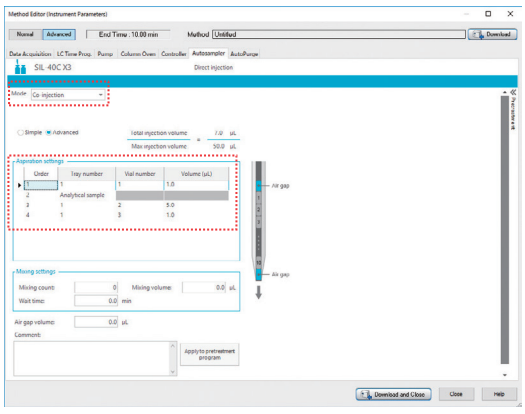

Fig. 3 Settings for Co-Injection Function (Advanced)

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3. Example of the Use of Co-Injection to Improve Peak Shape

The simultaneous analysis of lipid mediators and related substances is mainly performed in the context of biomarker discovery and in disease research fields. Because lipid analysis involves the use of sample solvents with a high organic solvent ratio, peak shapes for weakly retained components can become distorted during reverse-phase chromatography.

In this example, the co-injection function was used to improve the peak shapes for a mixed standard solution prepared with 100 % methanol as the solvent.

The effect of co-injecting water using the analytical conditions indicated in Table 1 is shown in Fig. 4. Co-injection improved both the peak shape and the tailing factor (S). It was particularly effective for components with fast retention times.

Table 1 Analytical Conditions

Column	: C8 column (2.1 mm I.D. × 150 mm L, 2.6 µm)
Mobile Phase A	: 0.1 % aqueous formic acid solution
Mobile Phase B	: Acetonitrile
Rinse Solution	: Acetonitrile
Other Conditions	: Compatibility with Shimadzu LC/MS/MS method package for lipid mediators
Sample	: Standard sample of lipid mediators and related compounds (mixture of 196 components in methanol solution)
Co-Injected Solvent	: Water (5, 10, and 15 µL)

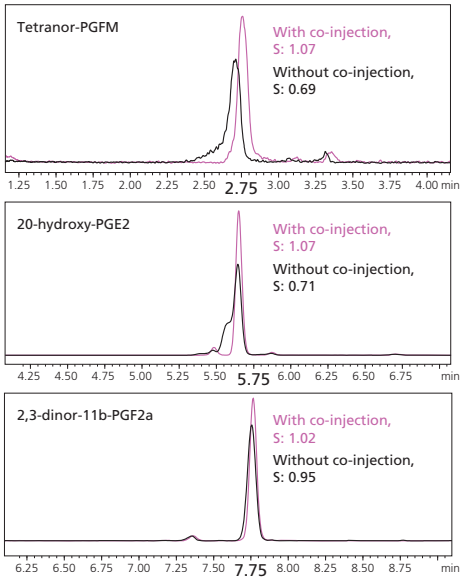

Fig. 4 MRM Chromatograms for Various Lipids
(with either 15 µL or no co-injection)
(The tailing factor S was calculated by the USP method.)

Fig. 5 shows the relationship between the quantity of co-injected solvent (water) and the improvement in peak shape. The greater the volume of co-injected water, the greater the improvement in the leading slope for both of the compound peaks, and in the resolution (Rs) for both components.

Further improvements in peak shape and resolution can be expected from optimizing the type and volume of co-injected solvent.

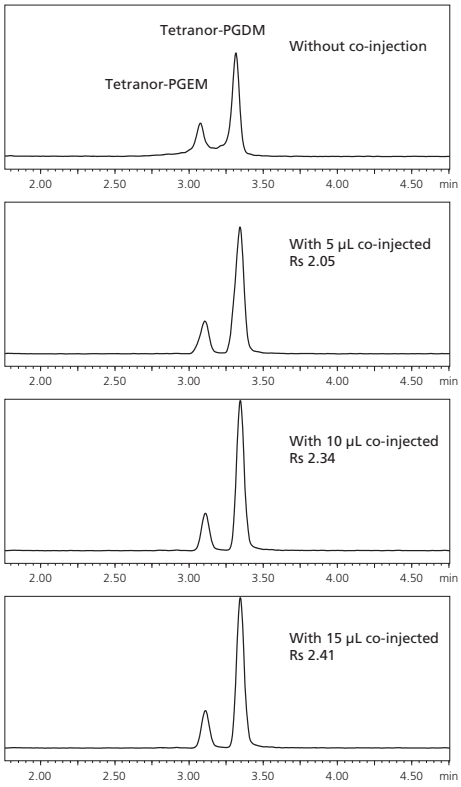

Fig. 5 Comparison of Resolution between Lipid Isomers
(with 5 ,10 ,15 µL or no co-injection)
(Resolution Rs was calculated by the USP method.)

4. Summary

- Autosamplers in the SIL-40 series are equipped as standard with a function for automatically pretreating samples inside the autosampler, including co-injection and dilution.
- For reverse-phase chromatography with samples that have a high organic solvent ratio, the use of the co-injection function to simultaneously inject a solvent with the sample can improve the shape of peaks with a short elution time, and improve the reliability of quantitative analysis results.

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Technical Report

Ultra-Fast Analysis of Drugs in Biological Fluids with the SIL-40 Autosampler

- Analytical Intelligence Part 5 -

Uchikata Takato[1], Davide Vecchietti[1]

Abstract:

There is increasing demand in modern clinical/pharmaceutical laboratories for high-throughput liquid chromatography-tandem mass spectrometry analysis for drug quantitation in biological fluids. The analysis of drugs and metabolites using LC-MS/MS typically requires analysis times of a few minutes per sample. In this report, we describe the use of the SIL-40 series autosampler (SIL-40) for the precise and ultra-fast quantitation of drugs in plasma samples within less than 20 sec, thanks to the extremely low injection cycle time, introducing real examples of increased productivity in the lab.

Keywords: Ultra-fast injection, High-throughput analysis, SIL-40 series

1. Importance of the Injection Cycle Time in High-Throughput Analysis

There is growing need for increased throughput of liquid chromatography-tandem mass spectrometry analysis in modern laboratories. For example, in clinical/pharmaceutical laboratories, the maximization of analytical throughput and associated higher efficiency facilitates more rapid reporting of results, increasing the effectiveness of all corrective actions for treatments such as dose adjustments.

Development of bioanalytical systems with higher throughput is also a prime concern for drug metabolism and pharmacokinetic (DMPK) evaluation, a crucial step in early drug discovery which deals with a large number of samples.

The analysis of drugs and metabolites using LC-MS/MS typically requires analysis times of a few minutes per sample. Recent advances in LC technology, such as improved LC column quality and higher pressure tolerance, allow the shortening of analytical cycle time to the range of several tens of seconds. Under these analytical conditions, autosampler injection cycle time plays a critical role in reducing overall analysis time and thereby achieving higher throughput.

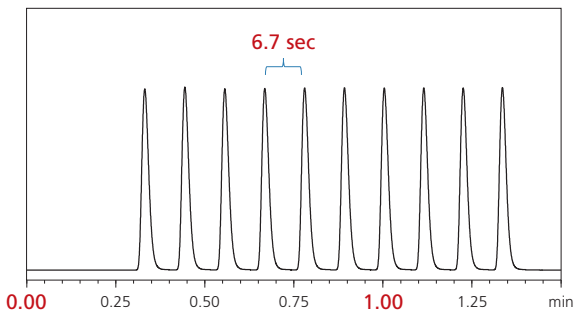


Fig. 1 UV chromatogram of caffeine obtained with the SIL-40, showing the injection cycle time

Table 1 Analytical conditions

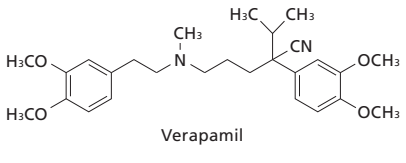
Mobile Phase:	Water / MeOH = 4 / 6 (v/v)
Column:	Shim-pack™ XR-ODS II (3.0 mm I.D.× 75 mmL., 2.2 µm)
Flow rate:	1.4 mL/min
Injection volume:	0.5 µL
Detection:	UV-VIS 273 nm

2. System Configuration

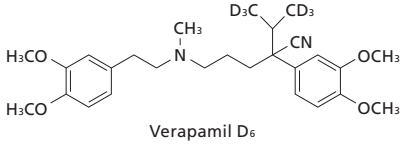
In this report, we describe the use of the SIL-40 for the precise and ultra-fast quantitation of drugs in plasma samples, with the goal of maximizing the throughput of a clinical laboratory.

Fig. 1 shows the ultra-fast injection of a caffeine standard solution using the SIL-40. An injection cycle under 7 seconds was achieved.

In order to evaluate the effectiveness of sample analysis with this ultra-fast injection performance, we carried out an ultra-fast analysis of drugs in blood plasma using the triple quadrupole mass spectrometer LCMS-8050 from Shimadzu. Fig. 2 shows the test compound and the internal standard. A 5 mmL guard column was used to reduce matrix effects in the short time window. Analysis conditions are shown in Tables 2 and 3. Analysis was performed with the system volume as low as possible (through direct connection of the column to the ESI source) to prevent post-column dilution. Even with extremely narrow columns (<5 sec), it was possible to collect enough data points with Shimadzu UFMS technology.



Verapamil



Verapamil D6

Fig. 2 Verapamil and IS chemical formulae.

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Table 2 Analytical Conditions

System:	Nexera XR
Column:	Shim-pack Velow EXP guard column cartridge (2.1 mm I.D., 5 mmL, 2.7 µm)
Column temperature:	Room temp.
Mobile phases:	A: water + 0.1% formic acid B: acetonitrile + 0.1% formic acid A / B = 27 / 13 (v/v)
Flow rate:	750 µL/min
Average cycle time:	18 sec
Injection volume:	0.5 µL

Table 3 MS/MS Acquisition Parameters

	MRM
Verapamil:	455.1>165.1, 105.3, 303.3
Verapamil D_6:	461.9>165.2, 150.2, 309.3

Plasma samples were spiked with an appropriate concentration of Verapamil from 0.39 to 100 µg/L and subsequently underwent protein precipitation in a plasma to precipitant solution ratio of 1:3. After vortex (1 min) and short incubation at room temperature (5 min), samples were centrifugated and 200 µL of supernatant was collected and transferred into micro-vials.

During analysis the needle was actively rinsed after sample aspiration using the SIL-40 rinsing pump (1 sec). As a result, the carryover was kept extremely low while achieving an overall analysis cycle time of under 18 sec.

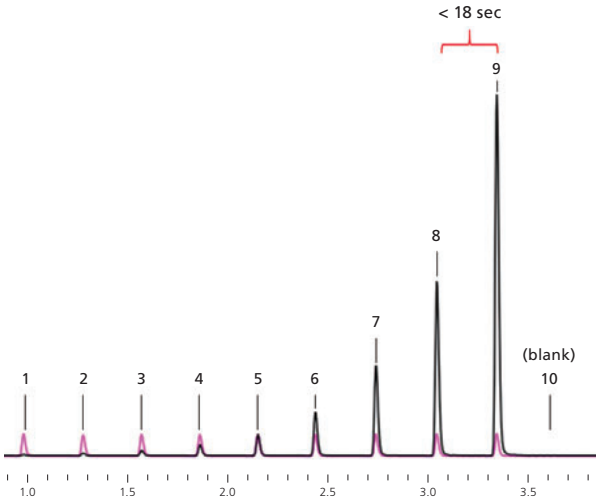

Fig. 3 Linearity over the bioanalytically relevant conc. range. Black: Verapamil chromatogram. Pink: Verapamil D_6 chromatogram. 1: Calib 1 (0.39 µg/L). 2: Calib 2 (0.78 µg/L). 3: Calib 3 (1.56 µg/L). 4: Calib 4 (3.12 µg/L). 5: Calib 5 (6.25 µg/L). 6: Calib 6 (12.5 µg/L). 7: Calib 7 (25 µg/L). 8: Calib 8 (50 µg/L). 9: Calb 9 (100 µg/L). 10: Blank.

The 9 peaks shown in Fig. 3 (peaks 1-9) represent the standard samples added to the blood plasma. The calibration curves obtained from these peaks show good linearity (R2=0.9998). In addition, after injection of the most concentrated sample, a blank solution was injected (peak 10). The blank solution did not produce a peak above the average noise level, demonstrating that carryover was negligible.

In addition, even after 300 injections, the analytical stability remained high (Fig. 4). The RSD% of the internal standard (shown in blue) was 2.4%, stable even under ultra-fast injection conditions with a cycle under 18 sec. This confirmed the ability to measure a large number of samples with ultra-fast injection.

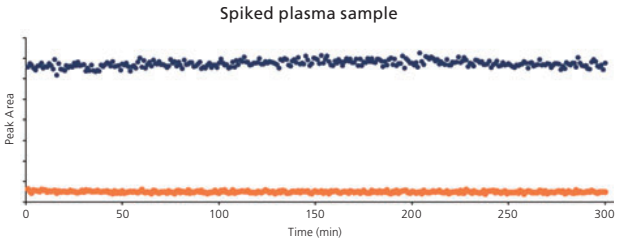

Fig. 4 Spiked plasma sample (0.39 µg/L), 300 consecutive injections. RSD% peak area without any smoothing: 2.4% for IS (blue), 9.3% for Verapamil (orange).

3. Conclusions

- The use of the SIL-40 in combination with the UFMS LCMS-8050 allows accurate drug quantitation in plasma samples.
- With a 5 mm column and active rinsing of the needle using a rinsing pump, it was possible to prevent carryover as well as matrix effects from non-target compounds in the samples, in addition to reducing the overall analysis cycle time to under 18 seconds.

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Technical Report

Eliminating the Effects of Room Temperature Fluctuations Using the Advanced TC-Optics Function in the SPD-M40 Photodiode Array Detector - Improving Baseline Stability and Analytical Precision

Hidetoshi Terada[1], Masato Watanabe[1]

Abstract:

Because of the detection principles involved, photodiode array detectors are affected by the environment in which they are installed. Consequently, room temperature fluctuations can cause baseline fluctuations. To eliminate the effects of room temperature fluctuations, the SPD-M40 includes a triple temperature control function (Advanced TC-Optics), which independently controls the temperatures of the detector cell, light source lamp, and spectrometer. As a result, baseline stability is obtained even when there are large room temperature fluctuations, enabling high analysis precision in high-sensitivity analyses, and in analyses over an extended period.

Keywords: Photodiode array detector, triple temperature control function, Advanced TC-Optics

1. Effects of Ambient Temperature Fluctuations on Photodiode Array Detectors

Due to the single-beam configuration*1) of photodiode array (PDA) detectors, their operating principles make them more likely to be affected by temperature fluctuations during measurements than UV-VIS detectors, with their double-beam configuration.*2)

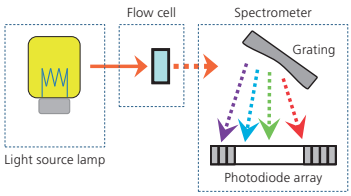

Fig. 1 Illustration of Detection by a Photodiode Array Detector

*1) Single-beam configuration: Light from the light source enters the cell directly, and then enters the detection unit.
*2) Double-beam configuration: Light from the light source is split into sample and reference light beams, with the sample beam entering the sample cell, and the reference beam used to correct for drift caused by the instrument.

Fluctuations in the surrounding air temperature where the PDA detector is installed can disrupt baseline stability by changing the light absorbance at measurement wavelengths. Such fluctuations can be caused by the following factors.

(1) Variations in source lamp light intensity
(2) Variations in mobile phase and target component absorbance inside the cell*3)
(3) Variations due to shifts of spectra (in the wavelength direction)

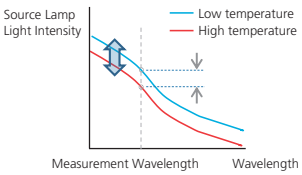

Fig. 2 Illustration of Temperature Effects on Source Lamp Light Intensities

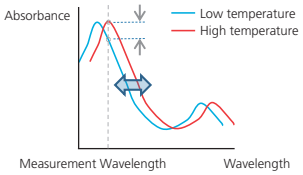

Fig. 3 Illustration of Temperature Effects on Spectra

*3) Temperature fluctuations can cause variations in the absorption spectra of mobile phases or target components inside the cell, in either the absorbance or wavelength direction, or in both directions.

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2. SPD-M40 Temperature Control Method

The SPD-M40 features an Advanced TC-Optics triple temperature control function, which not only controls the cell temperature, as available on previous models, but also controls the temperature of the light source lamp and the spectrometer independently. (Fig. 4)

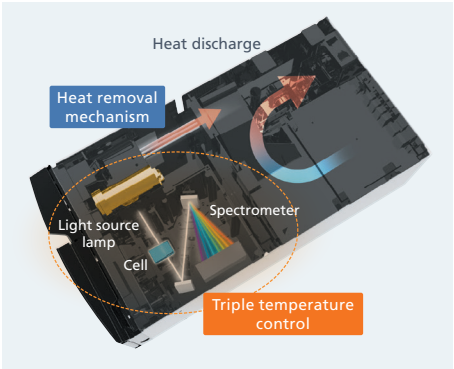

Fig. 4 SPD-M40 Triple Temperature Controlled Locations

The benefits obtained from controlling the temperature at each location are summarized in Table 1 All of these benefits help reduce detector absorbance fluctuations caused by fluctuations in the ambient temperature, so that HPLC analysis can be performed with a stable baseline, even if ambient temperatures fluctuate.

Table 1 Benefits of Temperature Control at Each Location

Temperature Controlled Location		Benefit
(1)	Light Source Lamp	Stabilizes source lamp light intensity
(2)	Cell	Inhibits changes in absorption spectra due to temperature variations in mobile phases or target components
(3)	Spectrometer	Inhibits absorbance changes due to shifts of spectra

Additional baseline stabilization and noise reduction are achieved by using a unique heat removal mechanism that removes heat from the light source lamp, an element that generates large amounts of heat.

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3. Effect of Ambient Temperature Fluctuations on the Baseline

Fig. 5 shows PDA detector baseline fluctuations caused by intentionally varying the ambient temperature in a thermostatic chamber. Analytical conditions and ambient temperature settings conditions are shown in Table 2. In addition to the SPD-M40, the SPD-M20A, which is Shimadzu's previous model, and another Vendor's PDA Detector were also verified in the same manner.

Due to the triple temperature control function, the baseline fluctuation in response to a 10 °C change in ambient temperature was an extremely small 0.2 mAU or less for the SPD-M40, ensuring excellent baseline stability. As a result, HPLC analysis can be performed with a stable baseline characterized by minimal undulations, even if the room temperature varies where the system is installed.

Table 2 Analytical Conditions

Column	: None	Cell temperature	: 40 °C
Mobile phase	: MeOH	Ambient temperature	: 20 to 30 °C (profile indicated in Fig. 5)
Flowrate	: 1.0 mL/min		
Detection	: 260 nm		

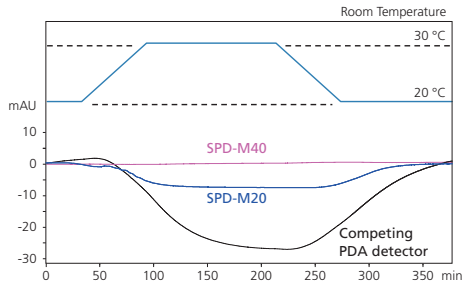

Fig. 5 Baseline Fluctuations in Response to Changes in Ambient Temperature

4. Effect of Ambient Temperature Fluctuations on Quantitative Analysis

To confirm the effect of baseline fluctuations on quantitative accuracy, samples were successively injected and analyzed as the ambient temperature was varied over 5 °C. Analytical conditions and ambient temperature settings conditions are shown in Table 3.

Table 3 Analytical Conditions

Mobile phase	: MeOH / Water = 70/30
Flowrate	: 0.2 mL/min
Column	: Shim-pack HRC-ODS (3.0 mmI.D. x 250 mmL)
Column temperature	: 40 °C
Detection	: 273 nm
Cell temperature	: 40 °C
Sample	: 5 mg/L Caffeine
Injection volume	: 1 µL
Ambient temperature	: 20 to 25 °C (profile indicated in Fig. 6)

The resulting chromatograms are shown in Fig. 6.

It is evident that the SPD-M40 baseline was unaffected by room temperature fluctuations, as the peaks were detected against a stable baseline that is essentially flat. In contrast, large baseline fluctuations affect peak detection against baseline drift.

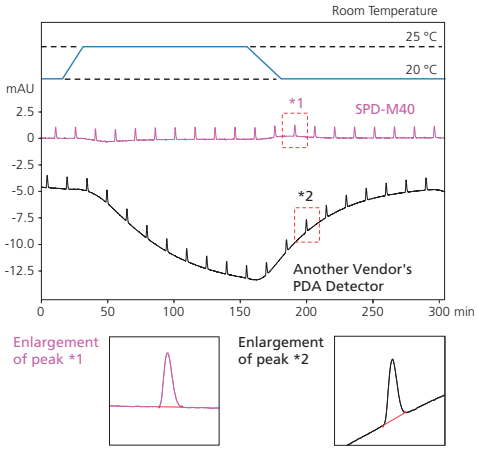

Fig. 6 Effect of Ambient Temperature Changes on Peak Integration

Table 4 indicates the reproducibility of peak area values for the peaks detected in the chromatograms above. The stable baseline with the SPD-M40 enables accurate peak integration, and provides good reproducibility even if room temperature fluctuations occur.

Table 4 Peak Area Reproducibility with Ambient Temperature Fluctuations

	SPD-M40	Another Vendor's PDA Detector
Peak Area Reproducibility (%RSD, n = 20)	0.62	1.87

5. Conclusions

- The SPD-M40 minimizes the effects of room temperature fluctuations where it is installed by using a triple temperature control function (Advanced TC-Optics), which independently controls the temperature of the detector cell, light source lamp, and spectrometer.
- The Advanced TC-Optics function minimizes baseline fluctuations, even when the room temperature fluctuates where the system is installed.
- The detector enables highly precise analysis by ensuring that the peaks can be detected against a stable baseline even if the room temperature varies. This is especially helpful for the quantitative analysis of trace amounts of target components, and in analyses over an extended period.

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Technical Report

Improved Linearity and Quantification Using the SPD-M40 Photodiode Array Detector

- Analytical Intelligence Part 4 -

Masato Watanabe[1], Hidetoshi Terada[1]

Abstract:

In principle, stray light generated during the UV-VIS and PDA detection process has a great influence on the linearity of the detector's response linearity. This report explains the influence of stray light upon detection and introduces the SPD-M40 photodiode array detector, which completely reduces the influence of stray light and achieves a linearity of 2.5 AU as a specification value (typical value is more than 2.5AU). Furthermore, in the low signal range, noise reduced noise has improved the detection accuracy of low concentrations, enabling the quantification of a wide concentration range. This enables simultaneous analysis and quantification of major components and impurities with different concentration ranges.

Keywords: Dynamic range, linearity, absorbance, stray light, noise

1. Principle of UV-VIS and PDA detectors

Unlike a UV-VIS detector, which separates light spectrally from a light source and irradiates the flow cell with only a specific wavelength to measure the absorbance of the target component, a PDA detector directly irradiates the flow cell with light from a light source containing various wavelengths (white light) and separates the light spectrally after passing through the flow cell. The optical flow diagrams are shown in Fig. 1.

A PDA detector can simultaneously measure the absorbance and the absorption spectrum, and can be used not only for quantitative analysis, but also for qualitative analysis.

2. Effect of stray light on absorbance

In general, absorbance is expressed in terms of the intensity of incident light on the flow cell and the intensity of transmitted light through the sample cell, according to Lambert-Bert's law.

$$A = -\log\frac{I}{I_0}$$

A : Absorbance
I : Real-time light intensity transmitted through the flow cell
I_0 : Incident light intensity

If the unexpected light is emitted by the spectroscope, the correct absorbance cannot be measured. Unexpected light during detection is commonly referred to as "Stray Light". The influence of stray light on the absorbance is expressed by the following equation.

$$A = -\log\frac{I+\Delta}{I_0+\Delta}$$

Δ: stray light intensity

If the absorbance in the flow cell is high, the transmitted light intensity from the cell will be small. In such cases, the effect of stray light intensity on the absorbance is more pronounced.

Fig. 2 illustrates the influence on the absorbance of stray light when the ratio of stray light intensity to incident light was changed to 0 - 0.5%. In cases when the stray light intensity is larger, the calculated absorbance value is smaller than the ideal value, and the linearity will be affected especially in the region exceeding 2AU.

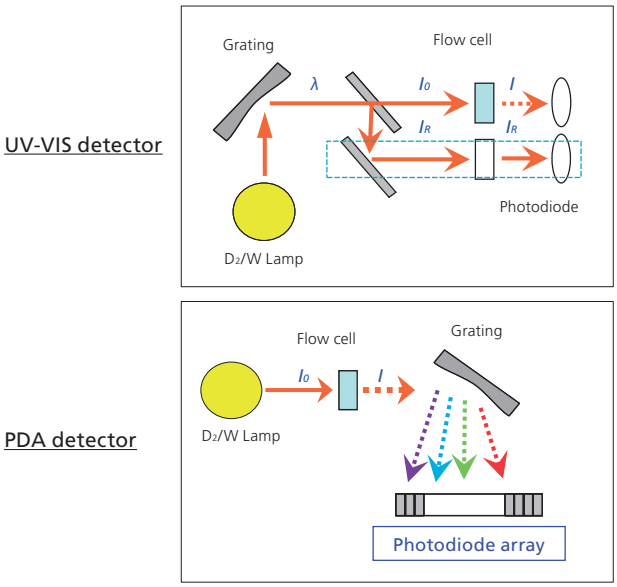

Fig. 1 Principle of UV-VIS and PDA detectors

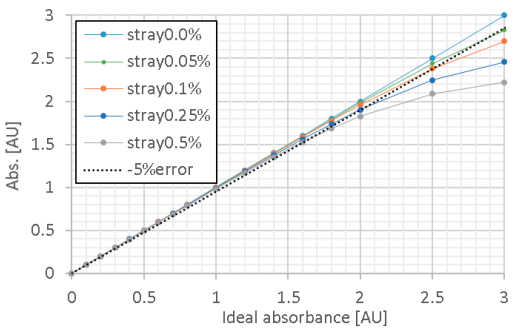

Fig. 2 Effect of stray light on absorbance

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Fig. 3 shows the effect of stray light intensity on absorbance as error rate using the results of Fig. 2. For example, when trying to obtain an absorbance linearity error within 5%, if the stray light has an intensity of 0.25% with respect to the incident light, the upper linearity limit is 2 AU; if the intensity is 0.1%, the upper limit is 2.5 AU.

Thus, the intensity of stray light greatly affects the quantitation of the target component.

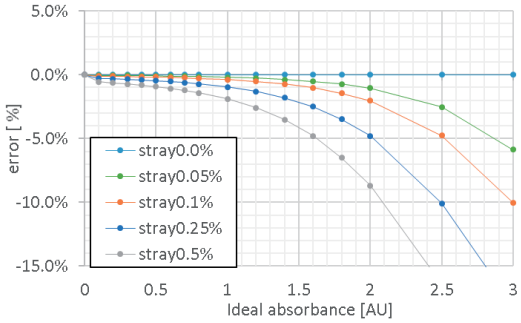

Fig. 3 Stray light intensity and absorbance error rate

3. Causes of stray light generation and reduction

Causes of stray light include reflection and scattering of light by the optical element itself and dirt attached to the optical element, reflection and scattering of light at the spectrometer, and unexpected reflection and dispersion of light at the grating.

In particular, a PDA detector emits white light from the lamp to the cell, and the transmitted light is dispersed and detected, so there is generally more stray light than for a UV-VIS detector.

Designed to reduce the effect of these causes of stray light, the SPD-M40 reduces overall stray light to a third of that in previous PDA detectors. It thereby achieves a linearity of 2.5 AU as a specification value, comparable to that of a UV-VIS detector (the actual value is typically more than 2.5 AU).

4. Noise reduction

The factor that predominantly determines the linear upper limit of absorbance is stray light. In contrast, the lower limit is primarily determined by the noise of the detector response. The SPD-M40 minimizes noise through e.g. optimization of the electrical system layout.

Fig. 4 shows a comparison of detector noise between the SPD-M40 and a conventional detector. When the time constant is small, the noise value is reduced significantly.

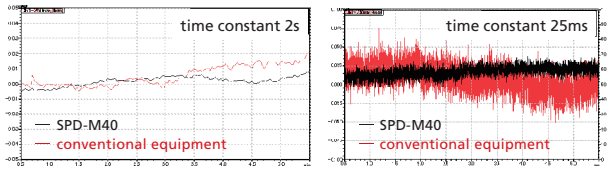

Fig. 4 Noise reduction

5. Improved dynamic range

Fig. 5 shows a chromatogram of a standard solution of ketoprofen, prepared by adjusting the analytical conditions and concentration to obtain a peak height with approximately 2.5 AU. Fig. 6 shows the calibration curve within a wide concentration range of 0.5 - 800 mg/L. With the SPD-M40, the linearity specification value is 2.5 AU; however, in practice, the value is significantly better. By reduced noise, it has become possible to simultaneously analyze high-concentration main components and trace impurities of 1 mAU or less with high accuracy. The coefficient of variation of the area value was 1% or less, even for the impurity with content that is about 0.1% quantified by area percentage, and good reproducibility was achieved.[1)]

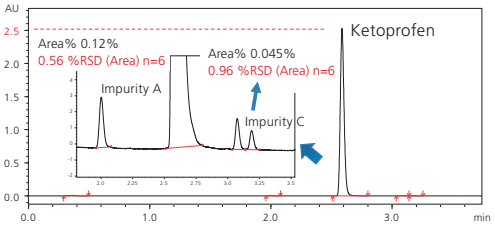

Fig. 5 Ketoprofen and impurity analysis

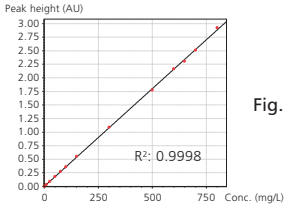

Fig. 6 Linearity of ketoprofen

6. Conclusion

- Stray light generated in the detection process has a significant effect on a detector's linearity range.
- The SPD-M40 photodiode array detector is designed to completely reduce the effects of stray light and offers wide linearity.
- In addition, it features significantly reduced noise. As a result, it is effective for analysis from low to high concentrations.

1) Application News No. L 538 "Impurity Analysis in Pharmaceutical Products with the Advanced Photodiode Array Detector SPD-M40"

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Technical Report

Using i-PeakFinder™, an Automatic Peak Integration Algorithm, to Provide Labor Savings and Improve the Efficiency of Analytical Operations

—Example of its Application to Organic Acid Analysis—

Keiko Matsumoto[1]

Abstract:

As the speed of analysis increases, the analysis time required per sample has shortened, which has improved productivity. On the other hand, there is concern that the increasing amount of data generated will require a much longer time for peak integration and data analysis processes. During data analysis, if contaminant peaks overlap with target component peaks, it may not be possible to process the samples adequately using conventional automatic peak integration methods. Manual peak integration 1) is troublesome and time consuming; and 2) can affect data consistency due to differences between results from different analysts. As a solution for such problems, the Technical Report entitled "A New Peak Integration Algorithm for LabSolutions (C191-E044)" described an Analytical Intelligence functionality called i-PeakFinder. The i-PeakFinder software simulate the operations of experts, and allows users with any level of knowledge or experience to acquire highly reliable data, without troublesome or time-consuming procedures. In the following example of the application of the algorithm to organic acid analysis, only simple settings are needed in order to accurately and simultaneously analyze the increasing amounts of data generated from faster sample analysis.

Keywords: Peak integration algorithm, i-PeakFinder, organic acid analysis, Shim-pack Fast-OA

1. Efficiency Bottleneck in Analytical Operations

Improved instrument and column performance has enabled faster analysis speeds and higher throughput. As analysis times per sample get shorter, the volume of data that must be processed continues to increase. On the other hand, peak integration remains a very time-consuming process. Consequently, there are concerns about the huge amount of time now required for data analysis.

When analyzing samples using low UV wavelength detection or under other HPLC conditions that do not provide high selectivity, and when analyzing foods or other samples with complicated compositions, it is not uncommon for peaks from contaminant components to overlap with the peaks from the target components being analyzed. In the case of organic acid analysis using electrical conductivity detection, negative peaks are detected for alcohol and other non-electrically conductive sample components.

Conventional automatic peak integration processes sometimes do not integrate target peaks appropriately if the chromatogram includes contaminant peaks that overlap with target component peaks or includes negative peaks (Fig. 2).

It is possible to perform a certain amount of batch peak integration using a time program (Fig. 3), but overlap by contaminant components must be treated by manual peak integration on an individual basis. Consequently, this does not avoid problems with 1) troublesome and time-consuming procedures and 2) data inconsistency due to differences between the results from different analysts. Therefore, given the increased quantity of data generated from faster sample analyses, there is a need for a peak integration method capable of accurate batch data analysis, and requiring only simple settings.

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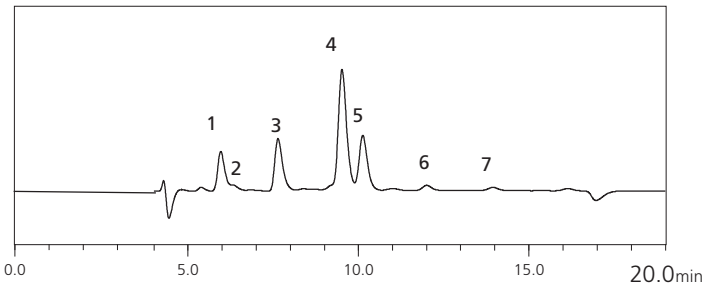
Shim-pack Fast-OA (Three 7.8mm I.D. × 100mm columns)



■ Peaks
1. Phosphoric acid
2. Citric acid
3. Malic acid
4. Succinic acid
5. Lactic acid
6. Acetic acid
7. Pyroglutamic acid

Fig. 1 Analysis of Organic Acids in Cooking Sake

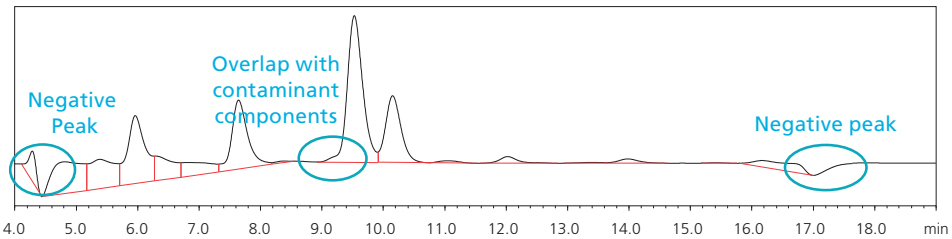


Fig. 2 Conventional Peak Integration Process

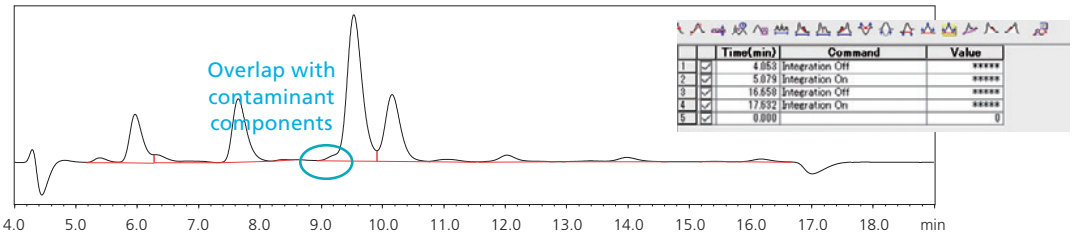


Fig. 3 Negative Peaks Eliminated by a Time Program for Peak Integration (Conventional Method)

2. Peak Integration Algorithm

Shimadzu's peak integration algorithm, shown in Table 1, is based on Analytical Intelligence functionality. The system and software simulate the operations of experts, and allow users with any level of knowledge or experience to acquire highly reliable data, without troublesome or time-consuming procedures.

i-PeakFinder requires only simple parameter settings and can automatically integrate peaks, including shoulder peak detection, even in complex chromatograms with waveforms that previously required manual peak integration by an analyst. In contrast, Peakintelligence, which is intended for specific method packages, uses AI technology to eliminate the need to adjust parameter settings prior to analysis. For this report, i-PeakFinder, which can be used for all LC and LCMS data, was used to analyze the data from an organic acid analysis.

Table 1 Applicability and Features of the Peak Integration Algorithm

	i-PeakFinder	Peakintelligence™
Applicability	New peak integration algorithm for LabSolutions. Can be used for LC, LCMS, and GC.	Optional LabSolutions Insight™ software. For primary metabolite and cell culture profiling LC-MS/MS method packages
Features	Analytical Intelligence functionality Fully automatic integration function detects peaks accurately, without the need to configure special parameter settings.	Analytical Intelligence functionality Enables expert-level data analysis, using expert peak integration skills learned by AI. No parameter setting adjustments are required prior to analysis.

ANALYTICAL INTELLIGENCE

Automated support functions utilizing digital technology, such as M2M, IoT, and Artificial Intelligence (AI), that enable higher productivity and maximum reliability.

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3. Automatic Peak Integration Using i-PeakFinder

i-PeakFinder, which requires simpler parameter settings than conventional methods, can be expected to provide the following benefits.

- Enables accurate shoulder peak detection.
- Baseline processing can be changed easily.
- Reliable peak tracking improves reproducibility.
- Enables correct peak integration, even with fluctuations from baseline drift.

It is easy to switch between the conventional method and i-PeakFinder. The peak integration method can be selected depending on the given situation. For example, the conventional method can be selected to maintain consistency with past data.

There are three basic parameter settings for peak detection: baseline processing, peak integration range, and peak detection threshold. The baseline processing setting can be selected based on the sample or the purpose of the test (Fig. 4).

If there are peaks that do not need to be detected in the first or second half of the chromatogram, specify [Integration range] settings that avoid those peaks, thereby enabling appropriate peak integration.

The [Detection Threshold] setting is used to determine the S/N ratio, based on estimated noise values calculated using Shimadzu's unique algorithm, and then detect peaks higher than a given threshold value. Increasing the [Detection Threshold] value results in the detection of multiple separate small peaks collectively, as a single peak. For comparison purposes, the chromatograms in Fig. 6 show the results of changing the [Detection Threshold] setting from 5 (default value) to 40. (The chromatograms are enlarged from the chromatogram in Fig. 5 between 4 minutes and 11.5 minutes.) Detection methods can be adjusted intuitively.

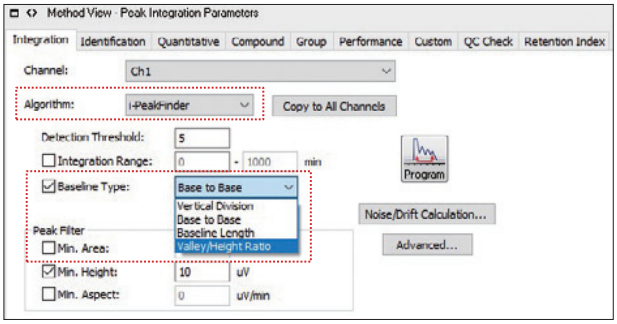

Fig. 4 Tab Page for i-PeakFinder Settings
(Method View [Integration Parameter] Tab Page)

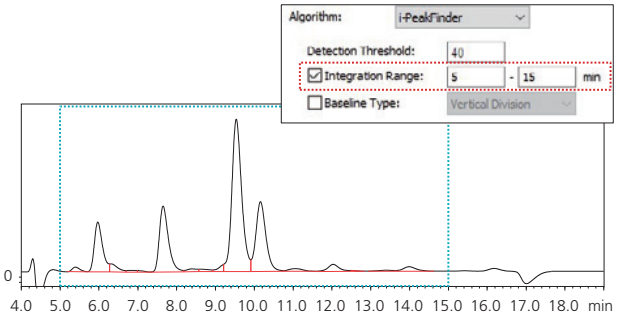

Fig. 5 Specifying the [Integration range] Settings

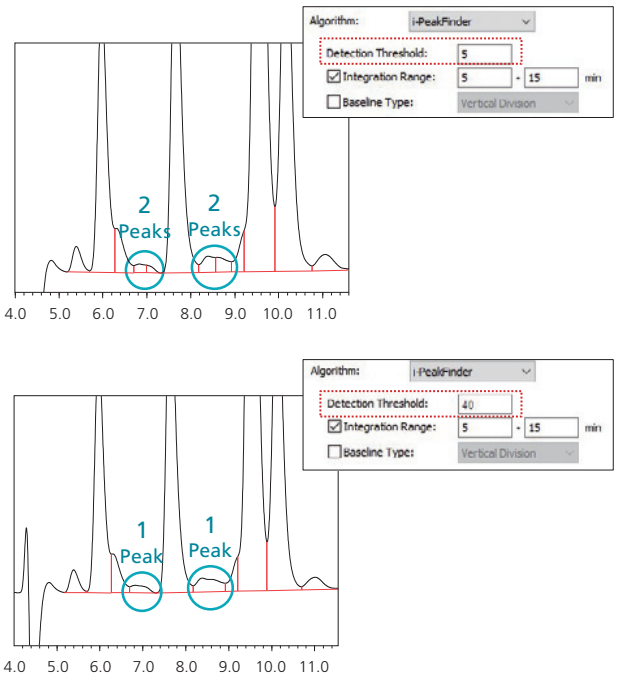

Fig. 6 Specifying the [Detection Threshold] Settings

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4. Example of the Use of i-PeakFinder for Organic Acid Analysis

Organic acids in cooking sake were analyzed using the post-column pH-buffered electrical conductivity detection method, which enables selective and high-sensitivity detection of organic acids, in combination with Shim-pack Fast-OA high-speed organic acid analytical columns. The analytical conditions (with overlapping injection) are indicated in Table 2. i-PeakFinder was specified as the peak integration algorithm, and peak integration was performed automatically. The corresponding peak integration results are shown in Fig. 7, and the area reproducibility results are shown in Table 3.

- [Integration range]: 5 to 15 min
- [Detection Threshold]: 40

The results show that baseline processing was the same for the entire chromatogram. Also, excellent area %RSD values of 1.5 % or less were obtained for most of the organic acids. i-PeakFinder integrates the peaks automatically and provides highly accurate results as soon as all the analysis steps are finished. This provides labor savings by improving the efficiency of analytical operations, regardless of the expertise level of the analysts (Fig. 8).

Table 2 Analytical Conditions

Column	: Shim-pack Fast-OA with three columns (100 mm L. × 7.8 mm I.D., 5 µm)
Guard column	: Shim-pack Fast-OA (G) (10 mmL. × 4.0 mm I.D.)
Mobile phase	: 5 mmol/L p-toluenesulfonic acid (Mobile phase, included in the mobile phase regent set for organic acid analysis)
Flow rate	: 0.8 mL/min
pH buffering solution	: 5 mmol/L p-toluenesulfonic acid 20 mmol/L Bis- Tris 0. 1 mmol/L EDTA (pH-buffering reagent, included in the mobile phase regent set for organic acid analysis)
Flow rate	: 0.8 mL/min
Detection	: Conductivity detector (CDD-10Avp)
Oven Temperature	: 50 °C
Injection volume	: 10 µL

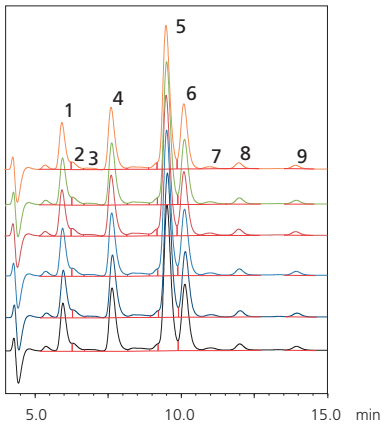

Fig. 7 Chromatogram of Cooking Sake ([Detection Threshold] = 40, n = 6)

Table 3 Area and Area Reproducibility Using i-PeakFinder for Organic Acids in Cooking Sake (n = 6)

	Organic Acid	Area (Mean Value)	Area Reproducibility (%RSD)
1	Phosphoric acid	145038	1.24
2	Citric acid	22133	0.96
3	Pyruvic acid	7734	1.34
4	Malic acid	209206	1.17
5	Succinic acid	515023	1.13
6	Lactic acid	236157	1.15
7	Fumaric acid	12480	3.14
8	Acetic acid	24466	0.83
9	Pyroglutamic acid	15195	1.76

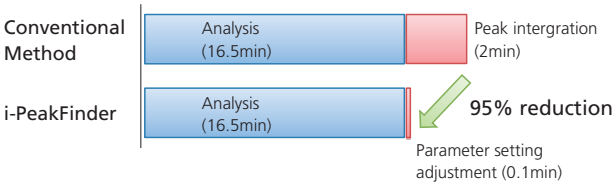

Fig. 8 Sample Analysis and Data Analysis Time per Sample (for Continuous Analysis of 50 Cooking Sake Samples)

Note: For details regarding Shim-pack Fast-OA columns for high-speed organic acid analysis, refer to the Technical Report entitled "High-Speed Analysis of Organic Acids Using Shim-pack Fast-OA and pH-Buffered Electrical Conductivity Detection (C190-E237A)."

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Technical Report

High-Speed Analysis of Organic Acids Using Shim-pack Fast-OA and pH-Buffered Electrical Conductivity Detection

Minori Nakashima[1], Katsuaki Koterasawa[1]

Abstract:

Organic acids are analyzed in a wide variety of fields, not only in food products, but also as counterions in pharmaceuticals, and ingredients in chemical products. Because post-column pH-buffered electrical conductivity detection is capable of analyzing organic acids selectively and with high sensitivity, this method is used to analyze organic acids in samples that contain many contaminants. However, shortening the analysis time when monitoring fermentation or analyzing intestinal flora has been problematic. Additionally, in research on the generation of alternative energies from biomass materials, the quantities of organic acids generated as metabolic products must be analyzed quickly in order to flexibly control the activity of microorganisms. This report describes techniques for achieving the high-speed analysis of organic acids with high selectivity, using the Shimadzu Shim-pack Fast-OA high-speed organic acid analytical column, in combination with the post-column pH-buffered electrical conductivity detection method.

Keywords: organic acid analysis, high-speed, post-column pH-buffered electrical conductivity detection, mobile phase reagent set for organic acid analysis

1. Analyzing Organic Acids by Detecting Electrical Conductivity

Organic acids absorb short wavelengths due to the carboxyl group they contain, which means that analysis is easily affected by contaminants, and that the detection method must be modified to achieve high sensitivity and specificity.

Shimadzu's unique post-column pH-buffered electrical conductivity detection method involves the successive addition of a pH-buffering reagent after column separation, in order to adjust the pH level to close to neutral. This not only reduces background noise, but also dissociates organic acids from the substance being analyzed. Consequently, electrical conductivity detection can then detect these organic acids with high sensitivity and selectivity.

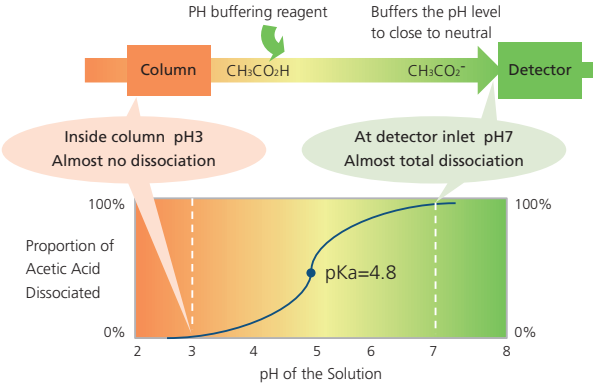
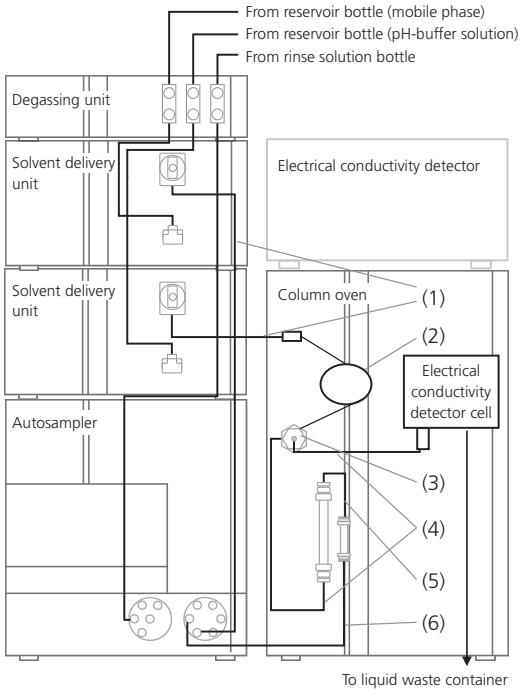


Fig. 1 Status of Organic Acids in the PH-Buffering Method

The post-column pH-buffered electrical conductivity detection system consists of flow channels for post-column buffering, and a CDD-10A$_{VP}$ electrical conductivity detector. A schematic of the tubing is shown in Fig. 2. Each organic acid component separated by the column is mixed with a pH-buffer solution in the mixer, and is then delivered in a dissociated state to the electrical conductivity detector, where it is detected with high sensitivity.



	Flow Channel Components
1	0.3 mm I.D. × 600 mm stainless steel tubing
2	0.1 mm I.D. × 2000 mm stainless steel tubing
3	MR-100 High-efficiency microreactor mixer
4	0.13 mm I.D. PEEK tubing (100 mm Length) *1
5	Stainless steel tubing with guard column
6	0.1 mm I.D. × 600 mm stainless steel tubing

*1: Before use, cut this from a commercially available 2-meter coil using a tube cutter.

Fig. 2 Tubing Diagram

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2. Retention Time Index for Organic Acid High-Speed Analytical Columns

Shim-pack Fast-OA columns are packed with an ion-exclusion polymer, and utilizing an acidic mobile phase, can separate mixture solution based on the pKa value of each sample component. The analytical conditions for this column are shown in Table 1, and the chromatograms of 25 organic acids are shown in Fig. 3. Because the column design is optimized for the high-speed analysis of organic acids, even short-chain fatty acids such as valeric acid are eluted within 10 minutes.

Reference retention time values for each organic acid component when the Shim-pack Fast-OA column is used are shown in Table 2 and Fig. 4. In the ion exclusion mode, the degree of separation depends on the column temperature and the mobile phase concentration. If separation of target components is inadequate, the table below can presumably be used as a reference for improving separation by changing the column temperature. However, it would be difficult to separate all the components in the table simultaneously.

Table 1 Analytical Conditions

Column	: Shim-pack Fast-OA (100 mm L. × 7.8 mm I.D., 5 µm)
Guard column	: Shim-pack Fast-OA (G) (10 mm L. × 4.0 mm I.D.)
Mobile phase	: 5 mmol/L p-toluenesulfonic acid
Flow rate	: 0.8 mL/min
pH buffering solution	: 5 mmol/L p-toluenesulfonic acid 20 mmol/L Bis- Tris 0. 1 mmol/L EDTA
Flow rate	: 0.8 mL/min
Detection	: Conductivity detector (CDD-10A$_{VP}$)
Injection volume	: 10 µL

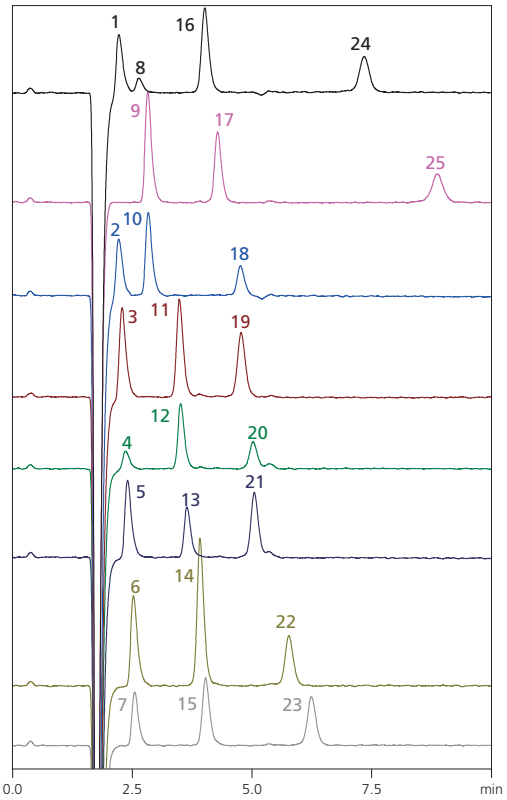

Fig. 3 Chromatogram of Standard Sample at 45 °C

Note 1: There is a dip in the baseline near the 1.8-minute point due to the sample solvent (water).
Note 2: Peak numbers and corresponding organic acids are as indicated in Table2

Table 2 Reference Retention Time Values for Organic Acids (with One Column)

	Organic acids	35°C	40°C	45°C	50°C
1	Phosphoric acid	2.209	2.228	2.250	2.272
2	Maleic acid	2.260	2.252	2.244	2.237
3	Alpha-ketoglutaric acid	2.333	2.321	2.310	2.299
4	Glucuronic acid	2.377	2.380	2.384	2.385
5	Citric acid	2.444	2.432	2.421	2.410
6	Tartaric acid	2.565	2.553	2.543	2.532
7	Pyruvic acid	2.577	2.573	2.570	2.566
8	Gluconic acid	2.654	2.657	2.659	2.660
9	Malonic acid	2.874	2.857	2.841	2.826
10	Malic acid	2.883	2.866	2.850	2.835
11	Succinic acid	3.573	3.531	3.490	3.451
12	Glycolic acid	3.545	3.531	3.519	3.505
13	Lactic acid	3.657	3.654	3.649	3.642
14	Formic acid	3.951	3.934	3.918	3.900
15	Glutaric acid	4.221	4.121	4.031	3.946
16	Fumaric acid	4.246	4.124	4.015	3.914
17	Acetic acid	4.329	4.307	4.284	4.260
18	Levulinic acid	4.950	4.850	4.754	4.662
19	Adipic acid	5.083	4.916	4.763	4.622
20	Pyroglutamic acid	5.238	5.116	5.012	4.916
21	Propionic acid	5.125	5.084	5.041	4.995
22	Isobutyric acid	5.879	5.823	5.763	5.696
23	Butyric acid	6.400	6.316	6.227	6.134
24	Isovaleric acid	7.594	7.464	7.328	7.182
25	Valeric acid	9.355	9.099	8.840	8.584

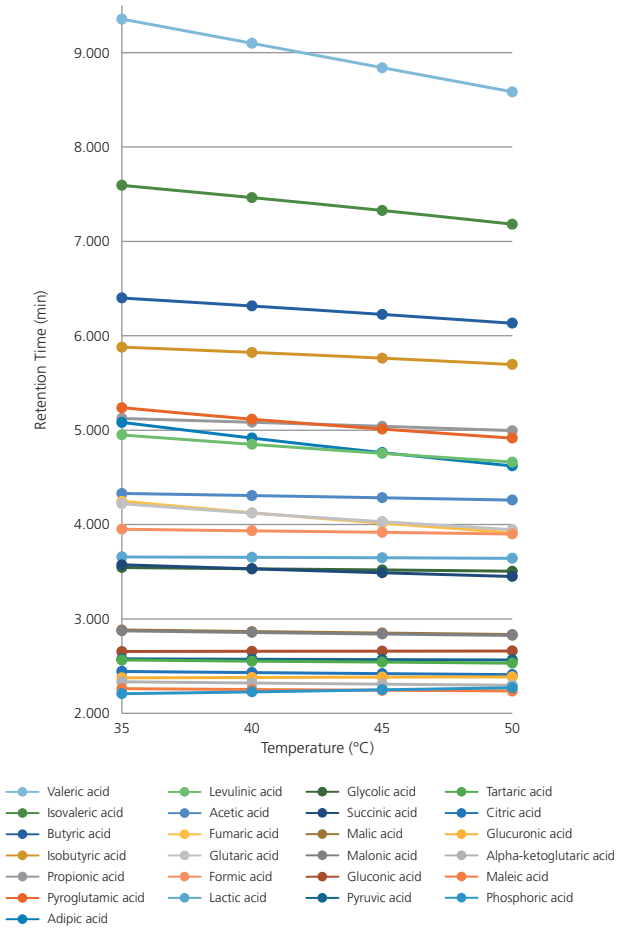

Fig. 4 Changes in Organic Acid Retention Times with Temperature

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3. Improving the Separation by Connecting Multiple Columns in Series

If separation with one column is inadequate, up to three Shim-pack Fast-OA columns can be connected in series to improve the separation. Table 3 indicates reference retention time values when two connected columns are used, and Table 4 indicates reference retention time values when three connected columns are used.

As an example, there is almost no difference between the retention times for lactic acid and formic acid, which makes it difficult to achieve full separation with only one column. Chromatograms for up to three connected columns are shown in Fig. 5. These illustrate that connecting the columns can improve the separation.

Table 3 Reference Retention Time Values for Organic Acids (with Two Columns)

	Organic acids	35°C	40°C	45°C	50°C
1	Phosphoric acid	4.017	4.051	4.090	4.126
2	Maleic acid	4.150	4.118	4.086	4.062
3	Alpha-ketoglutaric acid	4.292	4.260	4.219	4.178
4	Glucuronic acid	4.415	4.409	4.403	4.400
5	Citric acid	4.531	4.489	4.449	4.412
6	Tartaric acid	4.783	4.743	4.704	4.666
7	Pyruvic acid	4.821	4.796	4.772	4.755
8	Gluconic acid	4.988	4.986	4.973	4.957
9	Malonic acid	5.452	5.400	5.347	5.305
10	Malic acid	5.455	5.402	5.350	5.303
11	Succinic acid	6.906	6.796	6.688	6.585
12	Glycolic acid	6.860	6.813	6.764	6.724
13	Lactic acid	7.089	7.072	7.040	7.003
14	Formic acid	7.710	7.657	7.599	7.549
15	Glutaric acid	8.253	8.019	7.798	7.601
16	Fumaric acid	8.271	7.996	7.738	7.508
17	Acetic acid	8.503	8.435	8.361	8.293
18	Levulinic acid	9.787	9.556	9.320	9.096
19	Adipic acid	10.068	9.687	9.328	9.009
20	Pyroglutamic acid	10.345	10.076	9.822	9.601
21	Propionic acid	10.154	10.043	9.923	9.799
22	Isobutyric acid	11.738	11.589	11.423	11.257
23	Butyric acid	12.807	12.608	12.380	12.147
24	Isovaleric acid	15.296	14.991	14.640	14.310
25	Valeric acid	18.927	18.335	17.723	17.136

Table 4 Reference Retention Time Values for Organic Acids (with Three Columns)

	Organic acids	35°C	40°C	45°C	50°C
1	Phosphoric acid	5.852	5.908	5.969	6.037
2	Maleic acid	6.050	6.014	5.978	5.948
3	Alpha-ketoglutaric acid	6.268	6.210	6.174	6.124
4	Glucuronic acid	6.435	6.432	6.435	6.437
5	Citric acid	6.624	6.574	6.522	6.477
6	Tartaric acid	6.996	6.946	6.897	6.856
7	Pyruvic acid	7.047	7.022	6.997	6.976
8	Gluconic acid	7.303	7.289	7.292	7.282
9	Malonic acid	7.979	7.911	7.848	7.792
10	Malic acid	8.000	7.934	7.867	7.808
11	Succinic acid	10.168	10.016	9.868	9.734
12	Glycolic acid	10.085	10.031	9.974	9.922
13	Lactic acid	10.437	10.406	10.383	10.345
14	Formic acid	11.349	11.278	11.210	11.148
15	Glutaric acid	12.184	11.855	11.543	11.263
16	Fumaric acid	12.192	11.802	11.438	11.118
17	Acetic acid	12.540	12.455	12.363	12.272
18	Levulinic acid	14.478	14.135	13.814	13.501
19	Adipic acid	14.896	14.344	13.834	13.372
20	Pyroglutamic acid	15.290	14.912	14.558	14.244
21	Propionic acid	15.016	14.866	14.705	14.545
22	Isobutyric acid	17.383	17.181	16.959	16.730
23	Butyric acid	18.993	18.693	18.392	18.069
24	Isovaleric acid	22.707	22.261	21.792	21.314
25	Valeric acid	28.180	27.323	26.455	25.607

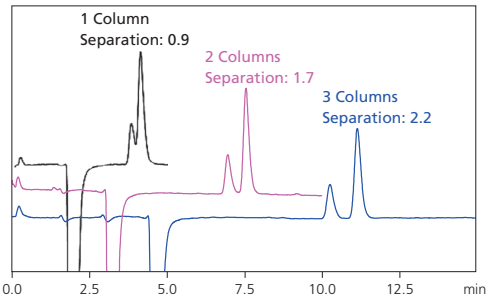

Fig. 5 Improvement in Separation by Connecting Multiple Columns

4. Example of a Standard Sample Analysis

The types of organic acids targeted will differ depending on the type of sample analyzed. Separation patterns for organic acids typically analyzed in various fields are shown in Figs. 6 to 9.

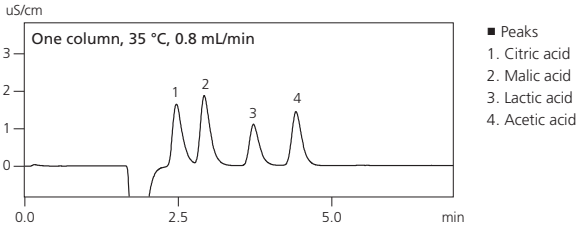

Fig. 6 Four Organic Acid Components Analyzed in the Food Industry

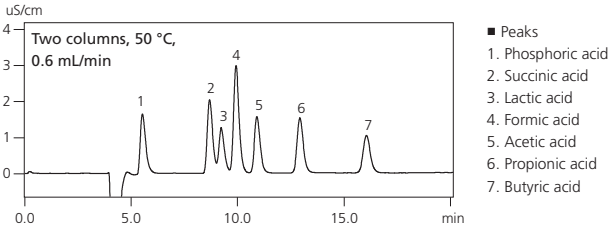

Fig. 7 Seven Organic Acid Components Analyzed in Intestinal Flora Research

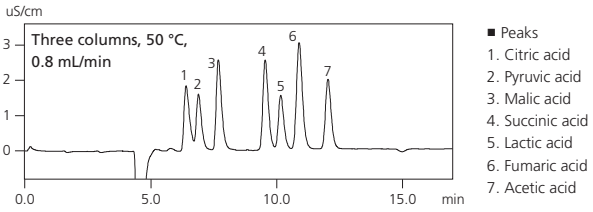

Fig. 8 Seven Organic Acid Components Analyzed in Culture Media Analysis

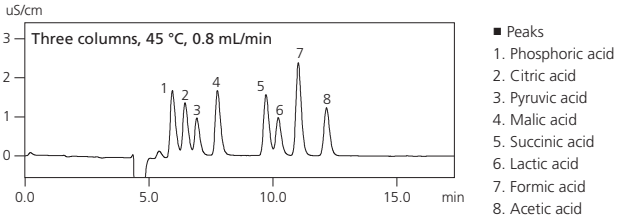

Fig. 9 Eight Organic Acid Components

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5. Analyzing Actual Samples

When used in combination with a Shim-pack Fast-OA high-speed organic acid analytical column, the post-column pH-buffered electrical conductivity detection method is ideal for applications that require the high-speed analysis of samples containing a large number of contaminants, such as in fermentation monitoring.

The results from an analysis of organic acids added to a culture solution are shown in Fig. 10 and the analytical conditions are shown in Table 5. Because this method can shorten analysis cycle times to 20 minutes or less, it can not only reduce mobile phase consumption and improve operating efficiency, but can also be used to control the status of cultures, or ensure a given number of repeated analyses.

Table 6 shows the calibration curve for the 7 organic acids. The calibration curve is shown in Fig. 11. A good linearity of R^2 = 0.999 or more was obtained for each component.

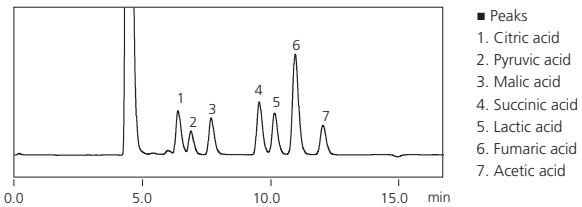

Fig. 10 Chromatogram of a Culture Solution

Table 5 Analytical Conditions

Column	: Shim-pack Fast-OA 3 columns in series (100 mm L. × 7.8 mm I.D., 5 µm)
Guard column	: Shim-pack Fast-OA (G) (10 mm L. × 4.0 mm I.D.)
Mobile phase	: 5 mmol/L p-toluenesulfonic acid
Flow rate	: 0.8 mL/min
pH buffering solution	: 5 mmol/L p-toluenesulfonic acid 20 mmol/L Bis- Tris 0. 1 mmol/L EDTA
Flow rate	: 0.8 mL/min
Column Temperature	: 50 °C
Detection	: Conductivity detector (CDD-10Avp)
Injection volume	: 10 µL

Table 6 Calibration Curve Concentration Range and Contribution Rate for Target Components

Compound	Calibration Curve Range (mg/L)	Contribution Rate (R^2)
Citric acid	5-100	0.99944
Pyruvic acid	5-100	0.99999
Malic acid	1-100	0.99997
Succinic acid	1-100	0.99997
Lactic acid	1-100	0.99992
Fumaric acid	1-100	0.99994
Acetic acid	1-100	0.99995

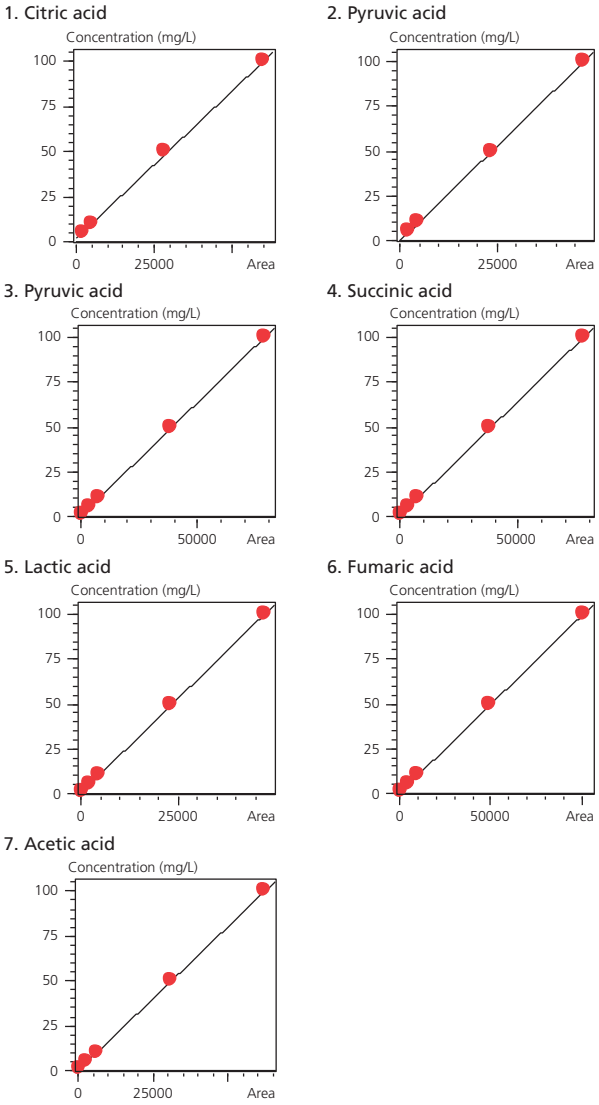

Fig. 11 Calibration Curves for Target Components

6. Conclusion

- Shim-pack Fast-OA high-speed analytical columns for organic acid analysis can shorten analysis cycle times.
- When the post-column pH-buffered electrical conductivity detection method is used in combination with a high-speed analytical column for organic acid analysis, organic acids can be selectively detected, with minimal impact from the contaminants in samples.

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Technical Report

New Analytical Intelligence Concept —Support for Automating Analytical Operations

The idea to support the novel workflow automation for analytical and testing operation

Satoshi Akita[1], Kyoko Watanabe[1]

ANALYTICAL INTELLIGENCE

Abstract:

Significant progress has been made in automating analytical operations in an effort to improve productivity and prevent human errors. Nevertheless, differences in the functionality, performance, and operability of instruments and software and also in the level of analytical chemistry knowledge and expertise can affect the reliability of results and the condition of instruments. Analytical Intelligence is a new concept for analytical instruments offered by Shimadzu. Analytical Intelligence consists of systems and software that simulate expert operators automatically determining whether or not conditions and results are good or bad, providing feedback to users, and solving common problems. It increases data reliability by compensating for any differences between users in their instrument knowledge or experience. This Technical Report bulletin describes the new Analytical Intelligence functionality included in the new Nexera™ series.

Keywords: Analytical Intelligence, Nexera series, auto-startup, FlowPilot, mobile phase monitor, auto-diagnostics, auto-recovery, i-PeakFinder, and i-PDeA II

1. Issues Involved in Improving Workflow for Analytical Operations

When systems equipped with autosampler-based automatic injection functionality and workstations with the capability of using such autosampler functionality to acquire data based on specified parameter settings became available, it resulted in automated instrumental analysis and it significantly changed how analytical operations are performed. Automated data acquisition enabled continuous analysis at night or at other times when facilities are closed, which not only dramatically improved operating efficiencies, but also reduced the risks of variability or errors associated with manual operations and improved data reliability.

However, analytical and testing operations often require fundamental knowledge about analytical chemistry and experience-based expertise. Experienced analysts have a good understanding of the principles underlying analytical techniques and systems and are able to apply their expertise gained from past experience to avoid problems and obtain highly reliable data. In contrast, it is difficult for analysts with minimal experience to predict potential risks in advance and analyze samples with corresponding countermeasures implemented. In addition, during data analysis, it is much more likely that an expert analyst will discover hidden problems in the data.

Overall operating efficiency taking into consideration data reliability and instrument uptime rate, etc., is dependent not only on analysis cycles, throughput, and other factors that can be resolved with instruments and software, but is also greatly dependent on the knowledge and skill level of users. Furthermore, whereas improving the knowledge and skill level of users requires a time-consuming process of training personnel, the number of expert analysts available in the analytical workplace is dwindling and the proportion of analysts with minimal experience is increasing. This trend is a major issue currently being faced by the analysis and testing industries.

2. Analytical Intelligence

ANALYTICAL INTELLIGENCE

That issue cannot be resolved by only shorter analysis times or higher throughput achieved through improvements in the basic performance of instruments or operability of software. It can only be truly solved if highly reliable results can be acquired at any time by any user, regardless of their knowledge or skill. Making that a reality requires an unprecedented new policy.

Analytical Intelligence is a new concept for analytical instruments offered by Shimadzu. Analytical Intelligence consists of systems and software that simulate expert operators automatically determining whether or not conditions and results are good or bad, providing feedback to users, and solving common problems. It increases data reliability by compensating for any differences in instrument knowledge or experience of users. This document describes the new Analytical Intelligence functionality included in the new Nexera series ultra high performance liquid chromatographs.

3. Analytical Intelligence Functionality in Nexera Series Systems

Typical liquid chromatography workflow involves several risks that could interrupt continuous analysis or compromise data reliability (Fig. 1).

- Column damage from sudden starting of solvent delivery
- Analysis interruption and column damage due to depletion of mobile phase
- Negative impact on chromatograms from using a defective column
- Retention time delay due to solvent delivery becoming unstable
- Poor quantitative reproducibility due to variability in peak integration.
- Poor quantitative accuracy due to overlapping peaks from coeluting components.

The next section explains how Analytical Intelligence helps minimize each of the above risks when using Nexera series systems. For more details, refer to individual Technical Report bulletins that describe the respective functionality.

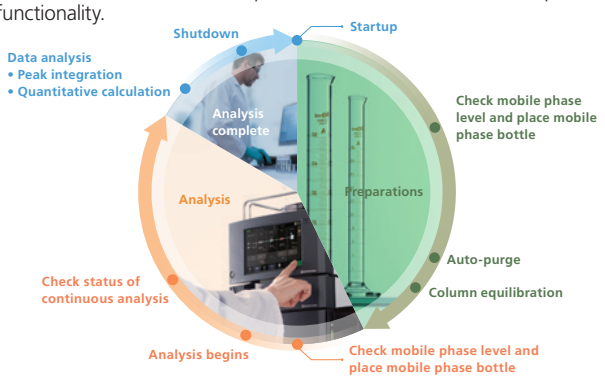

Fig. 1 Work flow of LC analysis

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3-1. Automatic Startup and FlowPilot

When starting up the system and equilibrating the column, expert analysts will gradually increase the flowrate as the column temperature is controlled to prevent exposing the column to any excessive pressure loads. Repeatedly starting solvent delivery too abruptly, without the above steps, can cause column damage. When the Nexera series auto-startup function starts up the system at the specified date and time, the FlowPilot function starts equilibrating the column by gradually increasing the mobile phase flowrate as the column temperature increases. That means the system automatically replaces the manual operations of expert analysts to avoid column damage and finishes preparing the system. (Refer to Technical Report C190-E227.)

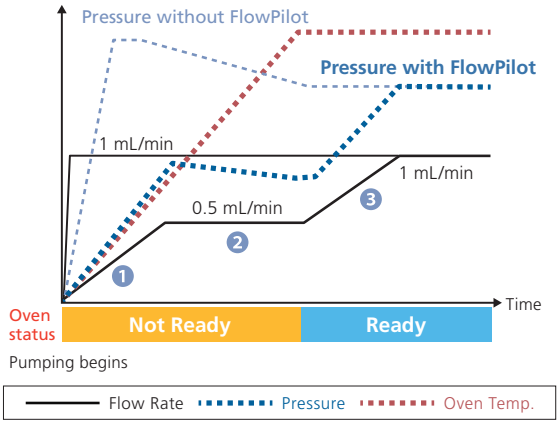

Fig. 2 Diagram of system pressure profile during start-up with the FlowPilot function

3-2. Mobile Phase Monitor Prevents Mobile Phase Depletion

Using the mobile phase monitor prevents depletion of mobile phase and eliminates the need to visually check the level. It also eliminates the need to perform bothersome consumption rate calculations.

The MPM-40 unit (Fig. 3), which comprises a weight sensor and controller, sends the current quantity inside the mobile phase bottle to a computer or smartphone in real time via a LAN connection. Dedicated MPMChecker software then graphically displays the remaining level (Fig. 4). When the remaining quantity of solution decreases to the specified level, a warning (orange) or error (red) signal is emitted to notify the user. It also stops the LC system if specified criteria are satisfied. LabSolutions prevents interruption of analysis due to insufficient mobile phase by comparing the predicted usage volume to the volume available before starting each analysis and notifies the user if there is not enough available (Fig. 5). The function also reduces the risk of bubbles getting inside the column and prevents loss of scarce samples caused by injecting samples when the mobile phase supply is depleted. (Refer to Technical Report C190-E226.)

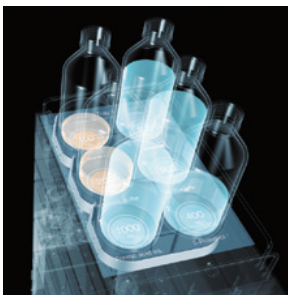

Fig. 3 MPM-40

Fig. 4 MPMChecker

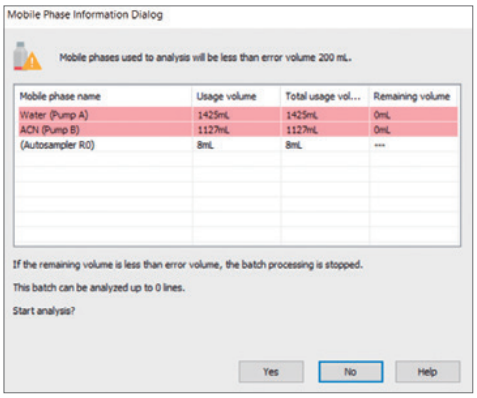

Fig. 5 Warning Window when Using LabSolutions™

3-3. Collective Management of Columns and Visualization of Column Condition CMD and Column Data Browser

Reliable quantitation is premised on separation by maximizing the inherent performance capabilities of columns. On the other hand, given that columns are consumables, their performance will gradually decrease as sample contaminant components accumulate and the column deteriorates over time. They can also become damaged suddenly, such as if insoluble substances from a sample are accidentally injected. To ensure columns with adequate performance are always used, column performance must be managed or usage history routinely recorded, which is not a simple matter if columns are shared by multiple users or the number of columns is large.

Therefore, the Nexera series column management function collects various information during column use, such as injection pressure, links the information to a LabSolutions data file, and stores it in a database. It can be used with all sorts of columns, regardless of the column type or brand, so that information about all columns can be checked at a glance using the column data browser (Fig. 6). A list of all registered columns can be displayed or the usage history or most recent chromatogram visually checked for the selected column, which means column condition can be confirmed without spending extra time.

Note: An optional column management device (CMD) and Nexera series-compatible LabSolutions DB/CS version software are required in order to use the new functionality for column management.

Fig. 6 Example of Column Data Browser Display

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3-4. Automatic Detection and Resolution of Solvent Delivery Problems during Analysis by Auto-Diagnostics and Auto-Recovery Functions

Due to degassing unit and solvent delivery unit performance improvements in recent years, problems occur much less often now than they used to, but in rare cases bubbles that form within HPLC/UHPLC flow channels can cause solvent delivery problems if they enter the solvent delivery pump. That can cause retention time and area value fluctuations, baseline instability, peak shape distortion, or other problems that can significantly reduce the reliability of quantitative results. If such a problem occurs, the user must manually stop the current analysis and implement corrective measures, such as purging the flow channels. That means that if air bubbles appear in flow channels during unattended operation, the same analysis must be repeated the next day.

The auto-diagnostics and auto-recovery functions included in Nexera series solvent delivery pumps detect abnormal pressure fluctuations (pulsation) caused by bubbles in flow channels and automatically purge the pump to restore the system to normal. Consequently, instead of the user, the instrument monitors and manages the relationship between easily overlooked chromatogram abnormalities and flowrate changes that can cause pulsation, thereby preventing analysis failures. (Refer to Technical Report C190-E225.)

Note: This function can also be switched OFF in settings.

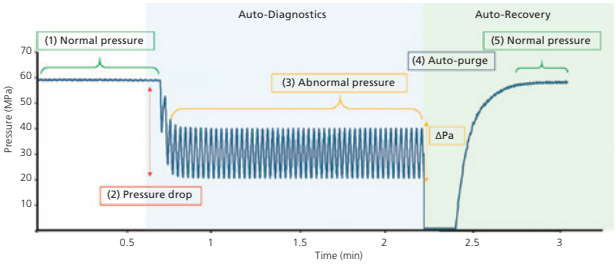

Fig. 7 Diagram of Auto-Diagnostics and Auto-Recovery Functions

3-5. Accurate Peak Detection without Manual Peak Integration Automatic Peak Integration Using i-PeakFinder

Given increasingly fast analysis capabilities and shorter data acquisition times, if manual operations are required for integrating chromatogram peaks, then the data processing step becomes a bottleneck that prevents truly improving operational efficiency. Therefore, automating the peak integration process is essential. However, for chromatograms with a large number of peaks from contaminant components and target components, automating the peak integration process while eliminating the effects of baseline fluctuations and unseparated peaks can require complicated steps, such as configuring detailed settings for a peak integration program. Also, manual peak integration processes are prone to causing differences between individual operators, which reduces the consistency of quantitation values.

i-PeakFinder, which is one of the peak integration algorithms available in LabSolutions, uses a completely automated integration function to accurately detect peaks, as shown below, without the need to specify special parameter settings (Fig. 8).

- Shoulder peaks can be detected very accurately.
- Baseline processing can be changed easily.
- Reliable peak tracking enables improved reproducibility.
- Peaks can be integrated correctly even with variability from baseline drift.

With applicable parameter settings available for a wide range of complex chromatogram patterns, i-PeakFinder can output highly accurate peak integration results even when processing large quantities of data at the same time.

i-PeakFinder is part of the standard functionality included with LabSolutions software, so it can be used for chromatograms obtained with non-Nexera series LC systems as well. (Refer to Technical Reports C191-E044 and C190-E243.)

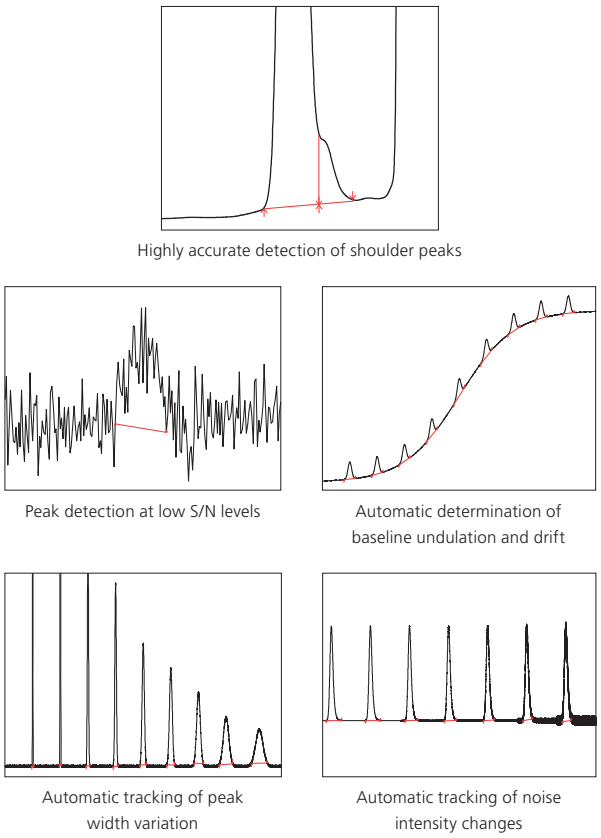

Fig. 8 Example of Automatic Peak Integration Using i-PeakFinder

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3-6. Separating Unseparated Peaks Using a PDA Detector Detecting Overlapping Peaks with i-PDeA II

If there are other peaks present near target component peaks, such as when analyzing multiple components simultaneously, checking for impurities simultaneously, or analyzing samples with many contaminant components, to ensure quantitative accuracy it is important to check for any peaks eluted together with target peaks and overlapping in the chromatogram. However, in reality, checking for such peaks is quite difficult unless a mass spectrometer or any other instrument with high selectivity is used for detection. Also, if unseparated peaks are discovered, it usually requires reassessing peak separation in the column.

The i-PDeA II (Intelligent Peak Deconvolution Analysis II) data analysis technique extracts target peaks from unseparated peaks by analyzing photodiode array (PDA) detector data using the chemometric multivariate curve resolution alternating least squares (MCR-ALS) method. The technology uses a PDA detector to identify overlapping peaks that were not adequately separated by the column and either separates those peaks in the chromatogram or determines the UV spectrum of each peak. Consequently, it can be used to check for impurity peaks hidden by key component peaks, extract chromatograms for individual components (Fig. 9 (b)), or confirm peak purity (Fig. 9 (c)). (Refer to Technical Report C191-0078.)

Note: This function can be used with LabSolutions and SPD-M20A/M30A/M40 detectors.

4. Conclusions

Based on a completely new concept, Analytical Intelligence consists of various supporting functionality that was developed for the purpose of promoting higher efficiency through workflow improvements, while also ensuring the reliability of data from instrumental analysis. Automatic operation of the system which simulates operation by an expert analyst reduces the risk of system problems and enables any problem to be solved if an unlikely event occurs. Consistent data analysis results are provided by automating the operations that tend to result in variability between individual operators and by automatically identifying overlapping peaks that are easily overlooked. Analytical Intelligence contributes to maximizing system utilization rates and improving operating efficiency by always acquiring data that is consistently highly reliable and by avoiding system problems, regardless of knowledge and skill level of users.

We also plan to add additional new Analytical Intelligence functionality in the future. In that way, the Nexera series systems currently in use can continue to be improved by adding new functionality as needed. Analytical Intelligence will undoubtedly significantly change how analysis and testing operations are performed.

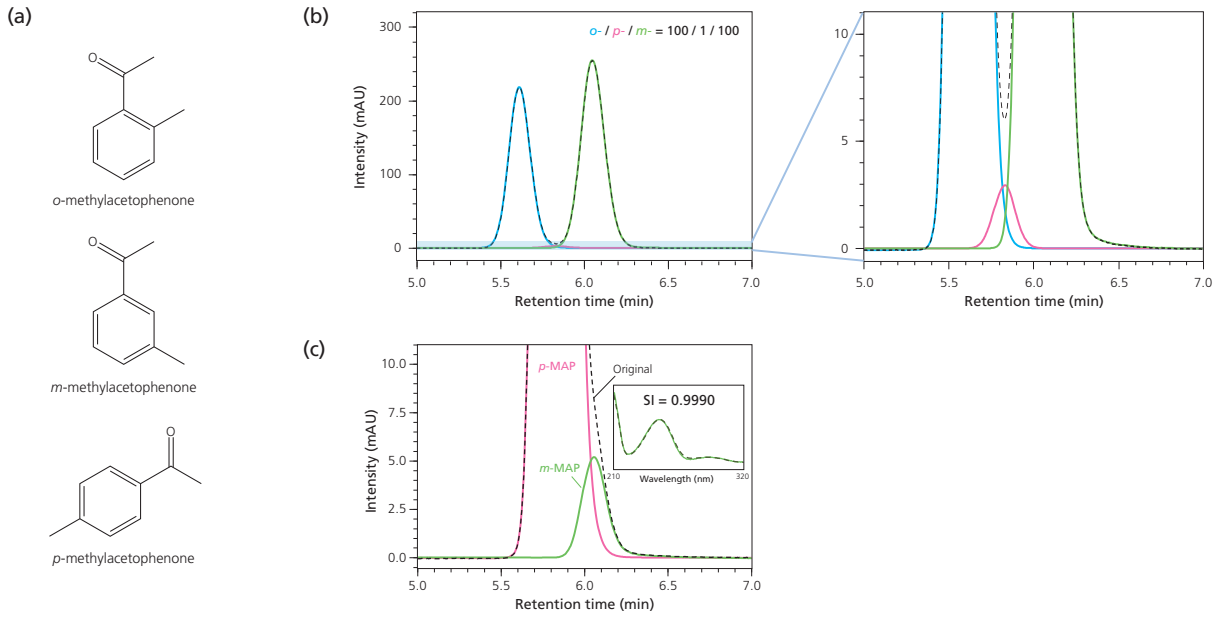

Fig. 9 (a) Positional Isomers of Methylacetophenone
(b) Results from Separating a Mixture Sample of o-MAP, m-MAP, and p-MAP
(c) Impurities in p-MAP Standard Sample

The Analytical intelligence logo, Nexera and LabSolutions are trademarks of Shimadzu Corporation.

First Edition: October, 2019

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Application News

No. L534

High Performance Liquid Chromatography

Impurity Analysis of Pharmaceutical Products Using Next-Generation LC Column "Shim-pack Arata™ C18"

When basic compounds are analyzed in a column packed with general octadecyl silyl (hereinafter, ODS) silica gel, it is known that analytical accuracy is affected by the peak shape. Although a large number of ODS columns with high separation performance have been commercialized in recent years, tailing or other peak shape abnormalities sometimes occur with columns for basic compounds due to the physical properties of the target compound. As an additional problem, the long time required for column equilibration is an issue under low ionic strength acidic mobile phase conditions, for example, when using 0.1 % formic acid in water. Because retention time will change over time if column equilibration is inadequate, stable resolution becomes impossible. The Shim-pack Arata C18 column was developed to solve these many problems. Since a satisfactory peak shape can be obtained with a simple system mobile phase, even with ionic compounds, higher analytical accuracy can be expected.

This article demonstrates that basic compounds and acidic compounds can be analyzed stably while maintaining a satisfactory peak shape by using the Shim-pack Arata C18 column, and introduces an example of application to impurity analysis of pharmaceuticals.

A. Nomura, T. Yamaguchi

Analysis of Basic Compounds and Acidic Compounds

Basic compounds and acidic compounds were analyzed using a low ionic strength organic acid mobile phase (0.1 % formic acid in water), which tends to cause deterioration of the peak shape of basic compounds. The tricyclic antidepressant amitriptyline was used as the basic compound, and benzoic acid was used as the acidic compound. The standard solution was prepared so that concentration of each compound in the mobile phase was 100 mg/L, and was analyzed under the conditions shown in Table 1.

Fig. 1 shows the obtained chromatograph. The symmetry factors of the peaks were 1.01 for amitriptyline and 1.00 for benzoic acid, indicating that satisfactory peak shapes could be obtained for both the basic compound and the acidic compound.

Table 1 Analytical Conditions

System	:	Nexera™ X2
Column	:	Shim-pack Arata C18 (75 mm L. × 3.0 mm I.D., 2.2 µm) (Figs. 1 and 3) Typical ODS (75 mm L. × 3.0 mm I.D., sub-2 µm) (Fig. 2)
Mobile Phase	:	0.1 % Formic acid in water/Acetonitrile = 70/30(v/v)
Flow Rate	:	0.4 mL/min
Injection Vol.	:	1 µL
Column Temp.	:	40 °C
Detection	:	SPD-M30A at 254 nm (Fig. 1) SPD-M30A at 280 nm (Figs. 2 and 3)

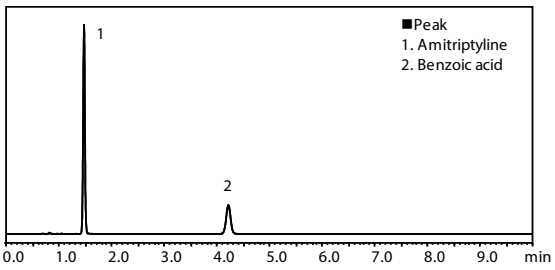

Fig. 1 Chromatograph of Basic Compound (Amitriptyline) and Acidic Compound (Benzoic Acid) by Shim-pack Arata C18 Column

Change of Retention Time and Symmetry Factor with Equilibration Time

With general ODS columns, excessive time may be required until retention time (RT) stabilizes, i.e., until equilibration is achieved, when the analysis is conducted using with a low ionic strength organic acid mobile phase such as 0.1 % formic acid in water. Therefore, the time required for equilibration under a low ionic strength organic acid mobile phase condition was evaluated with dextromethorphan and amitriptyline, which are basic drugs.

<Analytical Method>

The columns used were a general ODS column (new column, shipping solvent: acetonitrile) and a Shim-pack Arata C18 column (new column, shipping solvent: acetonitrile). The columns were equilibrated with the mobile phase without conditioning, and the standard solution was analyzed at set times. The analytical conditions are shown in Table 1. The standard solution was prepared with the mobile phase so that the concentration of each component was 100 mg/L.

<Results>

The transitions of the chromatograms when using the general ODS column and the Shim-pack Arata C18 column are shown in Fig. 2 and Fig. 3, respectively.

With the general ODS column, RT had not stabilized even after an equilibration time of approximately 720 min, and the symmetry factors of dextromethorphan and amitriptyline were 2.49 and 3.90, respectively. In contrast, with the Shim-pack Arata C18 column, RT became uniform at about 30 min after the start of equilibration, and the symmetry factors of dextromethorphan and amitriptyline were 1.19 and 1.07, respectively. After this point, there were no significant changes in either RT or the symmetry factor. These results demonstrate that the Shim-pack Arata C18 column enables quick equilibration while maintaining excellent peak shapes even when using a low ionic strength organic acid mobile phase. Based on this performance, the Shim-pack Arata C18 column is expected to provide outstanding stability in LC/MS (/MS) analysis.

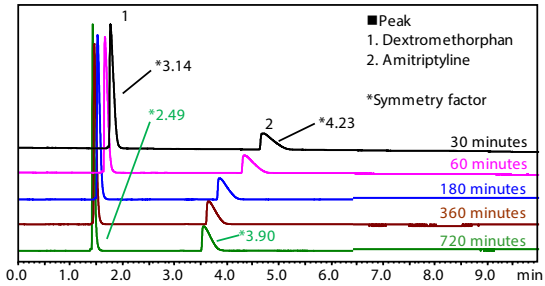

Fig. 2 Transition of Chromatogram of Dextromethorphan and Amitriptyline with General ODS Column

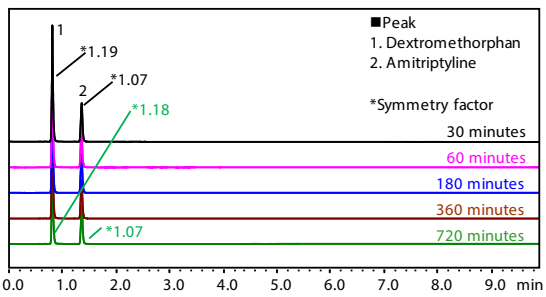

Fig. 3 Transition of Chromatogram of Dextromethorphan and Amitriptyline with Shim-pack Arata C18 Column

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Application to Impurity Testing of Drug Substances

Because impurity testing of drug substances is one critical type of test in the pharmaceutical product manufacturing process, high reliability and analytical accuracy are demanded in high performance liquid chromatography (HPLC) columns used in those tests.

<Analytical Method>

Amitriptyline prepared with the mobile phase to obtain a concentration of 100 mg/L was used as the model sample, and 0.1 % phosphoric acid in water was used as the mobile phase. The time until equilibration of a general ODS column and a Shim-pack Arata C18 was compared. Table 2 shows the analytical conditions. The organic solvent ratio was adjusted depending on the column so as to obtain the same main peak retention times.

Table 2 Analytical Conditions

System	:	Nexera X2
Column	:	Typical ODS (75 mm L. × 3.0 mm I.D., sub-2 µm) (Fig. 4) Shim-pack Arata C18 (75 mm L. × 3.0 mm I.D., 2.2 µm) (Fig. 5)
Mobile Phase	:	0.1 % Phosphoric acid in water/Acetonitrile = 70/30 (v/v) (Fig. 4) = 76/24 (v/v) (Fig. 5)
Flow Rate	:	0.4 mL/min
Injection Vol.	:	1 µL
Column Temp.	:	40 °C
Detection	:	SPD-M30A at 210 nm

<Results>

Fig. 4 shows a comparison of the chromatograms at 5 h and 12 h after the start of equilibration for the general ODS column. Retention of the peak of the main component, amitriptyline, and the peak of impurity 1 at around 3 min were weak. On the other hand, retention of the peaks of impurities 2 to 4 eluted at around 4 to 5 min tended to be strong. Furthermore, the peak of impurity 5 eluted at approximately 5.5 min at 5 h after the start of equilibration overlapped the main peak. Thus, the general ODS column required a long time to stabilize after the start of equilibration, and resolution also varied, as retention behavior differed depending on the substance.

Fig. 5 shows a comparison of the chromatographs at 5 h and 12 h after the start of equilibration for the Shim-pack Arata C18. RT of both the main peak and the impurity peaks coincided. Moreover, the Shim-pack Arata C18 not only displayed fast and stable column equilibration performance, but also maintained that performance for an extended time after equilibration.

The Shim-pack Arata C18 column enables stable analysis and does not affect the reliability of the HPLC impurity testing method.

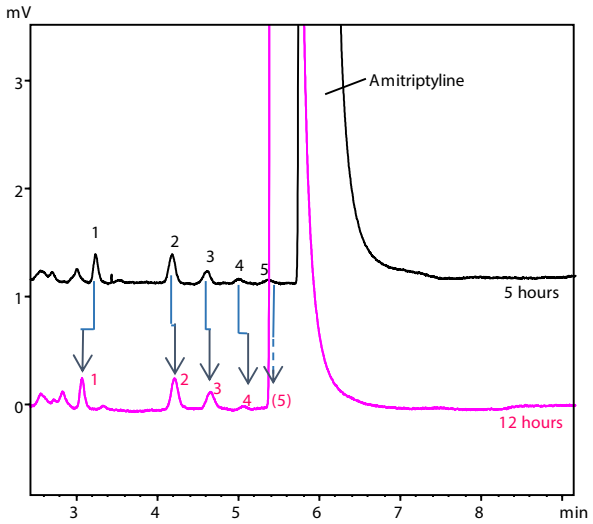

Fig. 4 Enlarged Chromatograms after Equilibration for 5 h and 12 h Using General ODS Column

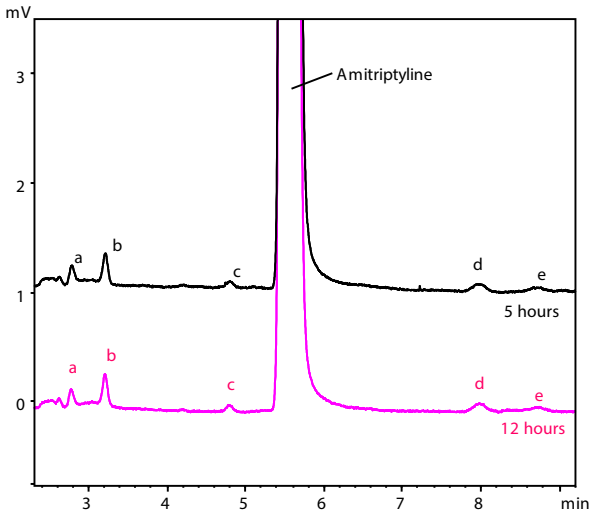

Fig. 5 Enlarged Chromatograms after Equilibration for 5 h and 12 h Using Shim-pack Arata C18

Conclusion

As introduced in this article, it was possible to analyze both basic compounds and acidic compounds with satisfactory peak shapes with a low ionic strength acidic mobile phase (0.1 % formic acid in water) by using the newly-developed Shim-pack Arata C18 column, and stable analysis was also possible in a short time. Highly reliable HPLC impurity testing in quality control of pharmaceuticals is possible by using the Shim-pack Arata C18 column.

* Shim-pack Arta C18 is classified as USP column category L1.

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Application News

No. L538

High Performance Liquid Chromatography

Impurity Analysis in Pharmaceutical Products with the Advanced Photodiode Array Detector SPD-M40

Impurities in pharmaceuticals must be appropriately controlled to ensure product safety. The ICH guidelines on impurities in pharmaceutical substances [1] and impurities in drug products [2] have established specific thresholds for impurities. If the content of impurity in pharmaceuticals is more than 0.1 %, it is necessary to identify its structure, perform the safety evaluation and report to the regulatory authorities.

Therefore, the accurate determination of trace impurities is of great importance in both pharmaceutical drug development and drug manufacturing.

The following procedure is commonly used to measure the concentration of impurities in pharmaceutical products using area percentage normalization: prepare and analyze the standard solution at a concentration such that the height of the main peak will be within the linear range of the detector, then analyze multiple dilutions of the sample.

Since the new photodiode array detector SPD-M40 has completely eliminated the effects of stray light during detection, it not only provides a wide dynamic range (linearity up to 2.5 AU as a specification value, typical value is more than 2.5 AU) but also achieves low noise and high sensitivity.

Moreover, the SPD-M40 has little fluctuation in its baseline thanks to Advanced TC-Optics (triple temperature control of the cell, light source, and optical system), making it ideal for analyzing trace impurities in pharmaceuticals.

Y. Zhou, H. Terada

Table 1 Analytical Conditions

Column	:	Shim-pack Velox™ C18 (100 mm L. × 3.0 mm I.D., 2.7 µm)
Mode	:	Low pressure gradient
Mobile Phase	:	A) 10 mM Sodium phosphate buffer (pH=2.6) B) Acetonitrile
Flow Rate	:	1 mL/min
Column Temp.	:	40 °C
Injection Volume	:	2 µL
Detection	:	SPD-M40 at 256 nm
Flow Cell	:	Standard cell

Table 2 Gradient Time Program

Time (min)	A. Conc	B. Conc
0	70	30
4	40	60
6	10	90
8	10	90
8.01	70	30
10	STOP	

Linearity

This section presents examples of impurity analyses with SPD-M40 using ketoprofen, a nonsteroidal anti-inflammatory drug.

Analytical conditions and gradient time programs for mobile phases are shown in Table 1 and Table 2, respectively.

To confirm the linearity of ketoprofen with SPD-M40, 0.5-800 mg/L of standard solutions were prepared and analyzed. Fig. 1 shows the chromatograms of the ketoprofen standard solutions, and Fig. 2 shows the calibration curve of ketoprofen.

Excellent linearity ($R^2 \geq 0.999$) was obtained over a wide range of concentrations, from 0.5 to 800 mg/L.

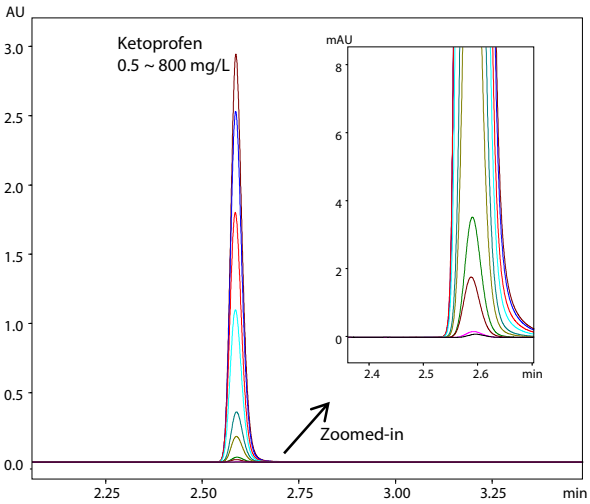

Fig. 1 Chromatograms of the Ketoprofen Standard Samples

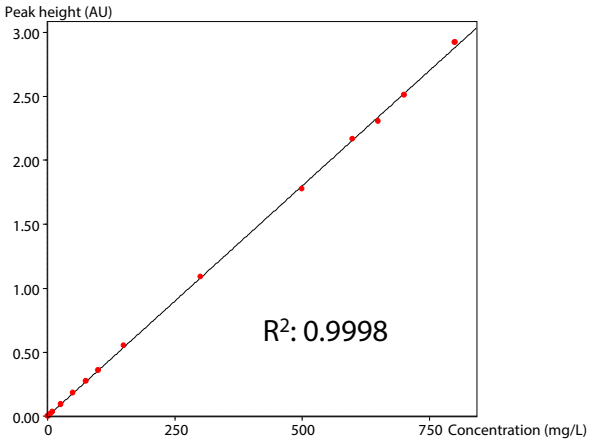

Fig. 2 Calibration Curve for Ketoprofen

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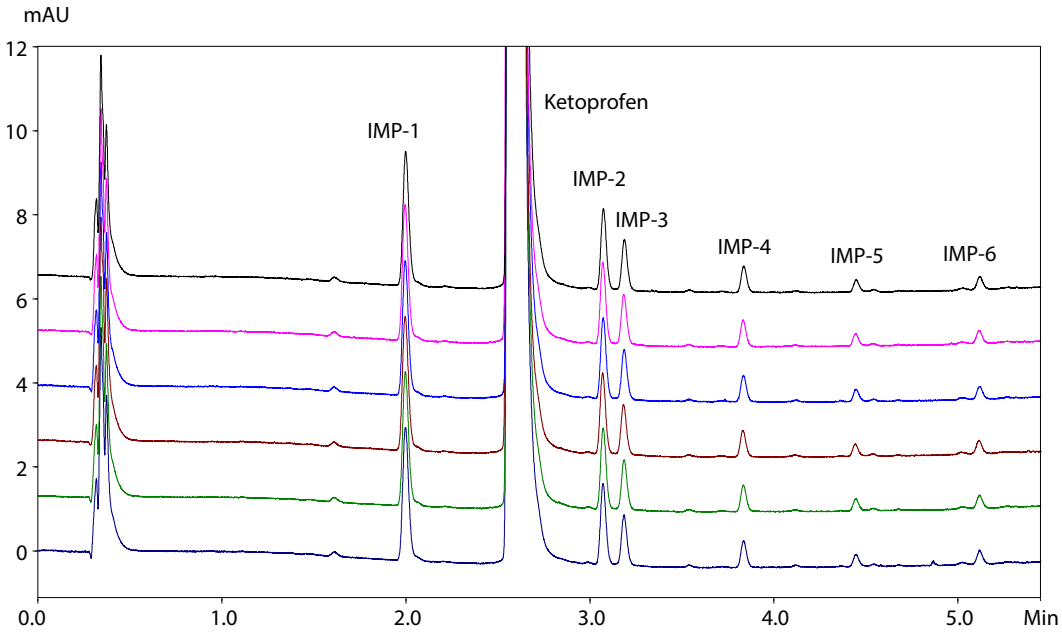

Fig. 3 Overlapped Chromatograms of Ketoprofen 700 mg/L Standard Solution (n = 6)

Impurity Analysis

Fig. 3 shows the chromatograms of six repeated analyses of 700 mg/L ketoprofen standard solution with peak heights of approximately 2.5 AU. Table 3 shows the area percentage and area coefficient of variation (%RSD) of ketoprofen and each impurity component.

The peak area RSD% of Impurity 1 (the only impurity with content >0.1 %) was less than 1 %, showing good reproducibility.

Table 3 Analytical Results for Each Component

No.	Compounds	Retention Time (min)	Area (%)	Area (%RSD)
1	Ketoprofen	2.583	99.704	0.001
2	Impurity 1	1.998	0.126	0.598
3	Impurity 2	3.072	0.075	0.543
4	Impurity 3	3.186	0.046	1.115
5	Impurity 4	3.834	0.025	1.644
6	Impurity 5	4.446	0.011	4.556
7	Impurity 6	5.118	0.012	3.355

Peak Purity and Spectral Confirmation

One of the main advantages of PDA detection for impurity analysis is the possibility of performing a peak purity assay and impurity spectral confirmation. Fig. 4 shows the purity curve of the ketoprofen peak. No impurities were detected within the peak elution interval. Fig. 5 shows the UV spectrum of Impurity 3 with an area percentage concentration of 0.046 %. This result shows that informative spectra can be obtained even at very low concentrations.

Results for pharmaceutical analyses obtained using SPD-M40 are highly reliable due to its high performance over a wide range of concentrations.

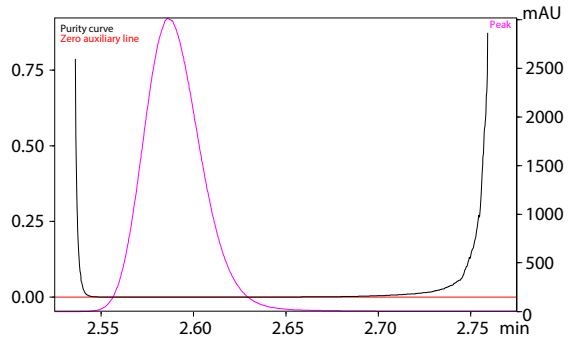

Fig. 4 Purity Curve of the Ketoprofen Peak

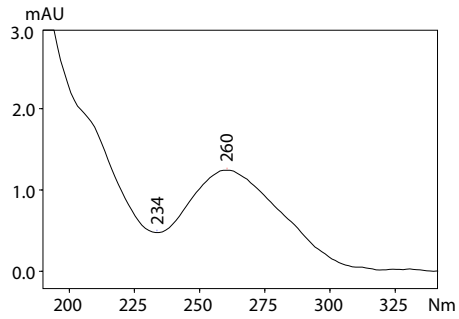

Fig. 5 UV Spectrum of Impurity 3

References

1) Revision of Guidelines for Impurities in New Drug Substances (Evaluation and Licensing Division, PMSB, dated December 16, 2002, Notification No. 1216001).
2) Revision of Guidelines for Impurities in New Drug Substances (Evaluation and Licensing Division, PMSB dated June 24, 2003, Notification No. 0624001).

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Application News

No. L539B

High Performance Liquid Chromatography

Improvement of Quantitative Performance for Ibuprofen Using UV Cut-Off Filter on SPD-M40

Ibuprofen is an example of a nonsteroidal anti-inflammatory drug (NSAID) and is widely used as an antipyretic or analgesic agent. It has been reported that during tests to confirm stability during storage, decomposition products were generated due to temperature, acidity, light irradiation, etc. of the surroundings. In particular, the area percentage of 4-Isobutylacetophenone, one of the decomposition products, increased by about 40 % after the 72-hour light irradiation test.

The Nexera™ Series photodiode array detector SPD-M40 is equipped with a UV cut-off filter that excludes light in the ultraviolet range, in order to ensure more stable detection of compounds that are prone to photodegradation. Here we introduce examples of improving the quantitation of ibuprofen using the UV cut-off filter function of the SPD-M40.(1)

＊This function was developed with the help of the comments by Lion Corporation.

H. Terada, K. Matsumoto

UV Cut-Off Filter

A photodiode array detector (PDA) irradiates the sample cell with white light (including ultraviolet light, which is relatively high-energy), spectrally separates the transmitted light, and measures the absorbance of a sample at a specific wavelength. Fig. 1 shows a diagram of a flow cell without the UV cut-off filter in use. Photodegradation can occur in the flow cell for analytes that are easily degraded by UV light.

As a consequence, accurate quantitation may be affected both by simultaneous determination of decomposition products with different light absorption characteristics, and by underestimation of the quantity of the target analyte.

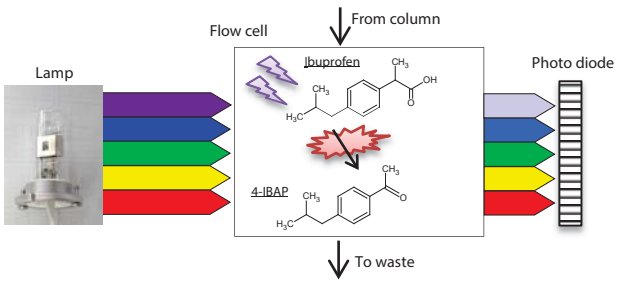

Fig. 1 **UV Cut-Off Filter Flow Cell (UV Cut-Off Filter Disabled)**

Fig. 2 shows a diagram of the flow cell with the UV cut-off filter in use. The short-wavelength, high-energy UV light can no longer enter the flow cell. Decomposition of the target analyte is suppressed due to the filter, and quantitation results are more reliable, no longer being affected by decomposition products.

Fig. 4 shows the chromatograms of the standard samples and the spectrum of the ibuprofen peak analyzed without using the UV cut-off filter. The calibration curve is shown in Fig. 5, and the error on each calibration point is shown in Table 2. In the low

The SPD-M40 detector uses a filter with a cut-off wavelength of around 240 nm. This completely cuts out incident light in the ultraviolet region <220 nm, and provides a 90 % reduction for wavelengths up to 240 nm.

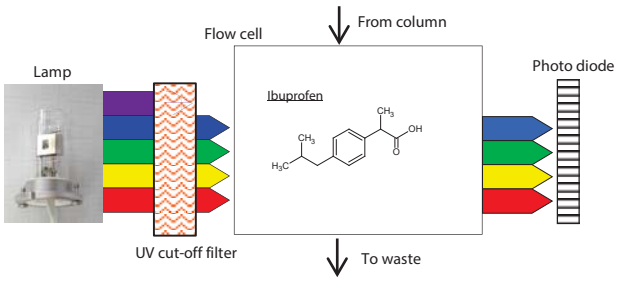

Fig. 2 **UV Cut-Off Filter Flow Cell (UV Cut-Off Filter Active)**

The user can choose whether to use the UV cut-off filter by modifying the method file (Fig. 3). It is easy to apply different conditions to each method even within the same analytical batch.

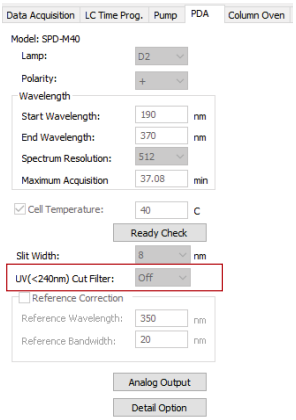

Fig. 3 **UV Cut-Off Filter Function Setting Screen for the SPD-M40**

Effects of UV Cut-Off Filter Use on the Analysis of Ibuprofen

Ibuprofen standard solutions (5 to 75 mg/L) were analyzed with and without the use of a UV cut-off filter. The analytical conditions are shown in Table 1.

Table 1 **Analytical Conditions**

Column	:	Shim-pack Velox™ C18 (3 mm × 100 mm, 2.7 µm)
Mobile phase	:	0.1 % Formic acid aq./Acetonitrile =2/3 (v/v)
Flow rate	:	0.4 mL/min
Column temp.	:	40 °C
Injection vol.	:	10 µL
Detection	:	SPD-M40 at 262 nm (190 - 400 nm)

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concentration range, the intercept of the calibration curve is relatively high due to the influence of decomposition products with large absorption coefficients. As a result, there is significant error on the calibration curve in the low concentration region.

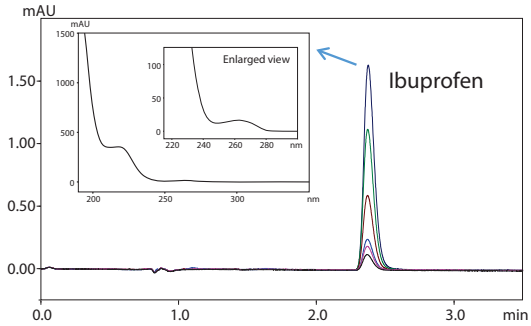

Fig. 4 **Chromatograms of Ibuprofen Standard Samples (5 to 75 mg/L) and Spectrum (75 mg/L) with UV Cut-Off Filter Disabled.**

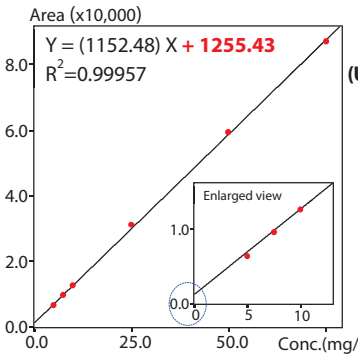

Table 2 Error on Calibration Points (UV Cut-Off Filter Disabled)

Conc. (mg/L)	Area	Error Rate (%)
5	6,418	**-10.40**
7.5	9,615	-3.28
10	12,692	-0.76
25	31,164	3.80
50	59,361	0.84
75	87,085	-0.70

Fig. 5 **Calibration Curve of Ibuprofen (UV Cut-Off Filter Disabled)**

Table 3 shows the results of quantitative analysis without using the UV cut-off filter for the 5 mg/L standard sample, which is the minimum concentration point on the calibration curve (LLOQ). After six consecutive repeats, the average result for the concentration of the analyte was >10 % lower than the expected value.

Table 3 Quantitative Results for 5 mg/L Sample (UV Cut-Off Filter Disabled)

	Retention Time (min)	Area	Conc. (mg/L)
1	2.366	6,462	4.517
2	2.380	6,319	4.394
3	2.376	6,508	4.558
4	2.378	6,339	4.411
5	2.377	6,371	4.439
6	2.378	6,468	4.523
Average	2.376	6411	**4.474**
RSD(%)	0.21	1.22	**1.51**

Fig. 6 shows the chromatograms of the standard samples and the spectrum of the ibuprofen peak analyzed with the use of the UV cut-off filter. The calibration curve is shown in Fig. 7, and the error on each calibration point is shown in Table 4. Since the short-wavelength UV light is eliminated by the UV cut-off filter, the influence of the decomposition products becomes negligible. Good linearity is achieved over the entire calibration range with reduced error and increased quantitative accuracy.

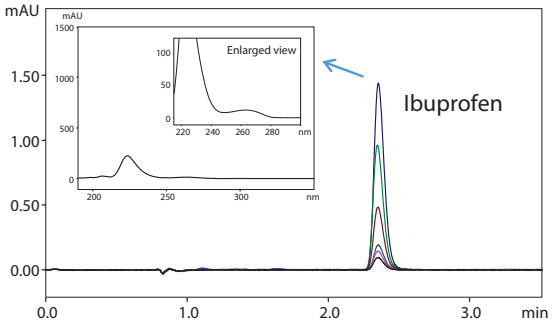

Fig. 6 **Chromatograms of Ibuprofen Standard Samples (5 to 75 mg/L) and Spectrum (75 mg/L) with UV Cut-Off Filter in Use.**

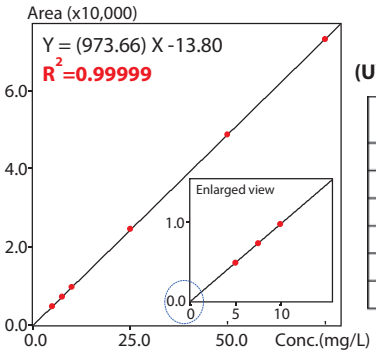

Table 4 Error on Calibration Points (UV Cut-Off Filter in Use)

Conc. (mg/L)	Area	Error Rate (%)
5	4,833	**-0.44**
7.5	7,290	0.02
10	9,657	-0.67
25	24,461	0.55
50	48,629	-0.08
75	73,003	-0.01

Fig. 7 **Calibration Curve of Ibuprofen (UV Cut-Off Filter in Use)**

Table 5 shows the results of the quantitative analysis using the UV cut-off filter for the 5 mg/L standard sample (LLOQ). The difference from the expected value is now much smaller, <0.1 %, compared to the previous case (UV cut-off filter off). There was also improved reproducibility (peak area).

The UV cut-off filter resulted remove this word to be useful for quantitative determination of components that are easily degraded by wavelengths in the UV range, particularly improving quantitation accuracy in the low concentration range.

Table 5 Quantitative Results for 5 mg/L Sample (UV Cut-Off Filter in Use)

	Retention Time (min)	Area	Conc. (mg/L)
1	2.354	4,882	5.028
2	2.352	4,843	4.988
3	2.350	4,844	4.990
4	2.353	4,859	5.005
5	2.353	4,829	4.974
6	2.351	4,840	4.985
Average	2.351	4,849	**4.995**
RSD(%)	0.06	0.38	**0.38**

Reference

(1) S. Farmer et al., "Forced Degradation of Ibuprofen in Bulk Drug and Tablets and Determination of Specificity, Selectivity, and the Stability Indicating Nature of the USP Ibuprofen Assay Method" Pharmaceutical Technology North America, **26** (5), 28-42 (May 2002)

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Third Edition: Aug. 2019

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Application News

No. L542

High Performance Liquid Chromatography

High-Speed Analysis of Linezolid following the Draft Guidance of International Harmonization of Pharmacopoeias

Linezolid is a new class of synthetic antibiotic with an oxazolidinone skeleton (Fig. 1). It was approved as a therapeutic agent in the United States in 2000 and in Japan in 2001. Linezolid was also listed in the Second Supplement to the 40th Edition of the United States Pharmacopeia (USP) published in 2017.

On the other hand, in international harmonization of the respective pharmacopeias, which is currently under study, the allowable range of changes in analytical conditions is specified in the draft guidance of international harmonization of JP/USP/EP [(1)]. Sharing a common allowable range of changes in high speed analysis conditions by all countries is important for achieving higher efficiency in drug development.

This article introduces an example of analysis of linezolid based on the USP and an example of its high-speed analysis based on the draft guidance of international harmonization of JP/USP/EP using Shimadzu Nexera™ Series and Shim-pack Scepter™ C18. It should be noted that this draft is based on the draft version published when public comment was solicited in July 2017 and may differ from the content which is finally adopted.

M. Oshiro

Outline of Shim-pack Scepter

Column selection is critical when high-speed analysis is applied. The separation performance of a column is proportional to the column length, but is inversely proportional to the particle size of the packing material. In other words, when the column size is shortened further while maintaining the same separation performance, smaller particles size must be used at the same time. When making these changes, it is possible to realize higher analysis speed with the same separation paturm by using the same packing material functional group and its modification condition.

Because the Shim-pack Scepter Series uses common packing materials with a wide range of particle sizes (1.9, 3, 5 µm) and also offers an extensive lineup of column size, it is possible to use the Scepter Series in applications from high-speed analysis to preparative analysis. As a result, seamless analysis method transfer is possible.

In this Application News, high-speed analysis using the column with 3 µm and 1.9 µm of particle size was examined.

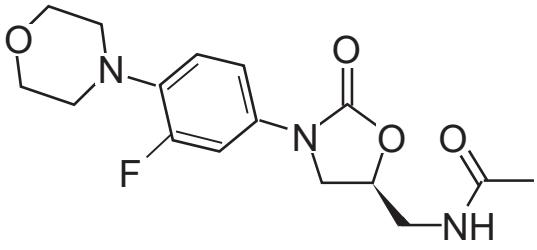

Fig. 1 Linezolid

High-Speed Analysis Conditions

As introduced in Application News L524, harmonization activities for method and pharmaceutical additive monographs in the Japanese Pharmacopoeia (JP), European Pharmacopoeia (EP), and United States Pharmacopeia (USP) are underway in the Pharmacopeial Discussion Group, and the draft guidance of international harmonization of JP/USP/EP on chromatography was published when public comment was solicited in 2017. This draft specifies the allowable range of changes in analytical test conditions, which is not included in the existing JP. Therefore, high-speed analysis may also become possible in the JP in the future.

Table 1 shows the USP analysis conditions used in the quantitative analysis method (Assay) for linezolid, together with the high-speed analysis conditions (UHPLC: ultra-high performance liquid chromatography) that were set based on the international harmonization draft. Fig. 2 shows the allowable range for adjustment of the analysis conditions for chromatography (gradient elution) in the draft used in setting the high-speed conditions. Only the principal items are excerpted here.

Table 1 High-Speed Analysis Conditions Based on International Harmonization Draft

	USP	UHPLC	Note
Column length (*L*) (mm)	75	50	User choice
Column diameter (*dc*) (mm)	4.6	2	User choice
Particle size (*dp*) (µm)	3	1.9	User choice
L/dp	25	26.3	-5.20 %
Flow rate (mL/min)	1.5	0.45	
Gradient factor		0.42	
Gradient (%B)	Time (min)	Time (min)	
20	0	0	
43	8	3.4	
100	18	7.6	
100	25	10.6	
20	25.01	10.61	
20	30	12.7	

Column dimensions: The ratio *L/dp* of the column length (*L*) and the particle size (*dp*) of the column to be changed shall be within the range of -25 % to +50 %. Columns for superficially porous particles are specified separately.
Flow rate: Adjustment of the flow rate is also necessary when the particle size is changed. The adjusted flow rate shall conform to the following equation.

$$F_2 = F_1 \times [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$$

F_1 : flow rate before change (mL/min)
F_2 : flow rate after change (mL/min)
dc_1 : internal diameter of column before change (mm)
dc_2 : internal diameter of column after change (mm)
dp_1 : particle size of column before change (µm)
dp_2 : particle size of column after change (µm)

When the particle size is to be changed from 3 µm or more to less than 3 µm, an increase in the linear velocity is allowed within the range where column efficiency does not decrease by 20 % or more.

Gradient time: The gradient volume changes in proportion to the column volume. The change is calculated using the following equation.

$$t_{G2} = t_{G1} \times (F_1 / F_2) [(L_2 \times dc_2^2) / (L_1 \times dc_1^2)]$$

t_{G1} : gradient time before change
t_{G2} : gradient time after change

Fig. 2 Allowable Range for Adjustment of Analysis Conditions for Chromatography in International Harmonization Draft (Gradient Elution, Principal Items)

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Evaluation of High-Speed Analysis Conditions

Fig. 3 shows the results of USP and UHPLC analyses of a linezolid solution (0.8 mg/L). Fig. 4 shows the enlarged chromatograms under these two conditions. Compound D was identified from the relative retention time to linezolid. Table 2 and Table 3 show USP analysis conditions and UHPLC analysis conditions respectively. Table 4 shows the results of an evaluation of a system suitability test based on the results of this study. Both the USP results and the UHPLC results satisfied the system suitability requirements.

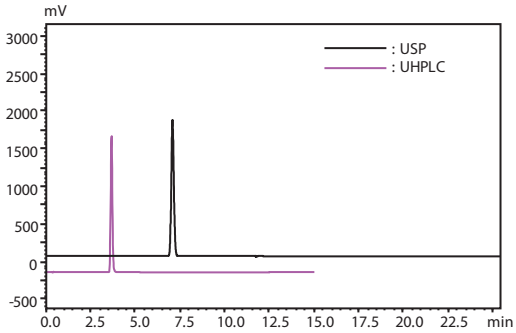

Fig. 3 Chromatograms of Linezolid

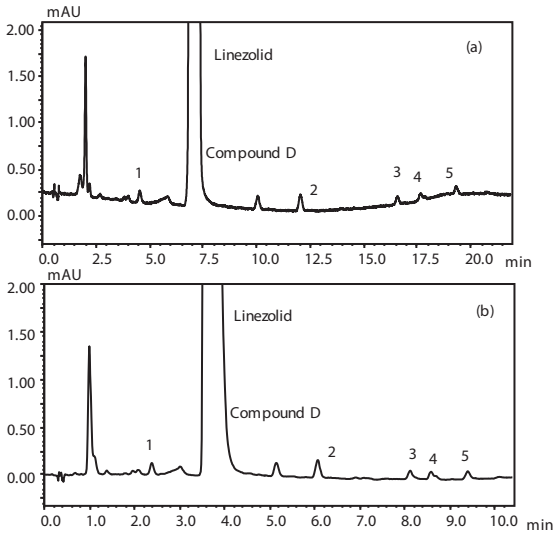

Fig. 4 Enlarged Chromatograms of Linezolid (a) USP, (b) UHPLC

Table 2 USP Analysis Conditions

Column	: Shim-pack Scepter C18 (75 mmL×4.6 mmI.D., 5 µm)
Mobile phase	: A) Methanol/Acetonitrile/1.4 g/L Monobasic potassium phosphate = 15/5/80 (v/v) B) Methanol/1.4 g/L Monobasic potassium phosphate = 50/50 (v/v)
Flow rate	: 1.5 mL/min
Time program	: B Conc.20 % (0 min) →43 % (8 min) →100 % (18 min) →100 % (25 min) →20 % (25.01-30 min)
Injection volume	: 10 µL
Column temp.	: 30 °C
Detection	: UV 254 nm

Table 3 UHPLC Analysis Conditions

Column	: Shim-pack Scepter C18 (50 mmL×2.0 mmI.D., 1.9 µm)
Mobile phase	: A) Methanol/Acetonitrile/1.4 g/L Monobasic potassium phosphate = 15/5/80 (v/v) B) Methanol/1.4 g/L Monobasic potassium phosphate = 50/50 (v/v)
Flow rate	: 0.45 mL/min
Time program	: B Conc.20 % (0 min) →43 % (3.4 min) → 100 % (7.6 min) →100 % (10.6 min) → 20 % (10.61-12.7 min)
Injection volume	: 2 µL
Column temp.	: 30 °C
Detection	: UV 254 nm

Table 4 Results of System Suitability Test

System suitability requirement		USP	UHPLC	Judgement
Resolution (between linezolid and compound D)	≥3.0	12	7.7	PASSED
Tailing factor	≤1.5	1.1	1.2	PASSED
Relative standard deviation	≤1.0 %	0.1	0.07	PASSED

Table 5 shows the relative retention times of impurities to linezolid used in this analysis under the USP and UHPLC conditions. It is possible to study high-speed analysis while maintaining the separation paturm more simply by using the Shim-pack Scepter Series.

Table 5 Comparison of Relative Retention Times for USP and UHPLC Conditions

Peak	Relative retention times to linezolid	
	USP	UHPLC
1	0.6	0.7
Compound D	1.4	1.4
2	1.7	1.7
3	2.3	2.2
4	2.5	2.3
5	2.7	2.6

Conclusion

An analysis of linezolid was conducted using the Shimadzu Nexera Series and Shim-pack Scepter C18. When high-speed analysis was used based on the USP and the international harmonization draft, both conditions satisfied the system suitability test. High-speed analysis while maintaining the separation paturm is possible more simply by using Shim-pack Scepter C18 in the analysis.

[Reference]
(1) International Harmonization (Stage 4), Pharmaceuticals and Medical Devices Agency.

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Application News

No. L550

High Performance Liquid Chromatography

Analysis of Meloxicam in Accordance with the United States Pharmacopoeia by Nexera™ XR

Meloxicam, inhibits the biosynthesis of prostaglandin, is utilized as an anti-inflammatory and analgesic for arthritis. In the United States Pharmacopoeia (USP), the HPLC method using a UV-Vis absorbance detector is adopted as the test method for meloxicam.

This article introduces an example of the analysis of meloxicam in accordance with the USP by Nexera XR, and compatibility with Prominence™ series using ACTO (Analytical Condition Transfer and Optimization) function.

T. Yoshioka

System Suitability Test

Table 1 shows the analytical conditions with the assay. Table 2 shows the method of preparing the solution used for system suitability test, and Fig. 1 shows the analysis results. The relative retention time for meloxicam related compound A[1] to meloxicam was 0.6, which is close to the reference value of 0.7. Table 3 shows the criteria and results for the system suitability test. It is evident that system suitability was satisfied for all items.

Table 1 Analytical Conditions (Assay)

Column	: Shim-pack™ GIST C18 (USP code:L1)
	: (150 mmL. × 4.6 mmI.D. , 5 µm)
Mode	: Isocratic
Mobile phase	: Methanol/solution A = 21/29
Flow rate	: 1 mL/min
Column temp.	: 45 °C
Injection volume	: 10 µL
Detection	: PDA detector (360 nm)

Table 2 Solution Preparation Method (Assay)

Solution A:
Mixture of a 0.1 % (w/v) solution of ammonium acetate adjusted with 10 % ammonia solution to a pH of 9.1

Diluent:
Methanol and 1 N sodium hydroxide (250:1)

System suitability solution:
0.08 mg/mL each of meloxicam and meloxicam related compound A. Prepared by dissolving in 50 % of the flask volume of diluent and diluting with water to volume.

Standard solution:
0.2 mg/mL of meloxicam. Prepared by dissolving in 50 % of the flask volume of diluent and diluting with water to volume.

Table 3 Results for System Suitability Test (Assay)

Test items	Criteria	Result	Judgement
Resolution between meloxicam related compound A and meloxicam	≥ 3.0	9.37	Passed
Tailing factor (meloxicam)	≤ 2.0	1.14	Passed
Relative standard deviation of peak area (n=6)	≤ 2.0 %	0.046 %	Passed

1) Isopropyl-4-hydroxyl-2-methyl-2*H*-1, 2-benzothiazune-3-carboxylate-1, 1-dioxide

2) 2-Amino-5-methyl-thiazole

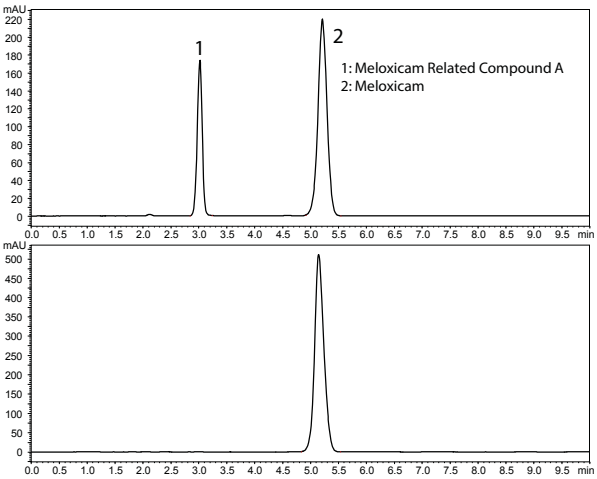

Fig. 1 Chromatograms from the Assay
Top: System Suitability Solution
Bottom: Standard Solution

Table 4 shows the analytical conditions for the impurity analysis. Table 5 shows the method of preparing the solution used for system suitability test, and Fig. 2 shows the analysis results. The relative retention times for meloxicam related compounds B[2] and A to Meloxicam were 0.5 and 1.3 respectively, which are close to the reference values of 0.4 and 1.4. Table 6 shows the criteria and results for the system suitability test. It is evident that system suitability was satisfied for all items.

Table 4 Analytical Conditions (Impurity Analysis)

Column	: Shim-pack GIST C18 (USP code:L1)
	: (150 mmL. × 4.6 mmI.D., 5 µm)
Mode	: High pressure gradient
Mobile phase	: A) SolutionA B) Methanol
Time program	: 40 %B (0 min) => 40 %B (2 min) => 70 %B (10 min) =>70 %B (15 min) => 40 %B (15.01 min) => 40 %B (18 min)
Flow rate	: 1 mL/min
Column temp.	: 45 °C
Injection volume	: 5 µL
Detection	: PDA detector (350 nm , 260 nm)

Table 5 Solution Preparation Method (Impurity Analysis)

Solution A:
0.1 % (w/v) solution of monobasic potassium phosphate adjusted with 1 N sodium hydroxide to a pH of 6.0

Diluent:
Methanol and 1 N sodium hydroxide (50:3)

System suitability solution:
0.08 mg/mL each of meloxicam, meloxicam related compound A, and meloxicam B. Prepared by dissolving in 10 % of the flask volume of diluent and diluting with methanol to volume.

Standard stock solution:
0.6 mg/mL of meloxicam. Prepared by dissolving in 25 % of the flask volume of diluent and diluting with methanol to volume.

Standard solution:
0.012 mg/mL of meloxicam in methanol from standard stock solution.

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Table 6 Results for System Suitability Test (Impurity Analysis)

Test items	Criteria	Result	Judgement
Resolution between meloxicam and meloxicam related compound A at 350 nm (system suitability solution)	≥ 3.0	7.18	Passed
Resolution between meloxicam and meloxicam related compound B at 260 nm (system suitability solution)	≥ 3.0	17.6	Passed
Relative standard deviation of peak area (n=6) (Standard solution)	≤ 10 %	0.30 % (350 nm)	Passed

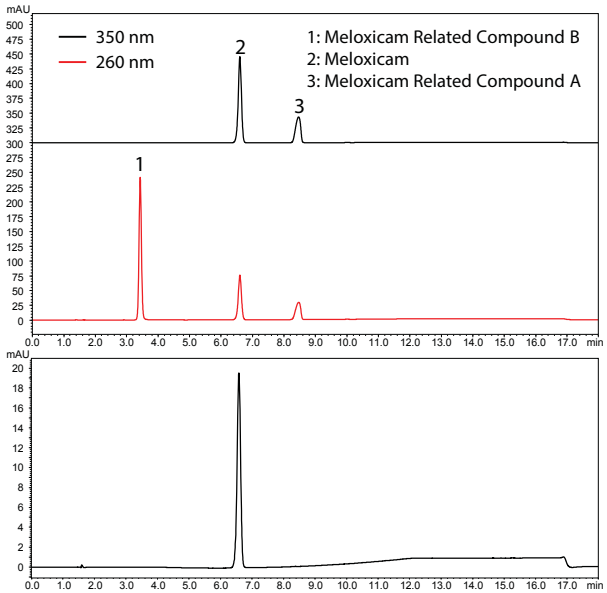

Fig. 2 Chromatograms from the Impurity Analysis
Top: System Suitability Solution
Bottom: Standard Solution

Compatibility with Prominence Series Using ACTO Function

In a gradient analysis, differences in the gradient delay volume between HPLC systems result different chromatograms. Fig. 3 shows a comparison of the chromatograms of system suitability solution (impurity analysis) using Nexera XR (mixer volume 180 µL) and Prominence (mixer volume 0.5, 1.6, and 2.7 mL). It is evident that the retention time changes depending on system differences. In such cases, the gradient start timing can be adjusted using ACTO function equipped in Labsolutions™. This enables a smooth method transfer without replacing piping, mixers, or other parts. Fig. 4 shows the results of a confirmation of compatibility with Nexera XR and Prominence using ACTO function. From this, it is evident that using ACTO function, comparable chromatograms can be obtained despite differing HPLC systems.

Nexera, Prominence, Shim-pack and LabSolutions are trademarks of Shimadzu Corporation in Japan and/or other countries.

References

United States Pharmacopeia 40-NF 35, 2017

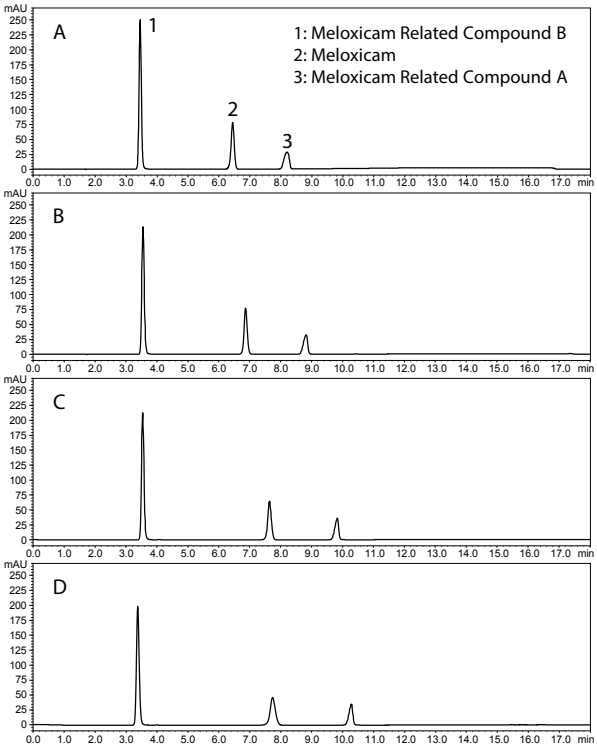

Fig. 3 Comparison of Chromatograms from Nexera XR and Prominence
A: Nexera XR (Mixer volume 180 µL)
B: Prominence (Mixer volume 0.5 mL)
C: Prominence (Mixer volume 1.7 mL)
D: Prominence (Mixer volume 2.5 mL)

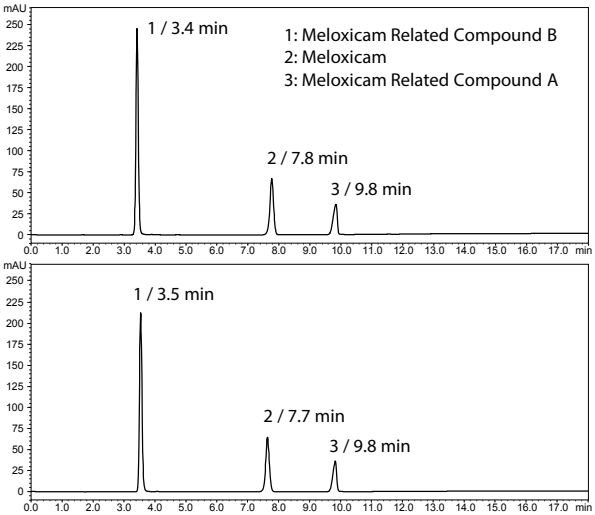

Fig. 4 Adjustment of Gradient Delay Volume by ACTO function
Top: Nexera XR (After adjustment of the gradient start timing)
Bottom: Prominence (Mixer volume 1.7 mL)

Summary

In this article, an analysis of meloxicam was performed in accordance with the USP using the Nexera XR. It was confirmed that system suitability was satisfied. Additionally, even when meloxicam is analyzed with an system with big gradient delay volume, comparable analysis can be performed by adjusting the gradient start timing using ACTO function.

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Application News

No. L559

High Performance Liquid Chromatography

High Efficiency in Workflow from Preparative HPLC to Analytical HPLC by Nexera™ Prep System

Preparative LC is widely used as a technique for separation and purification of a target compound from a mixture. Identification of the target compound peak and confirmation of fraction purity are extremely time-consuming, and automation of these processes can be expected to shorten the total working time for purification process. This article introduces a high throughput analysis conducted for simple fractionation using MS triggers and purity confirmation using an analytical/preparative LC-MS system, which is one configuration of the Nexera Prep brand.

K. Nakajima

Outline of Analytical/Preparative LC-MS System

The liquid handler (LH-40) used in this system has parallel preparative and analytical flow paths, enabling seamless reanalysis of the collected fractions. Fig. 1 and Fig. 2 show the flow line of the analytical/preparative LC-MS system used in this experiment. Fig. 1 shows the flow path when the system is used as preparative LC-MS. In this flow path, the target compounds injected from the LH-40 is separated by the preparative column, and part of the fraction is introduced into the LCMS™-2020. When the LCMS-2020 identifies peaks originating from the target compounds, an solenoid valve mounted on the tip of the LH-40 nozzle opens and the target compounds are collected respectively in the fraction collector.

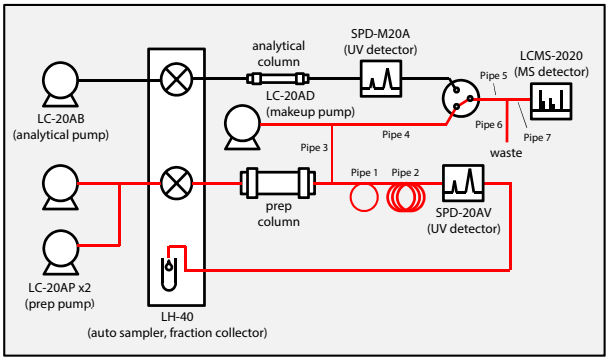

Fig. 1 Flow Diagram of Analytical/Preparative LC-MS During Fractionation

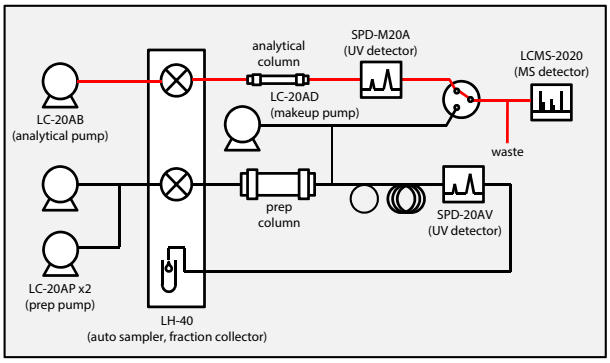

Fig. 2 Flow Diagram of Analytical/Preparative LC-MS During Analysis

Table 1 Configuration of Splitter Piping Used for LC-MS Trigger Fractionation

Pipe No.	Pipe 1	Pipe 2	Pipe 3	Pipe 4	Pipe 5	Pipe 6	Pipe 7
Material	PEEK	PTFE	PEEK	PEEK	PEEK	PEEK	PEEK
Diameter (mm)	0.25	1.0	0.025	0.25	0.13	0.13	0.13
Length (mm)	150	6000	40	1000	900	60	480

Fig. 2 is the flow diagram when the system is used as an analytical LC-MS. The LH-40 has a function that reinjects the fraction collected into the analytical flow path. In this system, gradient analysis is supported using a Shimadzu LC-20AB gradient solvent delivery unit, in which two pumps are incorporated in one compact module as solvent delivery pumps for analytical LC-MS, and introduction of a high pressure flow change-over valve in the downstream part of the analytical flow path allows to utilize the same LCMS-2020 in analytical LC-MS.

Both the preparative and analytical functions provided by this system can be controlled by the Shimadzu workstation LabSolutions™. All processes from switching of preparative LC-MS and analytical LC-MS during batch analysis, fractionation of the target compounds, and purity confirmation are conducted automatically in one system, realizing high efficiency in the entire workflow from fractionation to reanalysis.

Fractionation of Drugs by Preparative LC-MS

Purification was conducted by this preparative LC-MS system using a standard mixture consisting of 2 drugs, ketoprofen and indomethacin, as model compounds. Table 2 shows the fractionation conditions. A Shim-pack Scepter™ C18 with a particle diameter of 5 µm was used as the preparative column. MS detection was carried out in the high-speed positive/negative ionization switching scan mode (*m/z* range: 50 to 1,000). MS-triggered fractionation is conducted based on the XIC (extracted ion chromatogram) of ion species originating from the target compound. The XIC is drawn automatically by setting the molecular weight of the target compound. Fig. 3 shows the chromatograms obtained by preparative LC-MS. Two types of fractions, A and B, were detected by both the UV detector and the MS, and were successfully collected.

Table 2 Fractionation Conditions (Preparative LC-MS Flow Line)

Prep conditions		
Column	:	Shim-pack Scepter C18 (75 mm L. × 30 mm I.D., 5 µm)
Mobile phase	:	A: water (containing 1%(wt/v) formic acid) B: acetonitrile (containing 1%(wt/v) formic acid)
Flow rate	:	40 mL/min
Makeup	:	1.5 mL/min (methanol)
Time program	:	B conc. 10% (0 min) → 90% (6-8 min)
Column temp.	:	Ambient
Injection vol.	:	1000 µL (containing 10 mg/mL for each compound)
Detection	:	UV 250 nm (prep cell) MS (Posi. Nega. Scan *m/z* 50 - 1000)

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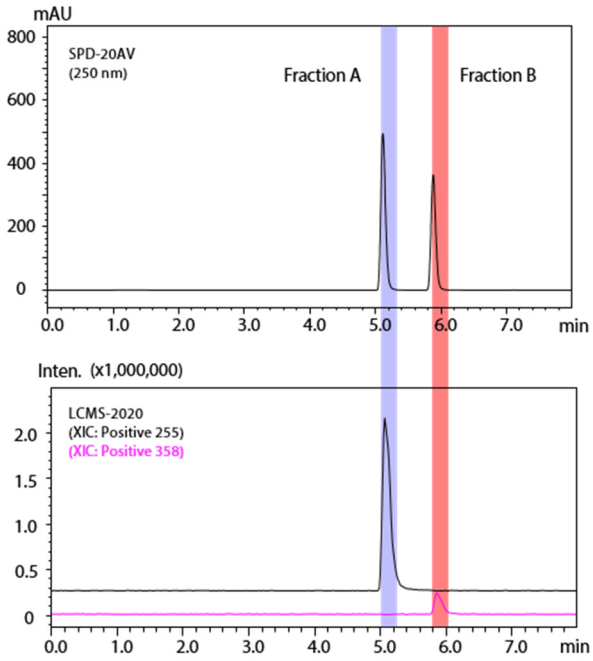

Fig. 3 Preparative Chromatograms of 2 Drugs
(Top: UV Detector Chromatogram, Bottom: LC-MS Chromatogram)

Purity Confirmation of Fractions by Analytical LC-MS

The obtained fractions A and B were analyzed by the analytical flow path (Fig. 2) of this system, and a purity confirmation test was conducted. Table 3 shows the analytical conditions. A large number of fractions are obtained in one fractionation in preparative LC, and high throughput analysis is required to confirm their purity. Here, a Shim-pack Scepter C18 with a particle size of 3 µm was used as the analytical column, aiming at a high separation capacity and good peak shapes in high throughput analysis.

Fig. 4 and Fig. 5 show the obtained chromatograms for ketoprofen and indomethacin, respectively. Purity of 99% or more was obtained for both ketoprofen and indomethacin in peak area normalization (UV chromatogram) (Table 4), and the analysis times for both compounds were within 3 min.

Table 3 Analytical Conditions (Analytical LC-MS Flow Path)

Analytical conditions	
Column	: Shim-pack Scepter C18 (50 mm L. × 4.6 mm I.D., 3 µm)
Mobile phase	: A: water (containing 1%(wt/v) formic acid) B: acetonitrile (containing 1%(wt/v) formic acid)
Flow rate	: 2.5 mL/min
Time program	: B conc. 10% (0 min) → 90% (2-3 min)
Column temp.	: Ambient
Injection vol.	: 1 µL
Detection	: UV 250 nm (prep cell) MS (Posi. Nega. Scan *m/z* 50 - 1000)

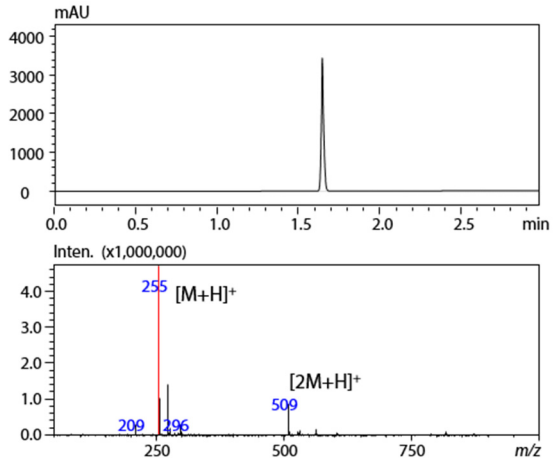

Fig. 4 Reanalysis Results of Fraction A (Ketoprofen)
(Top: UV Detector, Bottom: MS Spectrum of Detected Peak)

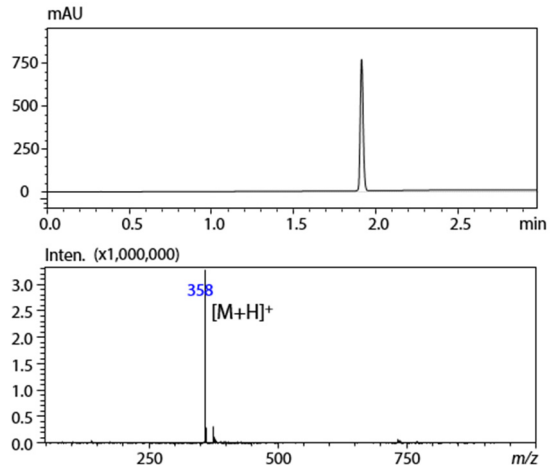

Fig. 5 Reanalysis Results of Fraction B (Indomethacin)
(Top: UV Detector, Bottom: MS Spectrum of Detected Peak)

Table 4 Purity of Target Compounds Contained in Fractions (Area Normalization, UV 250 nm)

	Area %
Ketoprofen*	99.8
Indomethacin	99.9

* Because the peak intensity was near the saturation point, the area normalization value is a reference value.

Conclusion

This article introduced a convertible system setup of preparative and analytical LC-MS using LH-40 liquid handler and its application. Use of the reinjection function of the LH-40 allowed to carry out all the processes from purification of the target compounds by preparative LC-MS to purity confirmation of the acquired fractions and acquisition of the MS spectrum in a single series batch analysis, significantly improving the efficiency in the conventional preparative LC workflow.

[Acknowledgement]
We wish to thank Mr. Atsushi Shimabukuro of ONO PHARMACEUTICAL CO.,LTD. for his generous guidance and cooperation in carrying out this research.

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Application News

No. L537

High Performance Liquid Chromatography

Increased Throughput with Nexera™ GPC system: Overlapped Injection and Simultaneous Determination of Polymer Additives

Measuring molecular weight distribution of polymer compound by size exclusion mode is one of the typical parts of HPLC and generally called gel permeation chromatography (GPC). Nowadays there is an increasing demand demand for high throughput analysis even in well-established GPC. Here we introduce a novel GPC analysis that affords both high throughput GPC results by overlapped injection using conventional size of columns and simultaneous determination of polymer additives.

Y. Watabe, K. Nakajima, H. Terada

GPC Analysis by Overlapped Injection and Simultaneous Determination of Polymer Additives

A refractive index detector (RID) that gives a response to a sample weight is commonly employed for GPC to calculate average molecular weight and polydispersity. On the other hand, A UV detector is used for determination of polymer additives such as antioxidants. Consequently, serially connected those two detectors were used for this study. In GPC, there is hardly any eluates prior to exclusion limit. So if the sample elution band from previous injection is managed to be overlapped within this no elution interval in present analysis, short analysis cycle time can be obtained and it provides increased throughput in sequential analyses. When two or more polymer additives are contained, correct determination of those small additives is difficult because complete separation of additives is almost impossible even using GPC columns of small exclusion limit due to small difference of molecular weight among those polymer additives. To address this difficulty, a photodiode array detector (PDA) that affords spectrometric information as well as chromatographic results was employed to give increased separation of polymer additives by peak deconvolution function within a single GPC analysis.

Fig. 1 and Table 1 show obtained chromatogram of polystyrene containing three antioxidants and employed analytical conditions respectively.

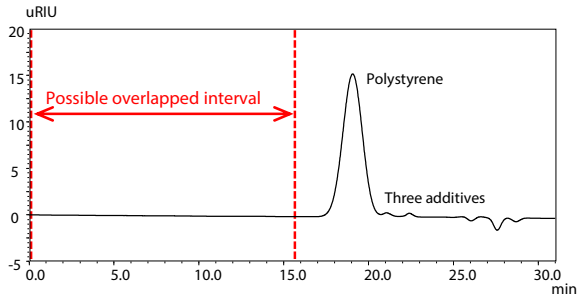

Fig. 1 Chromatogram of Polystyrene and Three Additives (RID)

Table 1 Analytical Conditions

Column	: Shim-pack™ GPC 805+GPC 801 (300 mmL. × 8 mmI.D. for each)
Mobile phase	: THF
Flow rate	: 0.8 mL/min
Column temp.	: 40 °C
Injection vol.	: 10 µL
Detection	: RID / PDA (220-400 nm)
Sample	: 0.5 % Polystyrene containing three additives
Cycle time	: 31 min
Overlap time	: 15.5 min

Increased Throughput by Overlapped Injection

Based on the chromatogram in Fig. 1, 0 to 15.5 term was assigned to overlapped interval due to no elution during it. Fig. 2 shows the comparison of sequential chromatograms obtained with/without overlapped injection. Almost 50 % of decreased analysis cycle time provided increased throughput of sequential GPC analyses.

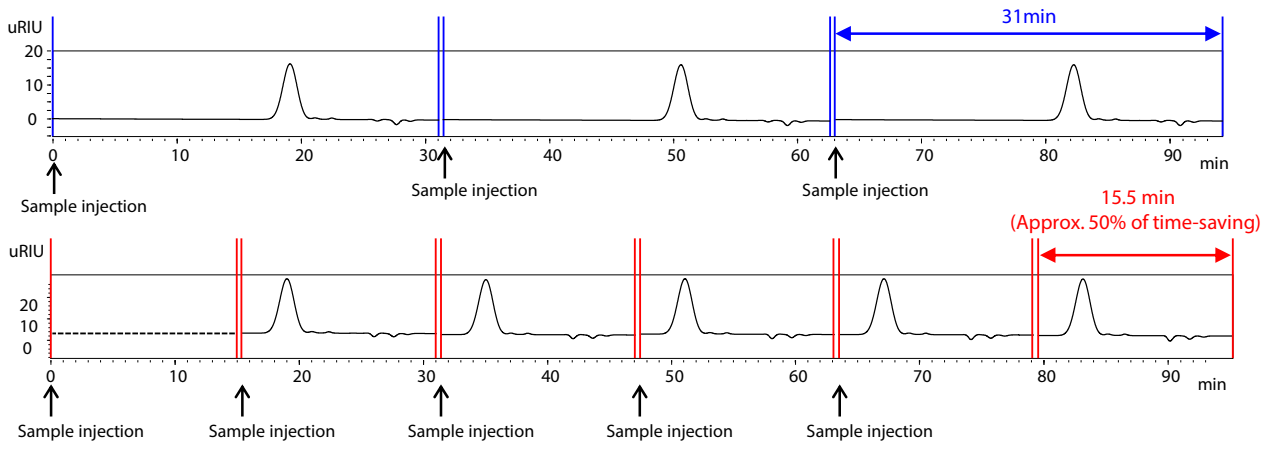

Fig. 2 Comparison of Sequential GPC Analyses With (Lower) / Without (Upper) Overlapped Injection (RID)

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Peak Deconvolution by *i*-PDeA II*

LabSolution™ workstation software equips two types of peak deconvolution functions named *i*-PDeA and *i*-PDeA II, those attempt to improve incomplete separation using spectral information obtained with PDA. The former handles two components cases using derivative spectra to cancel the effect from the one. The latter handles two or more components cases using a computer simulation to obtain chromatographic approximate solution of isolated peaks based on three-dimensional information from PDA. In this study, we employed *i*-PDeA II because incomplete separation band consists of three components. Fig. 3 shows a schematic image of peak deconvolution process.

* Shimadzu Technical Report C191-E042

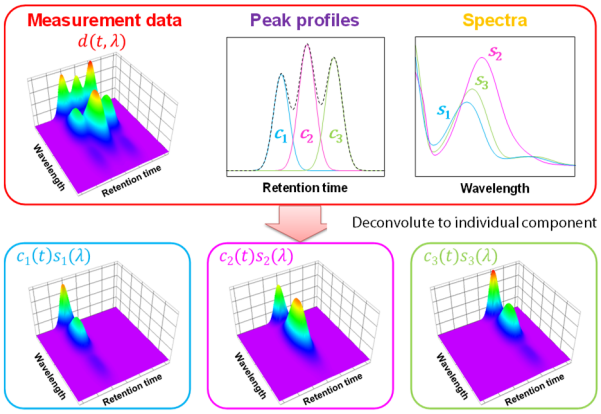
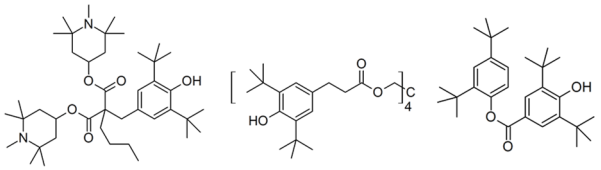


Fig. 3 Peak Deconvolution Process by *i*-PDeA II

Peak Deconvolution for Incomplete Separation of Polymer Additives

Fig. 4 shows the structural formulas of the three antioxidants added to the polystyrene. Fig. 5 shows the chromatogram at 240 nm and overlaid chromatograms of isolated respective compounds with *i*-PDeA II. Fig. 6 shows the UV spectra of three antioxidants after the peak deconvolution process. The original UV-chromatogram provides only two peaks, however three isolated peaks appeared after peak deconvolution process. Furthermore, the obtained peak areas can be applied to the quantitative determination because each peak area after the process directly comes from the original peak area contribution to incomplete peak separation band.



Tinuvin 144 Irganox 1010 Tinuvin 120

Fig. 4 Structural Formulas of Three Polymer Additives

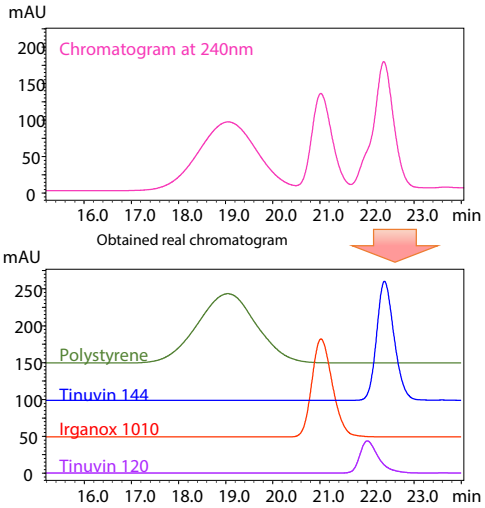


Fig. 5 Deconvolution Result of Three Polymer Additives (PDA)

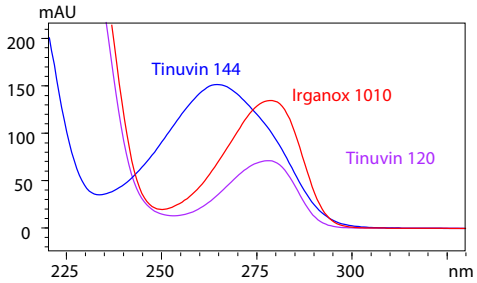


Fig. 6 UV Spectra of Three Additives Obtained After Peak Deconvolution

Determination After Peak Deconvolution

The calibration curves for the three additives were created in the range from 0.01 to 0.1 % (w/v) and applied to determination of the additives contained in the polystyrene. Linearity of calibration curve and determined result of each additive through consecutive six injections are summarized in Table 2. The GPC calculation results of the polystyrene based on RID chromatograms are shown in Table 3 as well. These results suggest that the value added high throughput GPC analysis can be performed using overlapped injection and *i*-PDeA II, which enhances separation performance of GPC column having small exclusion limit.

Table 2 Determination Results of Three Polymer Additives (n=6)

Additive	Irganox 1010	Tinuvin 144	Tinuvin 120
Linearity of calibration curve (r^2)	0.999	0.995	0.998
Determined content (mg/g)	49.2	23.1	27.4
%RSD	1.28	1.93	1.47

Table 3 GPC Calculation Results of Polystyrene (n=6)

	Number average molecular weight Mn	Weight average molecular weight Mw	Polydispersity Mw/Mn
Polystyrene	2.63×10^4	4.89×10^4	1.86
%RSD	1.41	0.89	0.52

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Application News

No. L540

High Performance Liquid Chromatography

High-Resolution and High-Speed Simultaneous Analysis of Preservatives in Cosmetics Using SPP Column

Cosmetics usually have a high water and oil content. For this reason, both bacteria and molds can proliferate in cosmetics stored at ambient temperatures, resulting in a change in the physical properties and safety of the product. The Pharmaceutical Affairs Law stipulates that cosmetics must have consistent properties and quality over more than three years, and those that may deteriorate within three years must display an "expiration date". To increase stability, many cosmetics contain antiseptic compounds such as parabens and 2-phenoxyethanol. However, these preservatives are not only bactericidal but can also cause allergic symptoms in humans, mainly skin conditions such as eczema and dermatitis. Strict regulations are therefore imposed in Japan and Europe on the amount of these ingredients that can be used in cosmetics.

This article introduces a method for the analysis of 24 types of cosmetic preservatives, either specified in the cosmetics standards established by the Japanese Ministry of Health, Labour and Welfare or regulated by the European Commission, using ultra-high performance liquid chromatography (UHPLC). Analyses were performed using the Nexera™ series UHPLC system equipped with a Shim-pack Velox™ C18 SPP (superficially porous particles, core-shell) column, which enabled both high resolution and reduced assay time.

Y. Toyota

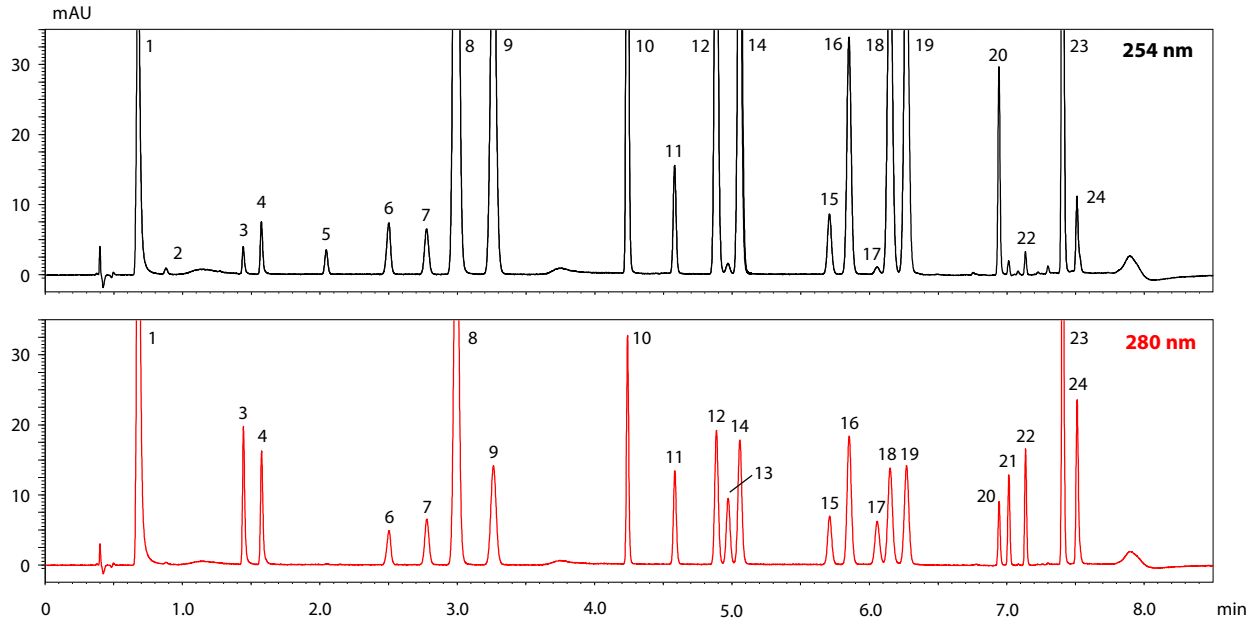
Analysis of Standard Solutions

We used the Shim-pack Velox C18 SPP column to analyze 24 types of compounds regulated by the Pharmaceutical Affairs Law of the Ministry of Health, Labour and Welfare and the European Commission.

All standard solutions were prepared at concentration 50 mg/L. Simultaneous chromatographic separation is shown in Fig. 1, and assay conditions are reported in Table 1. Separation and detection of all 24 components were possible in about 9 minutes (at 45 MPa).

Table 1 HPLC Analytical Conditions

Column	: Shim-pack Velox C18 (100 mm L × 3.0 mm I.D., 2.7 µm)
Mobile phase	: A) 25 mmol/L NaH_2PO_4 aq. (pH 3.8) B) MeOH/CH_3CN=9/1
Time program	: B Conc. 8 % (0 min) → 30 % (0.31 min-3.00 min) → 49 % (3.01 min-4.50 min) → 53 % (5.00 min-5.50 min) → 80 % (6.50 min-7.20 min) → 8 % (7.21 min-9.00 min)
Flow rate	: 1.0 mL/min
Column temperature	: 45 °C
Injection volume	: 1 µL
Detector	: SPD-M40 Photo diode array detector
Cell	: Semi-micro cell
Wavelength	: 190 to 800 nm (Monitor 254 nm and 280 nm)



Peaks:
1. 2-methyl-4-isothiazolin-3-one, 2. 2-bromo-2-nitro-1,3-propanediol, 3. salicylic acid, 4. isothiazolinones, 5. benzyl alcohol, 6. benzoic acid, 7. 2-phenoxyethanol, 8. sorbic acid, 9. methyl paraben, 10. ethyl paraben, 11. methyl benzoate, 12. isopropyl paraben, 13. 4-chloro-3-methylphenol, 14. propyl paraben, 15. ethyl benzoate, 16. 2-phenylphenol, 17. chloroxylenol, 18. isobutyl 4-hydroxybenzoate, 19. butyl paraben, 20. phenyl benzoate, 21. 2,4-dichloro-3,5-dimethylphenol, 22. clorofene, 23. triclocarban, 24. triclosan

Fig. 1 Chromatograms of Cosmetic Preservative Standard Solutions (50 mg/L Each)

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Shim-Pack Velox Column

Shim-pack Velox columns are the first columns equipped with Shimadzu Corporation's core-shell technology, which enables high-speed separation while keeping back pressure low through the use of superficially porous particles (SPP, core-shell). The SPP particles have a central, non-porous cores, and a porous layer on the surface containing the sample components. As a result, the average molecular migration distance within the particle is shortened, and the mass transfer diffusion (as a distribution of the migration distance of each molecule) is smaller than for fully porous particles. This structure gives sharp peak shapes compared to full-porous particles of the same particle size, resulting in a higher number of theoretical plates.

Fig. 2 shows a diagram of the stationary phase of the Shim-pack Velox C18 used in this study. This column is suitable for analysis in a wide range of fields, including pharmaceuticals, foods, and environmental science, due to its high end-capping rate and high hydrophobic retention.

Fig. 2 Representation of the Shim-pack Velox C18 Stationary Phase

Analysis of Commercially Available Lotions

Chromatograms of lotions A, B, and C are shown in Fig. 3, 4, and 5, respectively. Approximately 0.1 g of each sample was diluted with methanol in a 50 mL volumetric flask, filtered through a membrane filter, and then analyzed (1 µL injection).

Analytical conditions are shown in Table 1.

2-Phenoxyethanol, methylparaben and ethylparaben were detected in all samples analyzed (A, B and C). Propylparaben was also detected in lotion C. The amounts of each component present are shown in Table 2. All the compounds were below the maximum allowable content of 1000 mg/100 g specified in the Cosmetic Standards based on the Pharmaceutical Affairs Law.

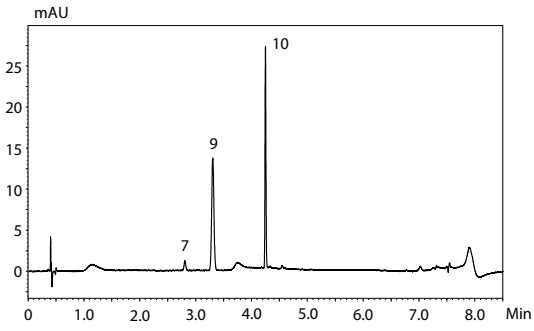

Fig. 3 Chromatogram of Lotion A

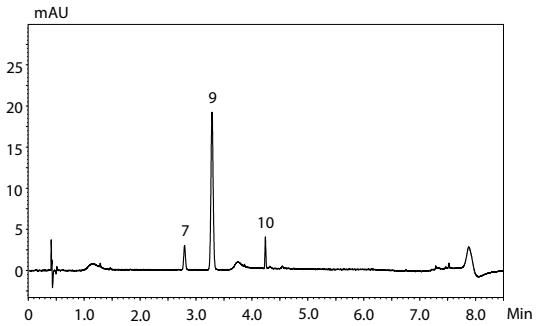

Fig. 4 Chromatogram of Lotion B

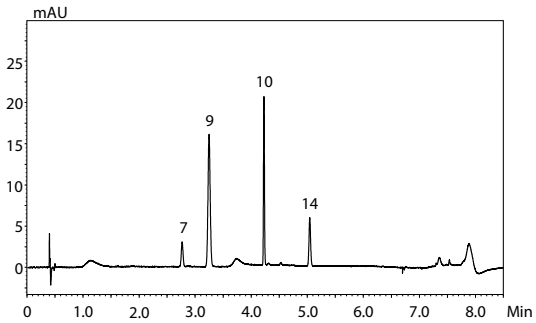

Fig. 5 Chromatogram of Lotion C

Table 2 Preservative Content Results for All Lotion Samples

Preservative	Amount present/ mg/100 g		
	A	B	C
Phenoxyethanol	99.0	224.5	261.6
Methylparaben	112.9	152.6	142.1
Ethylparaben	97.3	11.2	79.2
Propylparaben	-	-	41.5

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Application News

No. L541

High Performance Liquid Chromatography

High-Resolution and High-Speed Simultaneous Analysis of Regulated UV-Adsorbents in Cosmetics using SPP Column

Many cosmetics contain ultraviolet light absorbents (UV-absorbents) to protect the skin from ultraviolet light. In Japan, the standards for cosmetics based on the Pharmaceutical Affairs Law (Ministry of Health and Welfare Notice No. 331, 2000) regulate the types and allowable quantities of UV-absorbents that may be used in cosmetic products. Since regulations can vary significantly between different countries and regions, quantification with HPLC is used to determine compliance with import/export regulations.

This report presents an example of high-throughput analysis of 23 kinds of UV-absorbents using the Nexera™ LC system and Shim-pack Velox™ C18 SPP (superficially porous particles, core-shell) column. This is a larger number of compounds than covered in a previous report (Application News No. L381), as it also includes some residues regulated by EU agencies.

M. Oshiro

Analysis of Standards

Fig. 1 shows a 1 µL injection of a mixed solution of 23 UV-absorbents (100 mg/L each). The analytical conditions are shown in Tables 1 and 2. The 23 components of UV-absorbents that may be incorporated into cosmetics were analyzed (see Table 3 on the next page for details). All components showed good linearity with an R^2 value of 0.999 or higher within the range 1-100 mg/L.

Shim-pack Velox column enables high-speed separation while keeping back pressure low through the use of SPP technology. The system pressure in this analysis did not exceed 30 MPa.

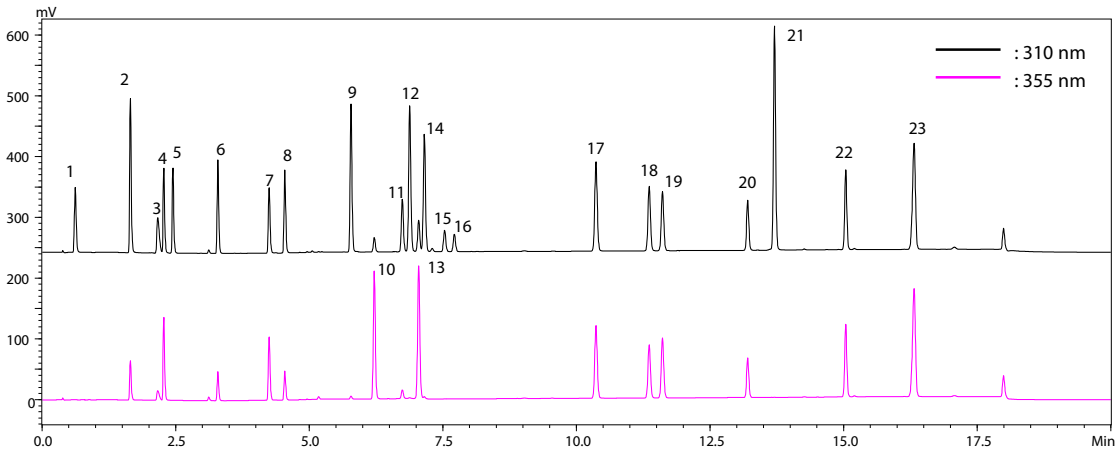
The flow rate of 1 mL/min enabled the accurate quantitation of 2,2'-methylenebis[6-(benzotriazol-2-yl)-4-tert-octylphenol) (peak 22) and bis-ethylhexyloxyphenol methoxyphenyl triazine (peak 23) within 20 minutes. This is a large reduction compared to the previous analytical time required for these two compounds.

Table 1 Analytical Conditions

Column	: Shim-pack Velox C18 (100 mm L. × 3.0 mm I.D., 2.7 µm)
Mode	: Low pressure gradient
Mobile Phase	: A) 0.1 % Formic acid in water B) Acetonitrile C) Methanol
Flow Rate	: 1 mL/min
Column Temp.	: 60 °C
Injection volume	: 1 µL
Detection	: 310 nm, 355 nm

Table 2 Time Program

Time (min)	A. Conc	B. Conc	C. Conc
0	90	10	0
2	60	40	0
4	25	0	75
14	0	0	100
17	0	0	100
17.01	90	10	0
20	90	10	0



1, 4-aminobenzoic acid
2, ferulic acid
3, 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid
4, 2,2',4,4'-tetrahydroxybenzophenone
5, ethyl 4-aminobenzoate
6, 2,4-dihydroxybenzophenone
7, 2,2'-dihydroxy-4,4'-dimethoxybenzophenone
8, 2-hydroxy-4-methoxybenzophenone
9, 4-methylbenzylidene camphor
10, hexyl 2-[4-(diethylamino)- 2-hydroxybenzoyl]benzoate
11, 2-ethylhexyl 2-cyano-3,3-diphenylacrylate
12, 2-ethylhexyl-4-dimethylaminobenzoate
13, 4-tert-butyl-4'-methoxydibenzoylmethane
14, 2-ethylhexyl 4-methoxycinnamate
15, 2-ethylhexyl salicylate
16, 3,3,5-trimethylcyclohexyl salicylate
17, 2-benzotriazol-2-yl- 4,6-di-tert-butylphenol
18, 2-(2H-benzotriazol-2-yl)- 4,6-di-tert-pentylphenol
19, 2,4-di-tert-butyl-6- (5-chloro-2H-benzotriazol-2-yl)phenol
20, drometrizole trisiloxane
21, 2,4,6- tris[4-(2-ethylhexyloxycarbonyl)- anilino]-1,3,5-triazine
22, 2,2'-methylenebis[6-(benzotriazol-2-yl)-4-tert-octylphenol]
23, bis-ethylhexyloxyphenol methoxyphenyl triazine

Fig. 1 Standard Chromatograms of 23 UV-Adsorbents (100 mg/L Each).

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Reproducibility

Table 3 shows the reproducibility of the retention times and peak area values over six repeated injections of a standard mixed solution (10 mg/L each) containing 23 UV-absorbents.

Table 3 Reproducibility of 23 UV-Absorbents (10 mg/L Each)

No.	Compound Name	Retention Time (%RSD)	Area (%RSD)
1	4-aminobenzoic acid	0.33	1.93
2	ferulic acid	0.25	1.48
3	2-hydroxy-4-methoxybenzophenone-5-sulfonic acid	0.41	1.98
4	2,2',4,4'-tetrahydroxybenzophenone	0.21	1.27
5	Ethyl4-aminobenzoate	0.23	1.06
6	2,4-dihydroxybenzophenone	0.13	1.05
7	2,2'-dihydroxy-4,4'-dimethoxybenzophenone	0.12	1.47
8	2-hydroxy-4-methoxybenzophenone	0.08	1.47
9	4-methylbenzylidene camphor	0.11	0.90
10	Hexyl 2-[4-(diethylamino)-2-hydroxybenzoyl] benzoate	0.10	0.98
11	2-ethylhexyl 2-cyano-3,3-diphenylacrylate	0.10	0.92
12	2-ethylhexyl-4-dimethylaminobenzoate	0.12	1.04
13	4-tert-butyl-4'-methoxydibenzoylmethane	0.12	1.31
14	2-ethylhexyl 4-methoxycinnamate	0.10	1.10
15	2-ethylhexyl salicylate	0.13	1.15
16	3,3,5-trimethylcyclohexyl salicylate	0.11	1.56
17	2-benzotriazol-2-yl-4,6-di-tert-butylphenol	0.11	0.90
18	2-(2H-benzotriazol-2-yl)-4,6-di-tert-pentylphenol	0.09	0.89
19	2,4-di-tert-butyl-6-(5-chloro-2H-benzotriazol-2-yl) phenol	0.10	1.19
20	drometrizole trisiloxane	0.08	0.94
21	2,4,6-tris [4-(2-ethylhexyloxycarbonyl)-anilino]-1,3,5-triazine	0.07	0.95
22	2,2'-methylenebis [6-(benzotriazol-2-yl)-4-tert-octylphenol]	0.05	0.90
23	bis-ethylhexyloxyphenol methoxyphenyl triazine	0.11	0.93

Analysis of Commercial Samples

Fig. 2 shows the analysis of a commercially available cosmetic cream. 100 mg of the sample was weighed out, and 5 mL of tetrahydrofuran added. An ultrasonic bath was used for extraction. After centrifugation, 0.5 mL of the supernatant was diluted to 10 mL with acetonitrile and filtered through a membrane filter with 0.22 µm pore size for analysis. The injection volume was 1 µL. All the components include with sample were below the maximum allowable content specified in the regulation.

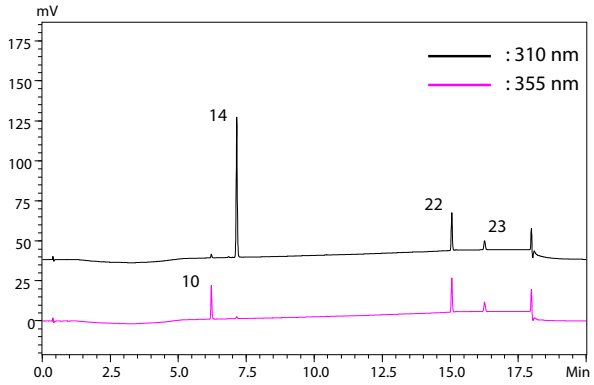

Fig. 2 Chromatograms of a Cosmetic Cream

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Application News

No. L546

High Performance Liquid Chromatography

Qualitative Analysis of UV-Absorbents in Cosmetics Based on UV-Vis Spectrum

Many cosmetic products contain ultraviolet absorbers (UV-absorbents) which protect the skin from ultraviolet rays. In Japan, ingredients of UV-absorbents and their allowable quantities are regulated under Standards for Cosmetics (2000, Ministry of Health, Labour and Welfare Notification No. 331) based on the Act on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical Devices (PMD Act). Because these standards differ depending on the country and region, compliance with the applicable regulations is verified by high performance liquid chromatography (HPLC) when importing/exporting cosmetics.

Against this background, Application News No. L541 introduced an example of high-speed analysis of 23 UV-absorbents. This article introduces a method for qualitative analysis of six UV-absorbents based on the UV-Vis spectrum.

T. Yoshioka

Analysis of Standard Substances

In this article, six UV-absorbents shown in Fig. 1 were focused on, and Fig. 1 shows the chromatogram of a mixed standard solution of six UV-absorbents (100 mg/L each). Table 1 shows the analytical conditions.

Fig. 2 on the following page shows the UV-Vis spectra of each UV-absorbent obtained by analyzing the mixed standard solution (100 mg/L of each UV-absorbent). A qualitative analysis of the UV-absorbents contained in cosmetics was conducted based on these spectra.

Table 1 Analytical Conditions

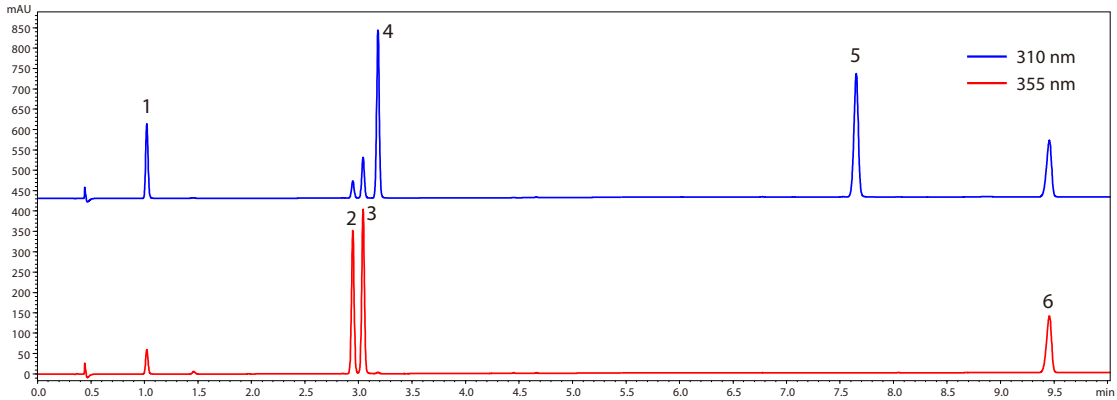
Column	: Shim-pack Velox™ C18 (100 mm L. × 3.0 mm I.D., 2.7 µm)
Mode	: High pressure gradient
Mobile phase	: A) 0.085% Phosphoric acid in water
	: B) Acetonitrile
Time program	: 60%B (0 min) → 60%B (0.5 min) → 90%B (4 min) → 100%B (10 min) → 60%B (10.01 min) → 60%B (15 min)
Flow rate	: 1 mL/min
Column temp.	: 60 °C
Injection volume	: 1 µL
Detection	: SPD-M40 (190-800 nm)

Linearity and Repeatability

Calibration curves for each UV-absorbent were prepared from the mixed standard solution of six UV-absorbents. Calibration curves for the calibration points of 1, 5, 25, 50, and 100 mg/L were prepared for each absorbent, and their linearity was evaluated. Repeatability of retention time and area was evaluated by a repeated analysis (n = 6) at 100 mg/L. Table 2 shows these results. Satisfactory linearity with a contribution ratio (R^2) of 0.9999 or more was obtained for all UV-absorbents. Repeatability was also satisfactory in terms of both retention time and peak area.

Table 2 Linearity and Repeatability of Six UV-Absorbents

No.	Compound	Linearity (R^2)	Retention time (%RSD)	Area (%RSD)
1	2-hydroxy-4-methoxybenzophenone	0.9999	0.24	0.35
2	4-*tert*-butyl-4'-methoxydibenzoylmethane	0.9999	0.10	0.34
3	2-ethylhexyl-4-methoxycinnamate	0.9999	0.09	0.29
4	2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid hexyl ester	0.9999	0.08	0.33
5	2,4,6-tris[4-(2-ethylhexyloxycarbonyl)-anilino]-1,3,5-triazine	0.9999	0.03	0.30
6	bis-ethylhexyloxyphenol methoxyphenyl triazine	0.9999	0.02	0.33



1. 2-hydroxy-4-methoxybenzophenone/2. 4-*tert*-butyl-4'-methoxydibenzoylmethane/3. 2-ethylhexyl-4-methoxycinnamate/4. 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid hexyl ester/5. 2,4,6-tris[4-(2-ethylhexyloxycarbonyl)-anilino]-1,3,5-triazine/6. bis-ethylhexyloxyphenol methoxyphenyl triazine

Fig. 1 Chromatogram of Mixed Standard Solution of Six UV-Absorbents (100 mg/L Each)

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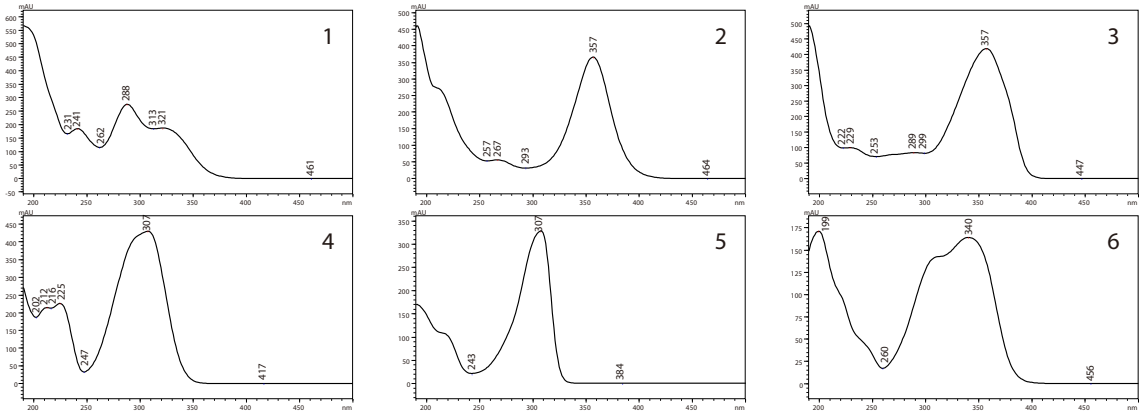

Fig. 2 UV-Vis Spectra of Six UV-Absorbents

Analysis of Cosmetic Product (Foundation)

100 mg of a commercial foundation was weighed, 5 mL of tetrahydrofuran (THF) was added, and ultrasonic extraction was conducted, followed by centrifugal separation, and 0.5 mL of the supernatant was adjusted to a constant volume of 10 mL with THF. The sample for analysis was then prepared by filtering that solution with a 0.22 μm pore size membrane filter.

Fig. 3 shows the chromatogram of the foundation. It was clarified that three types of UV-absorbents are contained in the foundation, and their contents satisfied the regulation values.

Fig. 4 shows the superimposed UV-Vis spectra of compound 4 in the analysis of the mixed standard solution and analysis of the cosmetic product. Similar spectra were obtained for the standard sample and the actual sample, demonstrating that qualification based on UV-Vis spectra is possible.

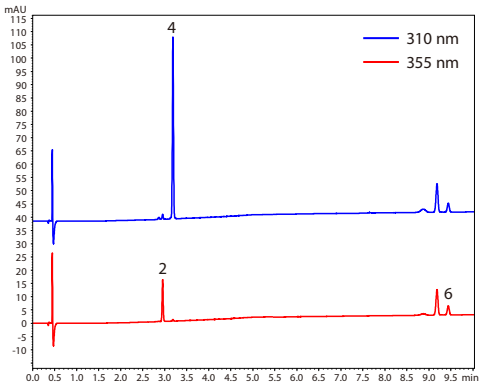

Fig. 3 Chromatogram of Foundation

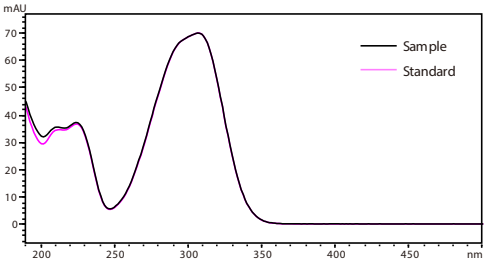

Fig. 4 Comparison of UV-Vis Spectra of Compound 4

Qualitative Analysis Based on UV-Vis Spectrum

In HPLC, retention time is used to qualify compounds, and for this reason, qualitative analysis is not a strong point of HPLC in comparison with NMR, LC-MS and similar techniques that can acquire information on the chemical structure. However, use of a photo diode array (PDA) detector makes it possible to obtain spectral information, and as a result, more reliable qualitative analysis also becomes possible with HPLC.

When the UV-Vis spectra of compounds are registered in the library file of the LabSolutions™ workstation manufactured by Shimadzu, it is possible to compare unknown spectra with the spectra in the library file. Moreover, quantitative evaluation using numerical values, and not simple visual comparison of the similarity between the spectra, is also possible.

The spectra of six UV-absorbents with concentrations of 100 mg/L were registered in the library, and were then compared with the spectra of 1 mg/L mixed standard solutions of each UV-absorbent and the foundation in the library. Table 3 shows the results. The spectra of each UV-absorbent at 1 mg/L shows high similarity with the 100 mg/L mixed solution, demonstrating that similar spectra can be obtained over a wide range of concentrations. Furthermore, because the spectra of the UV-absorbents contained in the foundation also displayed high similarity with the standard sample, it can be understood that using similarity is an effective technique for qualitative analysis.

Table 3 Comparison of Similarity of Spectra

No.	Similarity	
	Standard (1 mg/L)	Sample
1	0.999979	-
2	0.991281	0.998648
3	0.999114	-
4	0.999913	0.999988
5	0.999938	-
6	0.999957	0.999991

Similarities are calculated from the spectra from 230 nm to 500 nm.

Conclusion

This article introduced a qualitative analysis method based on the UV-Vis spectrum. Although this introduction is only a simple comparison of similarity, setting for automatic peak identification based on similarity and retention time is also possible. As demonstrated in this experiment, it is possible to enhance qualitative analysis capabilities by using a PDA detector.

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Application News

No. L549

High Performance Liquid Chromatography

Analysis of Cresol Positional Isomers by Using Shim-pack™ GIST Phenyl Column

Reversed phase chromatography (RPC), which is widely used in high performance liquid chromatography, is applicable to diverse compounds. The packing materials for columns used in RPC are substances that bond chemically with alkyl groups and other functional groups as a simple substance of silica gel. The octa decyl group (C18) and the octyl group (C8) may be mentioned as representative examples. The ODS (Octa Decyl Silyl) column is frequently used as the first choice when studying separation conditions, but separation of isomers is inadequate in some cases. Phenyl columns show different separation behavior from ODS columns. Due to the phenyl skeleton, in addition to the hydrophobic interaction, the π-π interaction also contributes to separation in the phenyl column, resulting in higher selectivity for aromatic compounds.

This article introduces an example of analysis of positional isomers of cresol by using a phenyl column that displays different separation characteristics from ODS columns among RPC columns.

A. Morita

Structures of Cresol Positional Isomers

Fig. 1 shows the structural formulas of cresol. Three types of positional isomers, ortho (*o*-), meta (*m*-), and para (*p*-), exist in cresol.

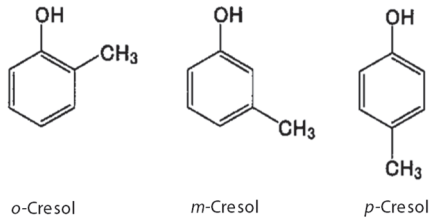


Fig. 1 Positional Isomers of Cresol

Features of Shim-pack Phenyl Column

Fig. 3 shows the lineup of the Shim-pack GIST Series and the features of the columns. The silica gel carrier of the Shim-pack GIST Series is high purity, completely porous spherical silica. Increased inertness not only improves analytical precision (peak shape), but also increases column durability. The Shim-pack GIST Phenyl column displays a π-π interaction owing to the phenyl skeleton. Because the phenyl group is bonded directly to the silica gel, the column has the characteristic that a larger difference in the electronic state of aromatic compounds can be recognized in comparison with general phenyl columns (alkyl phenyl group bonded columns).

Comparison of Separation by ODS Column and Phenyl Column

Fig. 2 shows the results of an analysis of standard solutions of *o*-, *m*-, and *p*-cresol (20 mg/L each) with a Shim-pack GIST Phenyl column and a Shim-pack GIST C18 column. Table 1 shows the analytical conditions. The linear velocity were matched and the organic solvent ratio was adjusted so that the retention time was approximately the same. The ODS column could not separate the *o*- and *m*-cresol. On the other hand, in the phenyl column, not only the hydrophobic interaction, but also the π-π interaction contribute to separation. As a result, selectivity for aromatic compounds is enhanced, and satisfactory separation could be achieved.

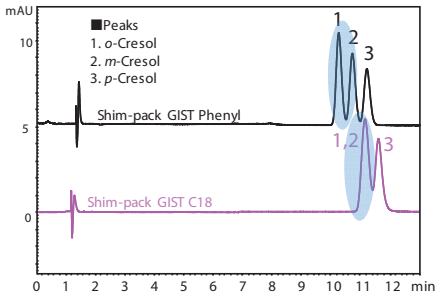


Fig. 2 Comparison of Chromatograms of Cresol Positional Isomers by Using Shim-pack GIST Phenyl (Top) and Shim-pack GIST C18 (Bottom)

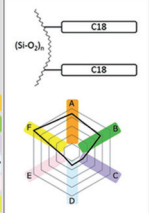
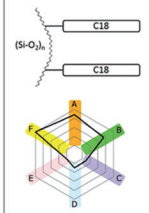
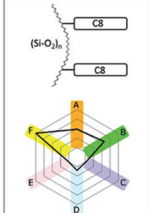
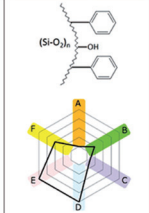
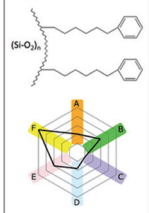
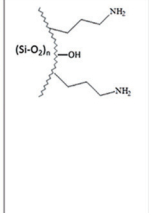
Solid phase	C18	C18-AQ	C8	Phenyl	Phenyl-Hexyl	NH2
A: Retentivity B: Hydrophobicity C: Stereoselectivity D: Hydrogen bond property E: π-π F: Inertness						
Functional group	Octadecyl group	Octadecyl group	Octyl group	Phenyl group	Phenylhexyl group	Aminopropyl group
Particle size	2μm, 3μm, 5μm	1.9μm, 3μm, 5μm	2μm, 3μm, 5μm	2μm, 3μm, 5μm	3μm, 5μm	3μm, 5μm
Pore size	10nm	10nm	10nm	10nm	10nm	10nm
Surface area	350m²/g	350m²/g	350m²/g	350m²/g	350m²/g	350m²/g
Carbon loading	14%	13%	8%	10%	9%	7%
End cap	Yes	Yes	Yes	No	Yes	No
Recommended pH range	1-10	1-10	1-10	2-7.5	1-10	2-7.5

Fig. 3 Lineup and Features of Shim-pack GIST Series

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Table 1 Analytical Conditions
(Top) Shim-pack GIST Phenyl, (Bottom) Shim-pack GIST C18

System	:	Nexera™-i
Column 1	:	Shim-pack GIST Phenyl (100 mm L. × 3.0 mm I.D., 2 μm)
Mobile Phase	:	Water / Methanol = 80/20 (v/v)
Flow Rate	:	0.4 mL/min
Column Temp.	:	40 °C
Injection Vol.	:	5 μL
Detection	:	UV-VIS detector (Nexera-i) at 254 nm
System	:	Nexera-i
Column 2	:	Shim-pack GIST C18 (100 mm L. × 2.1 mm I.D., 2 μm)
Mobile Phase	:	Water / Methanol = 70/30 (v/v)
Flow Rate	:	0.2 mL/min
Column Temp.	:	40 °C
Injection Vol.	:	2.5 μL
Detection	:	UV-VIS detector (Nexera-i) at 254 nm

Comparison of Organic Solvents by Using Phenyl Column

Methanol and acetonitrile are organic solvents that are frequently used as the mobile phase in RPC. Fig. 4 shows the chromatograms of standard solutions of *o*-, *m*-, and *p*-cresol (20 mg/L each) obtained by using a Shim-pack GIST Phenyl with methanol or acetonitrile as the mobile phase. Table 2 shows the analytical conditions.

Using methanol as the mobile phase facilitates the use of the π-π interaction. Acetonitrile (CH_3-CΞN) has a C-N triple bond and contains π electrons, whereas methanol (CH_3-OH) does not contain π electrons. The retentivity of methanol is greater than that of acetonitrile, as π electrons do not influence the π-π interaction between the solid phase phenyl group and the solute cresol when methanol is used.

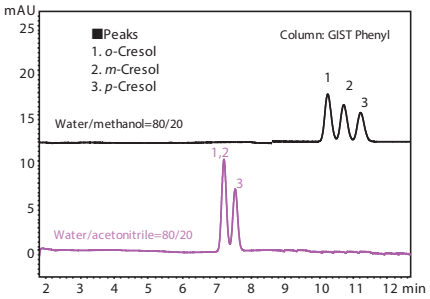

Fig. 4 Comparison of Chromatograms of Cresol Positional Isomers by Using Shim-pack GIST Phenyl
(Top) Methanol, (Bottom) Acetonitrile

Table 2 Analytical Conditions (Comparison of Organic Solvents)

System	:	Nexera-i
Column 1	:	Shim-pack GIST Phenyl (100 mm L. ×3.0 mm I.D., 2 μm)
Mobile Phase	:	Water / Acetonitrile = 80/20 (v/v), Water / Methanol = 80/20 (v/v)
Flow Rate	:	0.4 mL/min
Column Temp.	:	40 °C
Injection Vol.	:	5 μL
Detection	:	UV-VIS detector (Nexera-i) at 254 nm

Linearity of Calibration Curves

Fig. 5 shows calibration curves analyzed under the conditions of Table 1 (top). The calibration curves were prepared in the range of 5 to 20 mg/L. Satisfactory linearity with a contribution ratio $r^2 = 0.999$ or higher was obtained for all components.

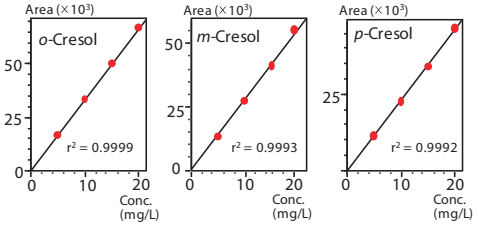

Fig. 5 Calibration Curves
Left: *o*-Cresol, Center: *m*-Cresol, Right: *p*-Cresol

Analysis of Cresol in Lignin Extracts

This section introduces an example of analysis of cresol in lignin extracts. Lignins remain as a residue component when cellulose and hemicellulose are removed from wood. Until now, lignins had been treated as a waste or used as a fuel. However, in recent years, lignophenol [*1], using cresol and other phenolic compounds obtained from lignins as plant biomass, has attracted attention as a basic material [(1)]. In the future, lignophenol is expected to be used as a substitute material for petroleum-derived plastics, which accelerate global warming. Fig. 6 shows the chromatograms obtained by analysis of lignin extracts A [*2] and B [*3] with standard addition of *o*-, *m*-, and *p*-cresols to the samples after 100× dilution with the mobile phase and filtration.

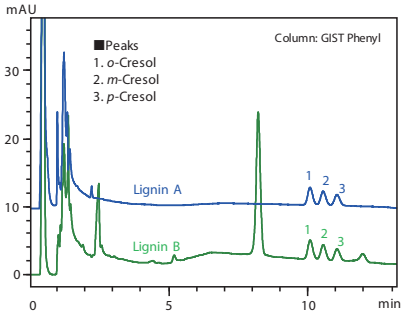

Fig. 6 Chromatograms of Lignin Extracts A and B
(Standard Addition of Cresol)

Conclusion

Shim-pack GIST Phenyl displays a π-π interaction owing to its phenyl ring structure, and is an effective choice as a column when the separation performance of ODS columns is inadequate. Effective use is expected, including use as a column in method scouting for comprehensive study of separation conditions.

*1 Lignophenol: A new chemical substance developed as a bioplastic raw material in the 1990s by Prof. Emeritus Masamitsu Funaoka of Mie University.
*2 Extract obtained by treating wood from needle- and broad-leaved trees with sodium sulfite.
*3 Liquid obtained from extract in *2 by further desulfonation (partial), oxidation, hydrolysis, demethylation, and adjustment to alkalinity.

References

(1) Shin-Kobe Technical Report, No. 17 (2007-2) Effects of Molecular Structures of Lignins on the Performance of Negative Electrodes in Lead-Acid Batteries: Hitachi Chemical Co., Ltd. (Japanese)

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First Edition: Sep. 2019

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Application News

High Performance Liquid Chromatography

No. L557

Analysis of Formaldehyde Using HPLC and Post-Column Derivatization with Acetylacetone

Formaldehyde is a useful ingredient for wood preservatives and resin products, but because it is a causative agent of sick house syndrome, it has attracted attention.

Shampoos, skin lotion, and foundation products used in everyday life are generally called cosmetics, and the ingredients used in them are subject to particularly strict regulation because they are applied to the human body. Japan's Standards for Cosmetics (Ministry of Health and Welfare Notification No. 331, 2000) list formaldehyde as one material which is prohibited to include in cosmetics. Moreover, in the EU, the content of formaldehyde in nail polish and other nail products is limited to no more than 5% under Regulation (EC) No. 1223/2009, Annex III.

In this article, formaldehyde in cosmetics was detected using the HPLC method and the post-column derivatization with acetylacetone, which is an established test method under the Methods of Analysis in Health Science (The Pharmaceutical Society of Japan, 2015).

This article introduces examples of analysis of formaldehyde using a Nexera™ Series Nexera XR ultra high performance liquid chromatograph.

M. Hayashida, A. Morita

System and Analysis Conditions

The Shim-pack™ GIST C18-AQ column used in this experiment achieves strong retention of high-polarity compounds such as formaldehyde, compared to general ODS column, and thus can maintain good retention and a superior peak shape in highly or 100% aqueous mobile phases.

Fig. 1 shows the flow path diagram of the system used in this analysis, and Fig. 2 shows the appearance of the system. Formaldehyde is detected by a process of separation with an ODS reversed-phase column, followed by online reaction with acetylacetone at 90 °C, and selective detection of the reaction product (3,5-diacetyl-1,4-dihydrolutidine) by using a PDA detector (414 nm). Table 1 shows the analysis conditions. Because temperature control up to 100 °C is possible with the CTO-40C column oven of the Nexera XR used in the analysis, the oven could be converted to use as a chemical reactor under the conditions of this analysis. Although the conventional chemical reactor (CRB-6A) can not be controlled from a workstation, the CTO-40C can. Integrated management of the CTO-40C such as setting the reaction temperature, logging the oven temperature, and the use time of consumables is performed by the workstation.

Table 1 Formaldehyde Analysis Conditions

	System	: Nexera XR
■	Separation	
	Column	: Shim-pack™ GIST C18-AQ (150 mmL. × 4.6 mm I.D. ; 5 µm)
	Mobile Phase	: 6 mmol/L Na_2HPO_4 (pH=2.1)*1
	Flow Rate	: 1.0 mL/min
	Column Temp.	: 30 °C
■	Injection Vol.	: 10 µL
■	Post Column Derivatization	
	Reaction Reagent	: Solution of acetyl acetone *2
	Flow Rate	: 0.5 mL/min
	Reaction Temp.	: 90 °C
■	Detection	: SPD-M40 at 414 nm

*1 Using phosphoric acid, adjust to pH = 2.1.

*2 Adjust to a constant volume of 1,000 mL while dissolving 150 g of ammonium acetate, 3 mL of acetic acid, and 2 mL of acetylacetone in ultrapure water.

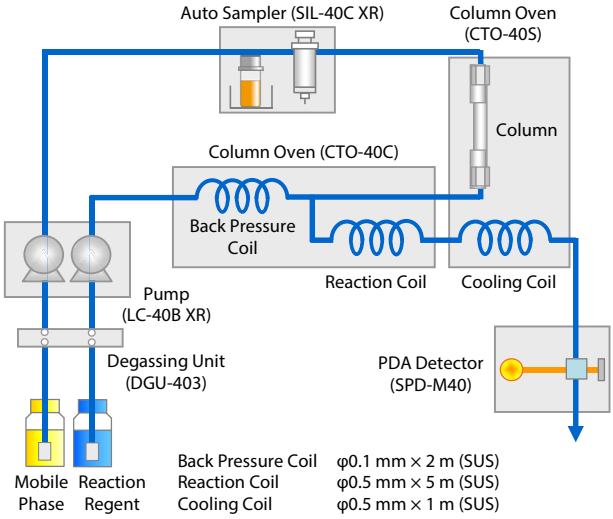

Fig. 1 Flow Path Diagram of Analytical System

Fig. 2 Image of Appearance of Analytical System

Derivatization of Formaldehyde by Acetylacetone

Formaldehyde was reacted with two molecules of acetylacetone in the presence of ammonium acetate and formed one molecule of 3,5-diacetyl-1,4-dihydrolutidine, as shown in Fig. 3. The analysis was conducted using this reaction product (derivative).

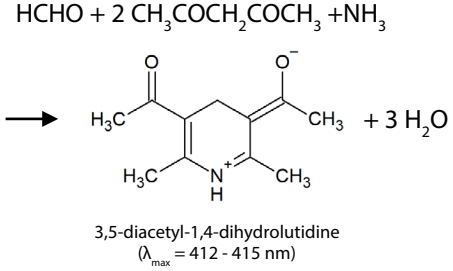

Fig. 3 Reaction of Formaldehyde and Acetylacetone

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Analysis of Formaldehyde Standard Solution

Fig. 4 shows the chromatogram obtained by analysis of the standard solution of formaldehyde. A peak corresponding to 3,5-diacetyl-1,4-dihydrolutidine, which was derivatized from formaldehyde, was detected at around 2.8 min. The formaldehyde standard solution was adjusted by diluting 100 mg/L (water medium) of the standard with ultrapure water. Because the mobile phase contains no organic solvents, the peak shape may be degraded when methanol, acetonitrile, or the other organic solvents were added to the standard solution.

Fig. 5 shows the calibration curve prepared with the formaldehyde standard solution for 5 points in the concentration range from 0.01 to 1.0 mg/L. Although preparation of a calibration curve for the concentration range of 1 to 4 mg/L is specified in the cosmetic test method, in this experiment the calibration curve was prepared for a lower concentration range than in that test method in order to quantify formaldehyde at the trace level.

Good linearity was obtained, as the coefficient of determination r^2 = 0.9999 or higher.

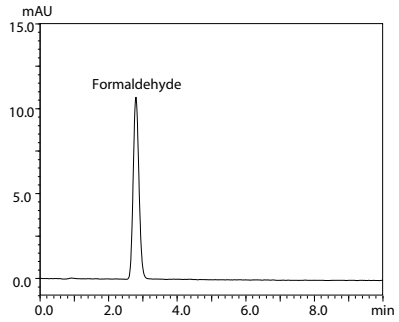

Fig. 4 Chromatogram of Formaldehyde Standard Solution (1.0 mg/L)

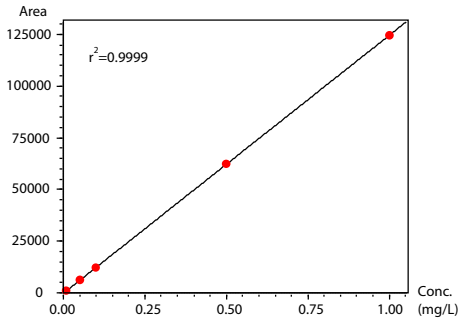

Fig. 5 Calibration Curve of Formaldehyde Standard Solution

Analysis of Formaldehyde in Shampoo, Conditioner, and Skin lotion

A shampoo, conditioner, and skin lotion which are sold commercially in Japan were used as cosmetics. The analysis was carried out after extracting samples from these 3 products with water. As mentioned above, use of formalin in cosmetics that are manufactured, sold, or distributed domestically in Japan is prohibited.

Fig. 6 to Fig. 8 show the chromatograms of the solutions extracted by pretreatment of the shampoo, conditioner, and skin lotion, together with the chromatograms of samples obtained by spiking those solutions with formaldehyde to a concentration of 0.1 mg/L. Table 2 shows the results of the spike and recovery tests. In all the samples of shampoo, conditioner, and skin lotion, the formaldehyde concentration was below the minimum concentration (1 mg/L) of the calibration point specified in the cosmetic test method.

In the pretreatment, 100 mL of ultrapure water was added to 1g of each sample, and after stirring, the solutions were filtered with a membrane filter (0.45 µm).

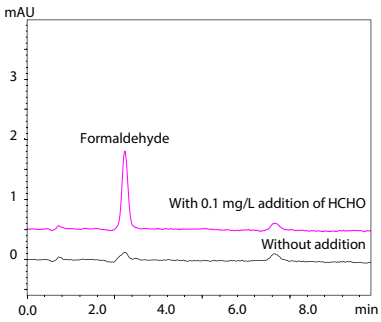

Fig. 6 Chromatogram of Commercially Available Shampoo

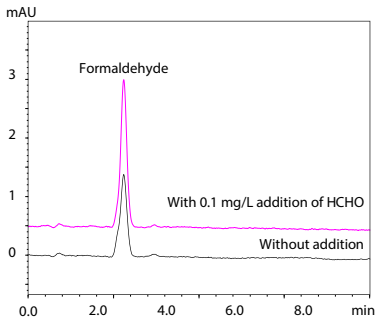

Fig. 7 Chromatogram of Commercially Available Conditioner

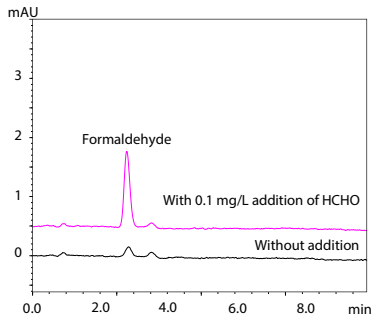

Fig. 8 Chromatogram of Commercially Available Skin lotion

Table 2 Concentrations of Formaldehyde in Shampoo, Conditioner and Skin lotion, and Recovery Rates of Each Pretreatment

Product	Formaldehyde (mg/L)	Recovery Rate (%)
Shampoo	0.025	101
Conditioner	0.167	104
Skin lotion	0.018	109

Conclusion

Formaldehyde was measured by the HPLC method and the post-column derivatization with acetylacetone using a Nexera XR ultra high performance liquid chromatograph. As detection by a wavelength with high selectivity was possible by derivatizing formaldehyde, the analysis was substantially unaffected by impurities in the sample.

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Application News

No. L560

High Performance Liquid Chromatography

Improvement of Peak Shape in Analysis of Basic Compounds and Reduction of Carryover by Multi-Rinse Function

Frequent hand disinfection is an effective measure against the spread of seasonal influenza. The main component of many hand disinfectants is ethanol, and some of them contain bactericidal components as well. The effective components in commercially-available disinfectants include chlorhexidine and benzethonium, both of which are strongly basic compounds.

When basic compounds are analyzed with a typical C18 column, tailing or other deterioration of the peak shape may occur due to the nonspecific interaction with remaining silanol on the surface of the packing materials. Because the Shim-pack Arata™ C18 is especially designed to suppress the interaction with silanol, a good peak shape can be expected when analyzing basic compounds, even if using a simple composition of mobile phase.

It is known that chlorhexidine is a compound that is extremely easily absorbed on the wetted parts of autosamplers, and for this reason, the results of subsequent analyses may be influenced by chlorhexidine carryover, particularly after analyzing a high concentration sample. The Nexera™ X3 system is equipped with multi-rinse function as a standard to reduce carryover by automatically rinsing the injection port, where carryover frequently occurs.

This article introduces an analysis of the two above-mentioned basic compounds using Nexera X3 and Shim-pack Arata C18, together with reduction of carryover by multi-rinse function.

Y. Zhou

Table 1 Analytical Conditions

System	: Nexera X3
Column	: Shim-pack Arata C18 (100 mm × 3.0 mm I.D., 2.2 µm) Typical C18 column (100 mm × 3.0 mm I.D., 2.2 µm)
Mobile phase	: A) 0.1% Formic acid in water B) 0.1% Formic acid in acetonitrile
Flow rate	: 0.7 mL/min
Column temp.	: 40 °C
Injection volume	: 1 µL
Detection	: UV 258 nm

Table 2 Time Program

Time (min)	A. Conc	B. Conc
0	95	5
3	50	50
6	50	50
6.01	95	5
10	95	5

Analysis of Chlorhexidine and Benzethonium

Fig. 1 shows the chromatograms when 1 µL of mixed standard solution of chlorhexidine diacetate (50 mg/L) and benzethonium chloride (200 mg/L) was injected, and Tables 1 and 2 show the analytical conditions. Typical C18 column and Shim-pack Arata C18 were used in this experiment. Table 3 shows the symmetry factor when the mixed standard solution was analyzed using these columns.

The symmetry factors using Shim-pack Arata C18 were 1.14 for chlorohexidine and 0.98 for benzethonium, whereas those obtained with typical C18 column were 1.85 for chlorohexidine and 2.17 for benzethonium. Thus, in comparison with the typical C18 column, better peak symmetry was obtained when these basic compounds were analyzed using the Shim-pack Arata C18.

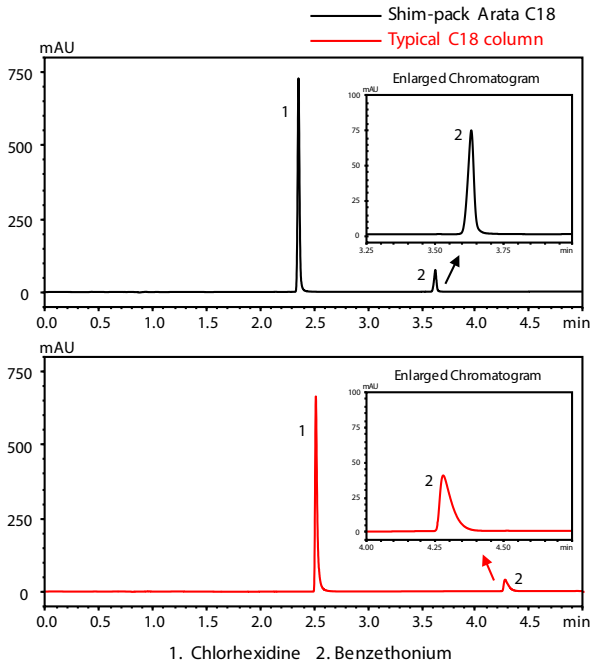

Fig. 1 Chromatograms of Mixed Standard Solution of Chlorhexidine Diacetate (50 mg/L) and Benzethonium Chloride (200 mg/L)

Table 3 Comparison of Symmetry Factors

Compound	Shim-pack Arata C18	Typical C18 column
Chlorhexidine	1.14	1.85
Benzethonium	0.98	2.17

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Reduction of Carryover by Multi-Rinse Function

Multi-rinse function makes it possible to rinse the external surface of the needle with a maximum of 2 rinse solutions and the internal surface of the needle with a maximum of 3 rinse solutions, and the rinse sequence can be set as desired. Although carryover occurs easily at the injection port, automatic rinsing of this part is also possible by multi-rinse function.

Here, we compared two carryovers. One was through the conventional rinse (only external rinse of the needle), the other was through the injection port rinse contained in the multi-rinse function. Water was immediately injected as blank after injection of the standard solution, and carryover was calculated from the respective peak areas. The rinse solutions used here were a mixture of water and acetonitrile, and an acetonitrile solution containing 0.1% formic acid.

Tables 4 and 5 show the analytical conditions. Fig. 2 shows the screen capture of settings on LabSolutions™ when multi-rinse function is used. Fig. 3 shows the chromatograms when 5 µL of blank was injected following a standard solution of chlorhexidine diacetate (10,000 mg/L) for the case of only using external rinse of the needle. Fig. 4 shows the chromatograms with multi-rinse function. Table 6 shows the result of carryover for both cases. With the settings shown in Fig. 2, in addition to needle external rinse, the internal surface of the needle and the injection port are also rinsed in the sequence of rinse solution R2, R1 after the analysis is completed. Following these rinsing operations, the solvent in the sample loop is replaced with R0 and equilibration for the next analysis is conducted for a hold time of 1 min.

Table 4 Analytical Conditions

System	:	Nexera X3
Column	:	Shim-pack Arata C18 (100 mm × 3.0 mm I.D., 2.2 µm)
Mobile phase	:	A) 0.1% Formic acid in water B) 0.1% Formic acid in acetonitrile
Flow rate	:	0.7 mL/min
Column temp.	:	40 °C
Injection volume	:	5 µL
Detection	:	UV 258 nm
Rinse solution R0	:	Water/acetonitrile=50/50 (v/v)
Rinse solution R1	:	Water/acetonitrile=50/50 (v/v)
Rinse solution R2	:	0.1% Formic acid in acetonitrile

Table 5 Time Program

Time (min)	A. Conc	B. Conc
0	80	20
2	80	20
2.01	60	40
3	60	40
3.01	80	20
6	80	20

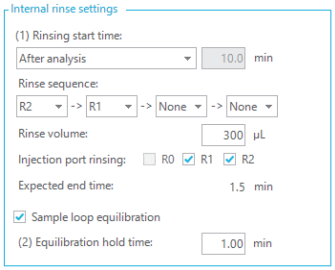

Fig. 2 Screen Capture of Needle Internal Rinse Settings (R0, R1, and R2: Types of Rinse Solutions)

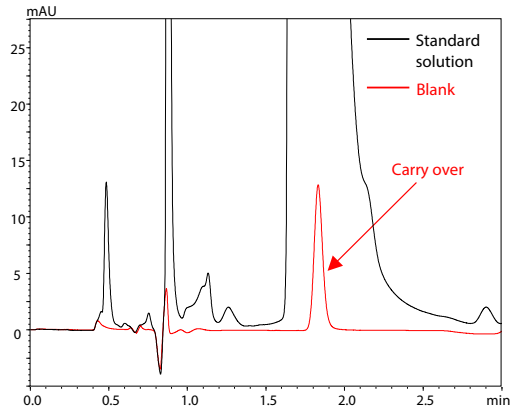

Fig. 3 Chromatograms When Only Using Needle External Rinse

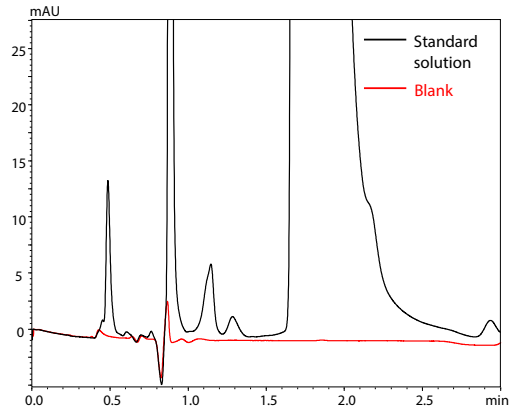

Fig. 4 Chromatograms When Using Multi-Rinse Function

The carryover when only using needle external rinse after injection of the standard solution of 10,000 mg/L of chlorhexidine diacetate was 2.31 mg/L, which is equivalent to 0.0231% of the standard solution. On the other hand, although the carryover when using needle internal/external rinse and injection port rinse was below the limit of detection, the reference concentration was 0.01 mg/L, which is equivalent to 0.0001% of the standard solution. Thus, carryover can be reduced to approximately 1/230 by multi-rinse function.

Table 6 Comparison of Carryovers

Rinse method	Concentration of carryover (mg/L)	Carryover (%)
Needle external rinse	2.31	0.0231
Needle internal/external rinse and injection port rinse	0.01	0.0001

Conclusion

When analyzing basic compounds with a simple composition of mobile phase such as 0.1% formic acid aqueous solution, an improved peak shape can be obtained by using Shim-pack Arata C18 column in comparison with that from typical C18 column. In addition, carryover can be greatly improved by multi-rinse function of Nexera. As demonstrated here, this function is a particularly useful for reducing carryover when high concentration samples are injected.

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Application News

No. L536

High Performance Liquid Chromatography

Analysis of Nucleic Acid Related Substances in Fish Meat and Automatic Calculation of Freshness (K Value) Using Multi-Data Report Function

Compared to the muscle tissue of livestock animals, the flesh of fish or shellfish is known to decay more rapidly due to its softness and high water content. Accurately determining the freshness of these fish and shellfish is extremely important in terms of food safety and security.

Changes in the concentration of ATP (adenosine triphosphate) in animals' muscles are widely used as an indicator of muscle freshness. Moreover, the so-called K-value is often used as a method for the freshness evaluation of fish.

In recent years, cases have been reported of allergies developing from histamine food poisoning. When a red fish such as tuna decays, histamine (a metabolite of histidine, which is a kind of amino acid) accumulates in high concentrations. Although it is possible to detect histamine using HPLC, the pretreatment (derivatization) is complicated, and a large system is required for automatic pretreatment. Therefore, it is useful to be able to measure the state of decay simply by analyzing ATP-related compounds using the Nexera™ LC system with a simple configuration.

In this report, we use the K-value to determine the freshness of tuna from HPLC analysis and demonstrate the use of the multi-data report function.

N. Iwata

Analysis of ATP-Related Compounds

A standard solution (10 µmol/L) of ATP-related compounds (hypoxanthine (Hx), inosine (HxR), IMP, AMP, ADP, ATP) *1 was analyzed. The chromatogram is shown in Fig. 1, and the analytical conditions are shown in Table 1.

A Shim-pack GIST C18 AQ column was used for chromatographic separation. This column shows high performance and durability even when a mobile phase with a composition close to 100 % aqueous solution is used.

Nucleobases and nucleotides represented by ATP-related substances are often separated by isocratic elution using a phosphate buffer, therefore it takes long time to analyze these compounds.

In this paper, the separation of these compounds was accomplished by gradient elution using mobile phase added ion pair reagent in order to retain a compound having phosphate group, and analytical time was shortened. In addition, in order to prevent pressure increase at the column due to clogging of contaminating compounds in sample, a column washing step was added.

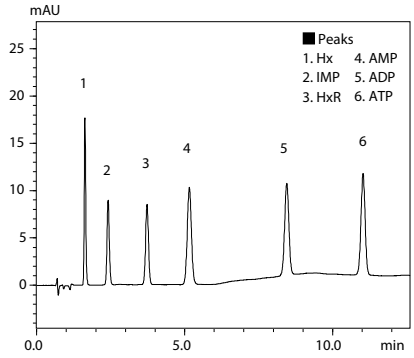

Fig. 1 Standard Solution of ATP-Related Compounds

Table 1 Analytical Conditions

Column	Shim-pack™ GIST 3 µm C18 AQ (100 mm L. × 3.0 mm I.D., 3 µm)
Flow rate	: 0.8 mL/min
Mobile phase *2	: A) Water/ acetonitrile=100/1 (v/v) containing 0.15 mol/L phosphoric acid, 0.225 mol/L triethylamine B) Water/ acetonitrile=80/20 (v/v) containing 0.15 mol/L phosphoric acid, 0.225 mol/L triethylamine
Time Program	: 0 %B (0-3.5 min) → 12 %B (11 min) → 100 %B (11.01- 18 min) → 0 %B (18.01-28 min)
Column temp.	: 30 °C
Injection volume	: 10 µL
Detection	: PDA 260 nm

*1: IMP : Inosine 5′-monophosphate, AMP : Adenosine 5′-monophosphate, ADP : Adenosine 5′-diphosphate, ATP : Adenosine 5′-triphosphate

*2: Phosphoric acid: 10.2 mL, Triethylamine: 31 mL } → mixture of Water/ Acetonitrile 1 L

Reproducibility

Table 2 shows the coefficient of variation (% RSD) of retention time and peak area over 6 repeats for each ATP-related compound. The coefficient of variation was <1% for both retention time and area value of every compound in the analysis.

Table 2 Coefficient of Variation (% RSD) (n=6)

Compounds	Retention time	Area
Hx	0.14	0.33
IMP	0.00	0.21
HxR	0.09	0.20
AMP	0.04	0.32
ADP	0.05	0.50
ATP	0.05	0.68

Estimation of the Freshness of Fish

Both fish and shellfish use ATP as the energy source in their muscle tissue. However, ATP is no longer synthesized after death, so the ATP concentration in the muscle tissue progressively decreases via enzymatic degradation in the following path: ATP → ADP → AMP → IMP → HxR → Hx.

The K-value of fish and shellfish muscles is defined as the percentage of the sum of the compounds that do not contain phosphoric acid (hypoxanthine, inosine) over the total amount of ATP-related components.

$$K = \frac{Hx + HxR}{Hx + HxR + IMP + AMP + ADP + ATP} \times 100$$

The above formula shows that smaller K-value is directly related to the freshness of the muscle tissue. The K-value is affected by various factors (storage temperature, time in storage etc.).

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Calibration Curve

Calibration curves were prepared for the 6 ATP-related compounds. Good linearity was obtained with $R^2 = 0.9999$ or greater for each component (Table 3).

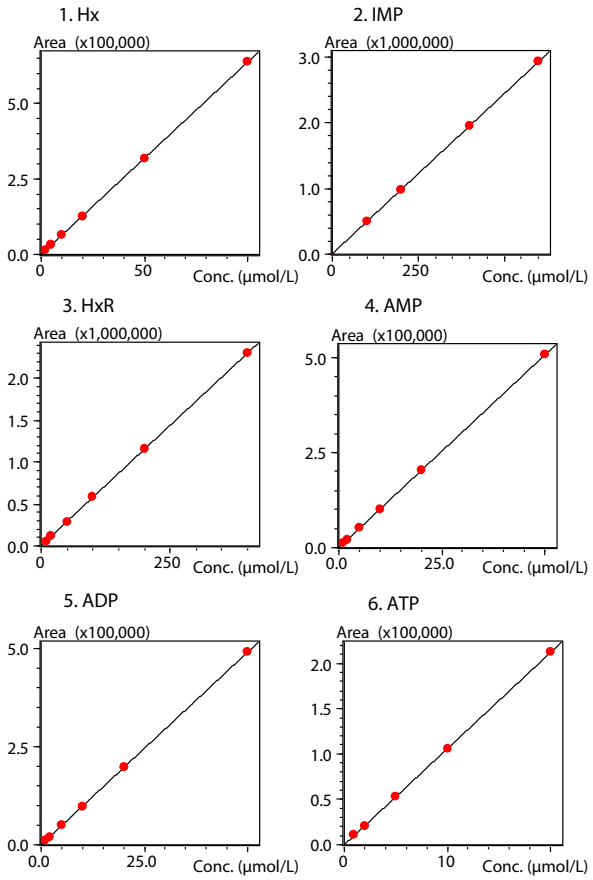

Fig. 2 Calibration Curves for ATP-Related Compounds

Table 3 Calibration Curve Concentration Range (μmol/L) and R^2 Value for Each ATP-Related Compound

Compounds	Conc. Range (μmol/L)	R^2
Hx	2-100	0.9999972
IMP	100-600	0.9999905
HxR	10-400	0.9999822
AMP	1-50	0.9999917
ADP	1-50	0.9999960
ATP	1-20	0.9999931

Sample Preparation

For our samples, we used commercially-available fresh albacore tuna and yellowfin tuna thawed from frozen. The sample preparation protocol was previously described in "Comparison of freshness changes in fresh and frozen black marlin via K-value", 2012 [1].

The homogenized sample was extracted with 10 % perchloric acid and then extracted twice with 5 % perchloric acid. The supernatant of the three extracts was ice-cooled, neutralized with KOH solution (10 N, 1 N, 0.1 N), and filtered prior to HPLC injection (Fig. 3).

Tuna Freshness Measurement

We analyzed samples immediately after purchase and after 1 to 3 days in order to measure changes in K-value. Fig. 4 shows the chromatograms for the fresh albacore tuna, and Fig. 5 shows the chromatograms for the yellowfin tuna. The lower diagrams in Fig. 4 and 5 show enlarged view.

Table 4 shows the change in K-value over time and the quantitative value (μmol/L) of each component.

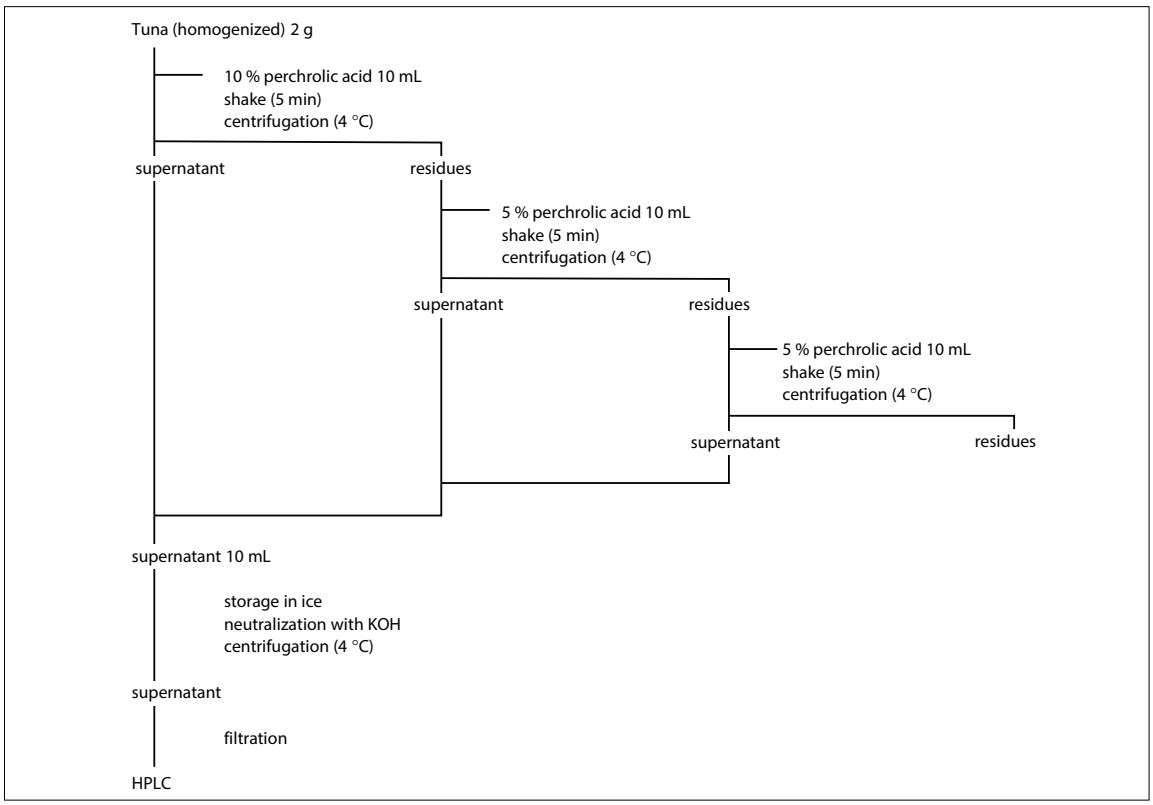

Fig. 3 Sample Preparation Procedure

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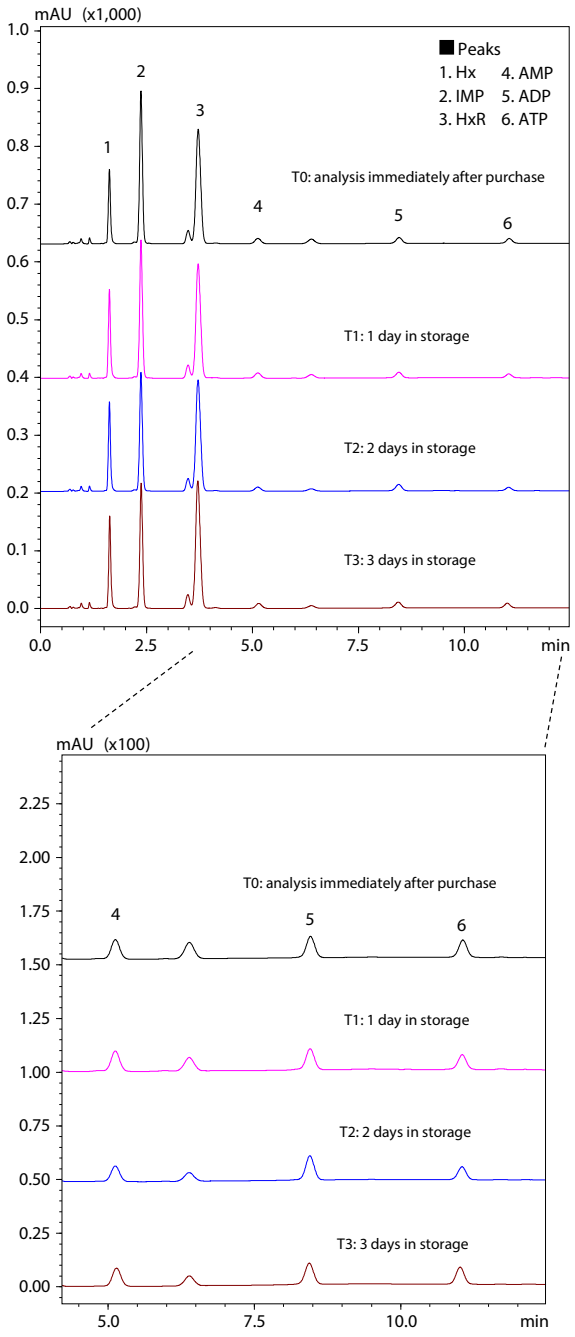

Fig. 4 Chromatograms of Fresh Albacore Tuna

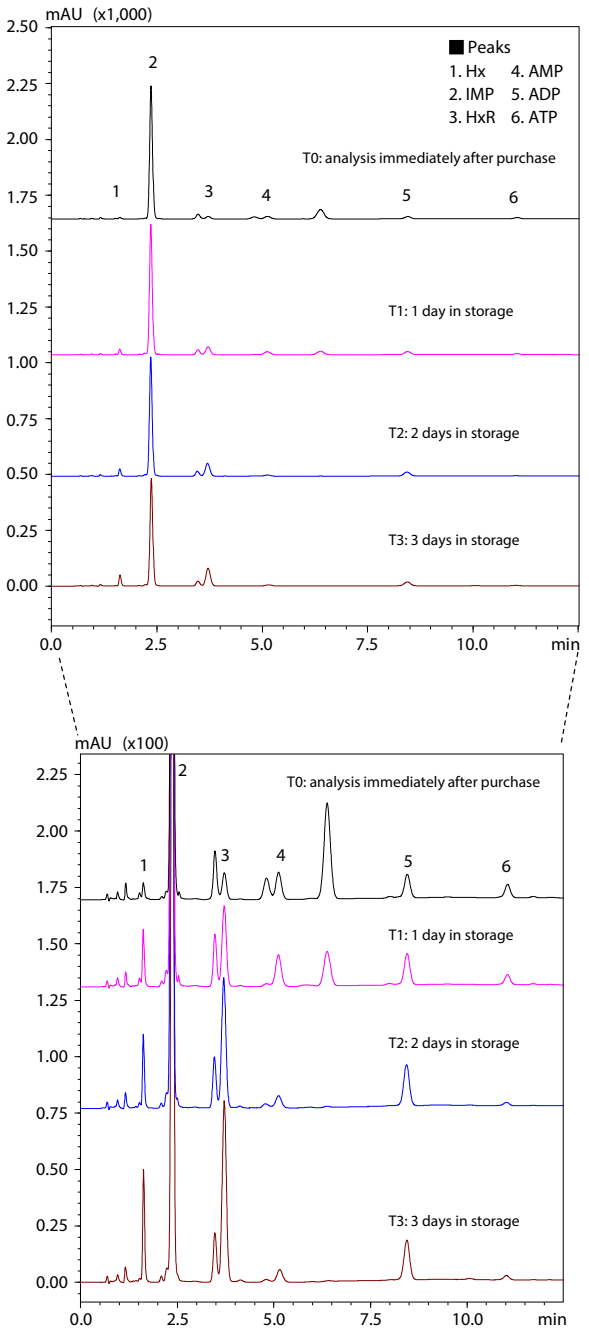

Fig. 5 Chromatograms of Yellowfin Tuna Thawed from Frozen

Table 4 Change in K-Value and Quantitative Value (μmol/L) for Each Component.

Albacore Tuna

days	K value (%)	Quantitative value (μmol/L)					
		Hx	IMP	HxR	AMP	ADP	ATP
0	53.8	68.970	252.281	256.739	9.052	10.552	7.882
1	56.9	83.502	229.872	254.640	9.286	9.962	6.728
2	60.2	83.825	195.520	249.527	7.197	12.054	5.841
3	61.5	87.607	203.348	279.969	8.561	10.889	7.659

Yellowfin Tuna

days	K value (%)	Quantitative value (μmol/L)					
		Hx	IMP	HxR	AMP	ADP	ATP
0	2.5	3.619	582.971	12.103	10.156	10.657	5.724
1	8.7	12.794	559.034	43.472	13.641	14.310	4.413
2	14.5	17.020	496.849	71.427	4.992	19.459	1.574
3	20.9	26.589	451.304	99.401	5.299	18.558	1.926

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Durability of the Column

The durability of the Shim-pack GIST C18 AQ column used for this report was evaluated. It was confirmed that column loading pressure (maximum pressure) does not rise even after 300 injections of tuna samples.

The theoretical plate number and symmetry coefficient before the 1st injection and after 300 injections of tuna sample were compared. There were no significant deterioration for both parameters (less than 10 %).

Table 5 summarizes the results for column load pressure (maximum pressure), theoretical plate number, and symmetry coefficient, comparing the status before the first injection and after the 300th injection.

Table 5 Reduction Rate of Each Parameter Before Actual Sample Injection and After 300 Injections.

Pmax. (MPa)

	Number of Injections		Decreasing rate (%)
	0	After 300	
Pmax.	22.15	22.15	0

Theoretical plate, N

	Number of Injections		Decreasing rate (%)
	0	After 300	
Hx	7692	7413	3.63
IMP	5692	5256	7.66
HxR	7679	7416	3.42
AMP	7057	6410	9.17
ADP	17048	16408	3.75
ATP	31533	28970	8.13

Symmetry

	Number of Injections		Deterioration rate (%)
	0	After 300	
Hx	1.165	1.163	-0.17
IMP	1.021	1.097	7.44
HxR	0.99	1.036	4.65
AMP	0.997	1.054	5.72
ADP	0.97	0.994	2.47
ATP	0.971	0.996	2.57

Use of Multi-Data Report Function *3

Multi-data report is one of the reporting functions that automatically inserts analytical results into a spreadsheet file. As soon as the batch analysis is finished, the results are automatically updated and reported. This function can be used also for data post-processing, generating a report from previously acquired data.

In this case, we created a multi-data report automatically from the tuna K-value measurement results (Fig. 6). By automatically generating a graph, it was possible to monitor the change in freshness of the tuna samples. The K-value of the fresh albacore tuna was 53.8 % on the day of purchase (T0). However, after two days of refrigerated storage (T48h), the K-value increased to >60 % due to the decay process. The thawed yellowfin tuna had a K-value of 2.5 % on the day of purchase (T0), suitable to be eaten raw. However, after three days of refrigerated storage (T72h), the K-value was >20 %, and no more suitable for raw consumption.

*3: The multi-data report function is supported by LabSolutions™ DB/CS

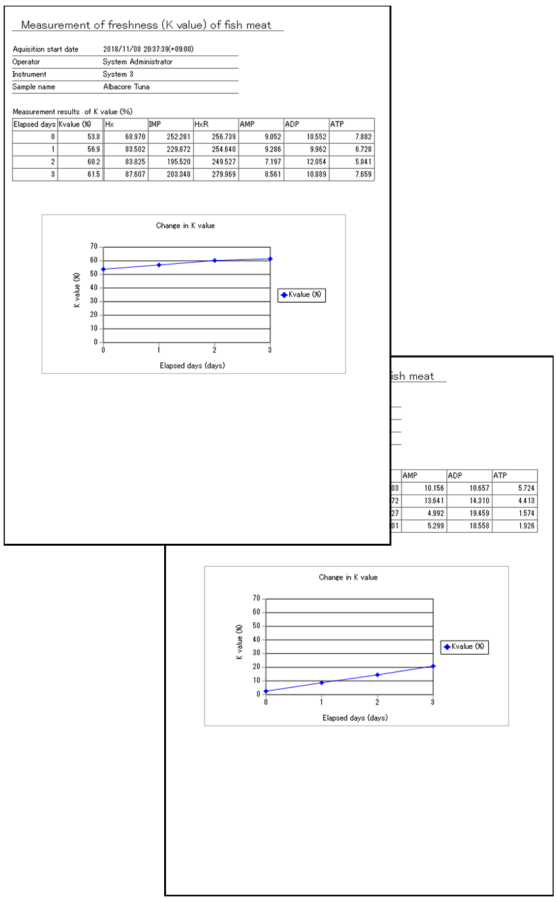

Fig. 6 Multi-Data Report Showing the Results of the Tuna Freshness Measurements

[References]

1) Usui Shigeru, Watanabe Etsuo, "Comparison of freshness changes in fresh and frozen black marlin via K-value", 2012. (Japanese only)

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First Edition: Apr. 2019

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Application News

No. L543

High Performance Liquid Chromatography

High-Speed Analysis of Methylated Catechin in Benifuuki Green Tea

It has been reported that the functional food Benifuuki green tea contains a large amount of methylated catechin as an antiallergic functional ingredient. [1] Methylated catechin is a kind of polyphenol which is contained in tea leaves, and is a partially methylated form of epigallocatechin gallate. It has also been reported that methylated catechin alleviates the discomfort of the eyes and nose caused by house dust and other forms of dust.

In 2018, "Determination of the O-methylated Catechin in 'Benifuuki' Green Tea (*Camellia sinensis L.*) - High-performance liquid chromatographic method" [2] was established as a Japanese Agricultural Standard (JAS) based on Japan's Act on Standardization and Proper Quality Labeling of Agricultural and Forestry Products (JAS Law).

This article introduces applications of analysis of methylated catechin in Benifuuki green tea leaves based on the JAS method and its high-speed analysis, using a Nexera™ X3 and Shim-pack™ GIST Series columns.

N. Iwata

Analysis of Methylated Catechin Standard Solution

A methylated catechin ((-) - epigallocatechin 3-(3″-*O*-methyl) gallate (EGCG3″Me)) standard solution (10 mg/L) was analyzed. Tables 1 and 2 show the analytical conditions for the JAS method and the high-speed analysis, respectively. The upper part of Fig. 1 shows the chromatogram using a Shim-pack GIST C18 analytical column (particle size: 5 µm) according to the JAS method, and the bottom portion shows that using a Shim-pack GIST-HP C18 column (particle size: 3 µm) for high-speed analysis in the same Shim-pack Series.

Analysis time and mobile phase consumption could be reduced to approximately 1/4 respectively using GIST-HP C18 column with the particle size of 3 µm.

Table 1 Analytical Conditions of JAS Method-Compliant

System	:	Nexera X3
Column 1	:	Shim-pack GIST C18 (150 mm L×4.6 mm I.D., 5 µm)
Flow rate	:	1.0 mL/min
Mobile phase	:	A) 0.2% Phosphoric acid in water B) Methanol/Acetonitrile=18/5 (v/v)
Time Program 1	:	23%B (0-12 min) →70%B (12.01-20 min) → 23%B (20.01-30 min)
Column temp.	:	40 °C
Injection volume	:	10 µL
Detection	:	UV 272 nm

Table 2 Analytical Conditions of High-Speed Analysis

System	:	Nexera X3
Column		Shim-pack GIST-HP C18 (75 mm L×3.0 mm I.D., 3 µm)
Flow rate	:	1.0 mL/min
Mobile phase	:	A) 0.2% Phosphoric acid in water B) Methanol/Acetonitrile=18/5 (v/v)
Time Program	:	23%B (0-3 min) →70%B (3.01-5 min) → 23%B (5.01-7.5 min)
Column temp.	:	40 °C
Injection volume	:	4 µL
Detection	:	UV 272 nm

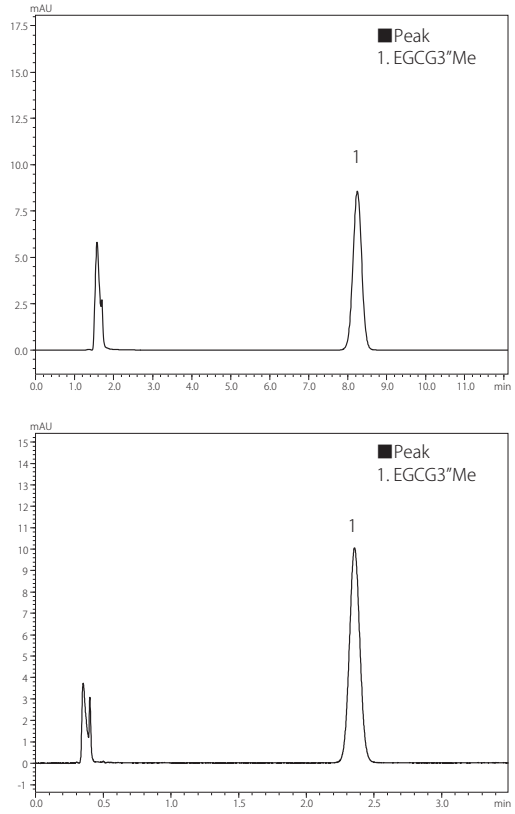

Fig. 1 Chromatograms of Methylated Catechin Standard Solution
Upper: JAS Method-Compliant Analysis,
Bottom: High-Speed Analysis

Calibration Curves

Fig. 2 shows the calibration curves for 1 to 50 mg/L in the respective analyses. Satisfactory linearity was obtained in both cases, with the contribution ratio $R^2 = 0.99999$ or more.

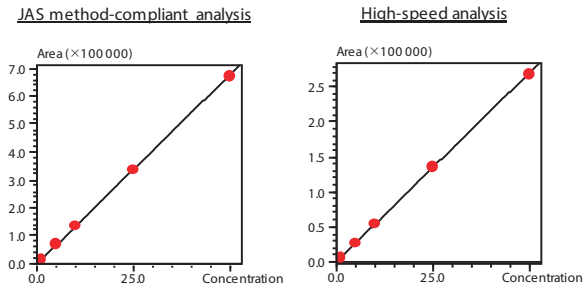

Fig. 2 Calibration Curves
Left: JAS Method-Compliant Analysis,
Right: High-Speed Analysis

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Reproducibility

Table 3 shows the coefficients of variation (%RSD) for retention time and area in analyses repeated six times using 1 mg/L of the standard solution, which is the lowest concentration of the calibration curve. In both analyses, coefficients of variation of 1% or less were obtained for both retention time and area.

Table 3 Coefficients of Variation in Analyses Repeated Six Times (%RSD)

Analytical condition	Column	Retention time	Area
JAS method-compliant	Shim-pack GIST C18 (particle size: 5 µm)	0.05	0.79
High-speed analysis	Shim-pack GIST-HP C18 (particle size: 3 µm)	0.02	0.53

Analysis of Benifuuki Green Tea

The test specimen was a commercially-available Benifuuki green tea. Pretreatment was carried out referring to the JAS method. [2),(3)]

Fig. 3 shows the pretreatment protocol. Extraction from the powdered specimen was performed using a mixed solution of phosphoric acid and ethanol at 30 °C. Next, extracts were filtrated with a filter paper and a 0.2 µm membrane filter, and diluted 10 times with water prior to HPLC injection.

Fig. 4 shows the chromatograms of the Benifuuki green tea. The upper part of Fig. 4 shows the chromatogram using a Shim-pack GIST C18 analytical column (particle size: 5 µm) according to the JAS method, and the bottom portion shows that using a Shim-pack GIST-HP C18 column (particle size: 3 µm) for high-speed analysis in the same series. Methylated catechin was separated from the other compounds in both analyses.

The contents of methylated catechin were calculated from the quantitative values. As a result, the specimen contained 12 g/kg of methylated catechin in both the JAS method-compliant analysis and the high-speed analysis.

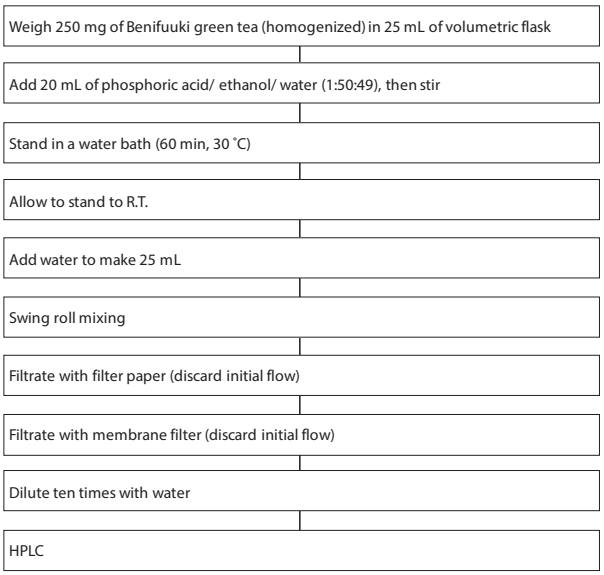

Fig. 3 Pretreatment Protocol

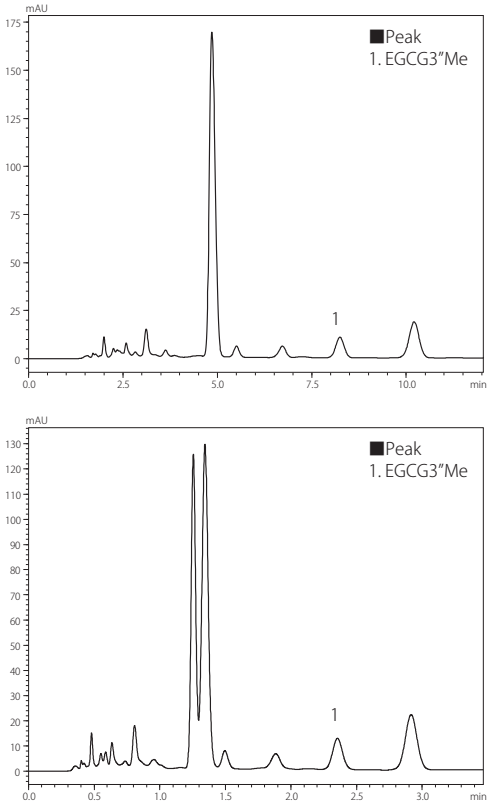

Fig. 4 Chromatograms of Benifuuki Green Tea Upper: JAS Method-Compliant Analysis, Bottom: High-Speed Analysis

Conclusion

Analyses of methylated catechin in Benifuuki green tea were carried out using the Nexera Series and Shim-pack GIST C18 columns. Using a high-speed analytical column in the same series, it was possible to reduce analysis time and mobile phase consumption to approximately 1/4 respectively while maintaining the same separation performance.

In both of the analyses described above, the Benifuuki green tea used in this experiment was found to contain 12 g/kg of methylated catechin.

[References]
(1) Mari Maeda-Yamamoto, Mitsuaki Sano, Nahomi Matsuda, Toshio Miyase, Keiko Kawamoto, Naoko Suzuki, Masayasu Yoshimura, Hirofumi Tachibana and Katsuhiro Hakamata, The Change of Epigallocatechin-3-*O*-(3-*O*-methyl) gallate Content in Tea of Different Varieties, Tea Seasons of Crop and Processing Method, Nippon Shokuhin Kagaku Kogaku Kaishi (published by Japanese Society for Food Science and Technology), 48, 64-68 (2001).
(2) Japanese Agricultural Standard, Determination of the O-methylated Catechin in 'Benifuuki' Green Tea (*Camellia sinensis L.*) - High-performance liquid chromatographic method (JAS 0002), Ministry of Agriculture, Forestry and Fisheries (March 29, 2018).
(3) Yuji Homura, Yusuke Hiejima, Takashi Kodama, Masumi Tanaka, Hideki Horie, Tadanao Suzuki and Akemi Yasui, Validation of Method for Determining *O*-methylated Catechin in 'Benifuuki' Green Tea (*Camellia sinensis L.*) by Interlaboratory Study, Nippon Shokuhin Kagaku Kogaku Kaishi, 63(7), 312-318 (2016).

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Application News

No. L544

High Performance Liquid Chromatography

Simple and Quick Analysis of Theanine in Tea by Automatic Pre-Column Derivatization Method

Theanine, which has an *umami* taste, accounts for more than half of the free amino acids in tea and is an important component that determines the flavor of tea. It is known that there is a strong correlation between theanine content and tea quality, especially for green tea. Theanine also has various physiological effects, such as relaxing effect, stress-reducing effect, premenstrual syndrome (PMS) alleviation, and so on. With the recent health food boom, theanine is getting attention day by day.

Application News L529A introduced amino acid analysis by the automatic pre-column derivatization method using the co-injection mode of the i-Series. This article introduces an example of simple and quick analysis of theanine and other major amino acids, by the same method as L529A using the Nexera™ Series.

T. Yoshioka

Simultaneous Analysis of Amino Acids

In the Nexera Series, an automatic pretreatment function is provided in the autosampler, and its co-injection mode was used here. Primary amino acids were derivatized into fluorescent substances in the needle by using *o*-phtalaldehyde (OPA) to analyze. Fig. 1 shows the setting of the co-injection mode.

Fig. 2 shows the chromatogram of an amino acid mixed standard solution that contains theanine and 19 proteinogenic amino acids except proline. All 20 amino acids were separated within 15 min. The analytical conditions are shown in Table 1 to Table 4.

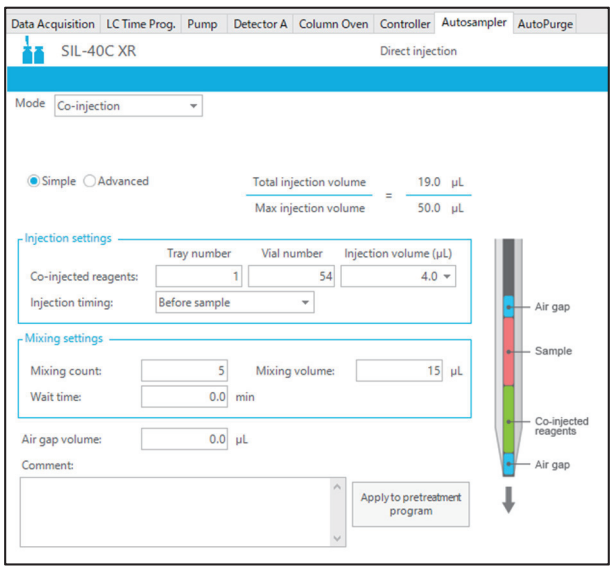

Fig. 1 Pretreatment (Co-Injection) Setting Screen of Nexera Series

Table 1 Mobile Phase Preparation Method

- 20 mmol/L (sodium) acetate buffer (pH6)
  Add 2.67 g of sodium acetate trihydrate and 41 µL of acetic acid into 1 L of ultrapure water.
- 20 mmol/L (sodium) acetate buffer (pH5) containing 0.5 mmol/L EDTA-2Na
  Add 0.19 g of EDTA-2Na, 2.03 g of sodium acetate trihydrate, and 308 µL of acetic acid into 1 L of ultrapure water.

Table 2 Derivatizing Reagent Preparation Method

- 0.1 mol/L borate buffer (pH9)
  Add 0.62 g of boric acid and 0.20 g of sodium hydroxide into 100 mL of ultrapure water.
- MPA Reagent
  Add 10 µL of 3-mercaptopropionic acid into 10 mL of 0.1 mol/L borate buffer.
- OPA Regent
  Add 0.3 mL of ethanol into 10 mg of *o*-phthalaldehyde and dissolve completely. Then add 0.7 mL of 0.1 mol/L borate buffer and 4 mL of ultrapure water.
- MPA / OPA Solution
  Mix equal volume of MPA regent and OPA regent.

Table 3 Analytical Conditions

Column	: Shim-pack™ XR-ODS II 100 mmL.×3 mmI.D., 2.2 µm
Mode	: Low pressure gradient
Mobile phase	: A) 20 mmol/L (sodium) acetate buffer (pH6) B) Water / Acetonitrile = 1 / 9 C) 20 mmol/L (sodium) acetate buffer (pH5) containing 0.5 mmol/L EDTA-2Na
Flow rate	: 1.0 mL/min
Column temp.	: 40 °C
Injection volume	: 1 µL
Detection	: Fluorescence detector Ex. 350 nm, Em. 450 nm

Table 4 Time Program

Time (min)	A.conc	B.conc	C.conc
0	95	5	0
0.2	93	7	0
1	93	7	0
4	87	13	0
5	0	15	85
7.5	0	30	70
12	0	35	65
14	0	45	55
14.01	0	95	5
17	0	95	5
17.01	95	5	0
19.5	95	5	0

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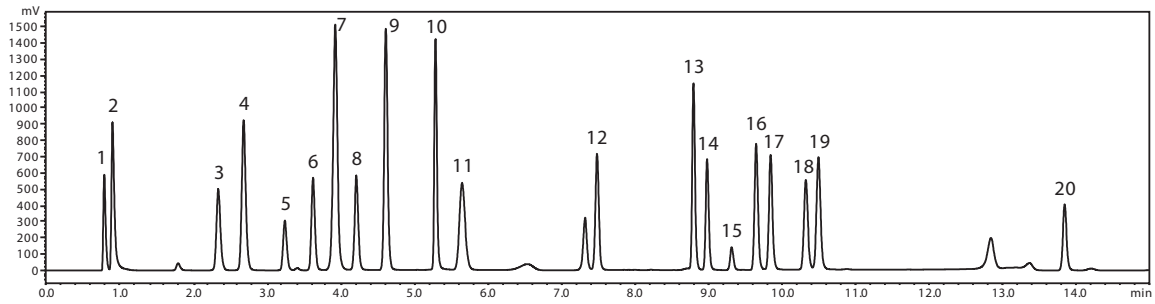

1, Asp 2, Glu 3, Asn 4, Ser 5, Gln 6, His 7, Gly 8, Thr 9, Arg 10, Ala 11, Theanine 12, Tyr 13, Met 14, Val 15, Cys2 16, Trp 17, Phe 18, Ile 19, Leu 20, Lys

Fig. 2 Chromatogram of Amino Acid Mixed Standard Solution (25 µmol/L Each)

Linearity and Repeatability

Calibration curves for theanine, aspartic acid, glutamic acid, serine, glutamine, and arginine, which are the major amino acids in tea, were prepared from the chromatogram of the amino acid mixed standard solution. Calibration curves for each of these compounds were prepared in the concentration ranges of 0.25, 1.25, 2.5, 12.5 and 25 µmol/L, and their linearity (R^2) was evaluated. Area repeatability was also evaluated by a repeated analysis (n=6) for 12.5 µmol/L. Table 5 shows these results.

Table 5 Linearity of Calibration Curves and Repeatability of Each Amino Acid

Amino acids	Linearity (R^2)	Repeatability (%RSD)
Theanine	0.9999	0.37
Aspartic acid	0.9998	0.61
Glutamic acid	0.9997	0.55
Serine	0.9998	1.26
Glutamine	0.9999	0.59
Arginine	0.9999	0.72

Example of Analysis of Teas

Each of the tea-leaves was extracted by the general methods to prepare analytical samples except green tea (PET bottle). These samples were diluted 50 times with ultrapure water, filtered with a 0.22 µm membrane filter, and then used in the analysis.

Fig. 3 shows the quantitative analysis results for the amino acids in the 5 types of tea, and Fig. 4 shows the chromatograms of teas. Judging from these data, the content of amino acids varies in accordance with the type of tea.

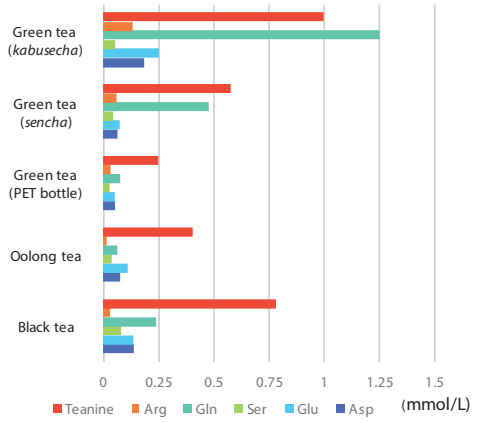

Fig. 3 Contents of Amino Acids in Teas

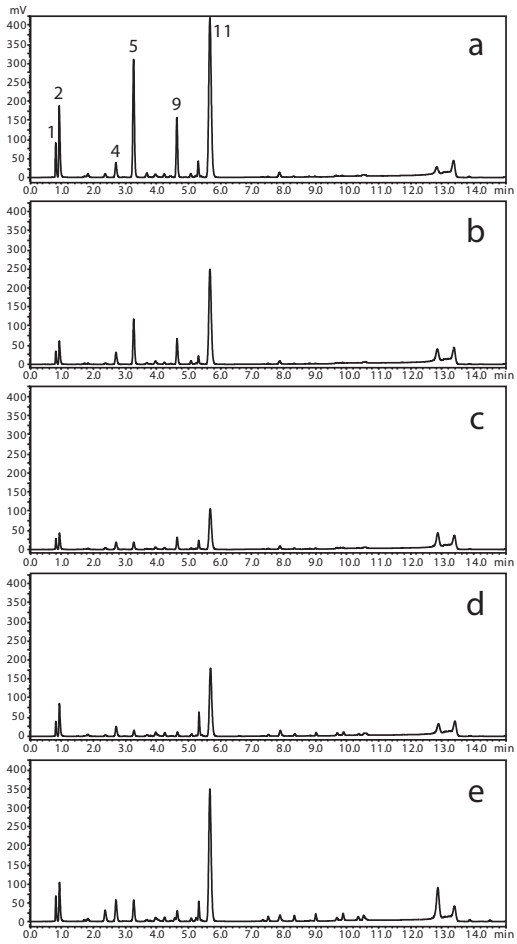

Fig. 4 Chromatograms of Teas
a: Green Tea (*kabusecha*), b: Green Tea (*sencha*), c: Green Tea (PET bottle), d: Oolong Tea, e: Black Tea

Conclusion

In this analysis, derivatization of amino acids was performed in the needle by using the co-injection mode installed in the Nexera Series as a standard feature. As a result, troublesome pretreatment operation and a complex equipment configuration are not necessary. Thus, it has become possible to perform simple and quick analyses of amino acids.

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Application News

No. L554A

High Performance Liquid Chromatography

Quick Estimation of the Freshness and the Level of Putrefaction in Fish Meat Using Nexera™ Dual Injection System

–Simultaneous Analysis of ATP-Related Compounds, Histamine and Amino Acids in Fish Meat–

The muscles in fish meat are easily spoiled due to its rough structure and large water content compared to those in farm-animal meat. Consequently, the accurate estimation of fish meat freshness is very important to maintain the safety of food. The variance of adenosine triphosphate (ATP), which is a source of energy for muscles of animals is commonly used as an index of animal meat freshness. In case of numerical estimation of fish meat freshness, the unique K value, related to ATP decomposition is commonly employed in Japan.

On the other hand, it is reported that histamine, one of the putrefactive amines, induces allergy through a food intoxication condition. Histamine (one of the metabolites of histidine) is highly accumulated in red fish meat in the putrefactive process.

Once histamine is generated, it is not possible to prevent food intoxication because histamine is resistant to heat and cannot be removed during cooking process. To address this issue, the Codex Alimentarius Commission (Codex), European countries, and elsewhere have established the official limitation for acceptable levels of histamine concentration.

Application News No. L536 introduced determination of the K value in fish meat and the resulting multi-data report on the changes in the freshness over time. This article introduces simultaneous determination of the K value as an indicator of freshness and histamine as an indicator of the level of putrefaction using the Nexera™ dual injection system. Under the analytical conditions described in this article, amino acids and nucleic acids known as nutritional compounds, including *umami* taste, contained in fish meat were able to be determined simultaneously as well.

N. Iwata

Dual Injection System Capable of Two Different Analyses Simultaneously

Generally, ATP-related compounds and histamine are not determined simultaneously so that two independent HPLC analyses are essential. Our novel system setup (Nexera dual injection system) affords a significant profit for easily being spoiled samples that have two types of functional compounds requiring different HPLC conditions. In addition, each obtained analysis data is stored as one data file. This facilitates integrate analysis and data management for the same sample.

The employed HPLC setup equipped photodiode array detector (PDA) and fluorescence detector on two independent analytical flow paths within the single HPLC setup. ATP-related compounds and amino acids including histamine were detected with PDA and fluorescence detector respectively.

Target Compounds

The target compounds were thirty-one compounds including six ATP-related compounds, histamine and, twenty-four amino acids (twenty protein compounds and four compounds related to fish meat). These compounds are shown in Table 1.

Table 1 List of ATP-related compounds, histamine and twenty-four amino acids analyzed

ATP-related compounds*1		10	Serine	21	Tyrosine
1	Hx	11	Glutamine	22	Valine
2	IMP	12	Glycine	23	Methionine
3	HxR	13	Histidine	24	Histamine
4	AMP	14	Threonine	25	Cystine
5	ADP	15	β-Alanine	26	Tryptophan
6	ATP	16	Arginine	27	Phenylalanine
Histamine and amino acids		17	Alanine	28	Isoleucine
7	Aspartic acid	18	Taurine	29	Leucine
8	Glutamic acid	19	Anserine	30	Lysine
9	Asparagine	20	Carnosine	31	Proline

*1: Hx: Hypoxanthine, HxR: Inosine, IMP: Inosine 5'-monophosphate, AMP: Adenosine 5'-monophosphate, ADP: Adenosine 5'-diphosphate, ATP: Adenosine 5'-triphosphate

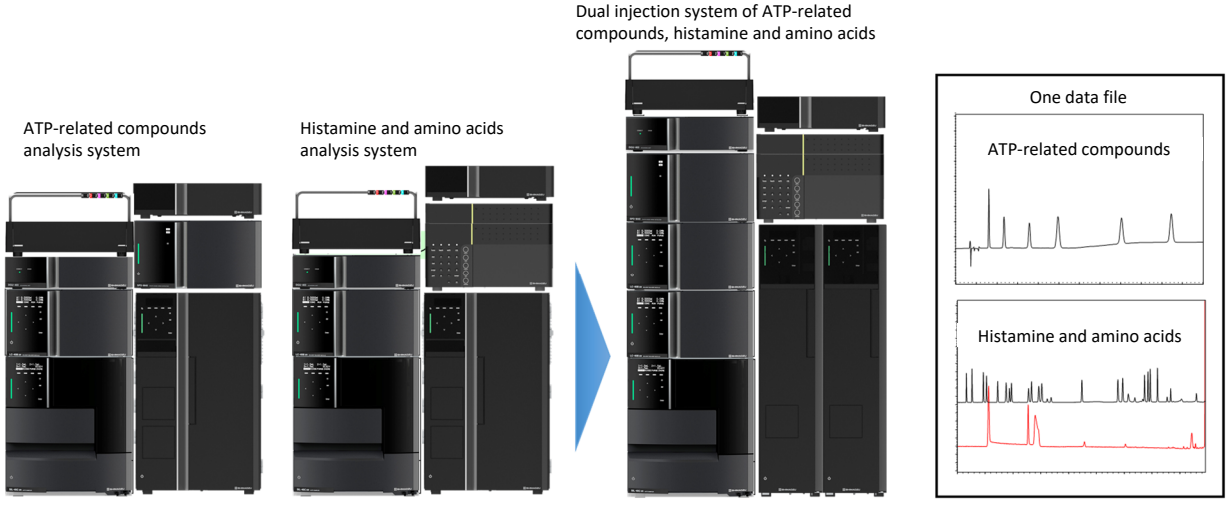

Fig. 1 Each analysis system (shown in left) and dual injection system (shown in right)

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Sample Preparations and Analytical Conditions

Tuna samples were prepared through deproteinization, extraction, and pH adjustment (Fig. 2).

Table 2 shows the analytical conditions. Improved analytical stability was obtained due to the gradient elution for ATP-related compounds whereas isocratic elution is employed in ordinary analysis. Amino acids including histamine were automatically derivatized with *o*-phthalaldehyde and 9-fluorenylmethyl chloroformate (Table 3 and 4). Therefore, a complicated manual pretreatment, e.g. dansyl chloride derivatization, is not required so analysis interval including derivatization can be kept constant.

In this article, TORAST™-H Glass Vial, a low-adsorption glass vial was used (Fig. 3). This vials are available in two sizes, 1.5 mL and 150 µL. The 1.5 mL vials were used for the derivatization reagents and the samples before derivatization. The sample was reacted with the derivatization reagents in the 150 µL vial because a mixing in the smaller vial provides good reaction efficiency. Fig. 4 shows the sample setting in the autosampler. The SIL-40 series for the Nexera system can contain three sample racks, and prevent incorrect setting of the samples using independent rack for each specific purpose as shown in Fig. 4.

Table 3 Overview of automatic pre-column derivatization

①	MPA*2 solution 20 µL
②	OPA reagent 20 µL
③	Sample 10 µL
④	Mix
⑤	FMOC reagent 5.0 µL
⑥	Mix
⑦	Injection

*2 Mercaptopropionic acid

Fig. 3 TORAST-H Glass Vial

Table 4 Preparation of derivatization reagents

- MPA solution
  Add 10 µL of 3 - mercaptopropionic acid into 10 mL of 0.1 mol/L borate buffer.
- OPA Reagent
  Add 0.3 mL of ethanol into 10 mg of *o* - phthalaldehyde and dissolve completely. Then add 0.7 mL of 0.1 mol/L borate buffer and 4 mL of ultrapure water.
- FMOC Reagent
  Dissolve 10 mg of 9 - fluorenylmethyl chloroformate into 50 mL of acetonitrile.

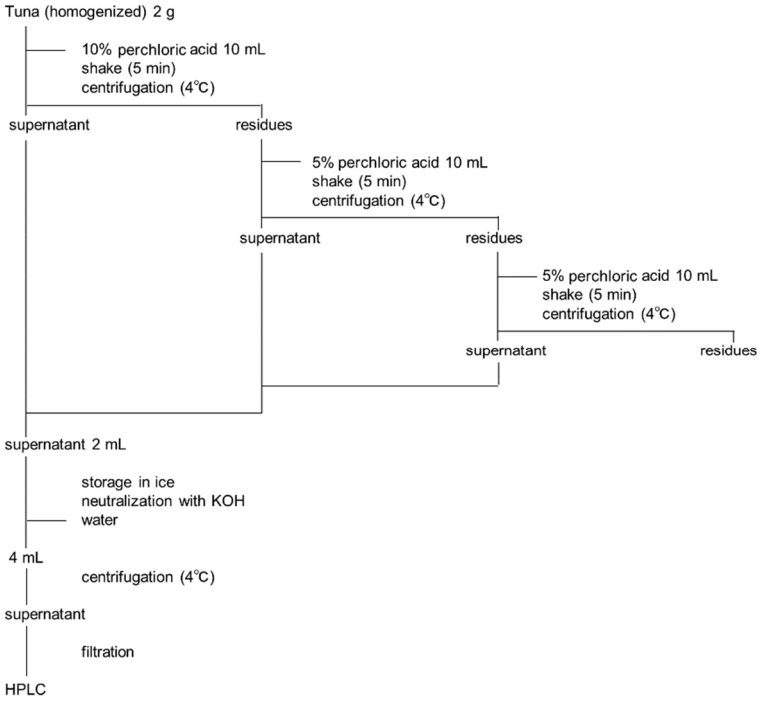

Fig. 2 Sample preparation protocol

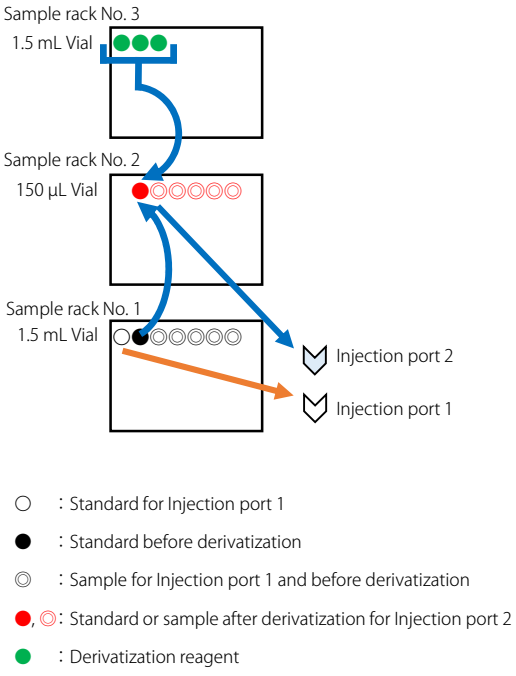

Fig. 4 Sample setting in the autosampler

Table 2 Analytical conditions

System	:	Nexera dual injection system	
		＜ATP-related compounds＞	＜Histamine and amino acids＞
Column	:	Shim-pack™ GIST 3 µm C18 AQ (100 mm L, 3.0 mm I.D., 3 µm)	Shim-pack Velox™ C18 (100 mm L, 3.0 mm I.D., 2.7 µm)
Flow rate	:	0.8 mL/min	0.8 mL/min
Mobile phase	:	A) Water/Acetonitrile=100/1 (v/v) containing 0.15 mol/L Phosphoric acid, 0.225 mol/L Triethylamine B) Water/Acetonitrile=80/20 (v/v) containing 0.15 mol/L Phosphoric acid, 0.225 mol/L Triethylamine	A) 20 mmol/L (Potassium) phosphate buffer (pH 6.5) B) Acetonitrile/ Methanol/ Water =45/40/15 (v/v/v)
Time program	:	0%B (0-4 min)→12%B (11.5 min)→100%B (11.51-18.5 min)→ 0%B (18.51-32 min)	5%B (0 min)→13%B (8 min)→25%B (15 min)→ 52%B (21.5 min)→100%B (21.51-27.50 min)→ 5%B (27.51-32 min)
Column temp.	:	30 °C	35 °C
Injection volume	:	10 µL	1 µL
Detection	:	PDA 260 nm	FL Ex: 350 nm, Em: 450 nm (Ch1) Ex: 266 nm, Em: 305 nm (Ch2)

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Examination of Extraction Solvent and Confirmation of Recovery Rates

A preparation protocol for extracting ATP-related compounds with perchloric acid has been reported for a long time. The extraction efficiency of histamine and amino acids were confirmed in the same way. As the operation blank test, histamine and histidine were extracted with water and perchloric acid. As a result, perchloric acid afforded stable extraction efficiency (Table 5).

Then, six tuna samples were spiked with histamine standard to make concentration of 10 mg/100 g, the threshold limit defined by Codex. 30 minutes later, sample preparation was performed. Table 6 shows the average recovery rates obtained from the results of six samples.

Table 5 Extraction efficiency of histamine and histidine by different extraction solvents

N	Extraction efficiency (%)			
	Histamine		Histidine	
	Water	Perchloric acid	Water	Perchloric acid
1	84.0	97.7	93.8	92.5
2	100.8	95.7	102.1	93.0

Table 6 Recovery rates of histamine (N=6)

N	Recovery rates (%)
1	96.8
2	98.3
3	99.8
4	99.8
5	101.0
6	103.0
Average (%RSD)	99.8 (2.14%)

Calibration Curve

Calibration curves were created for the thirty-one compounds to be analyzed. Good linearity was obtained with r^2 = 0.999 or greater for each compound. The concentration range of calibration curve and r^2 value for each compound are shown in Table 7.

Simultaneous Determination of ATP-related Compounds, Histamine and Amino Acids in Fish Meat, and Determination of K Value and Histamine Concentration

The raw yellowfin tuna under different storage days and temperatures were analyzed to confirm the K values and histamine concentrations. The storage temperature was 4°C and 25°C. In the tuna kept at 4°C for one day, the K value increased slightly by 2.6% and the freshness decreased compared to that immediately after purchase. Histamine was not produced after one day storage at 25 °C, which afforded 25.1% increase of K value.

The raw albacore tuna that had been refrigerated for 6 days afforded a K value of 70.4%, which reached the region called the putrefaction and histamine was detected. The histamine concentration was 2.1 mg/100 g, below the Codex threshold limit (Figure 5 and Table 8).

In addition, histamine was able to be separated from many amino acids such as histidine, alanine, taurine, anserine, carnosine and lysine, that are abundantly contained in fish meat. Table 9 shows the concentrations of ATP-related compounds and amino acids in the tuna samples.

Table 8 Change in K value and concentration of histamine in tuna over time and storage temperature

	days	Temperature (°C)	K value*3 (%)	Histamine (mg/100 g)
Yellowfin Tuna	0	4	36.1	N.D.
	1	4	38.7	N.D.
		25	61.2	N.D.
Albacore Tuna	6	4	70.4	2.1

*3 Definition formula for K value

$$\text{Formula} \quad \frac{Hx+HxR}{Hx+HxR+IMP+AMP+ADP+ATP} \times 100$$

Table 7 Concentration range of calibration curve and r^2 value

	Compound	Conc. range (μmol/L)	r^2		Compound	Conc. range (μmol/L)	r^2
1	Hx	1-300	0.99982	17	Alanine	0.25-100	0.99994
2	IMP	1-300	0.99983	18	Taurine	0.25-100	0.99995
3	HxR	1-300	0.99984	19	Anserine	0.25-100	0.99997
4	AMP	1-300	0.99987	20	Carnosine	0.25-100	0.99995
5	ADP	1-200	0.99998	21	Tyrosine	0.25-50	0.99995
6	ATP	1-200	0.99944	22	Valine	0.25-50	0.99995
7	Aspartic acid	0.25-50	0.99994	23	Methionine	0.25-50	0.99996
8	Glutamic acid	0.25-50	0.99995	24	Histamine	0.25-50	0.99999
9	Asparagine	0.25-50	0.99995	25	Cystine	0.25-25	0.99953
10	Serine	0.25-50	0.99995	26	Tryptophan	0.25-50	0.99993
11	Glutamine	0.25-50	0.99995	27	Phenylalanine	0.25-50	0.99995
12	Glycine	0.25-100	0.99996	28	Isoleucine	0.25-50	0.99995
13	Histidine	0.25-100	0.99968	29	Leucine	0.25-50	0.99996
14	Threonine	0.25-50	0.99994	30	Lysine	0.25-100	0.99995
15	β-Alanine	0.25-50	0.99991	31	Proline	1-25	0.99953
16	Arginine	0.25-50	0.99994				

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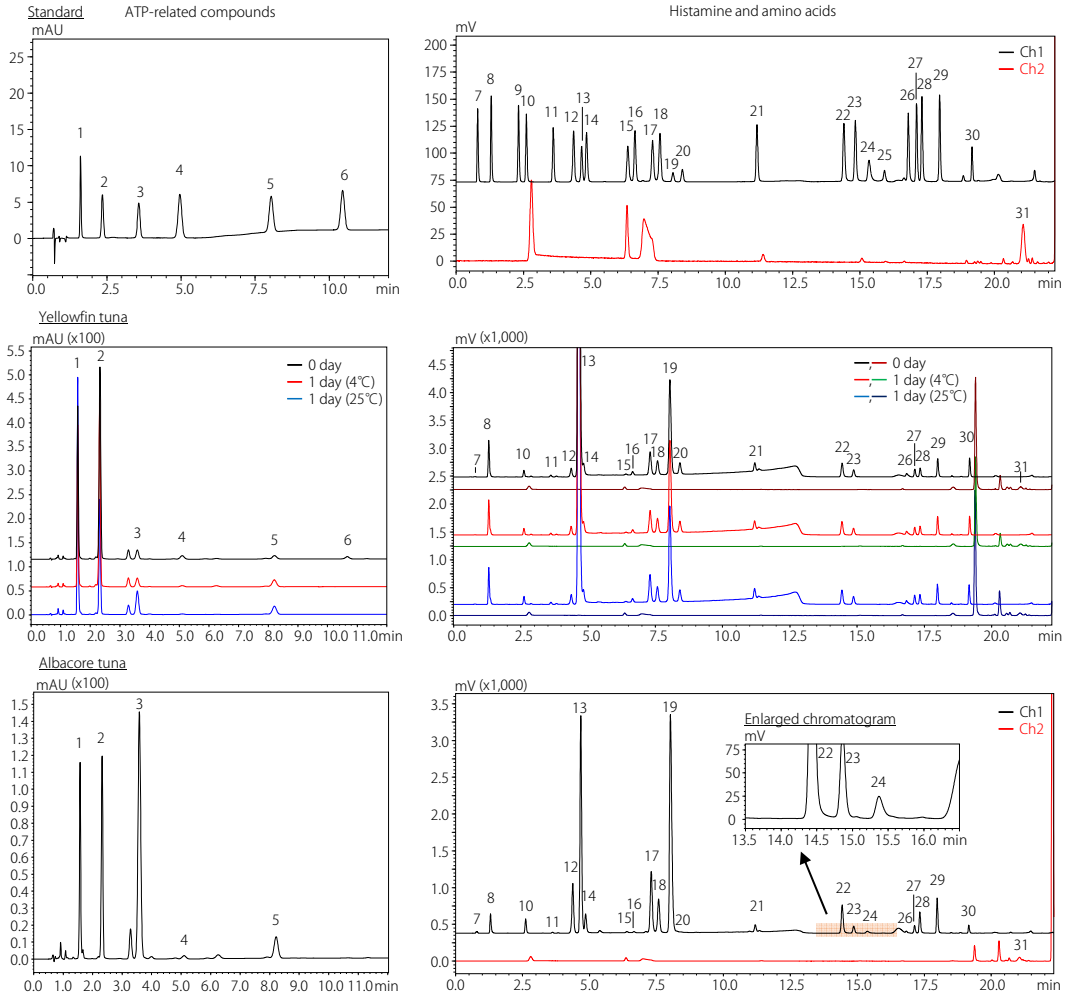

Fig. 5 Chromatograms of the standard solution and tuna sample solution. Peak identification as listed in Table 1.

Table 9 Concentration of ATP-related compounds and amino acids in yellowfin tuna

		Yellowfin Tuna (0 day) (μmol/L)*4
2	IMP	278.9
8	Glutamic acid	46.6
13	Histidine	(2416.0)
17	Alanine	55.7
18	Taurine	27.8
19	Anserine	(1062.5)
20	Carnosine	82.0
22	Valine	23.6
29	Leucine	20.4
30	Lysine	50.7

*4 Values in parentheses are outside the quantification range.

Summary

Simultaneous determination of the K value and histamine concentration was carried out using the Nexera dual injection system. The ATP-related compounds and histamine were able to be extracted by the same sample preparation.

It was shown that K value was changed by storage days and temperature. Histamine was also detected in the spoiled samples showing large K values. Histamine was able to be separated from many amino acids that are abundantly contained in fish meat.

Nexera dual injection system affords a significant profit for food sanitation inspection of easily being spoiled samples that have ATP-related compounds and histamine/ amino acids.

[Reference]

1) Usui Kazushige, Watanabe Etsuo, "Comparison of changes in freshness in fresh and frozen black marlin using the K-value," Bulletin No. 5 of the Kanagawa Prefectural Fisheries Technology Center, 11-14 (2012)

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Application News

No. L556A

High Performance Liquid Chromatography

USP-Compliant Analysis of Vitamins in Dietary Supplements
Analysis of Calcium Pantothenate by Nexera™ XR

The United States Pharmacopeia (USP) provides standards for quality control of dietary supplements and specifies testing methods and judgment standards for dietary supplements. Because many dietary supplements are distributed globally, USP compliance has become an important judgment standard for verifying the quality of supplements for consumers.

The substance that was analyzed here is pantothenic acid (pantothenate), which is a water soluble vitamin. Also called vitamin B_5, pantothenic acid is a substance in which β-alanine is bonded with pantoic acid. Many dietary supplements contain pantothenate in the form of its calcium salt.

"Oil and Water Soluble Vitamins with Mineral Tablets – Calcium Pantothenate" of USP40-NF35 describes two analysis methods using HPLC and one microorganism quantitation method. In the HPLC method in "Method 3," calcium pantothenate is detected with an ultraviolet-visible (UV-VIS) absorbance detector after separation with a reversed-phase ODS column.

This article introduces an example in which the calcium pantothenate in a dietary supplement was analyzed using a Nexera XR, which is part of the Shimadzu Nexera Series of ultra high performance liquid chromatographs. An analysis was also conducted with a Prominence™ Series HPLC, confirming that the same results can be obtained with that system, as reported here.

M. Hayashida, H.Yamamoto (SHIMADZU TECHNO-RESEARCH, INC.)

System Suitability Test

A system suitability test for quantitation of calcium pantothenate was carried out. Table 1 and Table 2 show the analysis conditions and judgment standard, respectively. Fig. 1 shows the chromatogram of the calcium pantothenate standard solution, and Table 3 shows the injection repeatability result for calcium pantothenate.

The relative standard deviation %RSD of the peak area did not exceed the standard value.

Table 1 **Calcium Pantothenate Analysis Conditions**

System	:	Nexera XR
Column	:	Shim-pack™ GIS C18 USP code: L1 (300 mm × 3.9 mm I.D., 5 µm)
Mobile Phase	:	5 g/L KH_2PO_4 (pH = 3.5)*/ Methanol =9/1
Flow Rate	:	2.0 mL/min
Column Temp.	:	50 ˚C
Injection Vol.	:	25 µL
Detection	:	SPD-M40 205 nm (190 - 800 nm)

* : Adjusted to pH = 3.5 using phosphoric acid.

Table 2 **Standard Value of System Suitability Test for Calcium Pantothenate**

Relative standard deviation (%RSD) (n = 6)	≤3

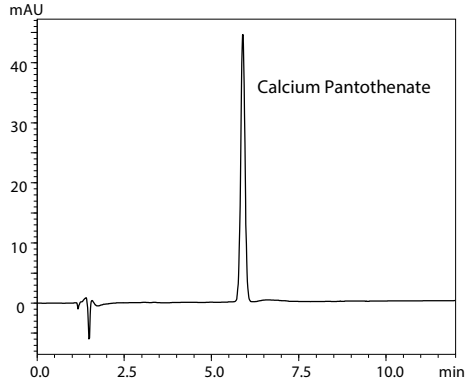

Fig. 1 **Chromatogram of Calcium Pantothenate Standard Solution (40 mg/L)**

Table 3 **Injection Repeatability of Calcium Pantothenate (n = 6)**

	%RSD	
	Retention time	Peak area
Calcium pantothenate	0.10	0.34

Analysis of Dietary Supplement

A multivitamin tablet, which is a commercially available dietary supplement, was analyzed. Fig. 2 shows the chromatogram of the multivitamin tablet, and Fig. 3 shows the pretreatment method.

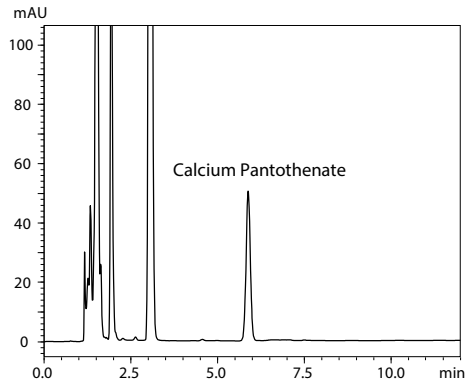

Fig. 2 **Chromatogram of the Multivitamin Tablet**

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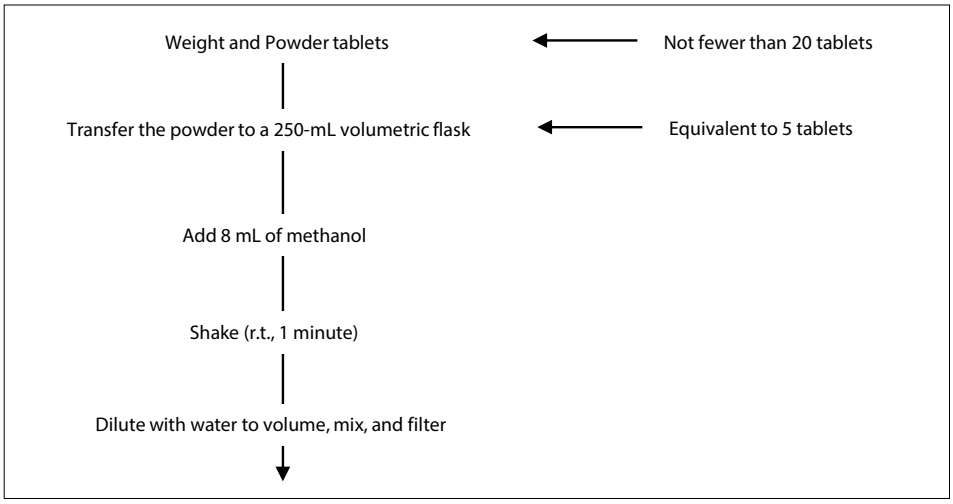

Fig. 3 **Pretreatment Procedure in Analysis of Calcium Pantothenate**

Compatibility with Prominence Series

The analysis carried out with a Prominence Series HPLC was also conducted in the same manner with the Nexera XR, as described above. The analysis conditions and pretreatment method were as shown in Table 1 and Fig. 3.

Fig. 4 shows the chromatogram of the standard solution of calcium pantothenate, Fig. 5 shows the chromatogram of the multivitamin tablet, and Table 4 shows the measurements of calcium pantothenate in the multivitamin tablet.

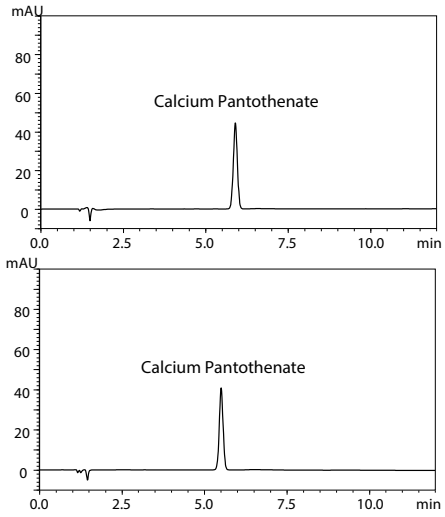

Fig. 4 **Chromatograms of Calcium Pantothenate Standard Solution (40 mg/L) (Top: Nexera XR, Bottom: Prominence)**

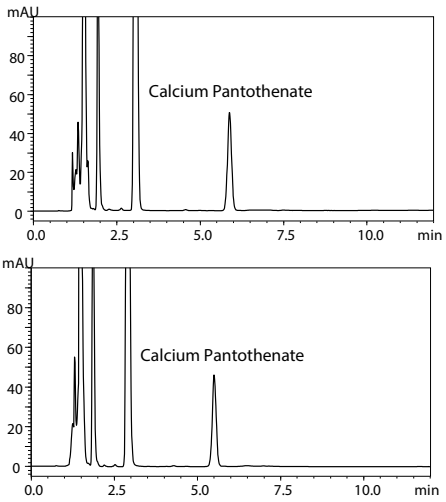

Fig. 5 **Chromatograms of the Multivitamin Tablet (Top: Nexera XR, Bottom: Prominence)**

Table 4 **Measurements of Calcium Pantothenate in the Multivitamin Tablet**

Calculated amount/tablet	Nexera XR	1.7 mg
	Prominence	1.7 mg
Labeled amount/tablet		1.5 mg

* : Standard specified in USP40-NF35: 90% to 150% of labeled amount.

Conclusion

The calcium pantothenate in a commercially available dietary supplement was analyzed under analysis conditions conforming to USP40-NF35 using a Nexera XR, which is a new product Nexera series, and a Prominence Series. This experiment confirmed that the same results as with the Nexera XR can also be obtained with the Prominence.

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Application News

No. L558

High Performance Liquid Chromatography

USP-Compliant Analysis of Vitamin in Dietary Supplement : Analysis of Cyanocobalamin by Nexera™ XR

The United States Pharmacopeia (USP) is used as one standard in quality control of dietary supplements.

Vitamin B_{12} is a general name for vitamins which contain cobalt. Although vitamin B_{12} is involved in blood formation and metabolism, it cannot be synthesized in the body and is rarely found in foods of plant origin. Because vitamin B_{12} deficiency occurs easily, vitamin B_{12} is included in many dietary supplements [1]. Generally, the amount of vitamin B_{12} is expressed as the cyanocobalamin equivalent value.

In USP40-NF35, "Oil and Water Soluble Vitamins with Mineral Tablets Cyanocobalamin" describes two analytical methods for cyanocobalamin, an analytical method by HPLC and a microbial assay [2].

Here, a USP40-NF35-compliant system suitability test for cyanocobalamin, representing vitamin B_{12}, was conducted using a Shimadzu Nexera XR. An analysis of a commercial multivitamin tablet was also carried out, and the compatibility of Nexera XR and Prominence™ was verified.

K. Matsuoka, H. Yamamoto*
(* : Shimadzu Techno-Research, Inc.)

Analysis Example: System Suitability Test

Table 1 shows the analytical conditions of the system suitability test for cyanocobalamin. Table 2 shows the reference value of the test. Fig. 1 shows the chromatogram of the cyanocobalamin standard solution, and Table 3 shows the results of repeatability for cyanocobalamin.

The relative standard deviation (%RSD) of the peak area satisfied the reference value, confirming that the Nexera XR possesses system suitability.

Table 1 Analytical Conditions for Cyanocobalamin

System	:	Nexera XR
Column	:	Shim-pack™ GIS-C18 (150 mm × 4.6 mm I.D., 5 µm; USP code L1)
Mobile Phase	:	Methanol / water =35:65
Flow Rate	:	0.5 mL/min
Column Temp.	:	20 °C
Injection Vol.	:	200 µL*
Detection	:	PDA 550 nm (190 - 800 nm)

* : With additional 500 µL sample loop

Table 2 Reference Value of System Suitability Test for Cyanocobalamin

Relative standard deviation (%RSD) (n = 6)	≦3

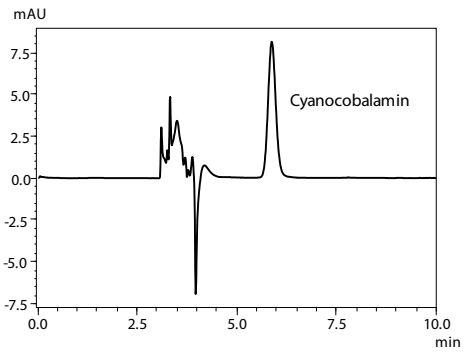

Fig. 1 Chromatogram of Cyanocobalamin (1 mg/L)

Table 3 Repeatability for Cyanocobalamin (n = 6)

	%RSD	
	Retention time	Peak area*
Cyanocobalamin	0.122	0.867

* : USP40-NF35 standard: 3% or less

Analysis of Commercial Supplement

Commercial supplement "Multivitamin Tablet" was analyzed. Fig. 4 shows the pretreatment workflow, and Fig. 2 shows the chromatogram of the commercial supplement.

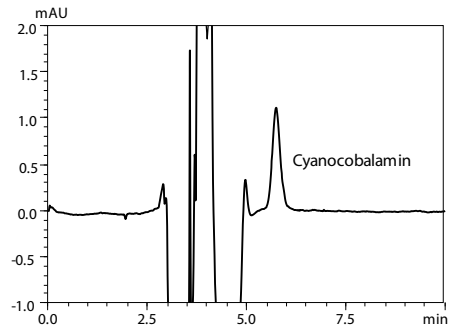

Fig. 2 Chromatogram of the Commercial Supplement

Absorption Spectrum of Cyanocobalamin

The retention time of the peak obtained from the analysis of the commercial supplement and the spectrum obtained from the peak were compared with those of the standard solution (Fig. 1) and identified.

Fig. 3 shows the spectra of cyanocobalamin in the standard solution and in the commercial supplement. The spectra have been normalized for easy comparison.

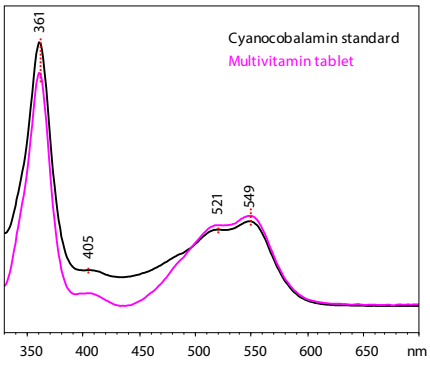

Fig. 3 Spectra of Cyanocobalamin

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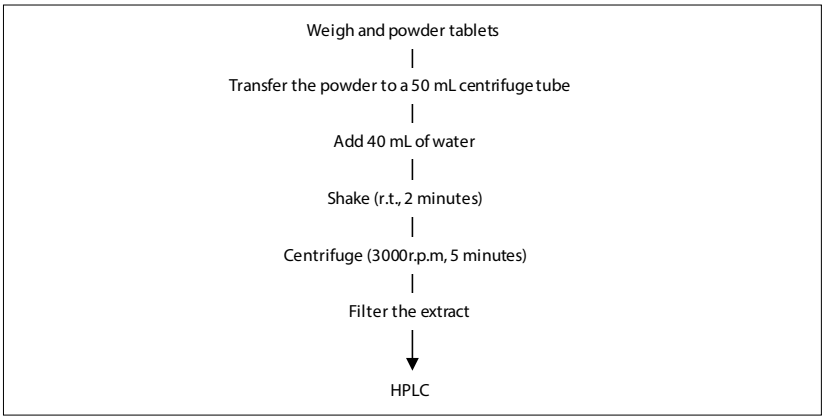

Fig. 4 Pretreatment Workflow in Cyanocobalamin Analysis

Compatibility with Prominence Series

The system suitability test and analysis of the commercial supplement described above were also carried out in the same manner with a Shimadzu Prominence series instrument. The analytical conditions and pretreatment workflow were the same as those in Table 1 and Fig. 4, respectively.

Fig. 5 shows the chromatograms of the cyanocobalamin standard solution, and Fig. 6 shows the chromatograms of the commercial supplement. Table 4 shows the quantitation values of cyanocobalamin in the commercial supplement by the two systems.

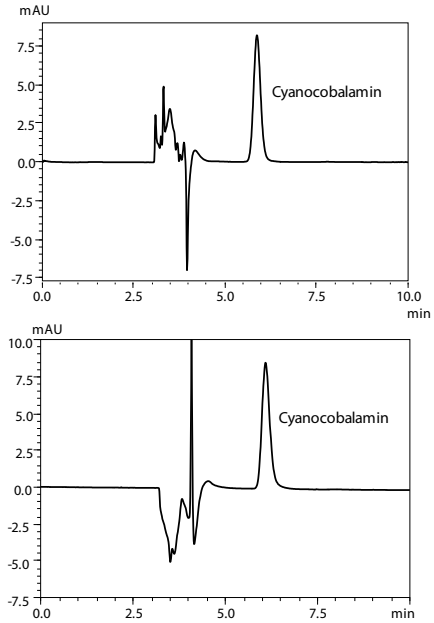

Fig. 5 Chromatograms of Cyanocobalamin (1 mg/L) (Top: Nexera XR, Bottom: Prominence)

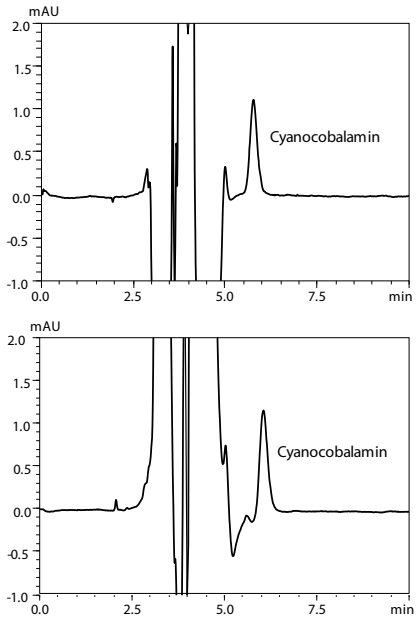

Fig. 6 Chromatograms of the Commercial Supplement (Top: Nexera XR, Bottom: Prominence)

Table 4 Quantitation Values of Cyanocobalamin in the Commercial Supplement

Calculated amount/tablet	Nexera XR	0.44 µg
	Prominence	0.49 µg
Labeled amount/tablet*		0.45-1.25 µg

* : USP40-NF35 standard: 90% to 150% of labeled amount

Conclusion

The Nexera XR met the system suitability requirement through cyanocobalamin analysis, a typical type of vitamin B_{12}. The obtained results were compatible to those from the existing Prominence.

<References>

(1) National Institutes of Biomedical Innovation, Health and Nutrition (https://hfnet.nibiohn.go.jp/contents/detail177.html)
(2) United States Pharmacopeia 40: 7384 “Oil and Water Soluble Vitamins with Mineral Tablets Cyanocobalamin”

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Application News

No. L547

High-Performance Liquid Chromatography

Monitoring Organic Acids during Fermentation with Shim-pack™ Fast-OA High-Speed Organic Acid Analytical Column

Organic acids are attracting attention not only as taste and flavor components in food, but also as raw materials for pharmaceuticals and chemical products, and have been analyzed in various fields. Organic acid analysis by HPLC has multiple separation modes such as ion exclusion, ion exchange, and reverse phase, and there are options for detection such as UV method and conductivity detection method. Ion-exclusion columns and post-column pH-buffered electrical conductivity detection are often used because they selectively detect organic acids while avoiding the matrix effects from the sample. However, the long analysis times are a problem. There is demand for shorter analysis times, especially in bio-production research and intestinal microbiota analysis, which involve analyzing only a limited number of target components. Furthermore, in the monitoring of fermentation conditions, it is necessary to quickly determine the amount of organic acids produced as metabolites by microorganisms in order to control the cultivation in accordance with their growth conditions. Here we report a case of monitoring organic acid content during fermentation using an ion exclusion column, Shim-pack Fast-OA.

M. Nakashima and K. Koterasawa

■ Analysis of Standard Sample

Using an acidic mobile phase in the ion-exclusion mode, Shim-pack Fast-OA columns separate solutions based on the pKa value of each sample component. The Shim-pack SCR-102H column also separated organic acids based on the same principle, but the Shim-pack Fast-OA column elutes organic acids more faster due to an optimized design, which includes an functional group bonding rate.

The analytical conditions are shown in Table 1, and the chromatograms of the five components of the standard organic acid mixture are shown in Fig. 1. It was confirmed that acetic acid could be eluted within 10 minutes.

Fig. 2 shows the elution times of the five standard organic acid mixtures and the pKa of each compound. As for the five components in this study, we found that the ion exclusion effect of the Shim-pack Fast-OA was effective and that the separation was achieved according to the difference in pKa.

Table 1 Analytical Conditions

Column:	Shim-pack Fast-OA 2 column in series (100 mm L × 7.8 mm I.D.)
Guard column:	Shim-pack Fast-OA (G) (10 mm L × 4.0 mm I.D.)
Mobile phase:	5 mmol/L p-toluenesulfonic acid
Flowrate:	0.8 mL/min
pH buffering solution:	5 mmol/L p-toluenesulfonic acid 20 mmol/L Bis-Tris 0. 1 mmol/L EDTA
Flowrate:	0.8 mL/min
Column temperature:	30 °C
Detection:	Conductivity detector (CDD-10Avp)
Injection volume:	10 µL

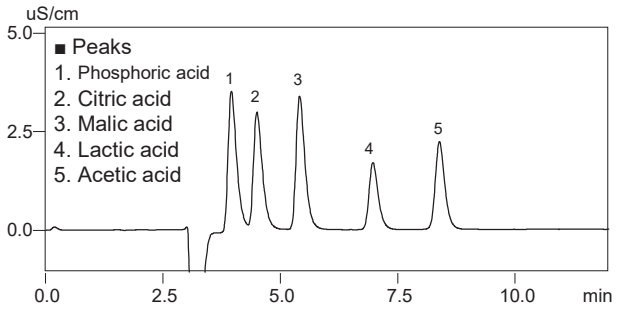

Fig. 1 Chromatogram of Standard Sample

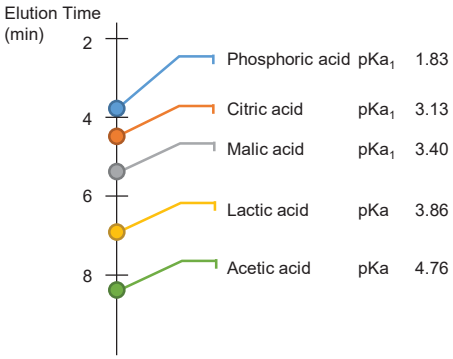

Fig. 2 Elution Time and pKa Values for Target Compounds (pKa values are from the Biology Data Book and Handbook of Chemistry)

■ Linearity and Reproducibility

Each organic acid component was evaluated in terms of contribution to linearity over the concentration range including 10, 50, 100, 500, and 1000 mg/L and in terms of area reproducibility for repeated analysis at 10 mg/L, the lowest concentration in the calibration curve. The results are shown in Table 2. Good results were obtained, with contribution rate (r^2) values higher than 0.9999, and RSD values lower than 1.5 %.

Table 2 Linearity and Area Reproducibility

	Linearity (r^2)	Area (%RSD)
Phosphoric acid	0.99994	0.981
Citric acid	0.99998	0.975
Malic acid	0.99999	0.777
Lactic acid	0.99997	1.322
Acetic acid	0.99999	1.190

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Sample Pretreatment

Organic acids were extracted from commercially available yogurt drinks by adding 5 mmol/L p-toluenesulfonic acid aqueous solution (mobile phase) and chloroform for protein removal and delipidation. The sample was filtered and analyzed by HPLC. The recovery rate was evaluated using yogurt to which the standard sample was added to make a standard sample concentration of 50 mg/L. The pretreatment protocol is indicated in Fig. 3. The resulting chromatogram is shown in Fig. 4, with corresponding recovery rates shown in Table 3. Selectively detecting organic acids by the pH-buffering method, excellent 96 to 118 % recovery rates were achieved.

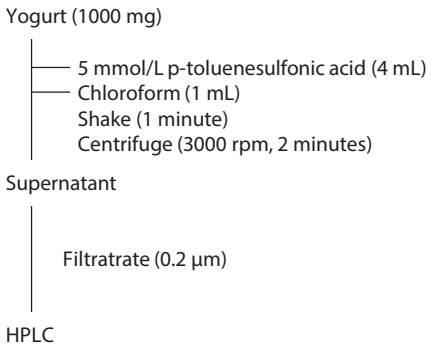

Fig. 3 Pretreatment Protocol

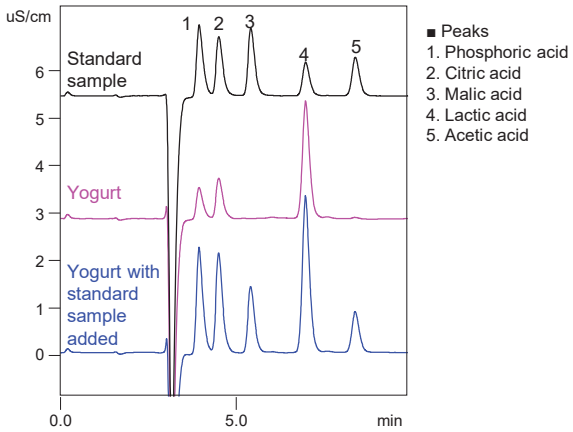

Fig. 4 Chromatogram of Pretreated Yogurt

Table 3 Recovery Rate of Each Component in Spiked Yogurt

	Recovery (%)
Phosphoric acid	102.0
Citric acid	102.2
Malic acid	96.3
Lactic acid	117.1
Acetic acid	100.9

Example of Monitoring Fermentation

Figure 5 shows the results of an evaluation of changes over time of phosphoric acid, citric acid, and lactic acid, which are the main acidic substances contained in home-fermented yogurt.

In general, microorganisms are said to metabolize the lactose to produce lactic acid, which gives yogurt its sour flavor as the fermentation progresses. In actuality, we confirmed that lactic acid increased from about 3.5 hours after the start of cultivation.

The metabolic and fermentation status of microorganisms can be checked by monitoring the organic acid contents present in environments where microorganisms are active (fermented foods or culture media).

With the method described above, the analysis time is 12 minutes, and it takes only about 20 minutes from sampling to confirmation of the results. This makes it possible to contribute to detailed fermentation monitoring and control, which was previously difficult to achieve.

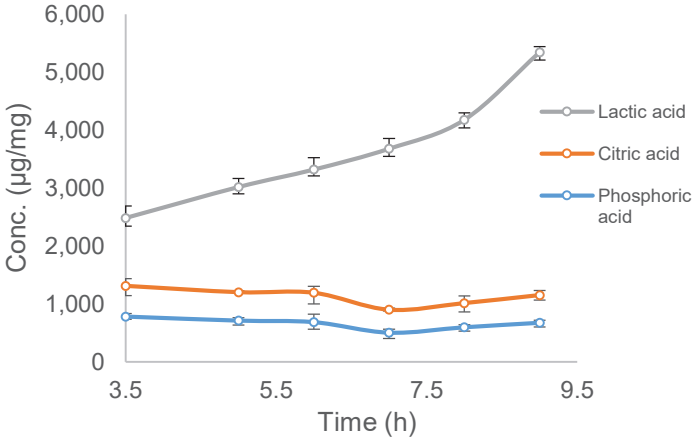

Fig. 5 Change in Acid Content in Yogurt Over Time (N = 3)

Summary

Using a Shim-pack Fast-OA column to analyze yogurt, we were able to confirm the primary organic acids in about 12 minutes. These results from rapid analysis of organic acids suggest that the method provides an effective means of ensuring an adequate number of repeated analyses for multianalyte processing and quickly controlling the fermentation process based on the fermentation status.

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Application News

No. L548

High-Performance Liquid Chromatography

Fermentation Processes Monitoring Using a Nexera™ Dual Injection System

When microorganisms decompose substances to produce useful materials, that process is called fermentation. Fermentation is used not only for producing foods, but in recent years is also being widely used in industrial fields. In such fields, organic acids, sugars, amino acids, and other groups of compounds are multilaterally measured to understand the fermentation process or optimize conditions. In the case of HPLC analysis, the appropriate separate mode and detection method can differ depending on the component class. This requires multiple independent pieces of equipment for each analysis.

This article introduces an example of monitoring a fermentation process using a dual injection system to evaluate two types of analysis simultaneously with only one system.

K. Koterasawa

Overview of Dual Injection System

Using the optional dual injection function available on Nexera SIL-40 series autosamplers, samples can be injected into two flow to run two independent analysis in one platform simultaneously. The two sets of results obtained by the system are integrated in one data file to ensure data traceability to specific samples. The corresponding method and batch files are also integrated in one file to simplify analysis operations.

Fig. 1 shows a flow channel diagram of a dual injection system used for two analyses. In one channel, organic acids are analyzed using an ion-exclusion column, post-column pH-buffering, and an electrical conductivity detector, whereas in the other channel, sugars are analyzed using a ligand exchange column and a refractive index detector. Because organic acid analysis and sugar analysis involve different column temperatures, columns are temperature-controlled separately in two CTO-40S column ovens.

Analysis of Organic Acids

Flow channel (1) was used to analyze organic acids. Fig. 2 shows a chromatogram for a standard mixture solution of organic acids (100 mg/L each of citric, malic, lactic, formic, and acetic acids) obtained using the analytical conditions shown in Table 1.

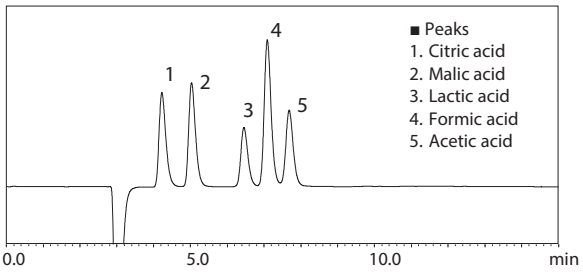

Fig. 2 **Chromatogram of Standard Organic Acid Mixture Solution**

Table 1 **Analytical Conditions for Organic Acid Analysis**

Column:	Shim-pack™ Fast-OA (100 mm L × 7.8 mm I.D., 5 µm) (two used)
Guard column:	Shim-pack™ Fast-OA (G) (10 mm L × 4.0 mm I.D., 12 µm)
Mobile phase flowrate:	0.8 mL/min
pH buffer solution flowrate:	0.8 mL/min
Mobile phase:	Aqueous 5.0 mmol/L *p*-toluenesulfonic acid solution
pH buffer solution:	Aqueous mixture of 5.0 mmol/L *p*-toluenesulfonic acid, 20 mmol/L Bis-Tris, 0.1 mmol/L EDTA 4H
Column temperature:	40 °C
Injection volume:	10 µL
Detector:	Electrical conductivity detector

For details regarding analysis using the Shim-pack Fast-OA column and post-column buffering, refer to the Technical Report, "High-Speed Analysis of Organic Acids Using Shim-pack Fast-OA and pH-Buffered Electrical Conductivity Detection" (C190-E237).

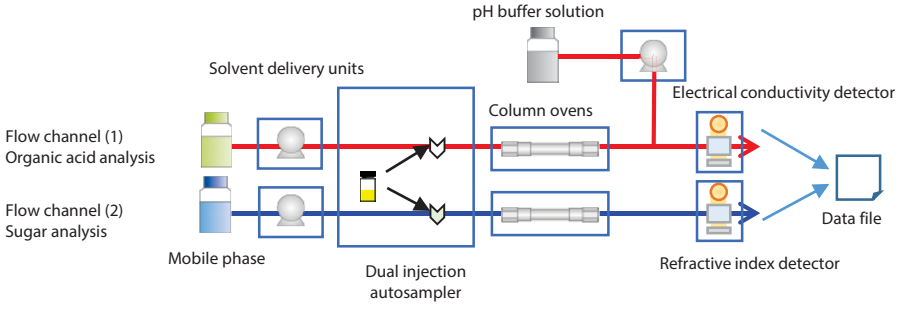

Fig. 1 **Dual Injection Flow Channel Diagram**

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Analysis of Sugars

Flow channel (2) was used to analyze sugars. Fig. 3 shows a chromatogram for a standard mixture solution of sugars (1000 mg/L each of glucose, fructose, mannose, and lactose) obtained using the analytical conditions shown in Table 2.

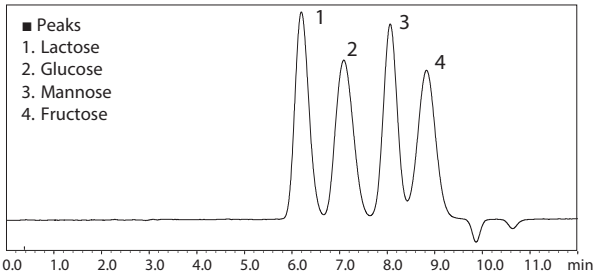

Fig. 3 **Chromatogram of Standard Sugar Mixture Solution**

Table 2 **Analytical Conditions for Sugar Analysis**

Column:	Shim-pack SCR-101C (300 mm L × 7.9 mm I.D., 10 µm)
Guard column:	Shim-pack guard column SCR (C) (50 mm L × 4 mm I.D., 10 µm)
Flowrate:	1.0 mL/min
Mobile phase:	Water
Column temperature:	80 °C
Injection volume:	10 µL
Detector:	Refractive index detector

Reproducibility

The average retention time and relative standard deviation of area (%RSD) values from six analysis repetitions are shown in Table 3 for the standard organic acid mixture solution (200 mg/L each) and in Table 4 for the standard sugar mixture solution (1000 mg/L each). For all components, the results indicated a %RSD of less than 1 % for both retention time and area.

Table 3 **Retention Time and Area Reproducibility of Organic Acids (n=6)**

Component	Average Retention Time (min)	Retention Time %RSD	Area Value %RSD
Citric acid	4.21	0.023	0.25
Malic acid	5.02	0.020	0.07
Lactic acid	6.44	0.018	0.39
Formic acid	7.08	0.015	0.34
Acetic acid	7.67	0.013	0.53

Table 4 **Retention Time and Area Reproducibility of Sugars (n=6)**

Component	Average Retention Time (min)	Retention Time %RSD	Area Value %RSD
Lactose	6.20	0.013	0.08
Glucose	7.10	0.055	0.09
Mannose	8.07	0.010	0.13
Fructose	8.83	0.008	0.11

Calibration Curves

Fig. 4 shows the calibration curves and Table 5 the calibration range and contribution rate for the organic acids. Fig. 5 shows the calibration curves and Table 6 the calibration range and contribution rate for the sugars.

The contribution rates over $R^2 = 0.9998$ obtained for the five organic acid components and four sugar components indicate good linearity.

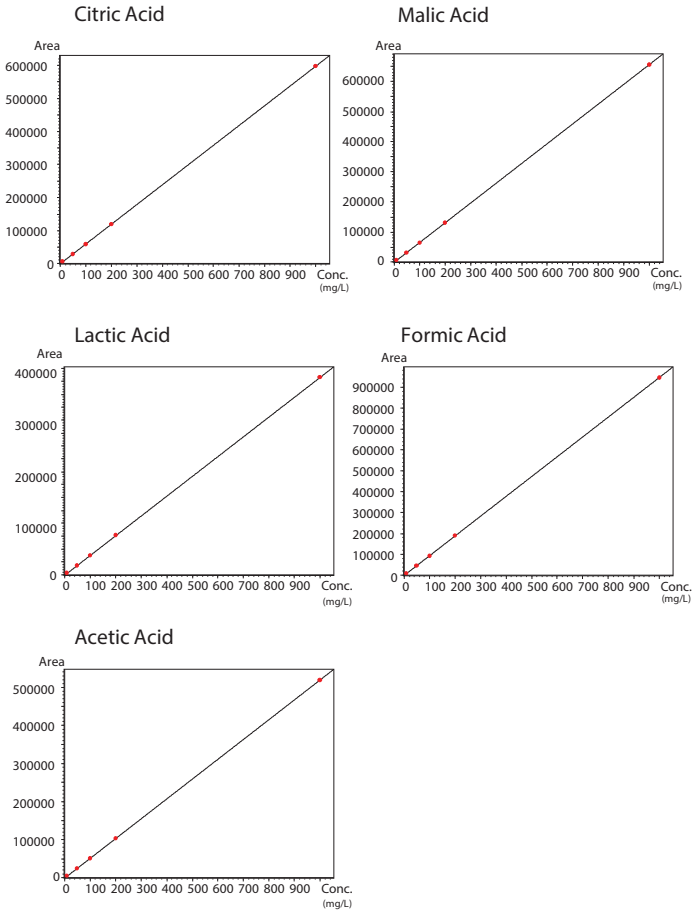

Fig. 4 **Calibration Curves for Organic Acids**

Table 5 **Linearity of the Organic Acids**

Compound	Range (mg/L)	Coefficient (R^2)
Citric acid	10-1000	0.9999
Malic acid	10-1000	0.9999
Lactic acid	10-1000	0.9999
Formic acid	10-1000	0.9999
Acetic acid	10-1000	0.9999

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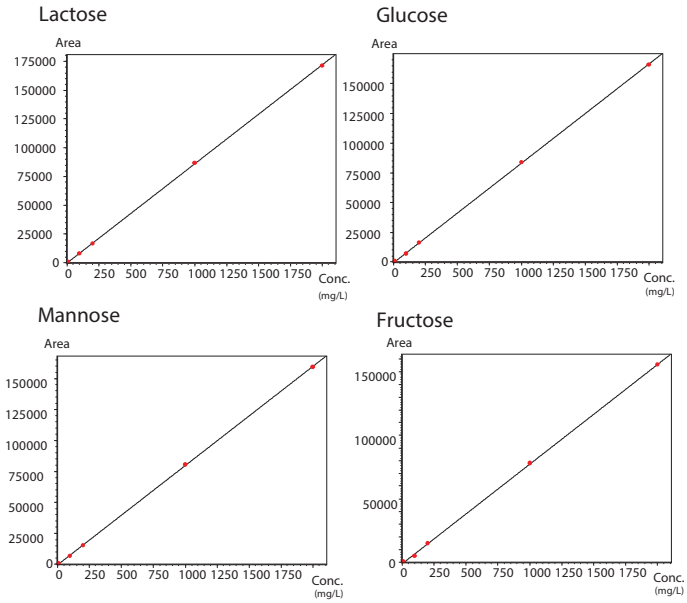

Fig. 5 **Calibration Curves of Sugars**

Table 6 **Linearity of the Sugars**

Compound	Range (mg/L)	Coefficient (R^2)
Lactose	10-2000	0.9999
Glucose	10-2000	0.9999
Mannose	10-2000	0.9998
Fructose	10-2000	0.9998

Evaluation of Carryover

Carryover was evaluated for organic acids (citric acid and lactic acid) and a sugar (lactose). To rinse the autosampler, the interior and exterior of the sample needle were rinsed with water.

Organic acid carryover evaluation results are shown in Fig. 6 and sugar carryover evaluation results in Fig. 7. Carryover was 0.0055 % for citric acid, 0.0069 % for lactic acid, and 0.0098 % for lactose, which are sufficiently low to confirm that carryover did not affect quantitation results.

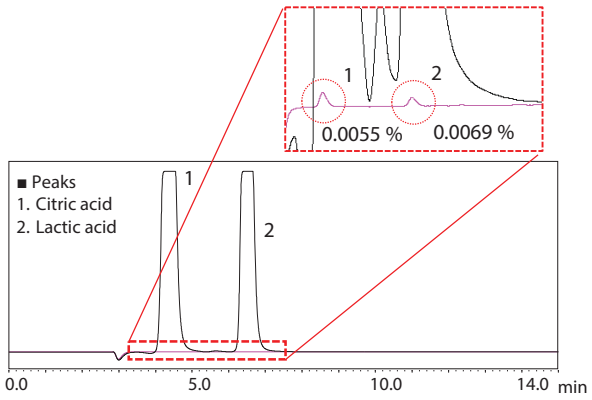

Fig. 6 **Evaluation of Citric and Lactic Acid Carryover**

Black line: Aqueous standard mixture solution of 100 g/L citric acid and lactic acid
Red line: Blank

Black line: 20 g/L aqueous lactose solution
Red line: Blank

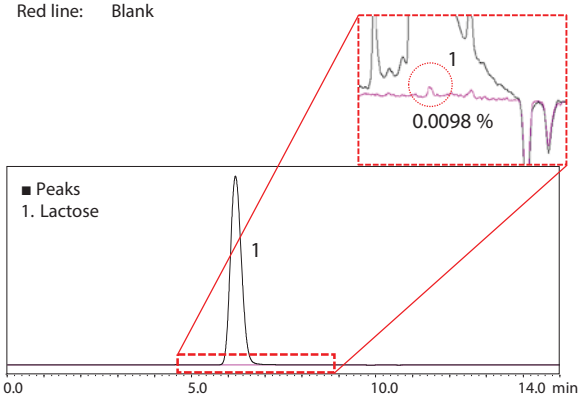

Fig. 7 **Evaluation of Lactose Carryover**

Sample Pretreatment

Yogurt was added to milk and fermented by heating it to 40 °C in a commercial yogurt maker. Samples were then obtained by sampling the mixture at fixed intervals after starting fermentation. Samples were pretreated according to the following procedure.

(1) Weigh 1 g of yogurt. Add 4 mL of 5 mmol/L aqueous *p*-toluenesulfonic acid solution and 1 mL of chloroform.
(2) Shake vigorously for one minute and then separate by centrifuge for one minute at 10,000 rpm.
(3) Collect the supernatant and filter it through a 0.45 µm pore filter.
(4) Use the filtrate diluted by ten times as the sample for analysis.

Analysis of Yogurt

For this article, samples were analyzed at fixed intervals (0.0, 1.0, 2.0, 3.5, 5.5, 7.0, and 8.5 hours) after fermentation started.

The chromatogram from analyzing organic acids in the sample acquired 3.5 hours after fermentation started is shown in Fig. 8 and the chromatogram from analyzing sugars in the same sample is shown in Fig. 9. Organic acids detected were citric and lactic acids and the sugar detected was lactose. These results were used for monitoring the fermentation process.

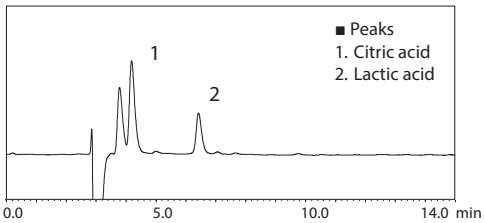

Fig. 8 **Chromatogram from Analyzing Organic Acids in Yogurt after 3.5 Hours of Fermentation**

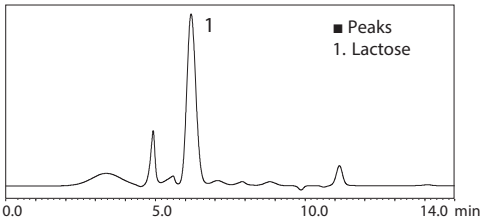

Fig. 9 **Chromatogram from Analyzing Sugars in Yogurt after 3.5 Hours of Fermentation**

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Evaluation of Yogurt Recovery Rate

The sample taken 3.5 hours after fermentation started was used to evaluate the recovery rate of added organic acids and sugars. In step (1) of the pretreatment process, 1 g of yogurt was weighed. Then 2.6 mL of a 5 mmol/L aqueous *p*-toluenesulfonic acid solution, 0.7 mL of a 400 mg/L aqueous organic acid mixture solution, 0.7 mL of a 2000 mg/L aqueous sugar mixture solution, and 1 mL of chloroform were added to the yogurt. Chromatograms with and without the standard organic acids added are shown in Fig. 10, the recovery rates of organic acids are indicated in Table 7, chromatograms with and without the standard sugars added are shown in Fig. 11, and the recovery rates of sugars are indicated in Table 8. Good recovery rates between 94.6 and 101.8 % were obtained for both organic acids and sugars.

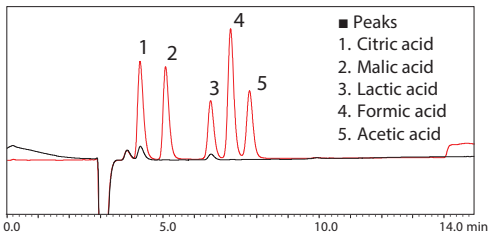

Fig. 10 **Chromatograms of Organic Acids**
(Red: With standard added; Black: Without standard added)

Table 7 **The recovery rate of target component (Organic acid)**
(Additive concentration: 56 mg/L, calculated as concentration after pretreatment)

Compound	Actual measurement of Spiked sample (mg/L)	Actual measurement of Unspiked sample (mg/L)	Recovery Rate (%)
Citric acid	65.9	10.0	99.8
Malic acid	57.0	Not detected	101.8
Lactic acid	61.4	8.4	94.6
Formic acid	56.4	Not detected	100.6
Acetic acid	56.1	Not detected	100.3

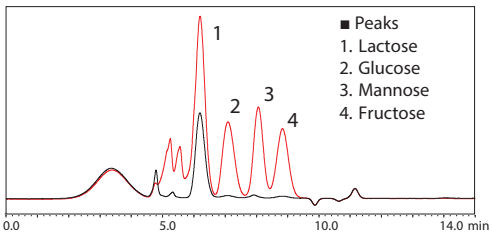

Fig. 11 **Chromatograms of Sugars**
(Red: With standard added; Black: Without standard added)

Table 8 **The recovery rate of target component (Sugar)**
(Additive concentration: 280 mg/L, calculated as concentration after pretreatment)

Compound	Actual measurement of Spiked sample (mg/L)	Actual measurement of Unspiked sample (mg/L)	Recovery Rate (%)
Lactose	544.0	257.1	102.4
Glucose	282.4	Not detected	100.9
Mannose	301.5	Not detected	107.7
Fructose	298.6	Not detected	106.6

Time-Course Evaluation of Changes during Yogurt Fermentation

Samples taken at fixed intervals (0.0, 1.0, 2.0, 3.5, 5.5, 7.0, and 8.5 hours) after fermentation started were analyzed to confirm the quantities of organic acids and sugars they contain.

The change in organic acid content after each fermentation interval is shown in Fig. 12 and the change in sugar content in Fig. 13. These results indicate that lactose is decomposed to form lactic acid as fermentation progresses.

Note that the indicated concentration values were converted based on the original yogurt solution.

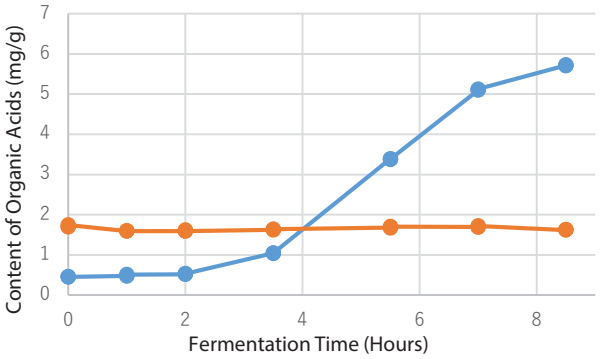

Fig. 12 **Content of Organic Acids in Yogurt**
(Blue: Lactic acid; Orange: Citric acid)

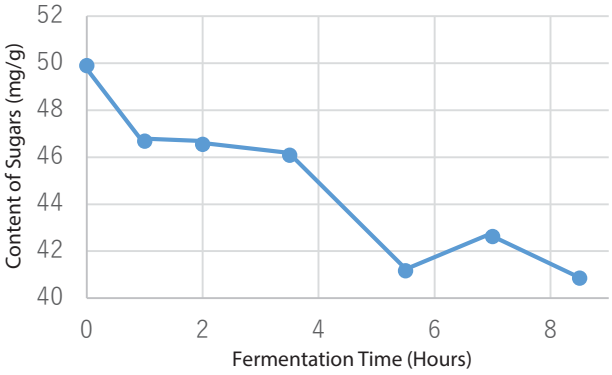

Fig. 13 **Content of Sugars in Yogurt**

Summary

Using the Nexera series dual injection system enabled two different types of analyses (organic acid analysis and sugar analysis) to be performed simultaneously in one system.

Using the dual injection system, we were able to monitor the progress of fermentation by evaluating time-course changes in organic acid and sugar content levels during fermentation. The dual injection system makes it easy to analyze multiple aspects of samples by analyzing samples and data from multiple groups of compounds simultaneously.

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First Edition: Aug. 2019

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Application News

No. L555

High Performance Liquid Chromatography

Improvement of Productivity in Research on Intestinal Microbiota by Shim-pack™ Fast-OA High-Speed Organic Acid Analysis Column

Recent research suggests that intestinal microbiota that grows in the intestinal tract contributes to maintaining and improving the health of the host. In the field of research on intestinal microbiota, mass spectrometry is used for comprehensive measurements of metabolites, and HPLC is utilized in cases where the target is clear (for example, quantitation of short-chain fatty acids formed by microbial metabolism).

This article introduces an example of measurement of short-chain fatty acids in feces of monkeys by using a high-speed organic acid analysis column.

M. Nakashima, T. Hattori

Analysis of Short-Chain Fatty Acids Standard Solution

Shim-pack Fast-OA is an ion exclusion mode column that separates acidic compounds such as short-chain fatty acids. Because retention time changes depending on the mobile phase concentration and temperature, conditions are optimized by adjusting the column temperature according to the target compound of the analysis. Fig. 1 shows the retention times of short-chain fatty acids at temperatures from 35 °C to 50 °C. Although there are no temperature-dependent changes in the elution order, retention becomes weaker as the temperature is increased. In this study, 50 °C was used, as this temperature enables rapid analysis with no loss of separation performance.

Table 1 shows the analysis conditions when analyzing 6 components of a mixed short-chain fatty acid standard solution using a Shim-pack Fast-OA and a Shim-pack SCR-102H, which is also an ion exclusion mode column, and Fig. 2 shows the chromatograms. When the Shim-pack SCR-102H was used, the analysis time for one cycle was about 30 min, but with Shim-pack Fast-OA, elution of valeric acid with a retention time within 10 min was confirmed.

The Shim-pack SCR-102H has the advantage that high separation performance is possible owing to its column length of 300 mm. However, when the target is short-chain fatty acids, analysis time becomes an issue. Although it is difficult to achieve a reduction in analysis time in ion exclusion chromatography by changing the analysis conditions, analysis time can be shortened effectively by using Shim-pack Fast-OA, which has a smaller particle size than conventional columns and allows selection of the number of columns depending on the analysis target.

Table 1 Analysis Conditions

Column	: Shim-pack Fast-OA (100 mm L. × 7.8 mm I.D., 5 µm) Shim-pack SCR-102H (300 mm L. × 8.0 mm I.D., 7 µm)
Guard column	: Shim-pack Fast-OA (G) Shim-pack SCR-102H (G)
Mobile phase	: 5 mmol/L p-toluenesulfonic acid (Mobile phase reagent set for organic acid analysis, mobile phase)
Flow rate	: 0.8 mL/min
pH buffering solution	: 5 mmol/L p-toluenesulfonic acid 20 mmol/L Bis-Tris 0.1 mmol/L EDTA (Mobile phase reagent set for organic acid analysis, pH buffering reagent)
Flow rate	: 0.8 mL/min
Column temperature	: 50 °C
Detection	: Conductivity detector (CDD-10Avp)
Injection volume	: 10 µL

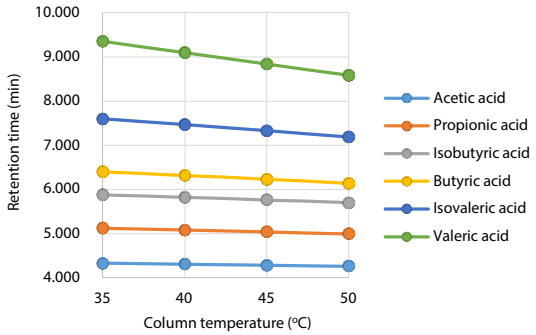

Fig. 1 Retention Behavior of Short-Chain Fatty Acids

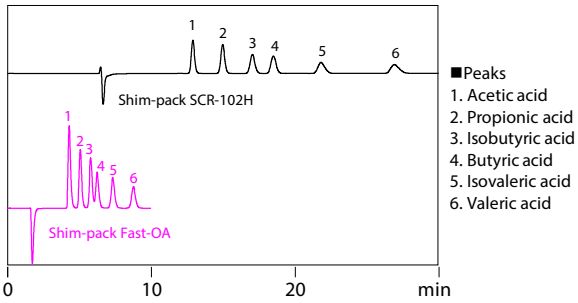

Fig. 2 Chromatograms of Standard Sample (500 mg/L each)

Calibration Curve

Calibration curves for each of the short-chain fatty acids were prepared in the concentration range of 5, 10, 50, 100, 500, and 1000 (mg/L). Table 2 shows the results of an evaluation of the linearity contribution ratio and area value reproducibility at 5 mg/L. Satisfactory results were obtained, with contribution ratio (r^2) values 0.999 or higher and area value reproducibility (%RSD) of 5% or less.

Table 2 Linearity Contribution Ratio and Area Value Reproducibility

	Linearity (r^2)	Reproducibility (N=6, %RSD)
Acetic acid	0.9997	1.36
Propionic acid	0.9997	3.51
Isobutyric acid	0.9997	2.48
Butyric acid	0.9998	2.24
Isovaleric acid	0.9999	3.40
Valeric acid	0.9997	1.95

Samples and Pretreatment

The monkey feces used as samples were frozen on site and stored in a frozen condition at -80 °C. Fig. 3 shows the pretreatment protocol. First, 700 µL of phosphate buffered saline (PBS) was added to 100 mg of the sample. The sample for use in the analysis was then prepared by mixing the solution, followed by centrifugal separation and ultrafiltration of the supernatant.

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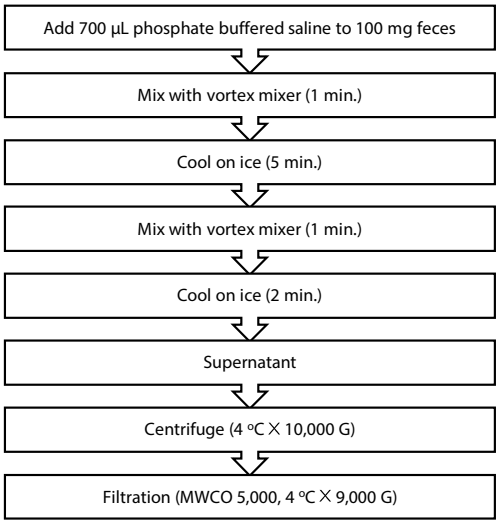

Fig. 3 Pretreatment Protocol for Monkey Feces Samples

Analysis of Monkey Feces

Feces samples from 5 monkeys were taken from points A, B, and C. Table 3 shows the details of the samples, and Fig. 4 shows a schematic illustration of the sampling locations.

Table 3 Details of Samples

Sample No.	Sampling location	Monkey	Time until freezing
1	A	Unknown	Half day to full day
2	B	Male, age 4 years	Immediately after excretion
3	B	Male, age 2 years	Immediately after excretion
4	C	Female, adult	Immediately after excretion
5	C	Female, adult	Immediately after excretion

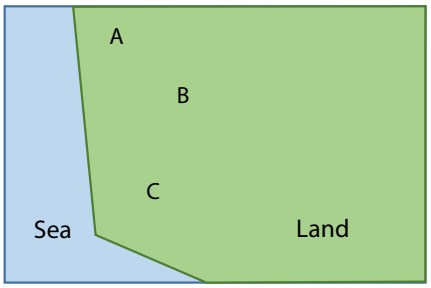

Fig. 4 Schematic Illustration of Sampling Locations

After pretreatment by the protocol in Fig. 3, the above-mentioned 5 samples were analyzed by HPLC under the conditions in Table 1 using Shim-pack Fast-OA columns. Fig. 5 shows the chromatogram of Sample 2. A large peak originating from the buffer solution used in the pretreatment, which contained phosphoric acid, was observed around t0 (around 2.5 min). However, peaks of the short-chain fatty acids eluted after acetic acid (Fig. 5, after 4 min), which are particularly important in research on intestinal bacterial flora, could be quantified with no problems.

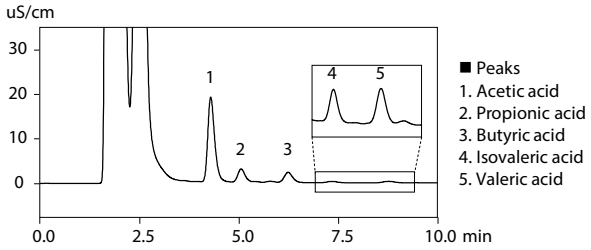

Fig. 5 Chromatogram of Sample 2

Table 4 shows the amounts of each of the short-chain fatty acids contained in Samples 1 to 5. In the case of Sample 1, half day to full day passed from excretion until sampling and freezing, and virtually no short-chain fatty acids were detected. This is thought to be due to volatilization of the short-chain fatty acids during the period from excretion to sampling, or metabolism by microorganisms in feces. Based on this, for accurate measurement, collection of samples as soon as possible after excretion and storage under conditions that prevent microbial metabolism and volatilization are critical for correct measurement of the effect of intestinal microbiota from feces samples.

Table 4 Amount of Short-Chain Fatty Acids Contained in Samples

	Amount of compounds ($\times 10^{-2}$ mol/kg)					
	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid
Sample 1	0.042	-----	-----	-----	-----	-----
Sample 2	5.6	0.99	-----	1.0	0.16	0.23
Sample 3	5.5	0.96	-----	0.90	0.19	0.34
Sample 4	5.8	1.3	-----	1.2	-----	0.25
Sample 5	6.3	0.88	-----	0.84	-----	0.23

Conclusion

Short-chain fatty acids, which are an object of research in the field of intestinal microbiota, were analyzed by using a Shim-pack Fast-OA column. Quantitation of the target compounds was possible within a time of 10 min and was unaffected by contamination. As a result of measurements of the amounts of short-chain fatty acids contained in feces samples from 5 monkeys, virtually no short-chain fatty acids were found in one sample that was collected and frozen more than half a day after excretion, but it was possible to measure the existence of short-chain fatty acids with satisfactory sensitivity in the samples that were frozen immediately after excretion.

This Application News was prepared with the cooperation of Prof. Kazunari Ushida of the Chubu University Academy of Emerging Sciences, who provided valuable guidance and also supplied the monkey stool samples.
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Application News

Ion Chromatography

No. L553

US EPA 300 Method-Compliant Environmental and Water Analysis

Many countries and regions specify standard values for general inorganic anions such as fluoride, chloride, nitrate, nitrite, and sulfate ions in order to minimize the potential health effects of environmental water and drinking water.

A suppressor-type ion chromatograph is generally used in quantitation of these inorganic anions. In the United States, Environmental Protection Agency (EPA) Methods 300.0 and 300.1 specify the analysis method for inorganic anions in water by ion chromatography (IC).

In anion analysis, conductivity suppressor-type IC enhances sensitivity by replacing the sodium ions in the eluent, which are necessary and indispensable for separation, with hydrogen ion before detections of the target anions.

In this article, an EPA 300-compliant quantitative analysis of 7 general inorganic anions in various types of water samples was conducted using an HIC-ESP, which is a new Shimadzu ion chromatograph system equipped with an electrodialysis-type suppressor.

H. Guo, M. Oshiro

Analytical Conditions

Fig. 1 shows the results when a 50 µL mixed standard sample of the 7 anions prescribed in EPA 300 was injected. Table 1 shows the analytical conditions.

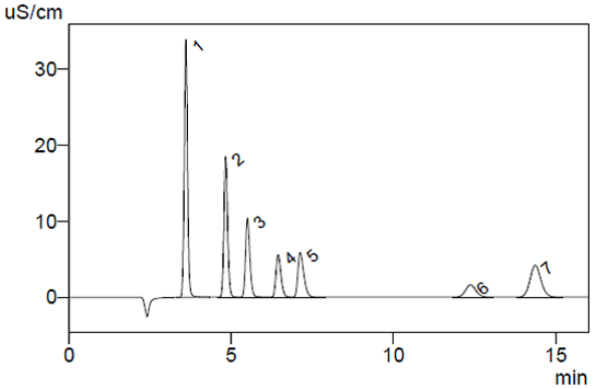

Fig. 1 Chromatogram of Mixed Standard Sample of Anions
Peaks: 1. F (5 mg/L), 2. Cl (5 mg/L), 3. NO_2 (5 mg/L), 4. Br (5 mg/L), 5. NO_3 (5 mg/L), 6. PO_4 (5 mg/L), 7. SO_4 (5 mg/L)

Table 1 Analytical Conditions

Column	: Shim-pack™ IC-SA2 (250 mmL. × 4.0 mmI.D.)
Mobile phase	: 1.8 mmol/L Sodium Carbonate 1.7 mmol/L Sodium Hydrogen Carbonate
Flow rate	: 1.0 mL/min
Column temp.	: 40 °C
Injection volume	: 50 µL
Detection	: Electro conductivity detector

Linearity and Detection Limit

Under EPA Method 300, advanced confirmation of linearity, the detection limit, the recovery rate, and repeatability is necessary for evaluation of the analysis method and system performance.

For the detection limit, in accordance with the procedure described in Method 300, an MDL (Method Detection Limit) standard sample was prepared, 7 continuous analyses were carried out, and MDL was calculated as (t) × (S). Here, t means the t value for the 99% confidence level (Student's t-value in t-test; in 7 continuous analyses, t = 3.14), and S means the standard deviation of 7 continuous analyses.

Table 2 shows the calibration curve range, linearity, MDL standard concentration, and calculated MDL. Fig. 2 shows the calibration curves of all of the anions.

Table 2 Linear Calibration Region, Linearity, MDL Standard, and MDL

Component	Calibration curve range (mg/L)	Coefficient of correlation (r^2)	MDL standard concentration (µg/L)	MDL (µg/L)
F	0.05-20	0.9999	10	3.31
Cl	0.1-100	0.9992	5	2.05
NO_2	0.05-20	0.9995	20	1.49
Br	0.05-20	0.9989	20	3.3
NO_3	0.05-20	0.9991	20	2.69
PO_4	0.05-20	0.9995	50	14.38
SO_4	0.05-20	0.9992	20	3.63

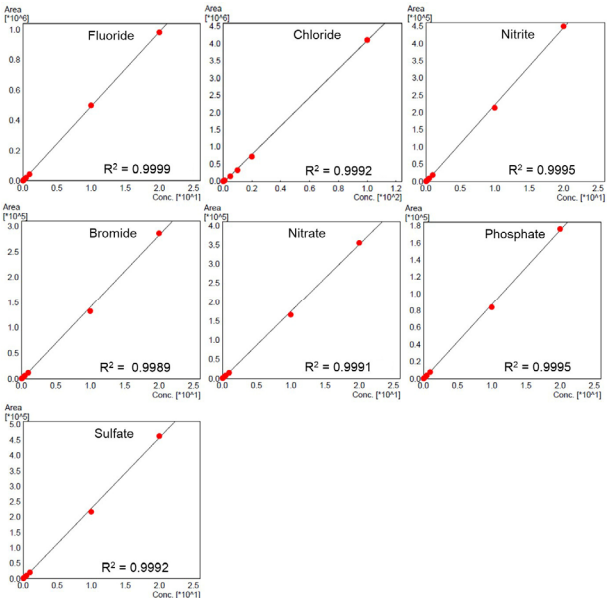

Fig. 2 Calibration Curves of 7 Anions in EPA Method 300

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Repeatability and Accuracy

The repeatability of the retention time and peak area was verified by using a mixed standard sample with a 10 mg/L of each ion. To also verify day-to-day repeatability at the same time, the test was conducted for a period of 4 days.

The repeatability of the retention time and peak area was verified from the results of 5 continuous analyses each day. Table 3 shows the results for the 1st and 4th days. The results showed excellent repeatability of the retentions times and peak areas of all components during the test period.

Table 3 Repeatability of Retention Time and Peak Area (One Day, Day-to-Day Repeatability)

Component	1st day		4th day	
	Retention time (%RSD)	Peak area (%RSD)	Retention time (%RSD)	Peak area (%RSD)
F	0.06	0.07	0.12	0.72
Cl	0.06	0.07	0.15	0.78
NO_2	0.06	0.14	0.18	0.70
Br	0.07	0.24	0.20	0.75
NO_3	0.08	0.20	0.22	0.97
PO_4	0.15	0.25	0.24	0.64
SO_4	0.13	0.18	0.30	0.63

To determine accuracy, blank ultrapure water (DI) and tap water were evaluated after standard spiking with a 2 mg/L concentration of each anion. Table 4 shows the results. Satisfactory recovery rates were obtained from the various water samples.

Table 4 Recovery Rates of Blank and Tap Water

Component	Spiked concentration (mg/L)	Recovery rate (%)	
		Ultrapure water	Tap water
F	2.0	93.9	99.4
Cl	2.0	94.0	96.2
NO_2	2.0	96.1	95.2
Br	2.0	97.1	91.2
NO_3	2.0	98.0	106.0
PO_4	2.0	98.4	103.0
SO_4	2.0	98.3	92.6

Analysis of Actual Samples

Fig. 3 shows the chromatograms of three types of water samples, and Table 5 shows the quantitation results.

Table 5 Results of Quantitation of Anions in Water Samples

Component	Concentration (mg/L)		
	PET bottle water	Water server water	Tap water
F	0.08	0.50	0.64
Cl	12.99	41.06	39.69
NO_2	N.D.	0.25	N.D.
Br	0.27	0.39	0.36
NO_3	2.66	8.93	8.87
PO_4	N.D.	N.D.	N.D.
SO_4	25.88	15.95	15.37

The negative peaks detected at around 2.5 min in the chromatograms are called water dip and originate from the water in the injected sample. Satisfactory separation between this peak and the fluoride peak, which has a short retention time, was obtained under these conditions, demonstrating that high quantitation accuracy is possible.

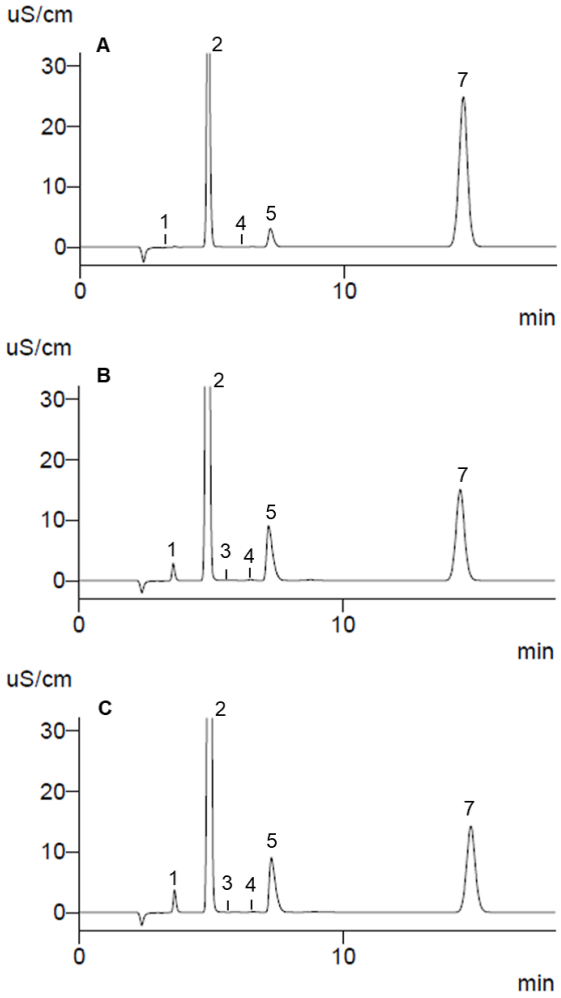

Fig. 3 Chromatograms of Water Samples
A: PET Bottle Water, B: Water Server Water, C: Tap Water
(Peaks: 1. F, 2. Cl, 3. NO_2, 4. Br, 5. NO_3, 6. PO_4, 7. SO_4)

* The samples were obtained and the analysis was conducted in the United States.

Conclusion

EPA 300-compliant analysis of inorganic anions with high sensitivity and high reliability is possible by using the Shimadzu HIC-ESP ion chromatograph system.

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