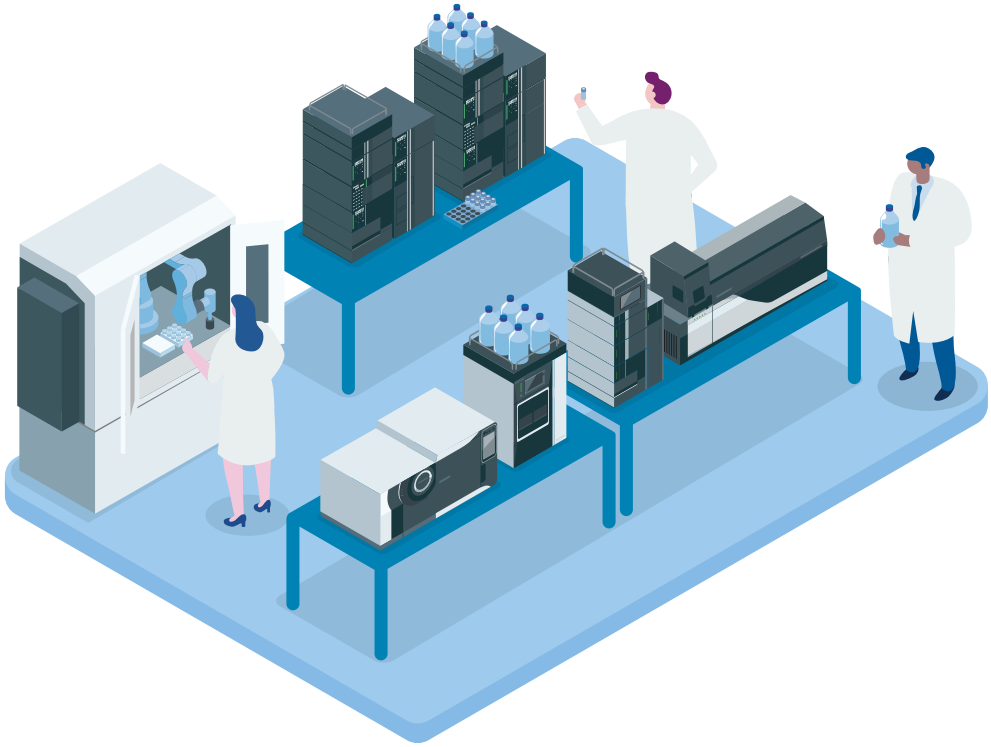
C190-E340

SHIMADZU
Excellence in Science

Automation and Efficiency Improvement Solutions (HPLC and LC-MS)



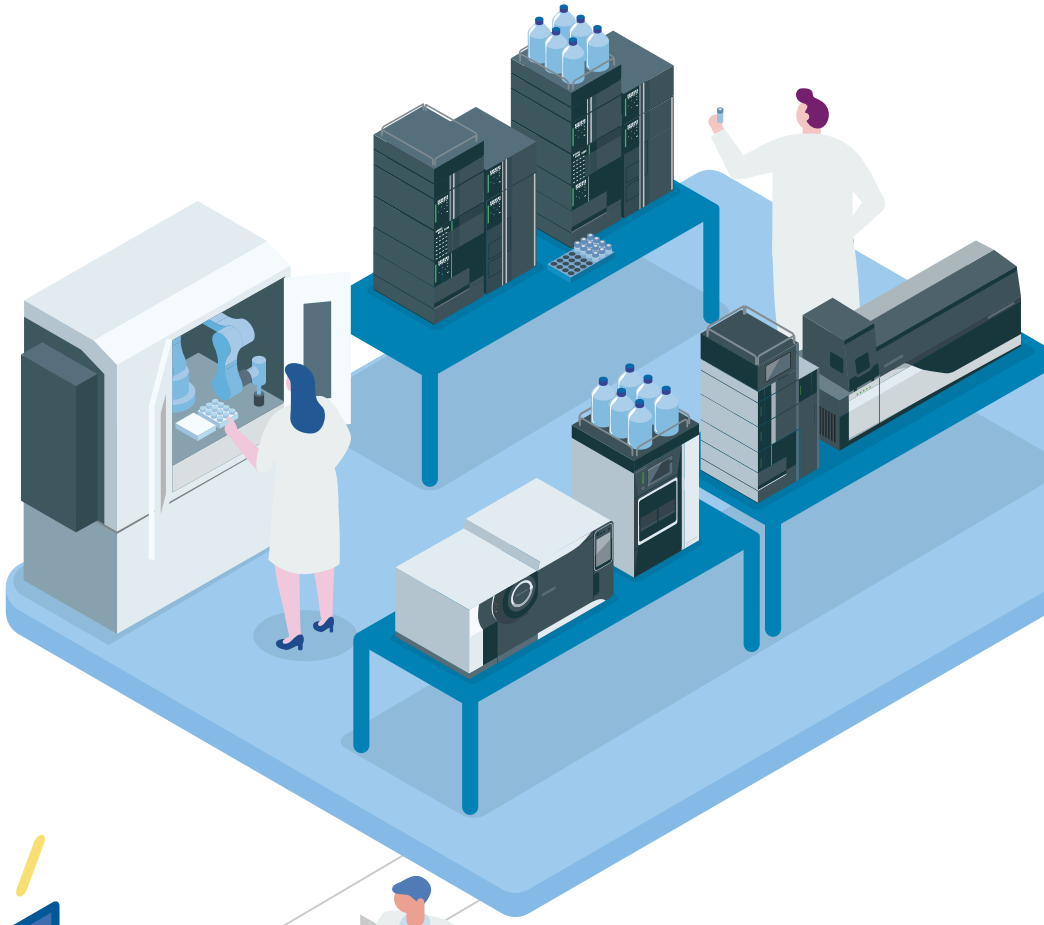
HPLC/LC-MS Analysis Workflow

Just as robots equipped with AI functionality are offering major changes to our lives, continuous advancements in Shimadzu HPLC, LC-MS, data analysis software, and pretreatment systems are significantly changing how day-to-day analytical operations are performed.

01

Sample Pretreatment

Multiple automation technologies (such as for dispensing liquids, centrifuging, and heating) can be used in combination based on the given analytical application, such as for automating pretreatment processes necessary for analyzing glycan modifications of antibodies. This will eliminate operating errors and pretreatment variability.



02

Starting/Managing the System

Functionality for automatically starting up the system, monitoring mobile phase reservoir levels, and self-diagnosing and restoring solvent delivery pumps can improve workflow efficiency and increase laboratory productivity.

04 Quantitative Analysis

Automatic dilution functionality included with the autosampler reduces the burden on analysts by automating sample dilution and calibration curve creation steps that previously required tedious pipetting operations.

03 Method Development

Gradient conditions and various other parameter settings are automatically optimized to satisfy specified criteria (e.g., separation level), such as by switching between different mobile phases and columns.

05

Sampling

Operator workloads can be reduced by using a flow vial autosampler to automatically acquire samples at fixed intervals and check them for trend changes over time during synthesis reaction monitoring or by automatically injecting them into the analytical instrument during formulation elution testing.

06

Preparative Purification

Functionality for automatically generating analysis schedules, used to help evaluate analysis scale parameter settings, and functionality for scaling up those settings once they are determined, helps ensure preparative chromatography can be performed with confidence, even by analysts without preparative experience.

Table of Contents

Sample Pretreatment

Profiling Antibody N-Linked Glycans in Culture Supernatant Using Fully Automated Sample Preparation Module for Glycan Analysis ····· P6

Starting/Managing the System

Fully Automated Workflow for HPLC Analysis Using Automatic Startup with FlowPilot Function - Analytical Intelligence Part 3 - ····· P8

Maximizing Analytical Efficiency with Real-time Measurement of Mobile Phase Consumption - Analytical Intelligence Part 2 - ····· P10

Use of Solvent Delivery Unit Equipped with Auto-diagnostics and Auto-recovery Functions to Enhance Lab Productivity - Analytical Intelligence Part 1 - ····· P12

Method Development (Gradient Conditions)

Automated Gradient Optimization Based on AI Algorithm for LC Method Development ····· P14

Automatic Optimization of Gradient Conditions by AI Algorithm - Application to LC Method Development for Simultaneous Analysis of Functional Components in Foods - ····· P16

Automatic Optimization of Gradient Conditions by AI Algorithm for a Synthetic Peptide and Impurities ····· P19

Automatic Optimization of Gradient Conditions by AI Algorithm for Impurity Analysis ····· P21

Method Development (AQbD)

Efficient Method Development Based on Analytical Quality by Design with LabSolutions™ MD software ····· P23

Efficient Method Development on Pharmaceutical Impurities Based on Analytical Quality by Design ····· P27

Efficient Method Development for a Synthetic Peptide and Related Impurities ····· P29

Quantitative Analysis

Simple Labor-Saving Calibration Curve Creation Using Autosampler Automatic Dilution Function ····· P34

Simple Labor-Saving Calibration Curve Creation Using Autosampler Automatic Dilution Function Part 2 ····· P37

Sampling

On-Line Monitoring of Flow Synthesis Reactions Using Nexera FV ····· P40

On-Line Dissolution Test of Loxoprofen Sodium Tablets ····· P42

Preparative Purification

Seamless Purification Workflow from Analytical to Preparative Using a Single Quad LC-MS System ····· P44

Streamlining of Preparative Purification Work by Nexera ASAPrep ····· P47

Application News

Fully Automated Sample Preparation Module for Glycan Analysis
High Performance Liquid Chromatograph

Profiling Antibody N-Linked Glycans in Culture Supernatant Using Fully Automated Sample Preparation Module for Glycan Analysis

Mayu Okajima, Shuichi Nakaya, and Takashi Nishikaze

User Benefits

- By simply placing the dedicated reagent kit and the culture supernatant in the system, it can automatically perform all processes, including antibody purification, glycan release, and fluorescent labeling.
- In addition to relieving operators from repetitive routine tasks, the system can also automatically pre-treat samples overnight.
- Using validated methods to further automate operations, the system enhances laboratory productivity.

■ Introduction

Antibody drugs and other biopharmaceuticals are manufactured using genetic engineering and cell culturing technologies, based on peptides or proteins derived from biological organisms as active ingredients. In recent years, many companies have been researching or developing biopharmaceuticals as therapeutic or diagnostic drugs.

Antibody drugs include N-linked glycans introduced by a post-translational modification. Due to the potential impact on the safety and efficacy of pharmaceuticals, these glycans are considered critical quality attribute of antibody drugs. However, the glycan has structural diversity as an inherent character and the diversity trends can be affected by slight variations in the cell strains and cultivation parameters used in manufacturing process. Therefore, the glycans attached to antibodies are strictly monitored and controlled during manufacturing processes using appropriate analytical methods that align with the intended purpose.

One method used to characterize glycan modifications is glycan profiling, which involves releasing glycans from antibodies using enzymes or other methods, followed by fluorescent labeling and analysis via HPLC, LC/MS, or other techniques. If the given samples are culture supernatants, the process of purifying antibodies has to be included. However, purifying antibodies, releasing glycans, and applying fluorescent labels is a labor-intensive process that can take 2 days or more when performed manually, making the procedure highly person-dependent.

■ Overview of the MUP-3100 System

By simply placing the Auto-EZGlyco mAb-N Kit for SHIMADZU, a dedicated consumable, along with the cell culture supernatant or other samples into the MUP-3100 fully automated sample preparation module for glycan analysis, and clicking the [Run] button in the control software, the module quickly, reliably, and automatically performs all the necessary steps, from antibody purification to labeled glycan preparation. The MUP-3100 is compatible with N-linked glycans in antibodies and can process up to 24 samples within 6 hours.

The MUP-3100 is equipped with the following features to support stable operation.

- ✓ Check the placement of reagents by image processing before automatic operations
- ✓ Check the attachment or detachment of pipette tips using sensors
- ✓ Save a video image if a problem occurs
- ✓ Automatically generate pretreatment reports

For more details about the MUP-3100 system, please refer to the brochure C190-E304.

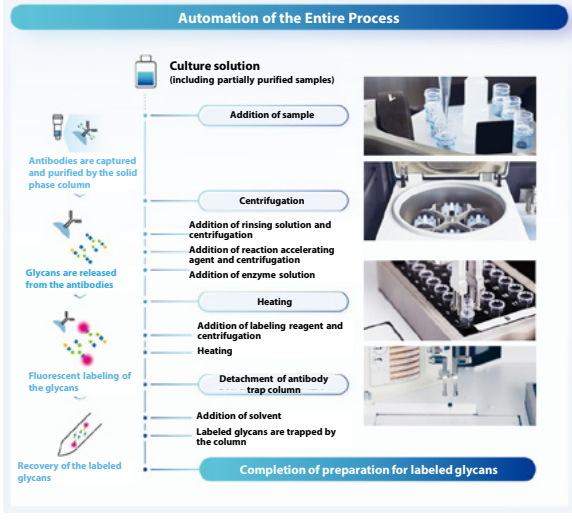


Fig. 1 MUP-3100 Fully Automated Sample Preparation Module for Glycan Analysis and Overview of Automatic Pretreatment Process Flow

■ Analysis of Antibody Glycans in Culture Supernatant

Culture Solution

Supernatant was collected on the third, fourth, fifth, and seventh day of culturing a CHO-MK cell line producing trastuzumab.

Sample Preparation

The culture supernatant was diluted to achieve an antibody concentration within the 20 to 80 µg/1200 µL range. The Auto-EZGlyco mAb-N Kit for SHIMADZU antibody capturing solution was used as the diluting agent.

LC Analysis

The glycan samples obtained through MUP-3100 pretreatment were analyzed under the conditions specified in Table 1. Due to 2-AB (2-aminobenzamide) labeling, the glycans were detected using a fluorescence detector.

Nexera™

Table 1 Analysis Conditions

System:	Nexera XR
Column:	Shim-pack™ GIST-HP Amide [Metal free] (100 mm × 2.1 mmI.D.,1.9 µm)*1
Mobile Phases:	A) 100 mM Ammonium formate (pH 4.4)
	B) Acetonitrile
Time Program	B Conc. 77.5 % (0 min) → 68.5 % (50 min) → 50 % (50.01-55 min) → 77.5 % (55.01-62 min)
Column Temp.:	40 °C
Flowrate:	0.5 mL/min
Injection Volume:	1 µL
Detection:	Fluorescence (Ex. 330 nm, Em. 420 nm) (using RF-20Axs, semi-microcell)
Vial:	TORAST PP Vial screw*2

*1 P/N: 227-30951-02
*2 P/N: 370-04050-01

Chromatograms obtained by analyzing supernatant samples from the third day of culturing are shown in Fig. 2. The glycan structures corresponding to the detected peaks were identified by comparing their retention times with those of standard samples or through LC/MS analysis.

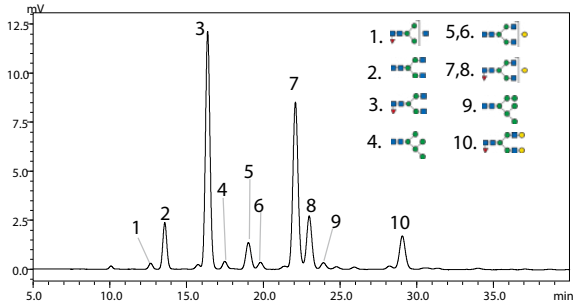


Fig. 2 Chromatogram of N-Linked Glycans in Antibodies Contained in the Supernatant from Culturing Trastuzumab-Producing Cells

● Mannose ▲ Fucose ● Galactose ■ N-Acetylglucosamine

Pretreatment Reproducibility

Six Lot 1 samples collected on the third day of culturing were pretreated using the MUP-3100 system and measured by HPLC to evaluate pretreatment reproducibility based on the area of each peak measured. The results demonstrated excellent pretreatment reproducibility, with 5 % or less RSD for major glycans and 8 % or less RSD for minor glycans.

Table 2 Reproducibility of Pretreatment for Culture Supernatant Samples (%RSD for n = 6 Measurements of Glycan Peak Areas)

Peak No.	Average Area	Area Value %RSD	Peak No.	Average Area	Area Value %RSD
1	5193	7.11	6	8062	4.20
2	43765	4.27	7	201774	4.21
3	236938	4.16	8	65241	4.11
4	7414	7.48	9	7947	7.79
5	32348	4.97	10	44387	4.58

■ Changes in Glycan Profiles Based on Culturing Duration

Fig. 3 shows chromatograms obtained from culturing days three to seven. Fig. 4 shows the change in the area of each glycan peak for each day of culturing, indicated as a ratio of the total area of 10 types of glycan peaks. From the third to fifth day of culturing, the peak ratio of the glycan No. 3, a non-galactosylated complex-type glycan, increased, while the peak ratio of galactosylated glycans and the glycan No.2, a non-fucosylated glycan, decreased. Furthermore, the results confirmed that the peak ratios for high mannose-type glycans No. 4 and No. 9 increased during the fifth to seventh days of culturing.

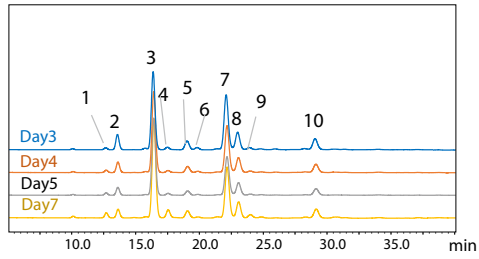


Fig. 3 Chromatograms for Respective Number of Days of Culturing

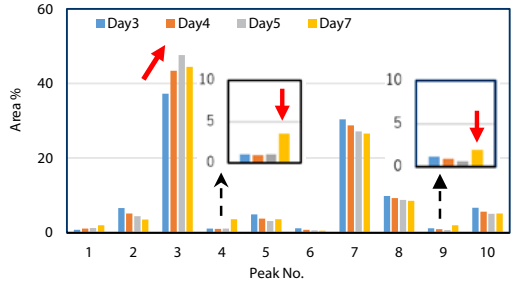


Fig. 4 Comparison of Glycan Profiles for Each Culturing Day

■ Conclusion

The MUP-3100 fully automated sample preparation module for glycan analysis was used to pretreat 24 supernatant samples from antibody-producing cell cultures within 6 hours. The resulting N-linked glycan samples were then analyzed using the Nexera HPLC system to confirm changes in glycan profiles over the elapsed days of culturing. The MUP-3100 offers a more efficient workflow compared to manual pretreatment methods and supports the development of manufacturing process for antibody drugs.

Acknowledgments

The CHO-MK cell lines for producing trastuzumab were provided by the Manufacturing Technology Association of Biologics. This research received support from the Agency for Medical Research and Development (AMED) under the grant numbers JP21ae0121014 and JP18ae0101057.

Technical Report

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.

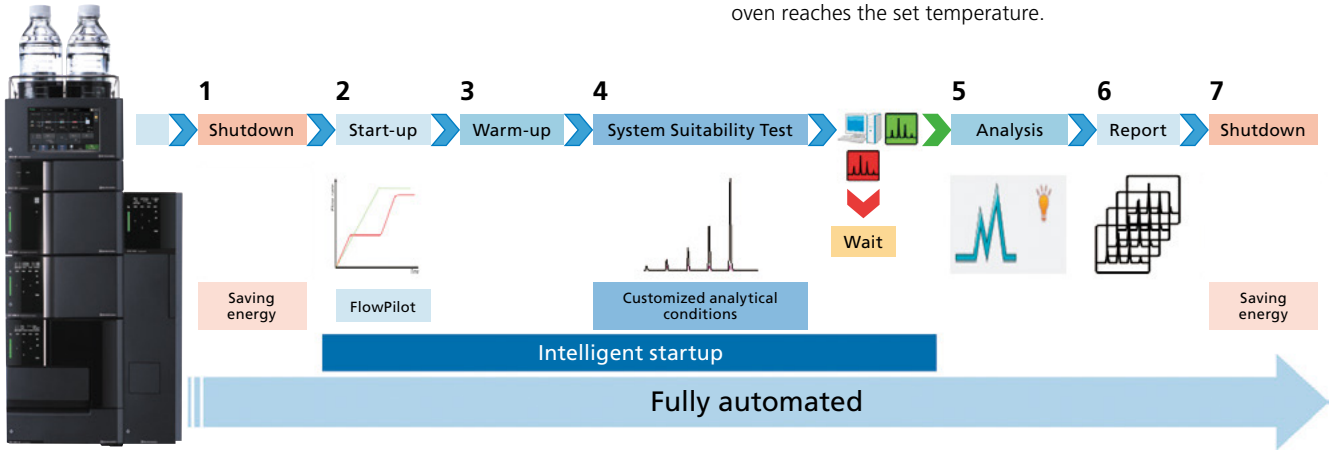


Fig. 1 Workflow diagram showing the fully-automated operation achievable with Nexera LC systems

1 Analytical & Measuring Instrument Division Life Science Business Department LC Business Unit

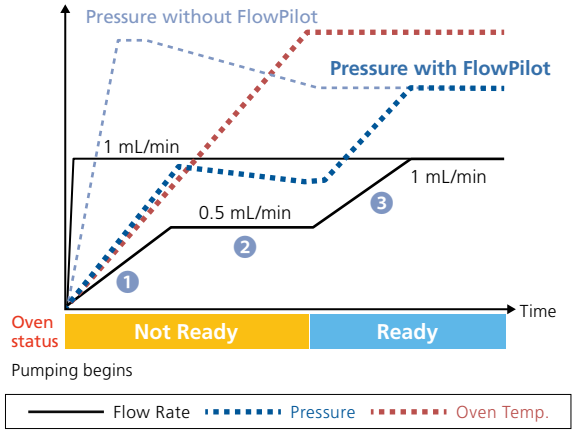


Fig. 2 Diagram of system pressure profile during start-up with the FlowPilot function

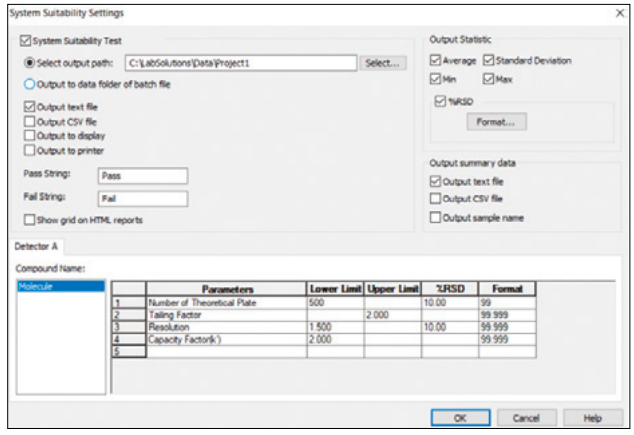


Fig. 3 System Suitability Test - example of 4 user-selected "pass/fail" criteria

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.

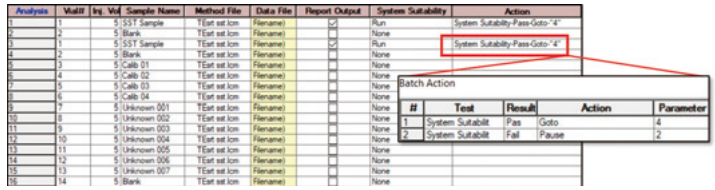


Fig. 4 System Suitability Test during batch analysis creation

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.

First Edition: May, 2019

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.

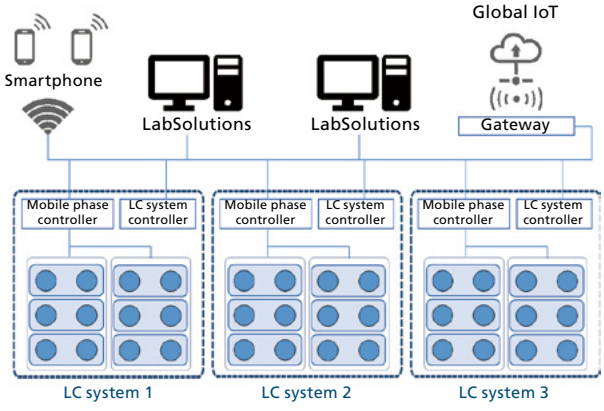


Fig. 2 Mobile phase monitoring technology integrated into laboratory workflow (representative system configuration)

1 Analytical & Measuring Instrument Division

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.

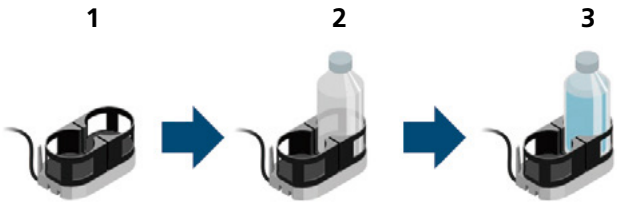


Fig. 3 Sensor calibration procedure

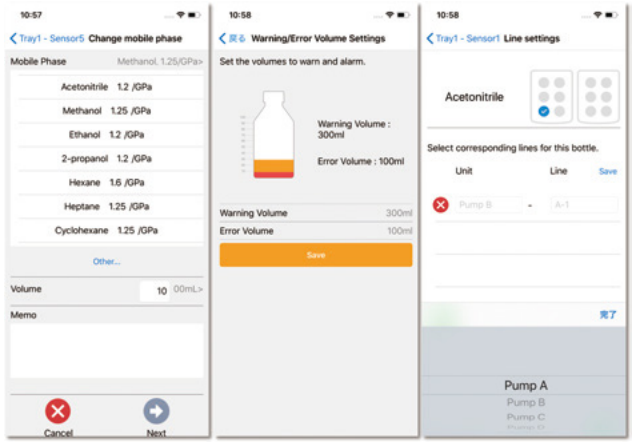


Fig. 4 Customizable settings for mobile phase monitoring

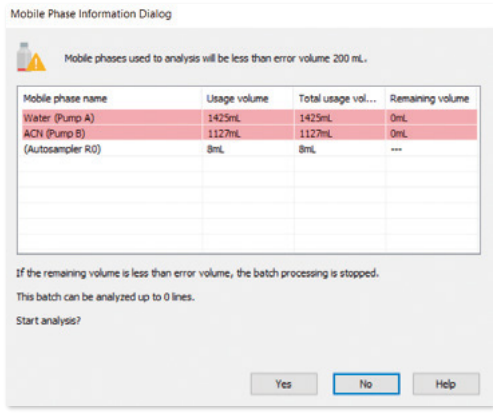


Fig. 5 Warning dialog before analysis start

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.

First Edition: April, 2019

Technical Report

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).

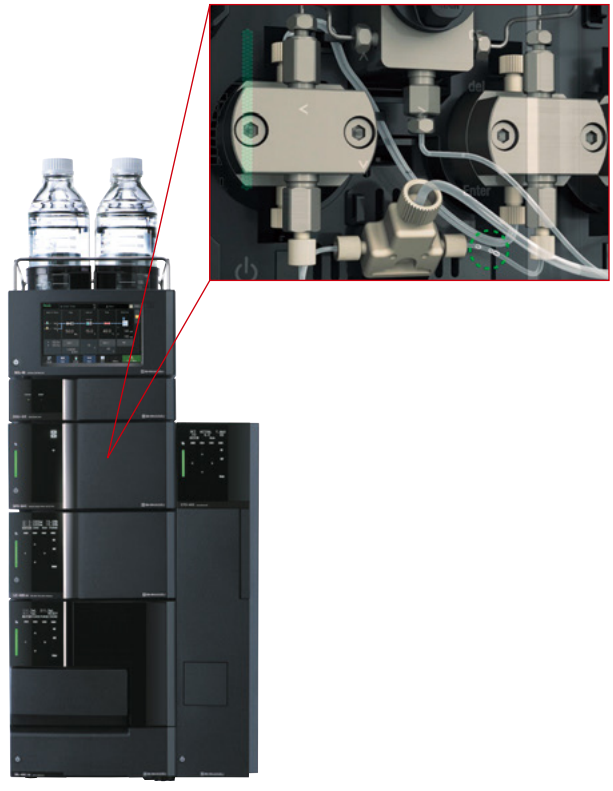


Fig. 1 Diagram of the Nexera™ solvent delivery unit flow lines

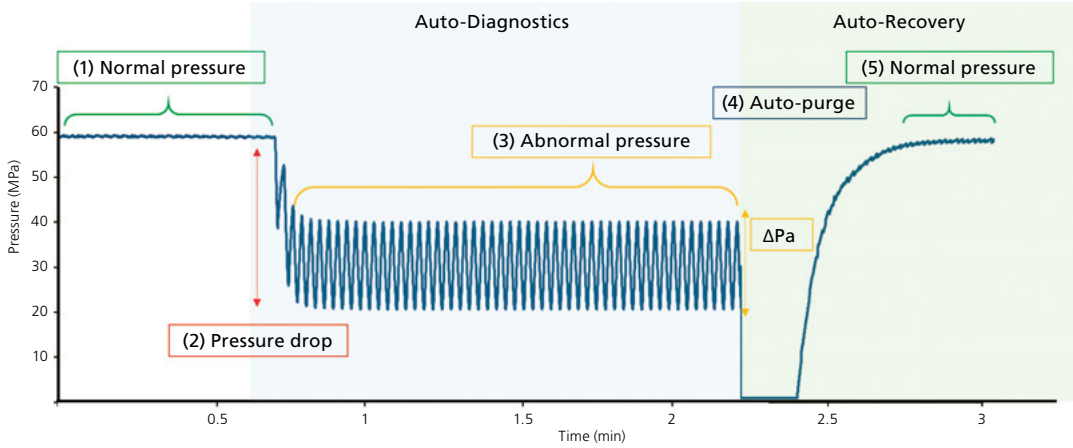


Fig. 2 Pressure changes during auto-diagnostics and auto-recovery. The time spent in each phase can vary depending on analytical conditions and user-defined settings.

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).

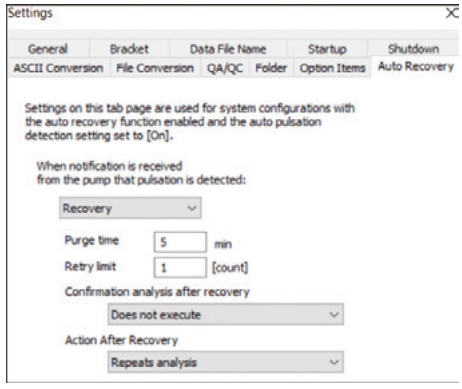


Fig. 3 LabSolutions auto-recovery settings window

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.

References

- S.R. Bakalyar, M.P.T Bradley, and R. Honganen, J. Chromatogr., 158, 277-293 (1978).
- Kagaku Binran, Kiso-hen II 8.7 Yokaido (General Chemistry Handbook, Fundamentals vol. II, 8.7 Solubility), edited by the Chemical Society of Japan, published by Maruzen Co. Ltd. (1984)

First Edition: April, 2019

Technical Report

Automated Gradient Optimization Based on AI Algorithm for LC Method Development

Shinichi Fujisaki[1]

Abstract:

In the process of method development, gradient optimization doesn't only require creating a lot of analysis schedules but also require human intervention for exploring the optimal condition based on the analysis results. LabSolutions MD, a dedicated software for supporting method development, has a unique AI algorithm for automatic optimization of gradient conditions and by setting criteria of resolution, it automatically searches for the gradient condition that meets the criteria. In this article, a case study demonstrates the application of automatic optimization of gradient condition for the simultaneous analysis of catechins, theaflavins, and gallic acid (15 compounds).

Keywords: method development, gradient optimization, AI, automatic, LabSolutions MD

1. Background

In the typical LC method development, the process begins with "preparation" which includes mobile phase preparation, installing columns, and creating analysis schedules, followed by running analysis. Then, the data is analyzed for the subsequent "preparation" and data acquisition. Method development is accomplished by repeating these processes over and over, but in addition to the massive amount of time required to repeatedly create analysis schedules, exploring the optimal condition based on the data analysis requires expertise in chromatography. In other words, typical method development requires "human intervention". Therefore, eliminating the involvement of human and automating such method development processes would be desirable to improve labor efficiency. LabSolutions MD has a unique AI algorithm to automatically optimize gradient conditions by repeating the process of "improvement gradient conditions by AI" and "correction analysis with improved condition". This enables anyone to explore the gradient conditions without "human intervention", leaving only the tasks of initial preparation of mobile phases and columns, and confirming the final result.

2. Automatic Optimization of Gradient Conditions by LabSolutions MD

Fig. 1 shows a comparison of the normal workflow of gradient optimization and automated workflow by AI algorithm of LabSolutions MD. The workflow of automatic optimization of gradient conditions consists of the following three phases.

1) Initial setting (① in Fig. 1)

2) Exploration of gradient conditions by AI (② in Fig. 1)

3) Determination of optimal condition (③ in Fig. 1)

In the "initial setting" phase, several gradient curves and the resolution criteria are set (Fig. 2 and 3). In the "exploration of gradient conditions by AI" phase, LabSolutions MD explores the gradient conditions to give better resolution based on the results from initial analysis (condition search and correction analysis in Fig. 1 ②). This process continues repeatedly until the resolution criteria is satisfied. In the "determination of optimal condition" phase, the gradient condition suggested by AI can be checked to sufficiently meet the criteria.

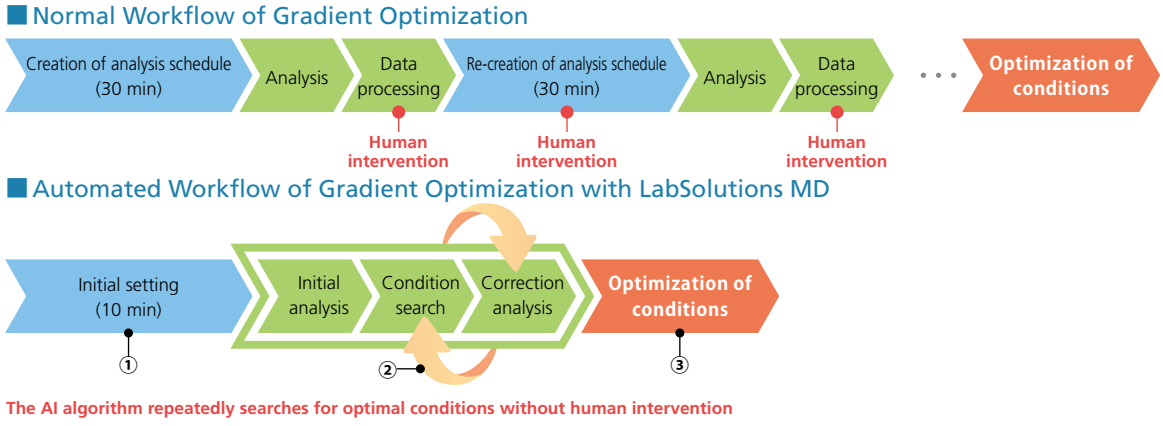


Fig. 1 Comparison of Normal Workflow and Automated Workflow with LabSolutions MD

[1] Analytical & Measuring Instruments Division

The advantages of using LabSolutions MD to automatically optimize gradient conditions are indicated below.

- Significant improvement of efficiency in the workflow of gradient optimization can be achieved without "human intervention".
- Anyone, regardless of experience in chromatography, can optimize gradient conditions by utilizing AI algorithm of LabSolutions MD.

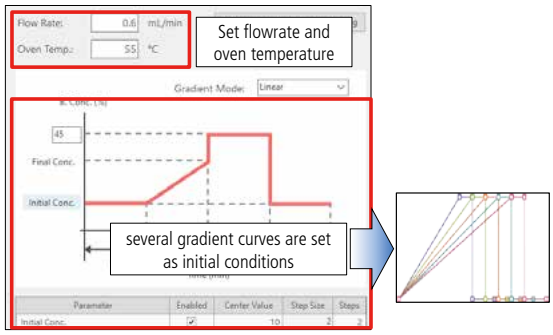


Fig. 2 Initial Setting for Gradient Optimization

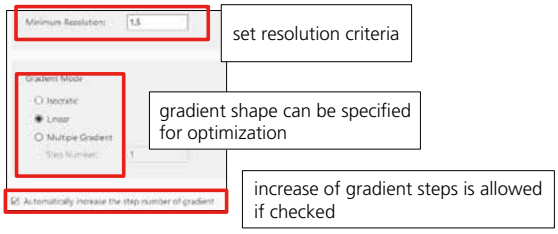


Fig. 3 Resolution Criteria Settings

3. Application to Functional Components in Foods

Fig. 4 shows an example of gradient optimization for simultaneous analysis of functional components in foods including catechins, theaflavins, and gallic acid (15 compounds). (Refer to Table 1 for analytical conditions.)

Table 1 Analytical Conditions

System : Nexera™ X3	
Sample : Catechin, Theaflavin and Gallic acid (15 compounds)	
C1) Gallocatechin	C8) Epicatechin gallate
C2) Epigallocatechin	C9) Catechin gallate
C3) Catechin	C10) Epicatechin 3-(3"-O-methyl) gallate
C4) Epicatechin	T1) Theaflavin
C5) Epigallocatechin gallate	T2) Theaflavin 3-gallate
C6) Gallocatechin gallate	T3) Theaflavin 3'-gallate
C7) Epigallocatechin 3-(3"-O-methyl)gallate	T4) Theaflavin 3,3'-digallate
	G1) Gallic acid
Mobile phase : Pump A : 0.2 % phosphoric acid in water Pump B : Acetonitrile	
Column : Shim-pack™ GISS C18 (100 mm × 3.0 mmI.D., 1.9 µm)*1 *1 227-30049-02 (Shimadzu GLC Part No.)	
Initial Settings :	
B Conc.	: 15 % (0 min) → 45 % (X min) → 15 % (X~X+5 min) *X = 6, 8, 10, 12, 14 (5 patterns)
Column Temp.	: 55 ℃
Flow rate	: 0.6 mL/min
Injection Vol.	: 5.0 µL
Detection	: Max plot 240-280 nm (SPD-M40, UHPLC cell)
Criteria of minimum resolution	: 1.5
Gradient shape for optimization	: Linear

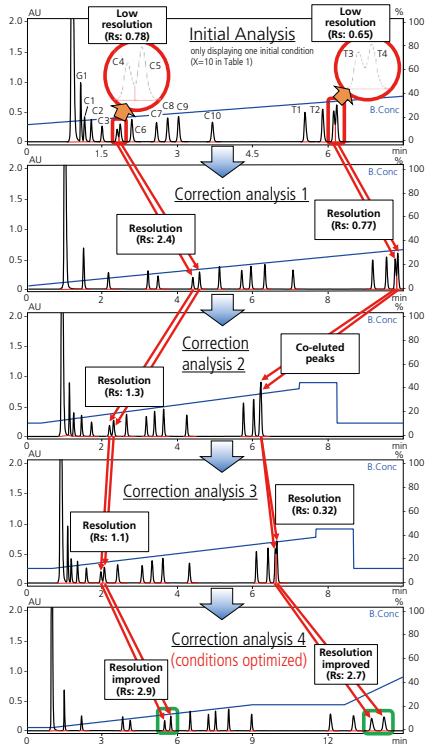


Fig. 4 Automatic Optimization of Gradient Conditions (blue lines show gradient curves)

The result of initial analysis shows the resolution between peaks C4 and C5 and between peaks T3 and T4 was not enough (shown in a red box at the top of Fig. 4). However, by using AI algorithm to repeatedly perform correction analyses, the gradient condition that satisfies the criterion (minimum resolution of 1.5) was finally discovered (shown in a green box at the bottom of Fig. 4). In this case, T3 and T4 were successfully separated by applying an isocratic elution after 9 minutes. Normally, exploring such gradient conditions requires human intervention for data analysis and knowledge about chromatography. In contrast, AI algorithm of LabSolutions MD enables anyone to easily find gradient conditions that satisfy specified criteria without relying on the intuition or experience.

4. Summary

When optimizing gradient conditions for method development, human intervention is normally required each time for creation of analysis schedules and data analysis. Therefore, automatic optimization of gradient conditions is in demand. LabSolutions MD, incorporated with unique AI algorithm, automatically explores gradient conditions that satisfy the resolution criteria, which can be expected to significantly improve the efficiency of method development.

First Edition: October, 2023

Application News

Software for Efficient Method Development "LabSolutions™ MD"

Automatic Optimization of Gradient Conditions by AI Algorithm

-Application to LC Method Development for Simultaneous Analysis of Functional Components in Foods-

Shinichi Fujisaki

User Benefits

- The AI algorithm of LabSolutions MD can automatically optimize gradient conditions to greatly reduce labor of LC method development.
- Anyone can optimize gradient conditions, regardless of their experience in chromatography.
- Comparison and evaluation of functional components, such as catechins and theaflavins, in tea leaves can be performed among different tea species.

■ Introduction

In the typical LC method development, the process begins with "preparation" which includes mobile phase preparation, column installation, and creation of analysis schedules, then analysis is started. After that, the acquired data is analyzed and "preparation" for the subsequent analysis is carried out, followed by starting the next analysis again. The method development progresses by repeating these processes, but in addition to the significant time required to repeatedly create analysis schedules, expertise in chromatography is necessary to explore optimal conditions based on data analysis. In other words, typical method development requires "human intervention". Therefore, eliminating human involvement and automating such method development processes would be desirable to improve labor efficiency. This article employs a fifteen-standard mixed solution of catechins, theaflavins, and gallic acid, which are functional components in tea leaves. The AI algorithm (See Technical Report C190-E309) equipped with LabSolutions MD, a dedicated software for supporting method development, was utilized for the automatic optimization of gradient conditions. Furthermore, the optimized method was applied to several tea leaves and comparisons were made among different tea species.

■ Analytical Conditions and Target Compounds

Analytical conditions and target compounds are shown in Table 1. Ten catechins, including Epigallocatechin gallate, Epigallocatechin, Epicatechin gallate, and Epicatechin, mainly present in tea leaves, along with four theaflavins and gallic acid (a total of fifteen compounds) were subjected to LC analysis. First, the gradient conditions of a mixed standard solution (ascorbic acid and EDTA-2Na were added as antioxidants at 1.76 g/L and 1.00 g/L, respectively) were automatically optimized by LabSolutions MD. Then, the optimized gradient conditions were applied to the analyses of four species of non-fermented green tea and two species of fermented black tea (pretreatment procedure: Fig. 1).

Table 1 Analytical Conditions and Target Compounds

System : Nexera™ X3	
Sample : Catechin, Theaflavin and Gallic acid (15 compounds)	
C1) Gallocatechin	C8) Epicatechin gallate
C2) Epigallocatechin	C9) Catechin gallate
C3) Catechin	C10) Epicatechin 3-(3''-O-methyl) gallate
C4) Epicatechin	T1) Theaflavin
C5) Epigallocatechin gallate	T2) Theaflavin 3-gallate
C6) Gallocatechin gallate	T3) Theaflavin 3'-gallate
C7) Epigallocatechin 3-(3''-O-methyl)gallate	T4) Theaflavin 3,3'-digallate
	G1) Gallic acid
Mobile phase:	
Pump A : 0.2% phosphoric acid in water	
Pump B : Acetonitrile	
Column : Shim-pack™ GISS C18 (100 mm × 3.0 mmI.D., 1.9 µm)*1	
*1 P/N : 227-30049-02 (Shimadzu GLC product number)	
Analytical conditions :	
B Conc.	: 15%(0 min)→45%(X*2 min) →15%(X~X+5 min)
	*2 : X = 6, 8, 10, 12, 14 (5 patterns)
Column Temp.	: 55 °C
Flow rate	: 0.6 mL/min
Injection Vol.	: 5.0 µL
Detection	: 242/272 nm (SPD-M40, UHPLC cell)
Parameters for automatic optimization of gradient conditions :	
Criteria of minimum resolution	: 1.5
Gradient mode for optimization	: Linear

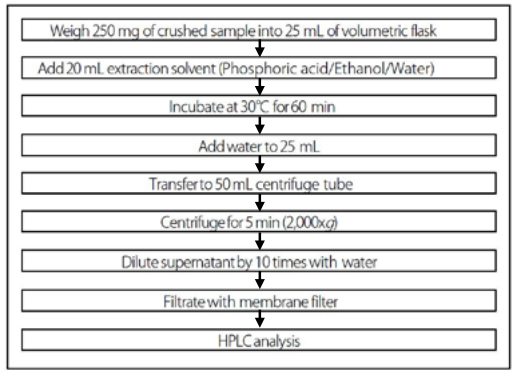


Fig. 1 Pretreatment Procedures for Tea Leaf

■ Automatic Optimization of Gradient Conditions

Fig. 2 shows the workflow of automatic optimization of gradient conditions using LabSolutions MD. This software has a unique AI algorithm to automatically explore gradient conditions that satisfy resolution criteria by alternately repeating "improvement of gradient conditions by AI (condition search)" and "analysis under improved conditions (correction analysis). For a mixture of catechins, theaflavins, and gallic acid (a total of fifteen compounds), gradient conditions were automatically searched with a minimum separation criterion of 1.5 (Fig. 3).

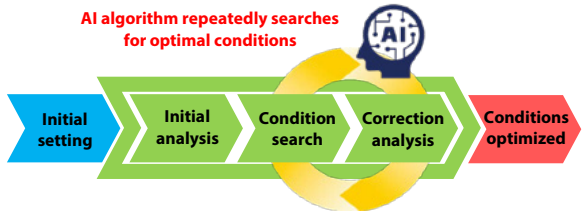


Fig. 2 Workflow for Automatic Optimization of Gradient Conditions by LabSolutions MD

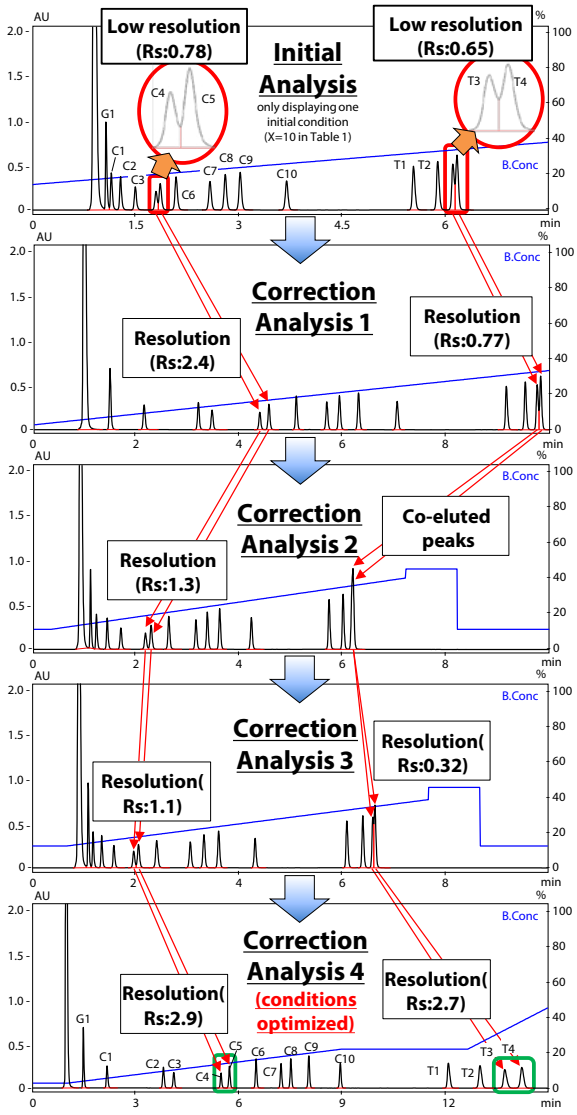


Fig. 3 Automatic Optimization of Gradient Conditions
(blue lines show gradient curves)

The result of initial analysis shows that the resolution between peaks C4/C5, and between peaks T3/T4 was not sufficient (shown in a red box at the top of Fig. 3). However, by using AI algorithm to repeatedly perform correction analyses, the gradient condition that satisfies the criterion (minimum resolution of 1.5) was finally discovered (shown in a green box at the bottom of Fig. 3). In this case, T3 and T4 were successfully separated by applying an isocratic elution after 9 minutes.

■ Application to Tea Leaves

The optimized method was applied to the quantitative analysis of extracts from six different species of tea leaves. The chromatograms of representative tea leaf extracts are shown in Fig. 4 and the graphs comparing the quantity of fifteen compounds are shown in Fig. 5. In addition, Table 2 lists the ranges of calibration curve, coefficients of determination, quantity (green tea A and black tea A), and repeatability (green tea A and black tea A) for the targeted fifteen compounds. Green teas A to D contained more catechins, including the four major catechins, than those in black tea, with the highest concentration of the functional component in all four green teas being Epigallocatechin gallate, known for its potential to inhibit elevated blood glucose. In green tea D, two methylated catechins were detected, which have garnered attention for their anti-allergic effects and ability to reduce hay fever. On the other hand, four types of theaflavins were detected in black teas A and B. Although both green tea D and black tea A were "Benifuki" species, the comparison between the two suggests that catechins were converted to theaflavins during fermentation.

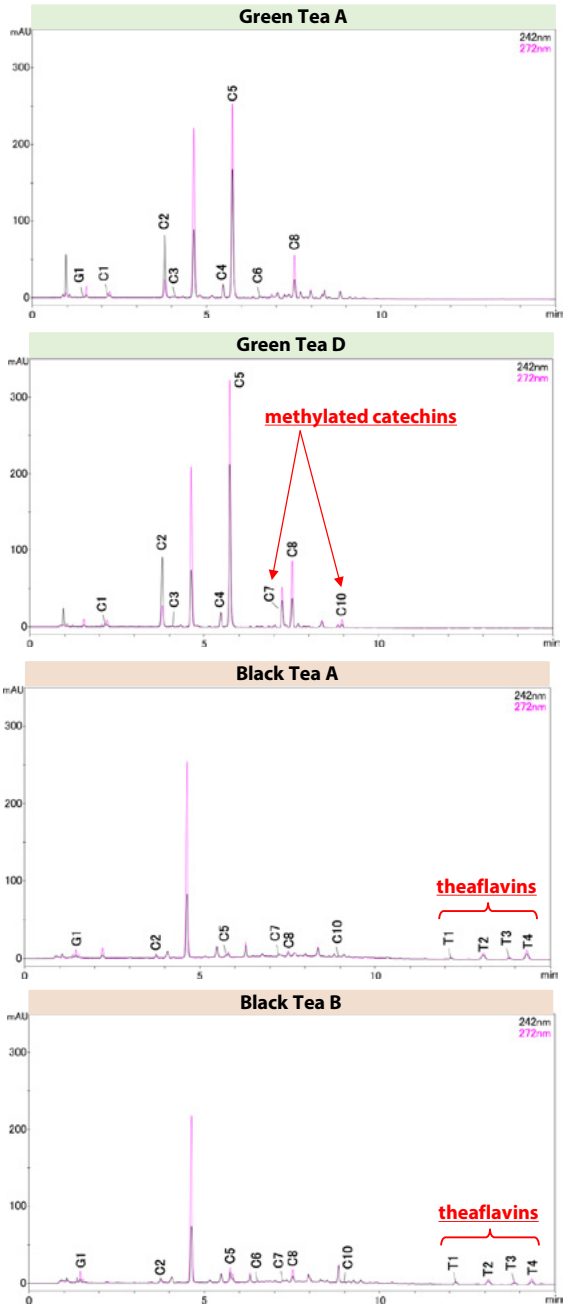


Fig. 4 Chromatograms of Representative Tea Leaf Extracts

■ Conclusion

Using a model sample of a mixture of fifteen standards solution of catechins, theaflavins, and gallic acid, all of which are functional components, the AI algorithm of LabSolutions MD was employed for automatic optimization of gradient conditions. As a result, the gradient conditions that satisfied the criteria (minimum resolution of 1.5) were automatically searched, resulting in significant labor savings. Furthermore, the optimized method was applied to tea leaf analysis to compare the quantity of functional components among different tea species. This method is expected to facilitate various scientific discussions on catechins and theaflavins.

<Acknowledgment>

The tea leaves used in this study were provided by the National Agriculture and Food Research Organization. We also thank Dr. Mari Yamamoto of the National Agriculture and Food Research Organization for her invaluable advice. We would like to express our sincere gratitude.

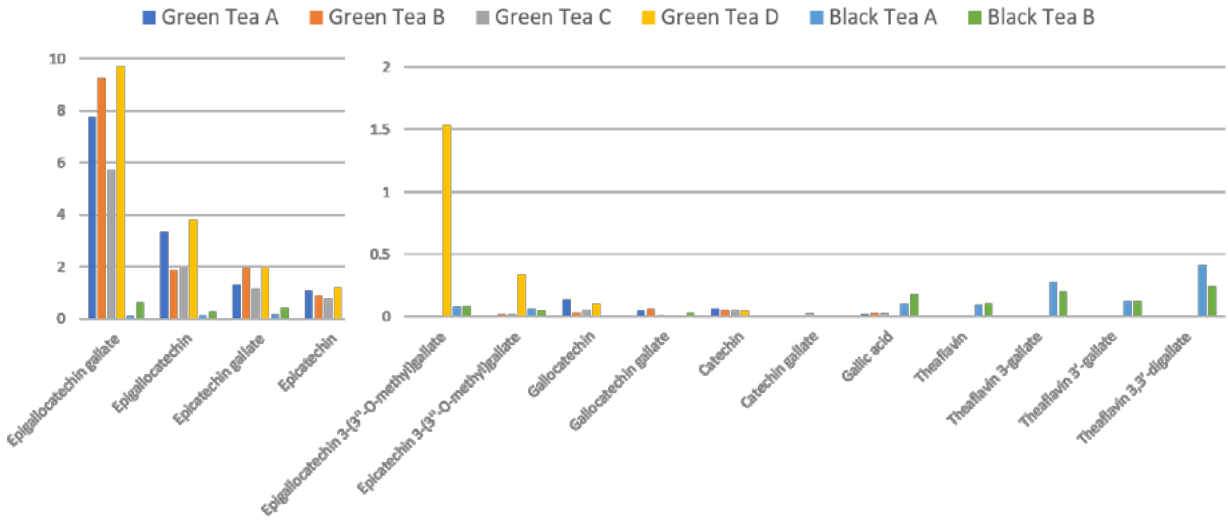


Fig. 5 Catechins and Theaflavins in Tea Leaves (g/100 g)

Table 2 Calibration Range, Coefficient of Determination, Quantity, and Repeatability (%RSD)

Compound		Calib. Range (mg/L)	Coefficient of Determination(r^2)	Quantity (g/100 g)		%RSD (n=6)	
				Green Tea A	Black Tea A	Green Tea A	Black Tea A
C5	Epigallocatechin gallate	1~100	>0.9999	7.74	N.D.*	0.09	-
C2	Epigallocatechin	1~100	>0.9998	3.35	0.16	0.73	0.26
C8	Epicatechin gallate	1~100	>0.9999	1.32	0.18	0.14	0.60
C4	Epicatechin	1~100	>0.9999	1.09	N.D.	0.31	-
C7	Epigallocatechin 3-(3''-O-methyl)gallate	1~100	>0.9999	N.D.	N.D.*	-	-
C10	Epicatechin 3-(3''-O-methyl)gallate	1~100	>0.9999	N.D.	N.D.*	-	-
C1	Gallocatechin	1~100	>0.9998	0.14	N.D.	0.72	-
C6	Gallocatechin gallate	1~100	>0.9999	N.D.*	N.D.	-	-
C3	Catechin	1~100	>0.9999	N.D.*	N.D.	-	-
C9	Catechin gallate	1~100	>0.9999	N.D.	N.D.	-	-
G1	Gallic acid	1~100	>0.9999	N.D.*	0.10	-	1.17
T1	Theaflavin	1~100	>0.9999	N.D.	0.10	-	0.57
T2	Theaflavin 3-gallate	1~100	>0.9999	N.D.	0.28	-	0.57
T3	Theaflavin 3'-gallate	1~100	>0.9999	N.D.	0.12	-	1.53
T4	Theaflavin 3,3'-digallate	1~100	>0.9999	N.D.	0.41	-	0.86

* Less than 0.1 g/100 g

01-00664-EN First Edition: Feb. 2024

index

Application News

Software for Efficient Method Development "LabSolutions™ MD"

Automatic Optimization of Gradient Conditions by AI Algorithm for a Synthetic Peptide and Impurities

Shinichi Fujisaki

User Benefits

- The AI algorithm of LabSolutions MD can automatically optimize gradient conditions to greatly reduce labor of LC method development.
- Anyone can optimize gradient conditions, regardless of their experience in chromatography.
- Gradient conditions that meet the resolution criteria for specified peaks are automatically searched (e.g., principal component and related impurities).

■ Introduction

In the typical LC method development, the process begins with "preparation" which includes mobile phase preparation, column installation, and creation of analysis schedules, then the analysis is started. After that, the acquired data is analyzed and "preparation" for the subsequent analysis is carried out, followed by starting the next analysis again. The method development progresses by repeating these processes, but in addition to the significant time required to repeatedly create analysis schedules, expertise in chromatography is necessary to explore optimal conditions based on data analysis. In other words, typical method development requires "human intervention". Therefore, eliminating human involvement and automating such method development processes would be desirable to improve labor efficiency. This article introduces an example of automatic optimization of gradient conditions to meet resolution criteria for synthetic peptide and related impurities using LabSolutions MD (Technical Report C190-E309), a dedicated software for supporting method development.

■ Target Compounds and Analytical Conditions

A target peptide and three related impurities with different sequences were used as a model sample of a synthetic peptide (Table 1). Full length peptide (FLP : beta-Melanotropin), deletion sequences of p.A1_K3del and p.A1_D5del as shorter length of products, and Met(O2) (methionine in FLP is oxidized to methionine sulfone) were prepared as a sample mixture. The analytical conditions are shown in Table 2. In this article, the resolution criteria were set for FLP, and LabSolutions MD automatically explored the gradient conditions to meet the criteria.

Table 1 Target Compounds

Name	Sequence
FLP	AEKKDEGPYRMEHFRWGSPPKD
p.A1_K3del	KDEGPYRMEHFRWGSPPKD
p.A1_D5del	EGPYRMEHFRWGSPPKD
Met(O2)	AEKKDEGPYR{Met(O2)}EHFRWGSPPKD

Note : Met(O2) = methionine sulfone

Table 2 Analytical Conditions

LC (Nexera™ X3)	
Mobile phase	
Pump A : 0.1% TFA (Trifluoroacetic acid) in water	
Pump B : Acetonitrile	
Analytical conditions	
B Conc.	: 5%(0 min)→60%(X*1 ~X+2 min) →5%(X+2~X+7 min)
Column Temp.	: 80 °C
Flow rate	: 0.6 mL/min
Injection Vol.	: 2 µL
Detection	: 220 nm (SPD-M40, STD cell)
Column : Shim-pack Scepter™ C8-120 (100 mm × 3.0 mmI.D., 1.9 µm)*2	
Criteria of automatic optimization of gradient conditions	
Resolution	: > 2.0 (FLP)
MS (LCMS-2050)	
Ionization	: ESI/APCI (DUIS™), positive mode
Mode	: SCAN (*m/z* 300-2000)
Nebulizing gas flow	: 2.0 L/min
Drying gas flow	: 5.0 L/min
Heating gas flow	: 7.0 L/min
DL Temp.	: 200 °C
Desolvation Temp.	: 450 °C
Interface voltage	: +1.0 kV

*1 X = 3, 4, 5, 6, 7 (5 patterns)

*2 P/N : 227-31034-03

■ Automatic Optimization of Gradient Conditions

Fig. 1 shows the workflow of automatic optimization of gradient conditions using LabSolutions MD. This software has a unique AI algorithm to automatically explore gradient conditions that satisfy resolution criteria by alternately repeating "improvement of gradient conditions (condition search)" and "analysis under improved conditions (correction analysis) by AI. For the criteria, "resolution" and "elution time of the last peak" can be set. The peaks to be optimized can be easily specified by simply entering the molecular weight of the target compound, as LabSolutions MD can calculate molecular weight by deconvolution of MS spectrum (Fig. 2). FLP (molecular weight : 2660) was specified as a target peak to optimize the separation from related impurities, with a minimum resolution criteria set at 2.0 (Fig. 2).

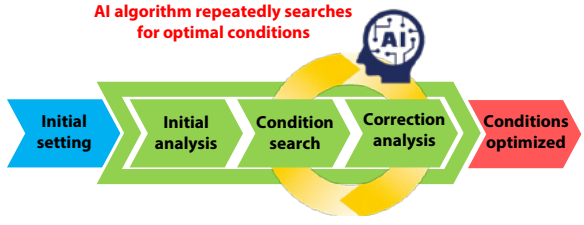


Fig. 1 Workflow for Automatic Optimization of Gradient Conditions by LabSolutions MD

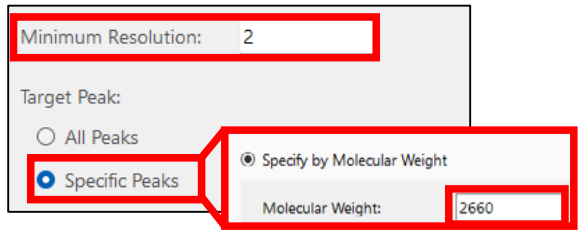


Fig. 2 Criteria Setting for Automatic Optimization

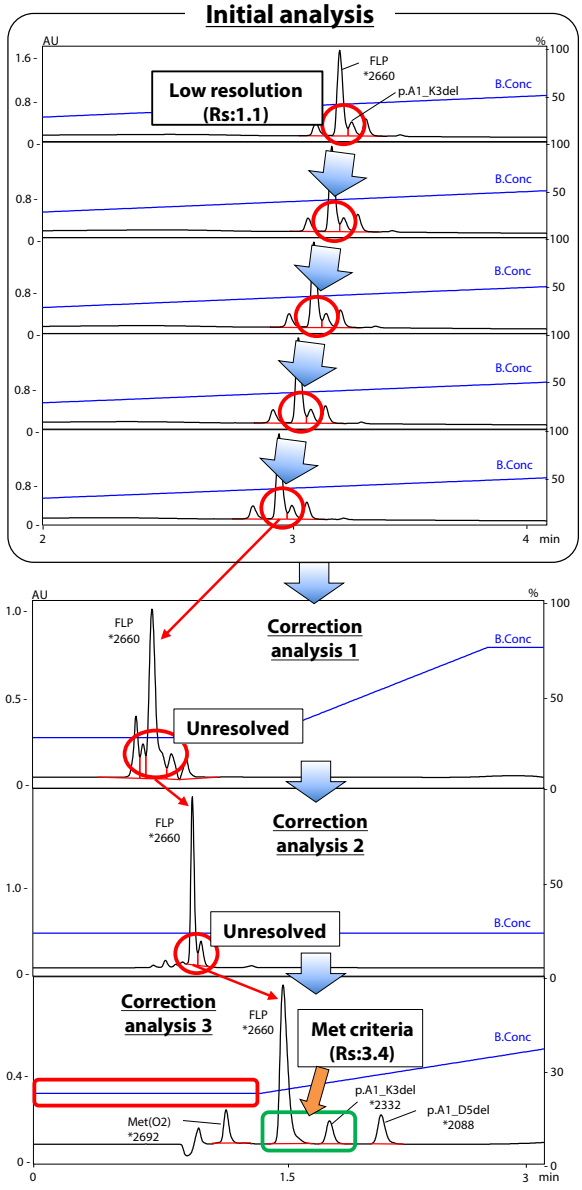


Fig. 3 Result of Automatic Optimization of Gradient Conditions
(blue line shows gradient conditions)
* : molecular weights estimated by deconvolution

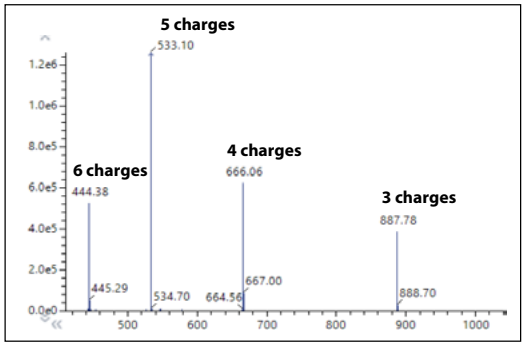


Fig. 4 MS Spectrum of FLP

The results of automatic optimization of gradient conditions and MS spectrum of FLP are shown in Fig. 3 and Fig. 4, respectively. Multiply-charged ions with charges from 3 to 6 were detected for FLP, and the molecular weight (2660) was calculated by deconvolution. In the initial analyses of the five gradient profiles (Table 1), FLP and related impurity (p.A1_K3del) were not sufficiently separated (red circles in Fig. 3 initial analyses). After repeated corrections by AI algorithm (3 times), the gradient conditions that met the criteria were automatically searched (correction analysis 3). In correction analysis 3, the resolution criteria (resolution > 2.0) was achieved (green box) by applying an isocratic elution (red box). By optimizing with LCMS-2050, molecular weight information for each compound can also be obtained, allowing for reliable optimization without missing changes in peak elution order.

■ Conclusion

Automatic optimization of gradient conditions using AI algorithm of LabSolutions MD was applied to synthetic peptide and related impurities. As a result, gradient conditions that met the criteria (resolution for FLP > 2.0) were successfully explored. This result indicates that significant labor saving in method development can be expected by LabSolutions MD. This article introduces an automatic optimization of gradient conditions in method development while LabSolutions MD also supports a series of workflow of method development, including screening of mobile phases and columns. For details, please refer to the application news, "Efficient Method Development for Synthetic Peptide and Related Impurities (01-00780)".

01-00814-EN First Edition: Nov. 2024

index

Application News

Software for Efficient Method Development "LabSolutions™ MD"

Automatic Optimization of Gradient Conditions by AI Algorithm for Impurity Analysis

Shinichi Fujisaki

User Benefits

- The AI algorithm of LabSolutions MD can automatically optimize gradient conditions to greatly reduce labor of LC method development.
- Anyone can optimize gradient conditions, regardless of their experience in chromatography.
- Gradient conditions that meet the resolution criteria for specified peaks are automatically searched (e.g., principal component and its related impurities).

■ Introduction

In the typical LC method development, the process begins with "preparation" which includes mobile phase preparation, column installation, and creation of analysis schedules, then the analysis is started. After that, the acquired data is analyzed and "preparation" for the subsequent analysis is carried out, followed by starting the next analysis again. The method development progresses by repeating these processes, but in addition to the significant time required to repeatedly create analysis schedules, expertise in chromatography is necessary to explore optimal conditions based on data analysis. In other words, typical method development requires "human intervention". Therefore, eliminating human involvement and automating such method development processes would be desirable to improve labor efficiency. This article introduces an example of automatic optimization of gradient conditions to separate montelukast (a small molecule drug) and its related impurities using LabSolutions MD (Technical Report C190-E309), a dedicated software for supporting method development.

■ Analytical Conditions and Target Compounds

The analytical conditions and target compounds are shown in Table 1. In this article, the criteria for resolution and elution time of the last peak were set for montelukast and related impurity (Imp1). LabSolutions MD automatically explored the gradient conditions to meet these criteria.

Table 1 Analytical Conditions and Target Compounds

System : Nexera™ X3	
Sample : montelukast	
Mobile phase:	
Pump A : 0.15% formic acid in water	
Pump B : 0.1% formic acid in acetonitrile	
Column : Shim-pack Scepter™ Phenyl-120 (100 mm × 3.0 mmI.D., 1.9 µm) *1	
Analytical conditions	
B Conc.	: 5% (0 min)→95% (X*2 ~X+2 min) →5%(X+2~X+7 min) *2 : X = 8, 9, 10, 11, 12 (5 patterns)
Column Temp.	: 30 °C
Flow rate	: 0.5 mL/min
Injection Vol.	: 10 µL
Detection	: 238 nm (SPD-M40, UHPLC cell)
Criteria of automatic optimization of gradient conditions	
Resolution	: > 3.0 (montelukast and Impurities)
Elution time of last peak	: < 15 min

*1 : 227-31064-03 (Shimadzu GLC product number)

■ Automatic Optimization of Gradient Conditions

Fig. 1 shows the workflow of automatic optimization of gradient conditions using LabSolutions MD. This software has a unique AI algorithm to automatically explore gradient conditions that satisfy resolution criteria by alternately repeating "improvement of gradient conditions by AI (condition search)" and "analysis under improved conditions (correction analysis). For the criteria, "resolution" and "elution time of the last peak" can be set. In this article, automatic optimization of gradient conditions was applied to montelukast (specified as the principal peak) and related impurity (Imp1) to meet the criteria of resolution (> 3.0) and maximum elution time of 15 minutes for the last peak (Fig. 2), considering the reduction of analysis time.

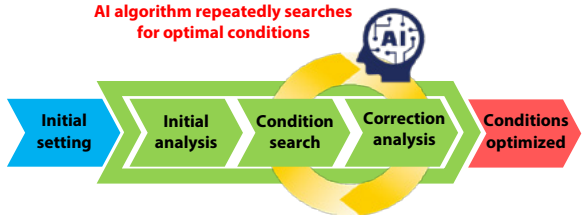


Fig. 1 Workflow for Automatic Optimization of Gradient Conditions by LabSolutions MD

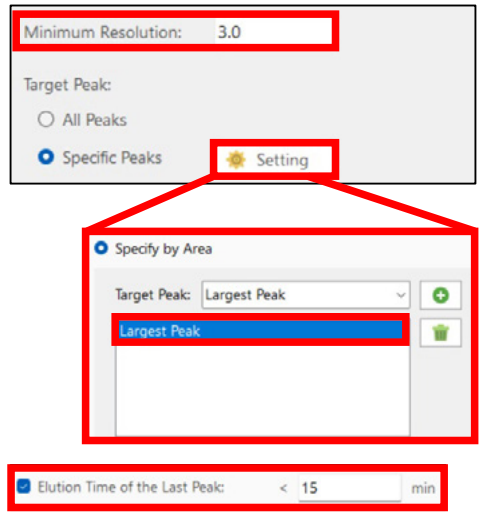


Fig. 2 Setting for Automatic Optimization

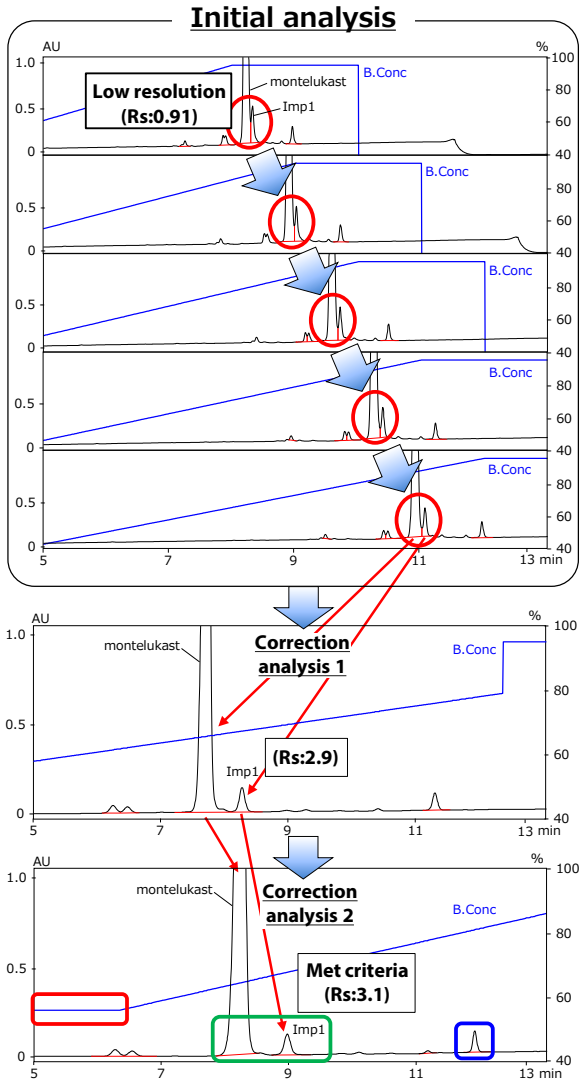


Fig. 3 Result of Automatic Optimization of Gradient Conditions
* blue line shows gradient conditions

The result of automatic optimization of gradient conditions is shown in Fig. 3. After the initial analyses were performed with the five different patterns of gradient curves (conditions are shown table 1), the resolution of montelukast and Imp1 was automatically optimized through two correction analyses. The result of initial analyses shows that the resolution of montelukast and Imp1 was not sufficient (red circles in the initial analyses in Fig. 3). After repeated correction analyses by the AI algorithm, the gradient conditions that finally met the criteria was automatically explored (correction analysis 2). In correction analysis 2, the resolution criteria (> 3.0) was achieved (green box) by inserting an isocratic elution (red box). The elution time of the last peak (blue box) also met the criteria (< 15 minutes).

■ Conclusion

Automatic optimization of gradient conditions using AI algorithm of LabSolutions MD was applied to montelukast and its related impurity. As a result, gradient conditions that met the criteria ("resolution for montelukast and related impurity > 3.0" and "elution time of the last peak < 15 minutes") were successfully explored. This result indicates that significant labor saving in method development can be expected by LabSolutions MD. This article introduces an automatic optimization of gradient conditions in method development while LabSolutions MD also supports a series of workflow of method development, including the screening phase and robustness evaluation phase. For details, please refer to the Technical Report "Efficient Method Development Based on Analytical Quality by Design with LabSolutions MD Software (C190-E284)".

01-00767-EN First Edition: Sep. 2024

index

Technical Report

Efficient Method Development Based on Analytical Quality by Design with LabSolutions™ MD software

Shinichi Fujisaki[1], Satoru Watanabe[1], Tomohiro Kawase[1], Yuichiro Fujita[1], Keiko Matsumoto[1]

Abstract:

This article describes the efficient development of robust analytical methods based on Analytical Quality by Design (AQbD) using LabSolutions MD with small-molecule drugs. Method development based on AQbD consists of 3 phases including screening, optimization, and validation. LabSolutions MD allows efficient method development by supporting every phase with dedicated functions such as experimental design, building of design space by automatic Peak Tracking function, and robustness evaluation.

Keywords: LabSolutions MD, AQbD, method development, method scouting, experimental design

1. Background

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) suggests AQbD approach for method development. It is recommended to acquire data by efficient experiments such as the use of experimental design and verify the parameters that have a large effect on analytical results and then build a design space to understand the effective domain of the parameters with respect to the analysis results. The risk-based approach ensures the development of robust, low-risk methods without relying on the user experience.

2. Overview of LabSolutions MD

LabSolutions MD supports efficient method development through each phase based on AQbD (Fig. 1). This software includes several functions for creating analysis schedule based on experimental design (Fig. 2) and for data analysis such as building design space and simulation of chromatograms (Fig. 3). It is possible to generate the analysis schedule that can switch mobile phases and columns automatically for screening. Furthermore, by utilizing experimental design, it is possible to find an optimum condition with reduced numbers of analysis. In figure 2, steps (1) to (6) show how to easily and quickly create the analysis schedule.

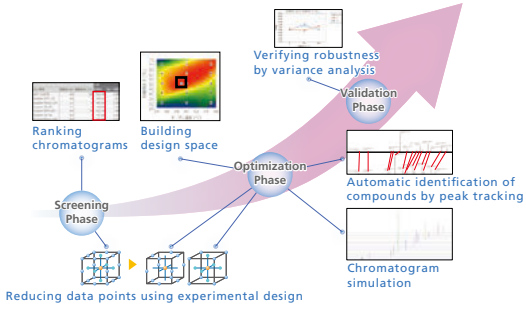


Fig. 1 Efficient Method Development Using LabSolutions MD

Mobile phases and columns can be selected directly by clicking the image in the software and analysis schedule including column equilibration is automatically generated. This doesn't only improve operational efficiency, but also reduces human errors. The experimental design can also be selected with a single click. Moreover, data analysis functions support automatic identification of target compounds by Peak Tracking function, visualization of design space to identify optimum condition, and simulation of chromatograms by predicting different analytical conditions. In addition, it is possible to identify parameters that have a large effect on separation by analysis of variance. This article describes the workflow of method development including screening, optimization, and validation with the sample of small-molecule drugs.

Fig. 2 Functions for Creating Analysis Schedule

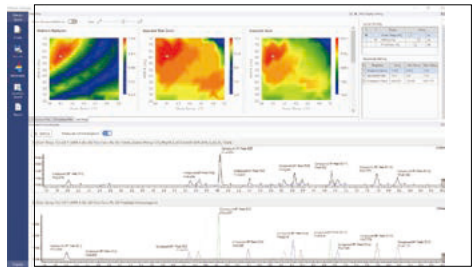


Fig. 3 Functions for Data Analysis

3. Analysis

3-1. Sample Information

Table 1 shows the small-molecule drugs used for method development and their physical properties. As a model case, small-molecule drugs which have different log P and pKa are selected.

Table 1 Sample Information

No.	Compounds	log P	pKa
1	Probenecid	3.21	3.4
2	(S)-(+)-Naproxen	3.18	4.15
3	Acetylsalicylic acid	1.19	3.49
4	Diclofenac sodium	4.51	4.15
5	Papaverine hydrochloride	3	6.4
6	Dibucaine hydrochloride	4.4	8.85
7	Amitriptyline hydrochloride	4.92	9.4
8	Indometacin	4.27	4.5
9	Antipyrine	0.38	1.4
10	Lidocain	2.44	8.01
11	Quinidine	3.44	8.56
12	Metoclopramide	2.62	9.27

3-2. Screening Phase

For screening phase, a total of 36 (2×3×6) data is acquired by using 2 aqueous mobile phases, 3 organic mobile phases, and 6 columns with full factorial design (Table 2). Because mobile phases and columns usually have a large effect on separation, these factors are screened in this initial phase of method development. pH of each aqueous mobile phase (Pump A) is automatically adjusted by Solvent Blending function as well as ratio of organic solvent (Pump B). Mobile phase selection and column switching are automatically implemented during analysis, strongly improving the overall efficiency of the method development phase.

Table 2 Mobile Phases and Columns in Screening Phase

Mobile phase:
- Pump A Buffer *1
  - A1 20 mmol/L (Sodium) phosphate buffer (pH 2.7)
  - A2 20 mmol/L (Sodium) phosphate buffer (pH 6.8)
- Pump B Organic solvent *2
  - B1 Acetonitrile
  - B2 Acetonitrile / Methanol = 50 : 50
  - B3 Methanol

Column:

1	Shim-pack Scepter C18-120	(100 mm × 3.0 mm I.D., 1.9 µm)*3
2	Shim-pack Scepter C8-120	(100 mm × 3.0 mm I.D., 1.9 µm)*4
3	Shim-pack Scepter C4-300	(100 mm × 3.0 mm I.D., 1.9 µm)*5
4	Shim-pack Scepter Phenyl-120	(100 mm × 3.0 mm I.D., 1.9 µm)*6
5	Shim-pack Scepter PFPP-120	(100 mm × 3.0 mm I.D., 1.9 µm)*7
6	Shim-pack GIST C18 AQ HQ	(100 mm × 3.0 mm I.D., 2.0 µm)*8

Analytical conditions:

Time program	:B.Conc. 5%(0 min) → 80%(8.01-11 min) → 5%(11.01-15 min)
Flow rate	:0.7 mL/min
Inj.vol.	:1.0 µL
Temperature	:40 ˚C
Detection	:Max plot 220- 400 nm (SPD-M40)

*1 The aqueous mobile phases below are automatically prepared by solvent blending function.

	Solvent	A1 ratio	A2 ratio
A	50 mmol/L Phosphoric acid water	16%	0%
B	50 mmol/L Sodium dihydrogen phosphate water	24%	24%
C	50 mmol/L Disodium phosphate water	0%	16%
D	Water	60%	60%

*2 The organic mobile phases below are automatically prepared by solvent blending function.

	Solvent	B1 ratio	B2 ratio	B3 ratio
A	Acetonitrile	100%	50%	0%
B	Methanol	0%	50%	100%

*3 P/N 227-31013-03
*4 P/N 227-31034-03
*5 P/N 227-31176-03
*6 P/N 227-31064-03
*7 P/N 227-31054-03
*8 P/N 227-30808-02

3-3. Chromatogram in Screening Phase

Fig. 4 shows chromatograms obtained by Shim-pack™ Scepter Phenyl-120. 14 peaks are eluted, including the impurities of quinidine and acetylsalicylic acid. It is confirmed that retention time and resolution factor of each compound changed significantly depending on pH of aqueous mobile phases, organic mobile phases, and columns.

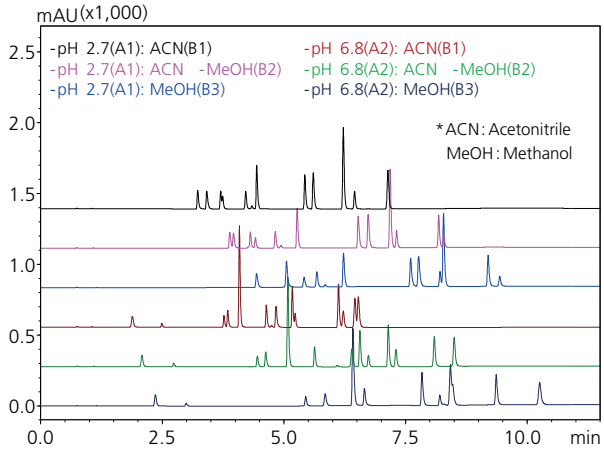


Fig. 4 Chromatogram with Shim-pack Scepter Phenyl-120

3-4. Quickly Find Optimum Condition

Because screening generates as many chromatograms as the number of conditions considered, they must be evaluated to determine which one is optimal. If all chromatograms had to be checked one by one, it would take a lot of time and cost. LabSolutions MD can quickly and easily find optimum condition using equation (1) below to quantitatively estimate the chromatographic separation.

$$(\text{Evaluation Value}) = P \times (Rs_1 + Rs_2 + \ldots + Rsp) \qquad \text{(Equation 1)}$$

Evaluation Value (E) is calculated as the number of peaks detected (P) multiplied by the sum of resolution factor (Rs) for all peaks. Fig. 5 shows Evaluation Value obtained from screening in an order from the highest to the lowest. It was confirmed that the highest Evaluation Value is obtained when using an aqueous mobile phase with pH 6.8, 50:50 acetonitrile/methanol as organic mobile phase, and Shim-pack Scepter Phenyl-120 column (Fig. 4: Green chromatogram).

			Response	
Column Nick Name	MPA pH	MPB A (%)	Evaluation Value	Minimum Resolution
Scepter-Phenyl-120	6.8	50	546.000	3.224
Scepter-C8-120	6.8	0	469.894	0.093
GIST-C18-AQ	2.7	0	465.124	1.075
GIST-C18-AQ	6.8	50	443.580	1.826
Scepter-C8-120	6.8	50	436.241	0.026
Scepter-Phenyl-120	2.7	50	419.659	1.743
Scepter-C18	2.7	0	419.338	1.518
Scepter-C18	6.8	50	396.000	4.326
Scepter-C4-300	2.7	0	394.239	0.402
Scepter-C18	6.8	100	384.553	2.046

Fig. 5 Ranking of the Conditions (mobile phases and columns) by Evaluation Value (Shown from the Highest to the Lowest)

3-5. Identification of the Parameters that have a Large Impact on Separation Using Analysis of Variance

Using the analysis of variance, it is possible to quantify how much each parameter such as mobile phase, columns, etc. affects separation. Identifying the parameters that have a large effect on separation helps to decide which should be further considered in the optimization phase, increasing the efficiency of method development.

Fig. 6 shows the results of analysis of variance with respect to each parameter. P value for "mobile phase A × mobile phase B" and "column" (in red) is both less than 0.05. The parameters with p value of 0.05 or less can be considered to be different (e.g. resolution factor) at each level, suggesting that they have a large effect on separation.

ANOVA Table

Display Plots	Effect	SSR	df	MS	F Value	p Value
☑	MPA pH x MPB B (%)	44817.9	2	22408.9	6.72	0.0141
☑	Column Nick Name	66312.0	5	13262.4	3.98	0.0302
☑	Column Nick Name x MPA pH	35853.2	5	7170.6	2.15	0.142
☑	Column Nick Name x MPB B (%)	50149.0	10	5014.9	1.50	0.265
☑	MPB B (%)	9123.7	2	4561.9	1.37	0.298
☑	MPA pH	3243.6	1	3243.6	0.973	0.347
	Error	33336.5	10	3333.7		
	Total	242835.8	35			

Fig. 6 Results of Analysis of Variance

3-6. Results of Screening

Table 3 shows the best condition obtained in screening phase with the highest Evaluation Value. The condition is obtained with aqueous mobile phase pH 6.8, 50:50 acetonitrile/methanol as organic mobile phase, and Shim-pack Scepter Phenyl-120. In optimization phase (3.7), further examinations are implemented for method optimization, including gradient program of pump and column oven temperature.

Table 3 Conditions with the Highest Evaluation Value

Mobile phase:	
Pump A	Buffer
A2	20 mmol/L (Sodium) phosphate buffer (pH 6.8)
Pump B	Organic solvent
B2	Acetonitrile / Methanol = 50 : 50
Column:	
4	Shim-pack Scepter Phenyl-120
Analytical conditions:	
Time program	:B.Conc. 5% (0 min) → 80% (8.01-11 min) → 5% (11.01-15 min)
Flow rate	:0.7 mL/min
Inj.vol.	:1.0 µL
Temperature	:40 °C
Detection	:Max plot 220- 400 nm (SPD-M40)

3-7. Optimization Phase

After the pH level of aqueous mobile phase and column are selected in the screening phase, analytical condition is further optimized by considering the parameters of mixture ratio of organic mobile phase (30, 40, 50, 60, 70 %), oven temperature (35, 40, 45 °C), and final concentration of gradient program (75, 80, 85 %). The effect on separation of these parameters are shown by design space with the mixture ratio of organic mobile phase on the vertical axis and oven temperature on the horizontal axis.

Building design space makes it possible to visualize the impact of the parameters on minimum resolution comprehensively. LabSolutions MD can suggest the most robust condition among the entire region of design space (black square in Fig. 7), which means a robust analytical method can be defined without relying on the user experience. Based on design space, it is confirmed that the most robust analytical condition is organic solvent mixture ratio of 50 %, oven temperature of 39 °C, and final concentration of gradient program of 80 %. Moreover, by clicking any point in design space (e.g. point A in Fig. 7), simulated chromatogram can be displayed (Fig. 8). This function of chromatogram simulation allows to check how the separation will change through any conditions quickly without running any analysis.

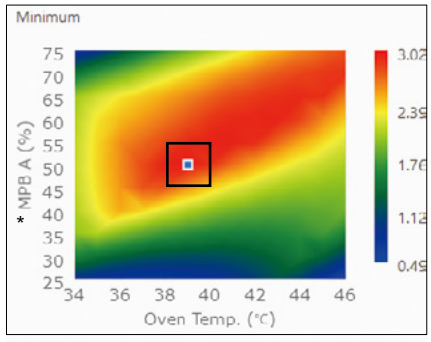


Fig. 7 Design Space of Minimum Resolution (Final Gradient Concentration of 80 %)
* Mobile Phase B A: Acetonitrile

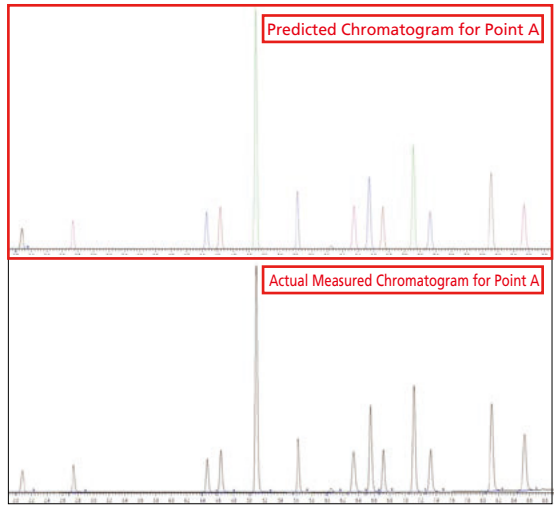


Fig. 8 Simulated Chromatogram and Actual Chromatogram at Point A (Fig. 7)

3-8. Automatic Identification of Compounds Using Peak Tracking

When analytical condition such as mobile phase, oven temperature, gradient program of pump is changed, retention time of each compound can also be different. It's a time consuming process to identify manually each compound through all the acquired data. LabSolutions MD can automatically identify target compounds by Peak Tracking function through all the data (Fig. 9). For example, similarity of UV spectrum, peak area, and other parameters can be used to automatically track each compound, contributing to quick recognition of peaks through all the data without any additional manual work.

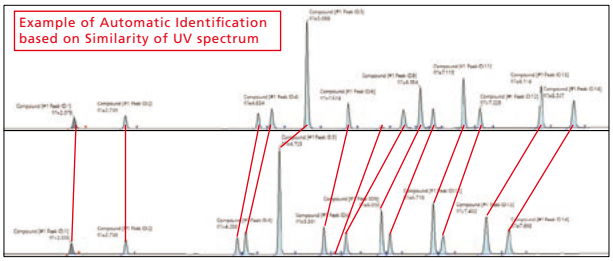


Fig. 9 Automatic Identification of Target Compounds Using Peak Tracking Function

3-9. Results of Optimization

Table 4 shows the condition determined through optimization phase. By building design space, optimum condition is decided with 50:50 acetonitrile/methanol as organic mobile phase, oven temperature of 39 °C, and final concentration of gradient program of 80 %. In the validation phase in 3.10, robustness of the optimized method is evaluated by checking the separation pattern when each parameter is changed in a small range.

Table 4 Optimized Condition

Mobile phase:	
Pump A	Buffer
A2	20 mmol/L (Sodium) phosphate buffer (pH 6.8)
Pump B	Organic solvent
B2	Acetonitrile / Methanol = 50 : 50
Column:	
4	Shim-pack Scepter Phenyl-120(100 mm × 3.0 mm I.D., 1.9 µm)*6
Analytical conditions:	
Time program	:B.Conc. 5%(0 min) → 80%(8.01-11 min) → 5%(11.01-15 min)
Flow rate	:0.7 mL/min
Inj.vol.	:1.0 µL
Temperature	:39 °C
Detection	:Max plot 220- 400 nm (SPD-M40)

3-10. Validation Phase

In Validation phase, LabSolutions MD can create sequential experimental design to perform robustness evaluation. Robustness evaluation is important to understand how the variations of parameters will affect results and ensure the reliability of method. LabSolutions MD creates sequential experimental design automatically by changing the parameters of optimized method in a small range to evaluate the robustness. Specifically, mixture ratio of organic mobile phase by 1 % (49, 50, 51 %) and oven temperature by 1 °C (38, 39, 40 °C) (white circle in Fig. 10) to verify the effect on separation. Fig. 11 shows chromatograms obtained for robustness evaluation. The effect of variation of the parameters on separation is very small, showing robustness of the optimized method constructed by design space. The optimized analytical method based on AQbD approach will be beneficial for next phases of method development.

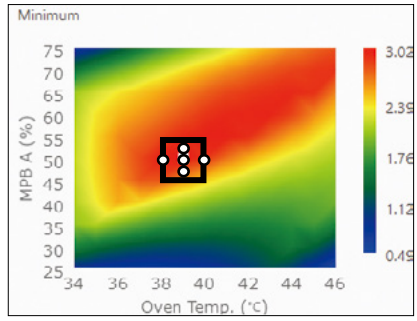
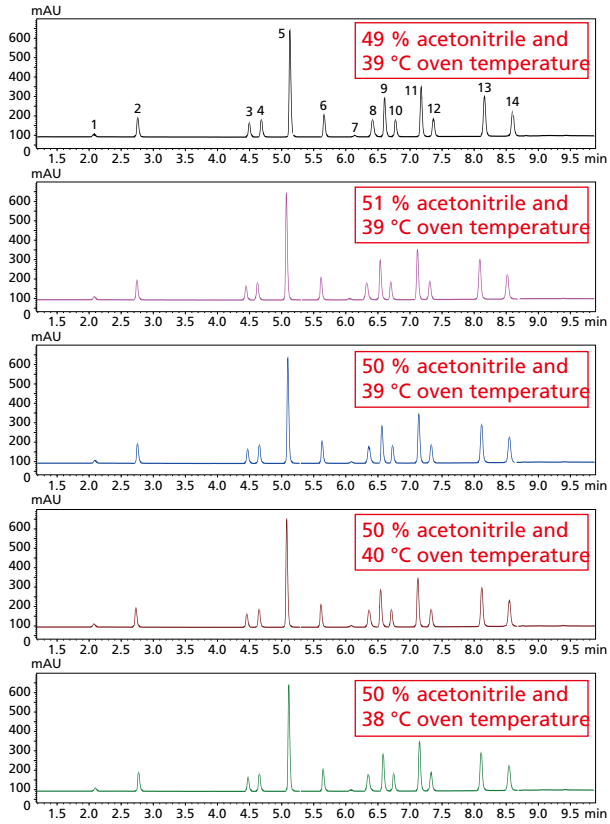


Fig. 10 Data Points of Robustness Evaluation
* Mobile Phase B A: Acetonitrile



1 : Acetylsalicylic acid
2 : Impurity of acetylsalicylic acid
3 : Antipyrine
4 : Metoclopramide
5 : (S)-(+)-Naproxen
6 : Probenecid
7 : Impurity of quinidine
8 : Quinidine
9 : Diclofenac sodium
10 : Indometacin
11 : Papaverine hydrochloride
12 : Lidocain
13 : Dibucaine hydrochloride
14 : Amitriptyline hydrochloride

Fig. 11 Chromatograms of Robustness Evaluation at each Data Point (White circle in Fig. 10)

index

Application News

LabSolutions™ MD : efficient method development based on Analytical Quality by Design

Efficient Method Development on Pharmaceutical Impurities Based on Analytical Quality by Design

S. Fujisaki, Y. Zhou

User Benefits

- Visualizing the resolution of API and impurities in the range of analysis conditions such as composition of mobile phase and column oven temperature comprehensively makes it efficient to find optimum analysis condition.
- Visualizing the area of analysis condition that satisfies robustness through different lot of columns makes robustness evaluation efficient.

■ Introduction

Since pharmaceutical impurities requires strict control to ensure safety, development of highly reliable analysis methods is necessary. LabSolutions MD, a new Shimadzu software for method development, supports efficient method development based on Analytical Quality by Design (AQbD). AQbD-based analysis method development consists of the phases of initial screening, optimization, and robustness evaluation. This article introduces an example of its use in optimization and robustness evaluation of the column and mobile phase selected in the initial screening in order to realize high efficiency in the development of a robust LC method for impurities on ketoprofen.

The resolution of each compound was evaluated by visualizing a "design space" after changing mobile phase composition, oven temperature and flow rate. In the step of robustness evaluation following optimization, resolution of each lot of columns was visualized and compared by design space to efficiently evaluate robustness among different lot of columns.

■ Analysis Conditions

Table 1 shows the analysis conditions used in the optimization study for separation of ketoprofen and its impurities. By varying the composition of mobile phase, column oven temperature and flow rate, the resolution of ketoprofen and its impurities was examined to find the optimal condition. Specifically, the acetonitrile ratio (B Conc.) was varied from 40% to 60% in increments of 5% (5 levels), the column oven temperature from 35 ˚C to 45 ˚C in increments of 5 ˚C (3 levels), and flow rate from 0.6 mL/min to 0.8 mL/min in increments of 0.1 mL/min (3 levels).

Table 1 Conditions of Optimization Study

System : Nexera™ X3 (Method Scouting System)	
Mobile Phase :	
Pump A : 0.1% formic acid in water	
Pump B : Acetonitrile	
Column :	
Shim-pack™ Velox C18 (100 mm × 3.0 mm I.D., 2.7 µm)*1	
Analytical Conditions (Isocratic)	
B Conc. (Acetonitrile)	: 40, 45, 50, 55, 60% (5 levels)
Column Temp.	: 35, 40, 45 ˚C (3 levels)
Flow Rate	: 0.6, 0.7, 0.8 mL/min (3 levels)
Injection Vol.	: 0.1 µL
Detection (PDA)	: 254 nm (SPD-M40, UHPLC cell)

*1 P/N: 227-32010-03

■ Separation of Ketoprofen and Impurities

First, Fig. 1 and Fig. 2 show the chromatograms when the acetonitrile ratio is at 60% and 40% (here, the column oven temperature and flow rate are constant at 40 ˚C and 0.7 mL/min, respectively). Imp1 to Imp3 in the chromatograms indicate impurities. When the acetonitrile ratio is 60%, Imp2 and Imp3 are eluted on the foot of the peak of ketoprofen, but at 40%, these peaks are mutually separated, suggesting that the acetonitrile ratio in the mobile phase has a large effect on separation. Next, the resolution when the acetonitrile ratio, column oven temperature, and flow rate are changed is visualized by design space.

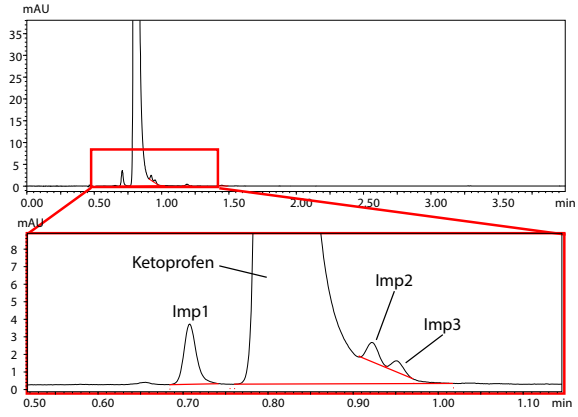


Fig. 1 Chromatogram at B Conc. (Acetonitrile) 60%, Oven Temperature 40 ˚C, Flow Rate 0.7 mL/min

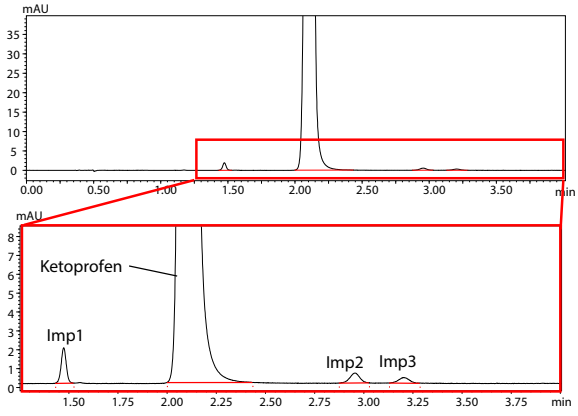


Fig. 2 Chromatogram at B Conc. (Acetonitrile) 40%, Oven Temperature 40 ˚C, Flow Rate 0.7 mL/min

■ Visualization of Resolution by Design Space

Fig. 3 shows the design spaces for resolution of ketoprofen and Imp2 (left) and also, Imp2 and Imp3 (right). The red region indicates higher resolution, and the blue region indicates lower resolution. LabSolutions MD is able to suggest the condition that provides better resolution as well as robustness. In Fig. 3, LabSolutions MD shows that the optimum condition for resolution of ketoprofen and Imp2 as well as, Imp2 and Imp3 is acetonitrile ratio at 40%, column oven temperature at 35 ˚C, and flow rate at 0.6 mL/min (blue square at the lower left in Fig. 3). By using design space, the effect of parameters on resolution can be understood easily to find the optimum condition.

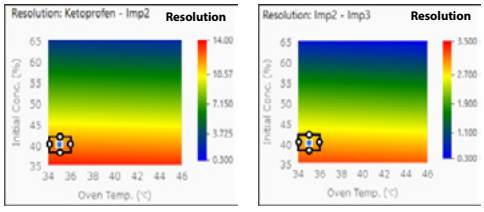


Fig. 3 Design Space for Resolution of Ketoprofen and Imp2 (Left) Imp2 and Imp3 (Right)
*Flow rate: 0.6 mL/min (optimum value suggested by LabSolutions MD),
*Initial Conc.: Acetonitrile ratio, *Executed with column of Lot 1

■ Robustness Evaluation

A robustness evaluation was carried out at the optimum condition (acetonitrile ratio at 40%, column oven temperature at 35%, flow rate at 0.6 mL/min).

The acetonitrile ratio was varied in 1% increments (39%, 40%, 41%) and the column oven temperature in 1 ˚C increments (34 ˚C, 35 ˚C, 36 ˚C) (shown in Fig. 3 by the blue point and 4 white circles, total of 5 points), and the effect on resolution was examined. Fig. 4 is the design spaces visualizing the resolutions of ketoprofen and Imp2 (left) and also, Imp2 and Imp3 (right). The resolution is higher than 8 (Ketoprofen and Imp2) and than 2 (Imp2 and Imp3) (shown by orange and red) within the entire region identified by Design Space. Furthermore, as shown in Fig. 5 and Fig. 6, design spaces are built in the same manner for additional two different column lots in order to assess robustness as well. The chromatograms obtained are shown in Fig. 7. In Figs. 4 to 6, it is found that resolution is higher than 8 (Ketoprofen and Imp2) and than 2 (Imp2 and Imp3) in the entire region, showing the optimized analytical method ensures robustness of all column lots.

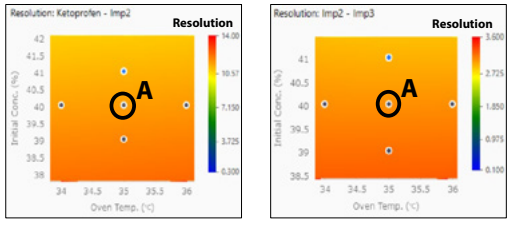


Fig. 4 Design Space for Resolution of Ketoprofen and Imp2 (Left) Imp2 and Imp3 (Right)
*The black dots (total: 5 points) in the figure are points where the analysis is executed (Column lot 1)

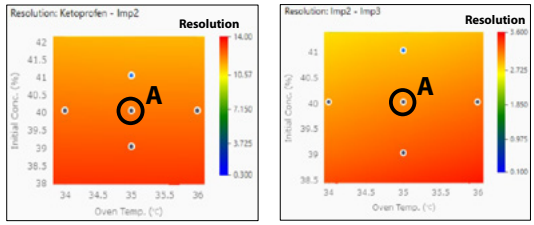


Fig. 5 Design Space for Resolution of Ketoprofen and Imp2 (Left) Imp2 and Imp3 (Right)
*The black dots (total: 5 points) in the figure are points where the analysis is executed (Column lot 2)

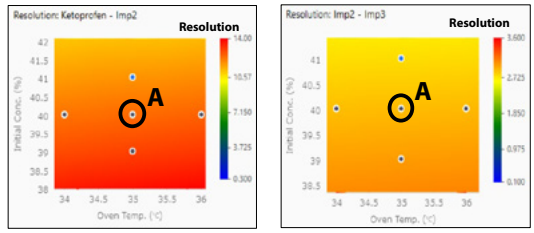


Fig. 6 Design Space for Resolution of Ketoprofen and Imp2 (Left) Imp2 and Imp3 (Right)
*The black dots (total: 5 points) in the figure are points where the analysis is executed (Column lot 3)

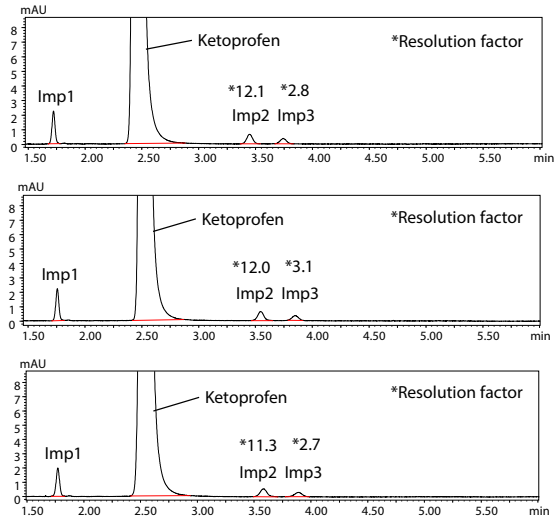


Fig. 7 Chromatograms Acquired with Columns of Different Lots (Point A in Fig. 4 to Fig. 6)
(Top: Lot 1, Middle: Lot 2, Bottom: Lot 3)

■ Conclusion

This article introduces efficient method development on pharmaceutical impurities by using LabSolutions MD. Visualizing resolution of API and impurities by design space enables efficient optimization and robustness evaluation of analytical method without relying exclusively on the user experience. It is also possible to evaluate robustness using different column lots by building design spaces for each lot. The process of development of pharmaceutical impurities introduced in this article can be also efficiently applicable to support method validation.

01-00335A-EN First Edition: Aug. 2022
Revision A: Oct. 2025

index

Application News

LabSolutions™ MD : Software for Efficient Method Development based on Analytical Quality by Design

Efficient Method Development for a Synthetic Peptide and Related Impurities

Shinichi Fujisaki

User Benefits

- LabSolutions MD improves the efficiency of the entire workflow for method development of synthetic peptide and impurities.
- Screening for multiple mobile phases and columns can be automated using mobile phase and column switching valves.
- Molecular weights of peptides and related impurities can be estimated and accurately tracked with LCMS-2050, a single quadrupole mass spectrometer.

■ Introduction

Peptide therapeutics, characterized by specific amino acid sequences crucial to their function, can be synthesized chemically, similar to small molecule drugs. The production of synthetic peptides involves multiple steps, including deprotection, activation, coupling, and the cleavage of the final sequence from the solid support. Impurities, such as those resulting from premature chain termination or missing amino acids, can affect the safety and efficacy of the final product. Therefore, these impurities must be separated by liquid chromatography (LC). In LC analysis, selecting the appropriate mobile phase and column across a wide range of combinations is critical for achieving optimal separation, as it significantly impacts the separation. However, since the separation pattern varies depending on the peptide chain lengths, amino acid compositions, and presence of modifications, optimizing separation for each peptide sequence is time-intensive. This study describes how to efficiently find the best separation conditions for peptides and related impurities utilizing LabSolutions MD, a dedicated software for supporting method development, through both screening and optimization phases.

■ Target Sample

A target peptide and five related impurities with different sequences were used as a model sample of a synthetic peptide (Table 1). Full length peptide (FLP : beta-Melanotropin), deletion sequences of p.A1del, p.A1_E2del, p.A1_K3del, and p.A1_D5del as shorter length of products, and Met(O2) (methionine in FLP is oxidized to methionine sulfone) were prepared as a sample mixture.

Table 1 Sequences of the Target Peptide and Related Impurities

Name	Sequence
FLP	AEKKDEGPYRMEHFRWGSPPKD
p.A1del	EKKDEGPYRMEHFRWGSPPKD
p.A1_E2del	KKDEGPYRMEHFRWGSPPKD
p.A1_K3del	KDEGPYRMEHFRWGSPPKD
p.A1_D5del	EGPYRMEHFRWGSPPKD
Met(O2)	AEKKDEGPYR{Met(O2)}EHFRWGSPPKD

Note : Met(O2) = methionine sulfone

■ Mobile Phases and Columns Screening

In the screening phase (analytical conditions : Table 2), the optimal combination of mobile phase and column was investigated. For the mobile phases, four aqueous solutions (0.1% TFA, 0.1% formic acid, 10 mmol/L ammonium formate (pH 4.0), and 10 mmol/L ammonium acetate (pH 5.0)), and three organic solvents (acetonitrile, methanol, and a 1 : 1 mixture of acetonitrile/methanol) were evaluated. For the columns, six columns with different stationary phases and pore sizes were tested. A comprehensive analysis schedule was created with a total of 72 (4 x 3 x 6) patterns of these mobile phases and columns to explore the optimal combination. LabSolutions MD can quickly and easily create an analysis schedule with different parameters such as mobile phase compositions and columns (steps (1) to (5) in Fig. 1). In addition, the mobile phases ((1) in Fig. 1) and the columns ((2) in Fig. 1) can be switched automatically using flow change valves, respectively. Furthermore, mobile phase blending function automatically prepares the mobile phases with the different mixture ratios of acetonitrile and methanol by simply clicking the desired mobile phases (step (1) in Fig. 1). This significantly reduces the amount of manual work and prevents human errors during preparation.

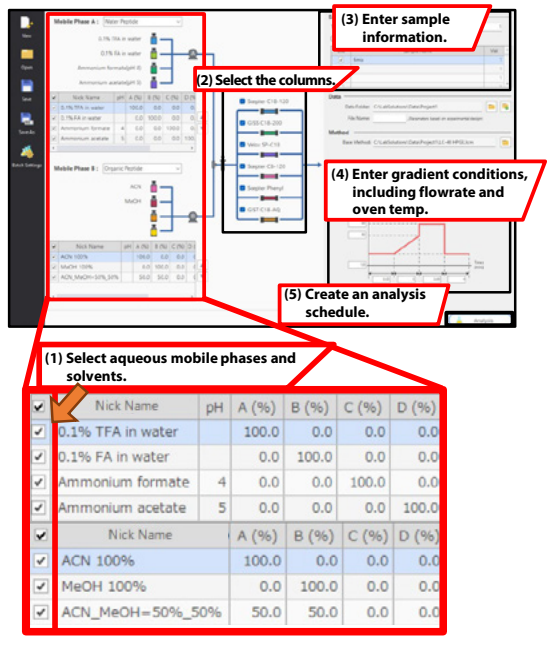


Fig. 1 Steps for Creating Analysis Schedule

Table 2 Analytical Conditions for Mobile Phases and Columns Screening

System	: Nexera™ X3 (Method Scouting System)
Column 1	: Shim-pack Scepter™ C18-120 *1
Column 2	: Shim-pack™ GISS C18 *2
Column 3	: Shim-pack Velox™ SP-C18 *3
Column 4	: Shim-pack Scepter C8-120 *4
Column 5	: Shim-pack Scepter Phenyl-120 *5
Column 6	: Shim-pack GIST-HP C18-AQ *6
	(100 mm × 3.0 mm I.D., 1.9 µm : column 1, 2, 4~6)
	(100 mm × 3.0 mm I.D., 1.8 µm : column 3)
Temperature	: 40 °C
Injection volume	: 2 µL (FLP : 1000 mg/L, other impurities : 100 mg/L)
Sample solvent	: Water
Mobile phases	
Pump A – Line A	: 0.1% TFA (Trifluoroacetic acid) in water
– Line B	: 0.1% formic acid in water
– Line C	: 10 mmol/L ammonium formate (pH 4) in water
– Line D	: 10 mmol/L ammonium acetate (pH 5) in water
Pump B – Line A	: Acetonitrile
– Line B	: Methanol
Flow rate	: 0.5 mL/min
Time program (%B)	: 10 % (0 min) →60 % (10 min)
	→10 % (10.01-15 min)
Detection	: 220 nm (SPD-M40, UHPLC cell)
System	: LCMS-2050
Ionization	: ESI/APCI (DUIS™), positive mode
Mode	: SCAN (*m/z* 300-2000)
Nebulizing gas	: 2.0 L/min (N_2)
Drying gas	: 5.0 L/min (N_2)
Heating gas	: 7.0 L/min (N_2)
DL temp.	: 200 °C
Desolvation temp.	: 450 °C
Interface Voltage	: 1.0 kV

*1 P/N：227-31013-03, *2 P/N：227-30049-02
*3 P/N：227-32002-02, *4 P/N：227-31034-03
*5 P/N：227-31064-03, *6 P/N：227-30808-02
(Shimadzu GLC product number)

■ Results of Mobile Phases and Columns Screening

Chromatograms acquired under different conditions of mobile phases (aqueous mobile phases : 0.1% TFA, 0.1% formic acid, 10 mmol/L ammonium formate, 10 mmol/L ammonium acetate / organic solvents : 100% acetonitrile, 100% methanol) on each column are shown in Fig. 2-7.

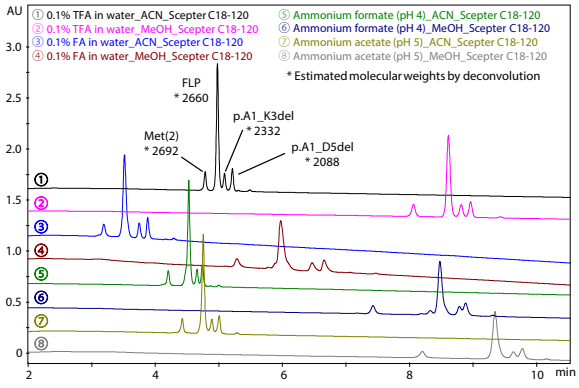


Fig. 2 Chromatograms Acquired by Scepter C18-120

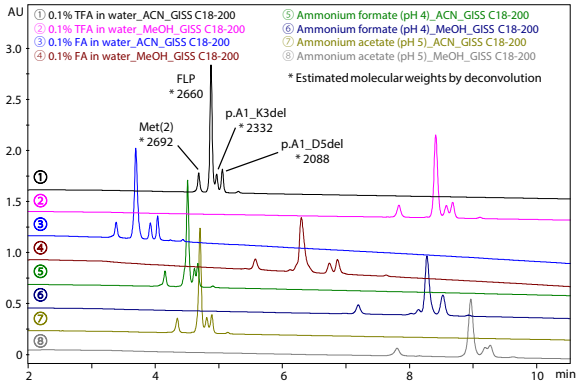


Fig. 3 Chromatograms Acquired by GISS C18-200

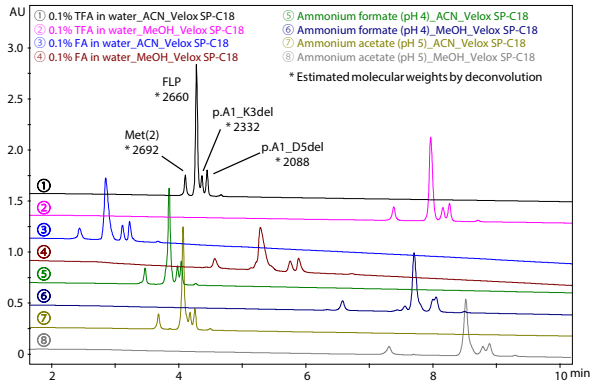


Fig. 4 Chromatograms Acquired by Velox SP-C18

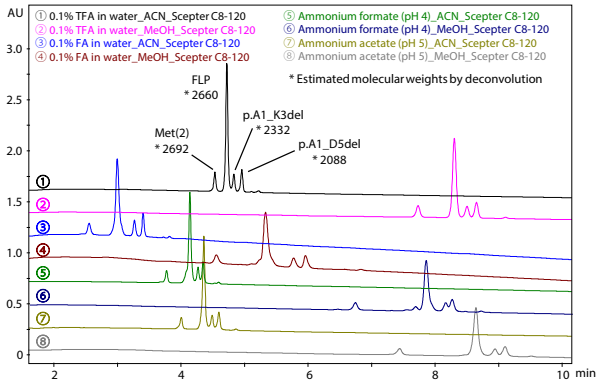


Fig. 5 Chromatograms Acquired by Scepter C8-120

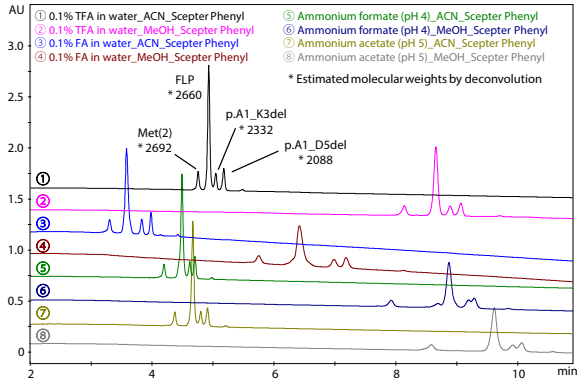


Fig. 6 Chromatograms Acquired by Scepter Phenyl

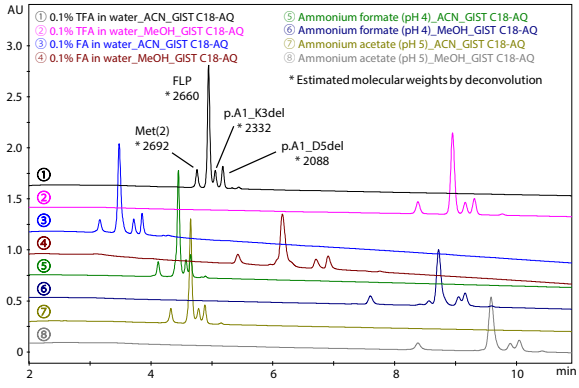


Fig. 7 Chromatograms Acquired by GIST C18-AQ

The results of mobile phases and columns screening indicated that the type of aqueous solution and the composition of organic solvent have a significant effect on separation of FLP and related impurities. Additionally, LabSolutions MD can calculate molecular weights (estimated by deconvolution of MS spectrum acquired by LCMS-2050) for each peak, facilitating the confirmation of synthesized compounds and the estimation of molecular weights for unknown impurities.

■ Quickly Find Optimal Condition

Many chromatograms are generated based on the number of conditions considered in the screening phase, and a certain level of chromatographic knowledge, along with significant effort, is required to evaluate which condition provides the desired separation. LabSolutions MD can quickly and easily find optimal condition using equation (Eq. 1) below to quantitatively evaluate the separation.

$$(\text{Evaluation Value}) = P \times (Rs_1 + Rs_2 + \ldots + Rs_{P-1}) \qquad \text{(Eq. 1)}$$

Evaluation Value is calculated as the number of peaks detected (P) multiplied by the sum of resolution factor (Rs) for all peaks. Fig. 8 shows Evaluation Value obtained through mobile phase screening and listed in the order from the highest to the lowest. The top three chromatograms with the highest evaluation values are shown in Fig. 9. The best chromatogram was obtained under the mobile phase composition of 0.1% formic acid and methanol using Scepter C8-120 column. However, in the conditions with the second and third highest evaluation values, p.A1_E2del was also detected, and a certain level of separation was achieved. Therefore, further investigation was conducted with different column oven temperatures (40, 50, 60, 70, and 80 °C) for these top three conditions.

MPA Nick Name	MPB Nick Name	Column Nick Name	Evaluation Value
0.1% FA in water	MeOH	Scepter-C8-120	26.889
Ammonium acetate (pH 5)	ACN_MeOH=50_50	Velox SP-C18	26.328
Ammonium formate (pH 4)	MeOH	Scepter-C8-120	26.073
Ammonium formate (pH 4)	ACN_MeOH=50_50	Scepter-C8-120	25.948
Ammonium formate (pH 4)	ACN_MeOH=50_50	Scepter C18-120	25.792
Ammonium formate (pH 4)	ACN_MeOH=50_50	Scepter Phenyl-120	25.575
Ammonium formate (pH 4)	ACN_MeOH=50_50	GIST-C18-AQ	25.502
Ammonium formate (pH 4)	MeOH	Scepter C18-120	25.318
Ammonium formate (pH 4)	ACN	Scepter-C8-120	24.890
Ammonium formate (pH 4)	ACN	Scepter Phenyl-120	24.860

Fig. 8 Ranking of Each Condition by Evaluation Value
(Top 10 Chromatograms Listed from the Highest to the Lowest)

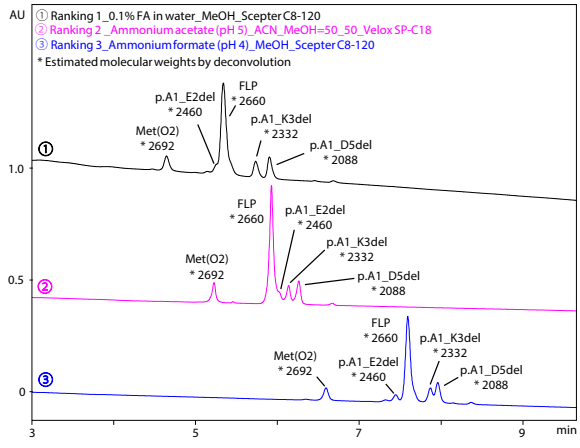


Fig. 9 Chromatograms of the Top Three Evaluation Values

■ Results of Column Oven Temperature Screening

Chromatograms obtained by varying the column oven temperatures at 40, 50, 60, 70, and 80 °C for the top three chromatograms (Fig. 9) are shown in Fig. 10-12, respectively. Higher temperatures improved separation in all conditions, indicating that column oven temperature is effective in improving the separation. In addition, it was confirmed that increasing the column oven temperature improves the resolution between p.A1del and FLP, which are the most difficult to separate. Fig. 13 shows the resolution results between p.A1del and FLP, listed in the order from the highest to the lowest. The highest resolution between p.A1del and FLP was observed with the mobile phase composition of 0.1% formic acid and methanol using Scepter C8-120 column (chromatogram ① in Fig. 10).

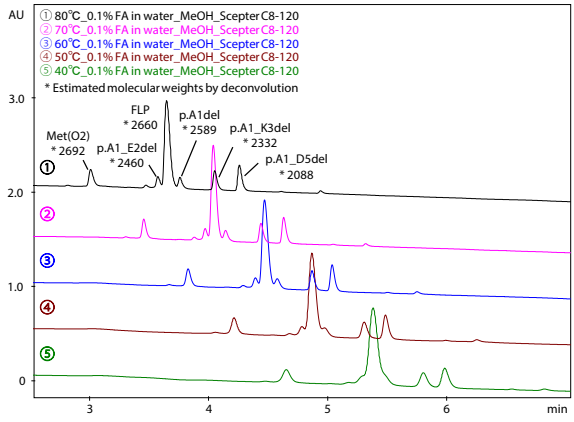


Fig. 10 Chromatogram with Different Column Oven Temperatures
80 °C(①), 70 °C(②), 60 °C(③), 50 °C(④), 40 °C(⑤)
(0.1% formic acid/ MeOH / Scepter C8-120)

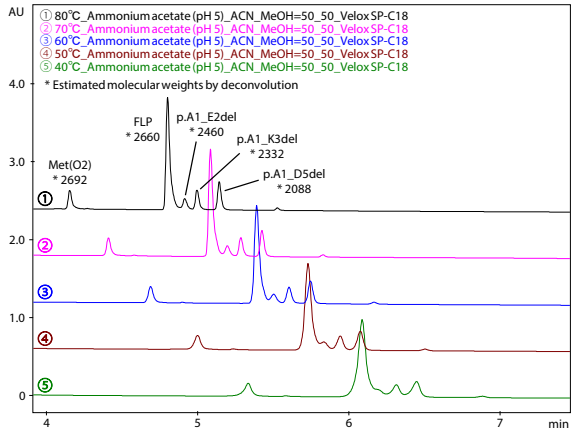


Fig. 11 Chromatogram with Different Column Oven Temperatures
80 °C(①), 70 °C(②), 60 °C(③), 50 °C(④), 40 °C(⑤)
(10 mmol/L ammonium acetate / ACN : MeOH=50:50 / Velox SP-C18)

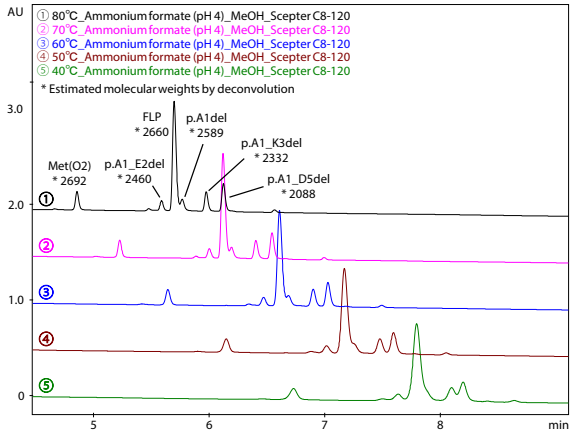


Fig. 12 Chromatogram with Different Column Oven Temperatures
80 °C(①), 70 °C(②), 60 °C(③), 50 °C(④), 40 °C(⑤)
(10 mmol/L ammonium formate / MeOH / Scepter C8-120)

MPA Nick Name	MPB Nick Name	Column Nick Name	Oven Temp.	Resolution (p.A1del)
0.1% FA in water	MeOH	Scepter-C8-120	80	1.492
0.1% FA in water	MeOH	Scepter-C8-120	70	1.396
0.1% FA in water	MeOH	Scepter-C8-120	60	1.238
Ammonium formate (pH 4)	MeOH	Scepter-C8-120	80	1.175
Ammonium formate (pH 4)	MeOH	Scepter-C8-120	70	1.172

Fig. 13 Ranking by Resolution between p.A1del and FLP
(Top 5 Chromatograms Listed from the Highest to the Lowest)

Next, the optimization phase was conducted to further improve separation and robustness by adjusting LC parameters, such as gradient conditions and flow rate.

■ Optimization Phase

Based on the optimal condition (aqueous solution : 0.1% formic acid , organic solvent : methanol, column : Scepter C8-120, column oven temperature : 80 °C), initial gradient concentration (5, 10, and 15% : Fig. 14), gradient time (5, 10, and 15 min : Fig. 14), and flow rate (0.5, 0.6, and 0.7 mL/min), were optimized to further improve the separation of FLP and related impurities. The obtained chromatograms are shown in Fig. 15-17. Higher initial gradient concentration and longer gradient time tended to improve the resolution between neighboring peaks, while the flow rate had a smaller effect on resolution. Then, peak tracking was performed for FLP and related impurities to visualize the resolution through design spaces.

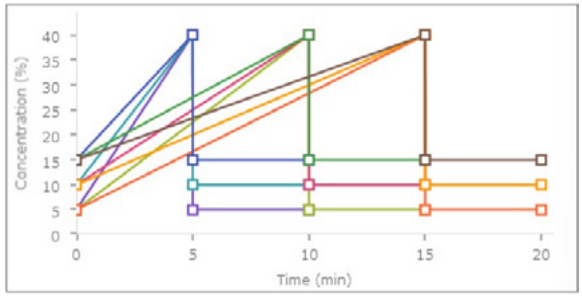


Fig. 14 Optimization of Gradient Conditions

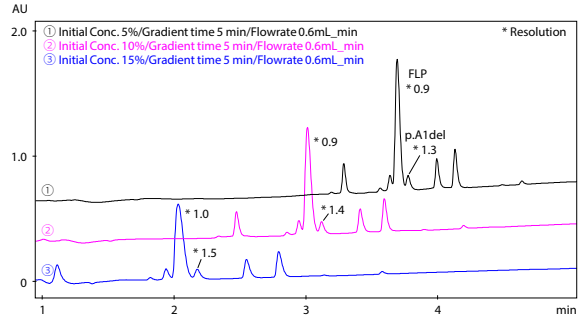


Fig. 15 Chromatograms with Different Initial Concentration
5%(①) , 10%(②) , 15%(③)

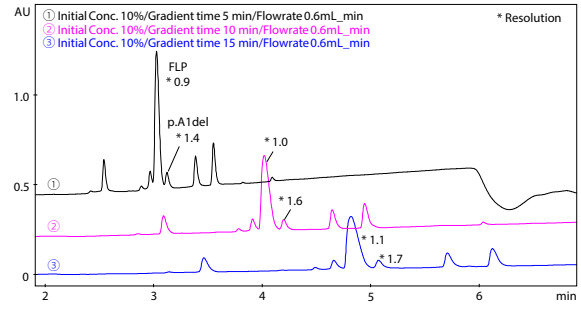


Fig. 16 Chromatograms with Different Gradient Time
5 min(①) , 10 min(②) , 15 min(③)

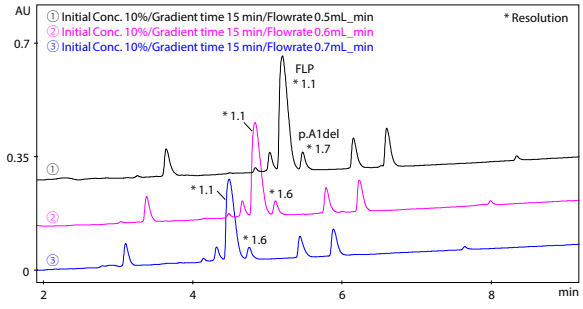


Fig. 17 Chromatograms with Different Flow Rate
0.5 mL/min(①) , 0.6 mL/min (②) , 0.7 mL/min (③)

■ Automated Peak Tracking by LCMS-2050

LC chromatograms obtained with a gradient time of 5 min, flow rate of 0.6 mL/min, and initial concentration of 5 % and 15 %, along with molecular weights for FLP and impurities, are shown in Fig. 18. The UV spectra for each impurity are displayed in Fig. 19. The similarity between UV spectra of Met(O2), p.A1del, p.A1_E2del, p.A1_K3del, and p.A1_D5del is greater than 0.99, suggesting that peak tracking based on UV spectra would be challenging. In contrast, LabSolutions MD enables peak tracking based on molecular weights acquired by LCMS-2050, facilitating accurate identification of compounds with similar UV spectra (Fig. 18). The estimated molecular weights for each compound show small mass errors compared to the theoretical values (Table 3), which can be used to confirm the molecular weights of known compounds as well as to approximate the molecular weights of unknown impurities.

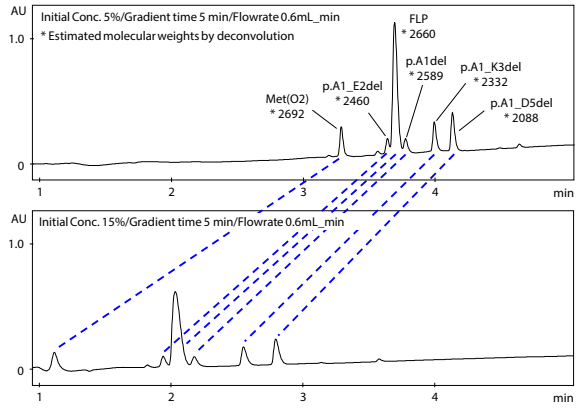


Fig. 18 LC Chromatograms at a Gradient Time of 5 min, Flow Rate of 0.6 mL/min, and Initial Concentration of 5 % (Upper) and 15 % (Lower)
(Dashed lines indicate tracking based on molecular weights.)

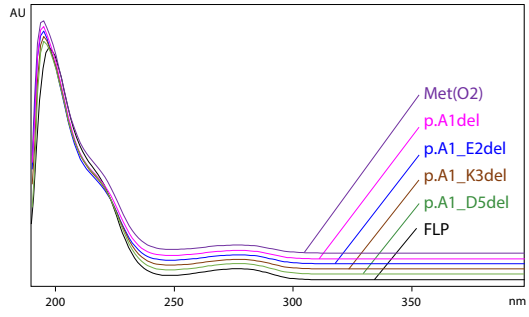


Fig. 19 UV Spectra of FLP and Impurities

Table 3 Estimated / Theoretical Molecular Weight of FLP and Impurities

Name	Estimated Molecular Weight	Theoretical Molecular Weight
FLP	2660	2660
p.A1del	2589	2589
p.A1_E2del	2460	2460
p.A1_K3del	2332	2332
p.A1_D5del	2088	2089
Met(O2)	2692	2692

Next, by visualizing resolution of FLP and each impurity with design spaces, the optimal condition that provides the best resolution and robustness was identified.

■ Design Space Evaluation for Optimal Condition

Design spaces of resolution of FLP and each impurity were shown (Fig. 20). The vertical axis represents gradient time, while the horizontal axis represents initial concentration. The red region indicates higher resolution, and the blue region indicates lower resolution. By visualizing the resolution through design spaces, it became evident that higher initial concentrations and longer gradient times improve the resolution of each peak.

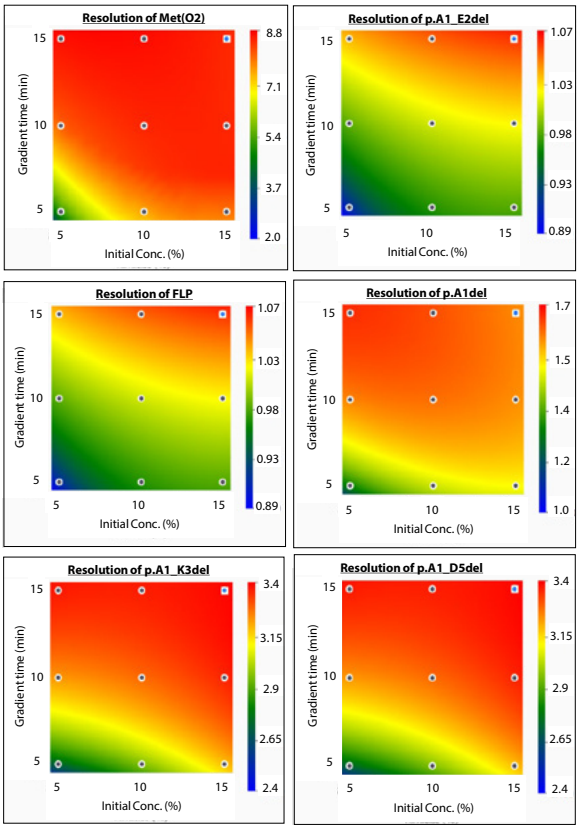


Fig. 20 Design Space for Resolution of FLP and Impurities
(Flow rate : 0.6 mL/min)

LabSolutions MD can automatically search for the optimal conditions that meet multiple criteria by overlaying design spaces. For example, Fig. 21 shows the area of the analytical conditions that satisfy the following requirements : resolution of FLP and p.A1del > 1.5, resolution of FLP and p.A1_E2del > 1.0, retention time of p.A1_D5del < 6 min to reduce analysis time, and retention time of Met(O2) > 3 min for proper retention. The region enclosed by the orange line is where the resolution of FLP and p.A1del < 1.5, the region enclosed by the pink line is where the resolution of FLP and p.A1_E2del < 1.0, the region enclosed by the yellow line is where the retention time of p.A1_D5del > 6 min, and the region enclosed by the brown line is where the retention time of Met(O2) < 3 min. Point A (red circle) in the remaining region (shown by the black hatching), represents the optimal conditions (initial concentration : 9%, gradient time : 11.5 min, flow rate : 0.6 mL/min) that satisfy all the criteria. By overlaying design spaces, the desired conditions can be easily identified.

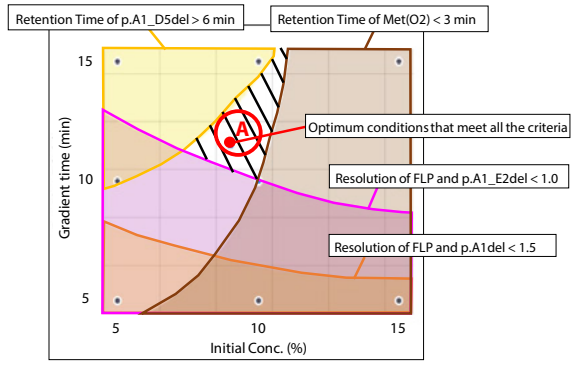


Fig. 21 Overlay of Design Spaces of Resolution and Retention Time

■ Chromatogram at Optimal Condition

The chromatogram obtained at optimal conditions (point A) is shown in Fig. 22. It shows that the resolution of FLP and p.A1del > 1.5, resolution of FLP and p.A1_E2del > 1.0, retention time of p.A1_D5del < 6 min, and retention time of Met(O2) > 3 min, which successfully satisfies the optimization criteria. By utilizing design spaces, the desired conditions can be easily identified without relying on chromatography experience.

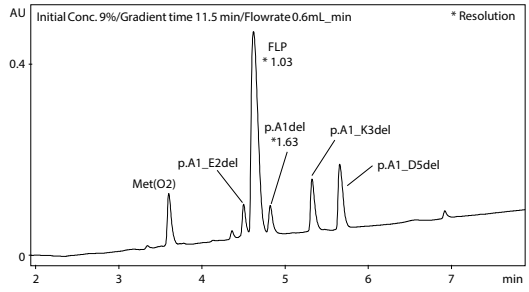


Fig. 22 Chromatogram at Optimal Conditions
(aqueous solution : 0.1% formic acid / organic solvent : MeOH / column : Scepter C8-120 / column oven temperature : 80 °C)

■ Conclusion

The separation patterns of synthetic peptides vary depending on mobile phase composition, column type, and various LC parameters, such as gradient conditions, column oven temperature, and flow rate. Separation behavior can also differ based on peptide structure, including length, amino acid composition, and the presence of modifications. Therefore, it is necessary to optimize the separation for each peptide sequence individually. However, optimizing analytical conditions through numerous analyses and data processing can be time-consuming. LabSolutions MD automates the entire workflow, including generating analysis schedules, preparing mobile phases, and processing the data, thanks to functionalities such as automated peak tracking, ranking of chromatograms, and design space visualization. This article introduced a case where the optimal separation conditions for synthetic peptides were efficiently identified through screening and optimization phases. LabSolutions MD also offers fully automated gradient optimization by AI algorithm to meet user-defined criteria. For more details, please refer to the application news, "Automatic Optimization of Gradient Conditions by AI Algorithm on Synthetic Peptide and Impurities : 01-00814".

01-00780A-EN First Edition: Oct. 2024
Revision A: Dec. 2024

High Performance Liquid Chromatograph Nexera™ XR

Application News

Simple Labor-Saving Calibration Curve Creation Using Autosampler Automatic Dilution Function

Natsuki Iwata and Ryota Kanamaru

User Benefits

- The autosampler's automatic dilution function eliminates manual dilution preparation, improving operational efficiency and productivity.
- Simply specify the desired dilution ratio in the batch table and use the same method file to automatically dilute solutions and create a calibration curve.
- Setting and management are easy when changing HPLC conditions since a single method file is used regardless of the dilution ratio.

■ Introduction

Dilution of standard and sample solutions for HPLC analysis is generally performed manually using pipettes. However, such work is labor-intensive and time-consuming.

In recent years, automation aimed at labor-saving has become increasingly desirable since if these dilution preparation operations could be automated, work efficiency and productivity would improve.

Nexera Autosamplers are equipped with a pretreatment function that automatically performs dilution, reagent addition, and co-injection. Using this automatic dilution function, it is possible to prepare a sample diluted at a user-defined factor and introduce it directly into the analytical column. This article introduces a simple method for creating calibration curves using the autosampler automatic dilution function.

■ Pretreatment Program and Operation Overview

A method file contains information such as LC parameters, analytical parameters, and the pretreatment program. The pretreatment program can set various dilution ratio, example 100-fold dilution. In addition, when the program is used with the batch add-in (Fig. 2 on the next page), a single method file can be used regardless of the dilution ratio, thereby preventing human errors such as setup mistakes.

The dilution factor and conditions related to the mixing process are configured using the LabSolutions™ workstation. The setup window for the autosampler pretreatment is shown in Fig. 1. Pretreatment program commands are shown in Table 1. In this article, the rinse solution was used as a diluent.

A volume corresponding to the dilution ratio is aspirated from the stock solution vial and dispensed with the diluent into an empty vial (mixing vial) previously set in the autosampler (final volume is 100 µL in this example). The solution in the vial is mixed using the aspiration/dispensing function (pipetting). Finally, a specific amount of the solution is aspirated and injected into the column.

Table 1 Pretreatment Program

Line	Command
1	a3=100/a2
2	n.drain
3	disp 600.0,rs
4	d.rinse
5	vial.n a0,a1
6	n.strk ns
7	aspir a3,ss
8	air.a 0.1,ss
9	d.rinse
10	vial.n rn,sn
11	n.strk ns
12	disp 100.1,rs
13	mix 1,5,40,ss,35
14	n.drain
15	disp 100.0,rs
16	d.rinse
17	inj.p
18	v.inj
19	wait 2.0
20	goto f0
21	end

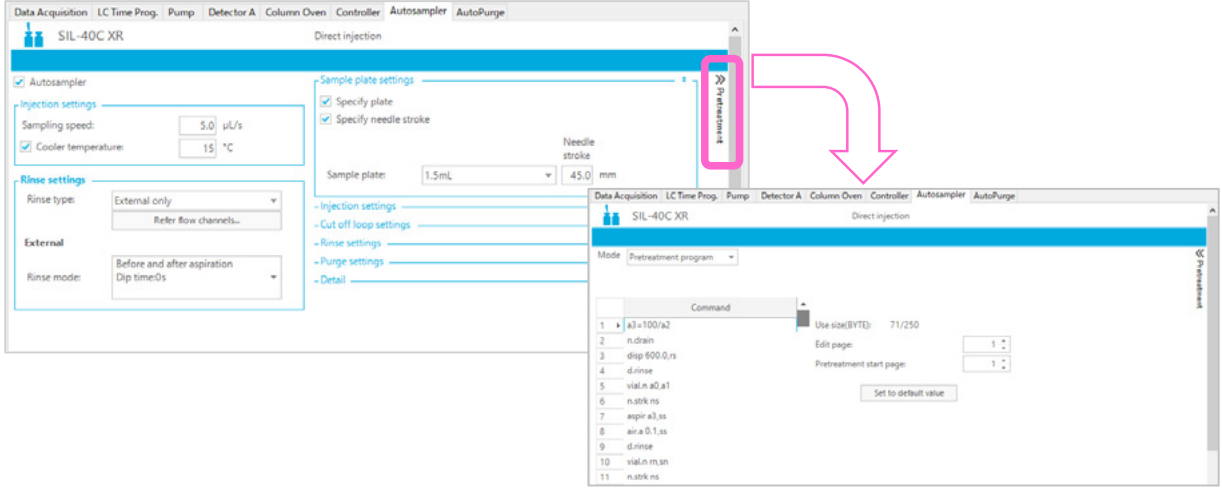


Fig. 1 Setup Window for Autosampler Pretreatment Program

■ Setting up a Batch Table

A batch add-in called the SIL pretreatment variable is pre-applied to LabSolutions to populate the batch table with the location of the stock solution vial and any dilution ratio*1. Fig. 2 shows the SIL pretreatment variable setup window for the batch table. Set the plate number (A0), vial number (A1), and dilution ratio of the stock solution vial (A2) in columns A0-A2 of the SIL pretreatment variables. Place empty vials for automatic dilution (mixing) at the locations specified by the tray and vial numbers in the batch table. If the sample type (standard) and level number are set as shown in Fig. 2, a calibration curve is automatically generated after the analysis. Note that separate analytical parameter settings are required.

*1 Contact Shimadzu for information about applying the batch add-in.

■ Automatic Dilution Analysis for a Caffeine Standard Solution

An automatic dilution analysis was performed using a 250 mg/L caffeine solution. Table 2 shows the analytical conditions. The pretreatment program is the same as in Table 1. 1.0 mL polypropylene vials were used for mixing, and septum vials were used for the stock solutions (standard and sample). Ultrapure water was used as the diluent for the rinse solution*2. Fig. 3 shows the chromatogram of caffeine (concentration after automatic dilution: 2.5 mg/L) diluted 100-fold with ultrapure water.

*2 For HPLC equipped with a multi-rinse function, use the rinse solution specified in the parameter settings of an autosampler.

Table 2 Analytical Conditions

System:	Nexera XR
Column:	Shim-pack™ GIST C18*3 (75 mm × 3.0 mm I.D., 2 µm)
Flowrate:	0.6 mL/min
Mobile Phase:	A) 10 mmol/L (Sodium) phosphate buffer (pH 2.6) B) Methanol
Time Program:	20 % B (0 min)→23 % B (3.30 min)→70 % B (3.31 - 4.30 min) →20 % B (4.31 - 5.50 min)
Column Temp.:	40 °C
Sample:	250 mg/L Caffeine aq.
Injection Volume:	4 µL
Needle Stroke:	45 mm
Vial for Mixing:	Shimadzu Vial, LC, 1 mL, Polypropylene*4
Vial for Stock Solution and Sample:	SHIMADZU LabTotal™ for LC 1.5 mL, Glass*5
Diluent:	Rinse solution (Ultrapure water)
Detection:	272 nm (SPD-M40)

*3 P/N: 227-30002-03, *4 P/N: 228-31600-91, *5 P/N: 227-34001-01

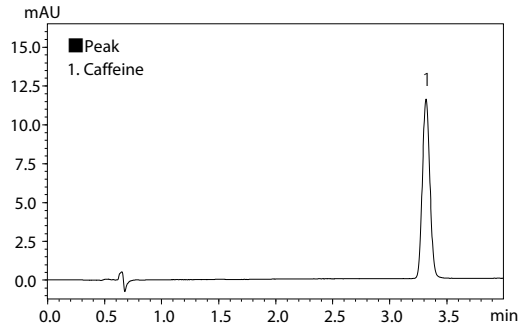


Fig. 3 Chromatogram for the Caffeine Standard Solution
(Dilution Ratio: 100,
Concentration of Caffeine after Automatic Dilution: 2.5 mg/L)

■ Repeatability

A standard solution prepared by automatically diluting 250 mg/L caffeine solution 500-fold with ultrapure water (the concentration after automatic dilution was 0.5 mg/L) was analyzed six times consecutively. The repeatability (%RSD) of the retention time and the peak area are shown in Table 3.

Table 3 Repeatability (%RSD) in Six Replicate Analyses

Retention time	Peak area
0.02	0.96

■ Calibration Curve

The calibration curve for caffeine (concentration range of 0.5-50 mg/L) using the autosampler automatic dilution function was showing an excellent linearity, with a coefficient of determination (r^2) of 0.999 or greater. The calibration curve is displayed in Table 4.

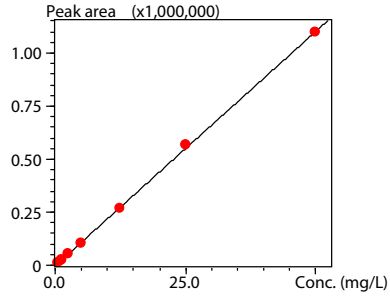


Fig. 4 Calibration Curve

Analysis	Tray Name	Vial#	Sample Name	Sample ID	SIL Pretreatment Variables	Data Comment	Sample Type	Level#	Inj. Volume	Method File
1	1	1	Caffeine	500	A0=1;A1=53;A2=500	A0: Plate No. A1: Vial No. A2: Dilution ratio	1:Standard:(1)	1	4	NS_GE3.lcm
2	1	2	Caffeine	200	A0=1;A1=53;A2=200	A0: Plate No. A1: Vial No. A2: Dilution ratio	1:Standard	2	4	NS_GE3.lcm
3	1	3	Caffeine	100	A0=1;A1=53;A2=100	A0: Plate No. A1: Vial No. A2: Dilution ratio	1:Standard	3	4	NS_GE3.lcm
4	1	4	Caffeine	50	A0=1;A1=53;A2=50	A0: Plate No. A1: Vial No. A2: Dilution ratio	1:Standard	4	4	NS_GE3.lcm
5	1	5	Caffeine	20	A0=1;A1=53;A2=20	A0: Plate No. A1: Vial No. A2: Dilution ratio	1:Standard	5	4	NS_GE3.lcm
6	1	6	Caffeine	10	A0=1;A1=53;A2=10	A0: Plate No. A1: Vial No. A2: Dilution ratio	1:Standard	6	4	NS_GE3.lcm
7	1	7	Caffeine	5	A0=1;A1=53;A2=5	A0: Plate No. A1: Vial No. A2: Dilution ratio	1:Standard	7	4	NS_GE3.lcm

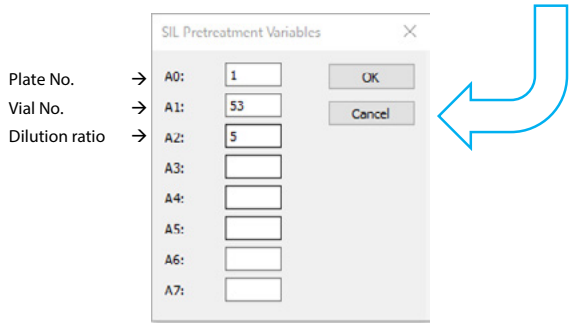


Fig. 2 Setup Window for Batch Table SIL Pretreatment Variables

■ Analysis of Caffeine in Beverages

Commercial green tea and coffee were used as samples. Samples were filtered through 0.2 µm membrane filters.

The pretreatment program and analytical conditions were the same as those in Tables 1 and 2. Figs. 5 and 6 show chromatograms obtained by diluting green tea and coffee 100-fold with ultrapure water using an automatic dilution function. Table 5 shows the analytical results (concentration after automatic dilution).

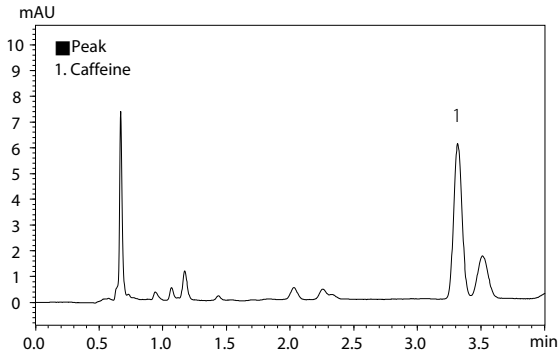


Fig. 5 Chromatogram of Green Tea Obtained by Automatic Dilution (Dilution Ratio: 100)

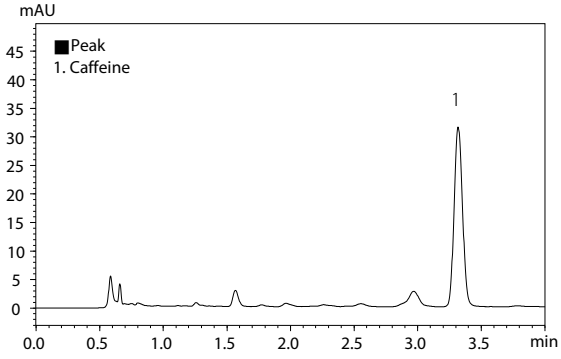


Fig. 6 Chromatogram of Coffee Obtained by Automatic Dilution (Dilution Ratio: 100)

Table 5 Analytical Results (n=6)

Sample	Concentration (mg/L)	%RSD
Green tea	1.32	0.72
Coffee	7.33	1.66

■ Automatic Calculation and Output of Content

The LabSolutions batch option can output directly the "sample concentration" just by specifying the dilution ratio. Fig. 7 shows the setup screen for the dilution ratios and automatic report output in the batch table. Fig. 8 shows the automatically generated report for the green tea analysis.

Sample Name	Dil. Factor	SIL Pretreatment Variables	Report Output	Report Format File
Caffeine	1	A0=1;A1=53;A2=500	☑	Report.lsr
Caffeine	1	A0=1;A1=53;A2=200	☑	Report.lsr
Caffeine	1	A0=1;A1=53;A2=100	☑	Report.lsr
Caffeine	1	A0=1;A1=53;A2=50	☑	Report.lsr
Caffeine	1	A0=1;A1=53;A2=20	☑	Report.lsr
Caffeine	1	A0=1;A1=53;A2=10	☑	Report.lsr
Caffeine	1	A0=1;A1=53;A2=5	☑	Report.lsr
Blank	1	A0=1;A1=54;A2=10	☑	Report.lsr
Coffee	100	A0=1;A1=52;A2=100	☑	Report.lsr
Blank	1	A0=1;A1=54;A2=10	☑	Report.lsr
Green Tea	100	A0=1;A1=51;A2=100	☑	Report.lsr

Fig. 7 Setup Screen for Dilution Ratio and Automatic Report Output in the Batch Table

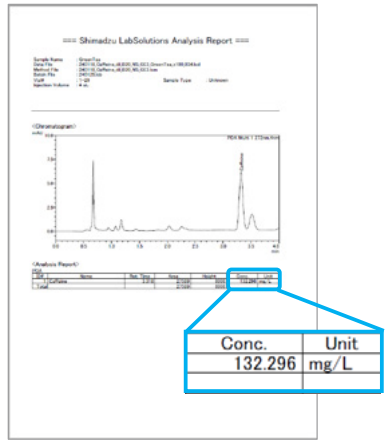


Fig. 8 Automatic Report Output (Green Tea Analysis)

■ Conclusion

By automatically preparing standard solutions for a calibration curve at any dilution ratios and using it for analysis, it was possible to generate an accurate calibration curve. Only stock solutions for standard and sample solutions need to be prepared, and subsequent work, from calibration curve creation to quantification , can be carried out automatically by the HPLC, thereby eliminating the labor needed for pretreatment. The calibration curve method described in this article is effective in improving the operational efficiency and productivity.

Contact Shimadzu for information about applying batch add-ins.

01-00717-EN First Edition: Aug. 2024

index

High Performance Liquid Chromatograph Nexera™ XR

Application News

Simple Labor-Saving Calibration Curve Creation Using Autosampler Automatic Dilution Function Part 2

Natsuki Iwata, Ryota Kanamaru, and Erika Baba

User Benefits

- The autosampler's automatic dilution function reduces manual dilution preparation and organic solvent consumption.
- Simply specify the desired dilution ratio in the batch table, and use the same method file to automatically dilute solutions and create a calibration curve.
- Setting and management are easy when changing HPLC conditions since a single method file is used regardless of the dilution ratio.

■ Introduction

The dilution of standard and sample solutions for HPLC analysis is generally performed manually, using pipettes. However, such work is labor-intensive and time-consuming. In recent years, automation for the purpose of labor-saving is desired to improve work efficiency and productivity. When the organic solvent is a diluent, a large amount of solvent is consumed to prepare standard solutions for calibration curves in volumetric flasks, but the volume of sample solution required for HPLC analysis is only a few tens of µL or less.

Using the automatic dilution function equipped with Nexera Autosamplers, it is possible to prepare a sample diluted at a user-defined factor and introduce it directly into the analytical column. The Application News 01-00717 describes a simple method for creating calibration curves using ultrapure water as a diluent. This article introduces the analysis using organic solvent as a diluent.

■ Pretreatment Program and Operation Overview

A method file contains information such as LC parameters, analytical parameters, and the pretreatment program. The pretreatment program can set various dilution ratios, such as a 100-fold dilution. In addition, when the program is used with the batch add-in (Fig. 2 on the next page), a single method file can be used regardless of the dilution ratio, thereby preventing human errors such as setup mistakes.

The dilution factor and conditions related to the mixing process are configured using the LabSolutions™ workstation. The setup window for the autosampler pretreatment is shown in Fig. 1. Pretreatment program commands are shown in Table 1. In this article, the rinse solution was used as a diluent.

A volume corresponding to the dilution ratio is aspirated from the stock solution vial and dispensed with the diluent into an empty vial (mixing vial) previously set in the autosampler (final volume is 100 µL in this example). The solution in the vial is mixed using the aspiration/dispensing function (pipetting). Finally, a specific amount of the solution is aspirated and injected into the column.

Table 1 Pretreatment Program

Line	Command
1	a3=100/a2
2	n.drain
3	disp 600.0,rs
4	d.rinse
5	vial.n a0,a1
6	n.strk ns
7	aspir a3,ss
8	air.a 0.1,ss
9	d.rinse
10	vial.n rn,sn
11	n.strk ns
12	disp 100.1,rs
13	mix 1,5,40,ss,35
14	n.drain
15	disp 100.0,rs
16	d.rinse
17	inj.p
18	v.inj
19	wait 2.0
20	goto f0
21	end

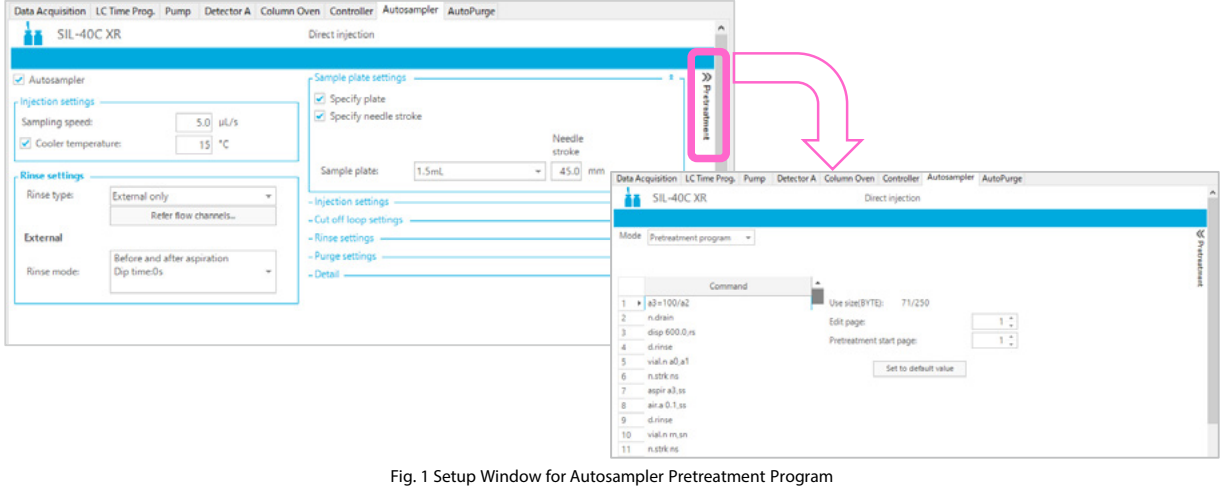


Fig. 1 Setup Window for Autosampler Pretreatment Program

■ Setting up a Batch Table

A batch add-in called the SIL pretreatment variable is pre-applied to LabSolutions to populate the batch table with the location of the stock solution vial and any dilution ratio[*1]. Fig. 2 shows the SIL pretreatment variable setup window for the batch table. Set the plate number (A0), vial number (A1), and dilution ratio of the stock solution vial (A2) in columns A0-A2 of the SIL pretreatment variables. Place empty vials for automatic dilution (mixing) at the locations specified by the tray and vial numbers in the batch table. If the sample type (standard) and level number are set as shown in Fig. 2, a calibration curve is automatically generated after the analysis. Note that separate analytical parameter settings are required.

*1 Contact Shimadzu for information about applying the batch add-in.

■ Automatic Dilution Analysis for Mixed Standard Solution

An automatic dilution analysis was performed using a mixed standard solution of coumarin and cinnamaldehyde at a concentration of 100 mg/L each (prepared with acetonitrile). Table 2 shows the analytical conditions. The pretreatment program is the same as in Table 1. 1.0 mL polypropylene vials were used for mixing, and septum vials were used for the stock solutions (standard and sample). Acetonitrile was used as the diluent for the rinse solution[*2]. Fig. 3 shows the chromatograms of mixed standard solution (concentration after automatic dilution: 1.0 mg/L each) diluted 100-fold with acetonitrile.

*2 For HPLC equipped with a multi-rinse function, use the rinse solution specified in the parameter settings of an autosampler.

Table 2 Analytical Conditions

System:	Nexera XR
Column:	Shim-pack™ GIST-HP C18[*3] (150 mm × 3.0 mm I.D., 3 µm)
Flowrate:	0.8 mL/min
Mobile Phase:	A) Water B) Acetonitrile
Time Program:	50 % B (0-2.0 min)→60 % B (4.0 min)→ 100 % B (4.1 - 5.0 min) →50 % B (5.1 - 5.5 min)
Column Temp.:	40 °C
Sample:	100 mg/L Coumarin and Cinnamaldehyde in Acetonitrile
Injection Volume:	5 µL
Needle Stroke:	45 mm
Vial for Mixing:	Shimadzu Vial, LC, 1 mL, Polypropylene[*4]
Vial for Stock Solution and Sample:	SHIMADZU LabTotal™ for LC 1.5 mL, Glass[*5]
Diluent:	Rinse solution (Acetonitrile)
Detection:	Ch1 (Coumarin): 276 nm, Ch2 (Cinnamaldehyde): 288 nm (SPD-M40)

*3 P/N: 227-30040-05, *4 P/N: 228-31600-91, *5 P/N: 227-34001-01

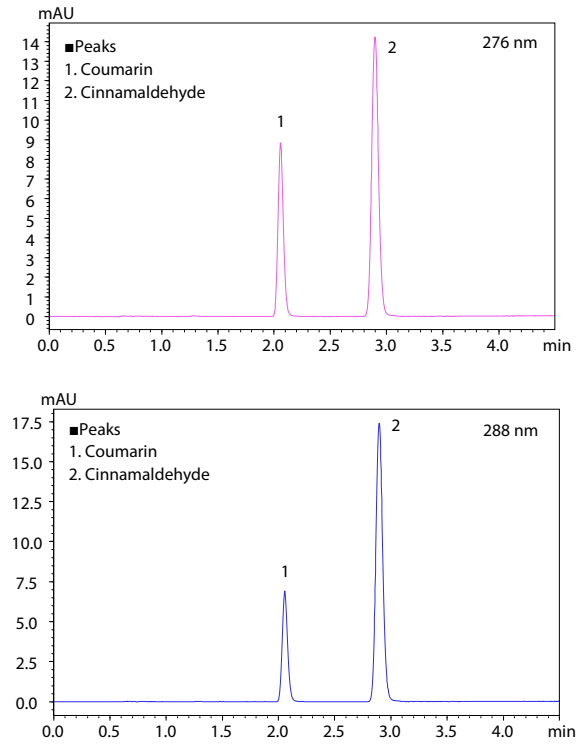


Fig. 3 Chromatograms for Mixed Standard Solution of Coumarin and Cinnamaldehyde
(Dilution Ratio: 100, Concentration after Automatic Dilution: 1.0 mg/L each)

■ Repeatability

The mixed standard solution (Dilution Ratio: 500, the concentration after automatic dilution was 0.2 mg/L each) was analyzed six times consecutively using the autosampler automatic dilution function. The repeatability (%RSD) of the retention time and the peak area are shown in Table 3.

Table 3 Repeatability (%RSD) in Six Replicate Analyses

Compound	Retention time	Peak area
Coumarin	0.09	1.46
Cinnamaldehyde	0.06	1.82

Analysis	Tray Name	Vial#	Sample Name	Sample ID	SIL Pretreatment Variables	Sample Type	Level#	Inj. Volume	Method File
1	2	3	2mix_100	x1000	A0=2;A1=51;A2=1000	1:Standard(I)	1	5	45_5.5m.lcm
2	2	4	2mix_100	x500	A0=2;A1=51;A2=500	1:Standard	2	5	45_5.5m.lcm
3	2	5	2mix_100	x200	A0=2;A1=51;A2=200	1:Standard	3	5	45_5.5m.lcm
4	2	6	2mix_100	x100	A0=2;A1=51;A2=100	1:Standard	4	5	45_5.5m.lcm
5	2	7	2mix_100	x50	A0=2;A1=51;A2=50	1:Standard	5	5	45_5.5m.lcm
6	2	8	2mix_100	x20	A0=2;A1=51;A2=20	1:Standard	6	5	45_5.5m.lcm
7	2	9	2mix_100	x10	A0=2;A1=51;A2=10	1:Standard	7	5	45_5.5m.lcm

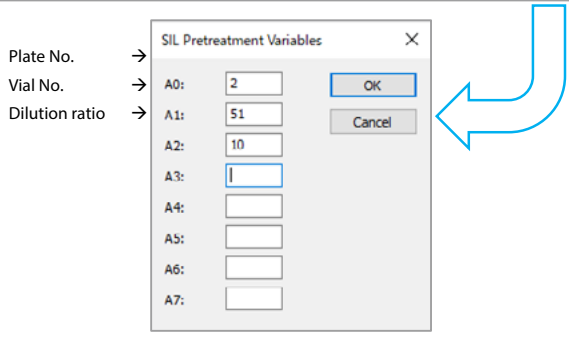


Fig. 2 Setup Window for Batch Table SIL Pretreatment Variables

■ Calibration Curve

Three calibration curves created with automatic dilution function for coumarin and cinnamaldehyde (concentration range of 0.1-10 mg/L each) were analyzed.

Excellent linearities with coefficients of determination (r^2) of 0.999 or greater were obtained. Calibration curves are displayed in Fig. 4.

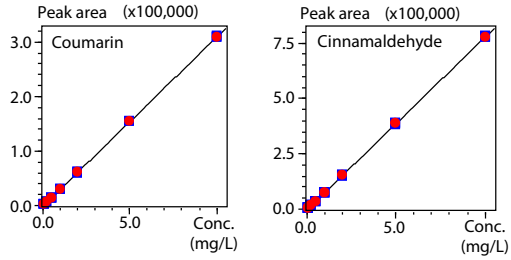


Fig. 4 Calibration Curves (n = 3)

■ Analysis of Cinnamon

A Sample of commercial *Cinnamomum cassia* was used. The pretreatment protocol is the same as the process up to filtration in Fig.3 from Application News No. 01-00233. Note that samples were manually diluted with acetonitrile at the final step of pretreatment in No. 01-00233-EN, but the sample was automatically diluted with an autosampler in this article. The pretreatment protocol is shown in Fig. 5. Acetonitrile was used as the extraction solvent. Lipids were removed using a dispersive solid phase extraction (dSPE) cartridge (Merck Supel™ QuE Z-Sep+). The cartridge eliminates the need to carry out conditioning before loading samples, which simplifies operations.

Fig. 6 shows chromatograms obtained by diluting *Cinnamomum cassia* extracts 100-fold with acetonitrile using an automatic dilution function. The two target compounds were well separated from the contaminants. The analytical results (concentration after automatic dilution) are shown in Table 4. Table 4 also shows the analytical results obtained when standard solutions for calibration curves were prepared manually, and the 100-fold dilution with acetonitrile of the pretreated cinnamon extract was performed manually.

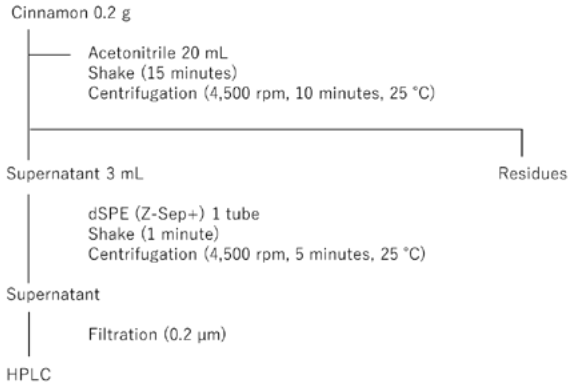


Fig. 5 Pretreatment Protocol

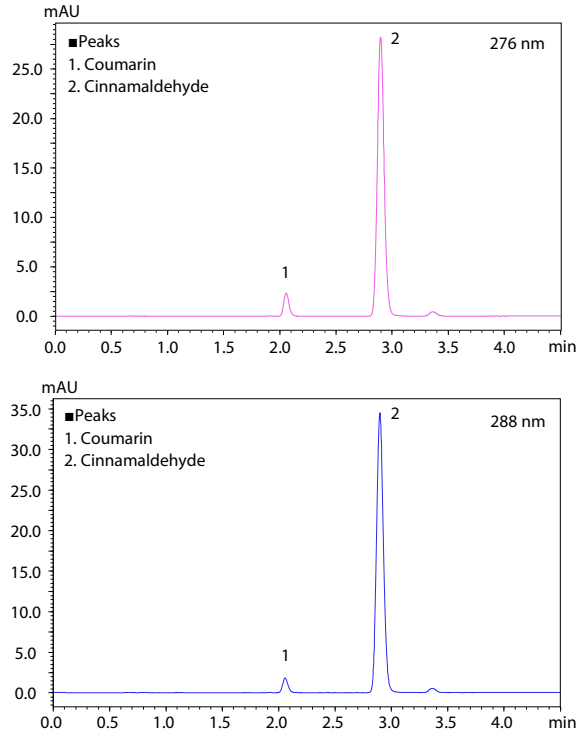


Fig. 6 Chromatograms of *Cinnamomum Cassia* Obtained by Automatic Dilution (Dilution Ratio: 100)

Table 4 Analytical Results (n = 3)

Compound	Concentration (mg/L)*6	
	Automatic	Manual
Coumarin	0.28 (0.72)	0.27 (0.38)
Cinnamaldehyde	1.90 (1.08)	2.02 (0.59)

*6 Numbers in parentheses indicate %RSD, n = 3.

■ Conclusion

By automatically preparing standard solutions for calibration curves at any dilution ratio and analyzing them directly, it was possible to create a calibration curve easily and accurately. In addition, when performing the determination of an actual sample, dilution could be performed automatically. It was confirmed that automatic determination was possible with only simple pretreatment. The calibration curve creation method described in this article is expected to lead to labor-saving for analysts and solvent-saving from a sustainability viewpoint.

Related Applications

1. Simultaneous Quantitative Analysis of Coumarin and Cinnamaldehyde in Cinnamon Produced in Different Regions
   Application News No. 01-00233
2. Simple Labor-Saving Calibration Curve Creation Using Autosampler Automatic Dilution Function
   Application News No. 01-00717

Contact Shimadzu for information about applying batch add-ins.

High Performance Liquid Chromatograph Nexera™ FV

Application News

On-Line Monitoring of Flow Synthesis Reactions Using Nexera FV

Ayano Tanabe, Katsumasa Sakamoto

User Benefits

- On-line monitoring of flow synthesis reactions can be performed.
- Simple operation with dedicated software allows automatic execution of analysis at specific sampling intervals and automatic report generation.
- Reliable experimental system setup can be established using HPLC as a part of flow synthesis system.

■ Introduction

In the pharmaceutical and fine chemical industries, there is an increasing demand to switch from conventional batch manufacturing for the sake of improvement of efficiency (labor and manpower savings), quality and safety, and reduction of environmental load.

ICH-Q13 guidelines for continuous production of drug substances and drug products recommend the application of Process Analytical Technology (PAT) for the purpose of maintaining controlled conditions for manufacturing. Consequently, the demand for PAT is increasing.

Nexera FV is an HPLC system setup for on-line analysis equipped with a flow through vial in the autosampler (Fig. 1). The system enables automated processes from continuous delivery of reaction solution into the flow through vial to resulting report creation of HPLC analysis.

This article introduces an on-line monitoring of flow synthesis reactions using Nexera FV, taking an example of the esterification of carboxylic acid.

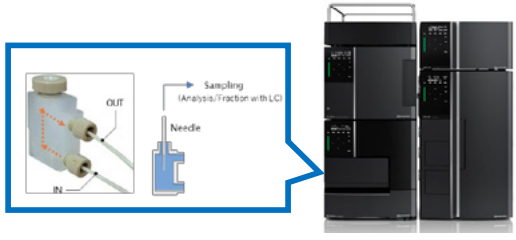


Fig. 1 Nexera FV (for flow synthesis) and flow through vial

■ Analytical conditions

An esterification reaction under acidic condition was investigated. Esterification by dehydration-condensation is a process in which a carboxylic acid reacts with an alcohol to form an ester, which is catalyzed by an acid. 3-phenylpropionic acid was used as the reactant of carboxylic acid and methanol as the alcohol, and AmberLyst™ 36WET, a strong acid catalyst, was packed in the reaction column and used for the reaction (Fig, 2).

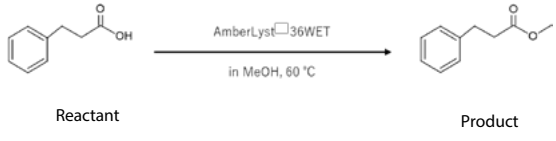


Fig. 2 Esterification reaction of phenylpropionic acid

In this study, on-line monitoring of the esterified reaction solution was performed by delivering 50 mmol/L phenylpropionic acid methanol solution through the reaction column (AmberLyst 36WET) heated to 60 °C at the flow rate of 0.1 mL/min (Fig. 3).

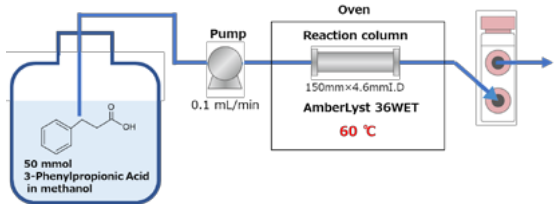


Fig. 3 Flow path diagram of flow synthesis monitoring

■ On-line monitoring with Nexera FV

Nexera FV introduces the reaction solution delivered from the flow synthesizer into the flow through vial for sampling. In this case, LC-40D pump and CTO-40C column oven of Shimadzu Nexera Series were used as the flow synthesizer and were connected to Nexera FV to introduce the reaction solution from the reaction column into the flow through vial (Fig. 4). The reaction solution was automatically injected from the flow through vial to HPLC according to the created analytical batch.

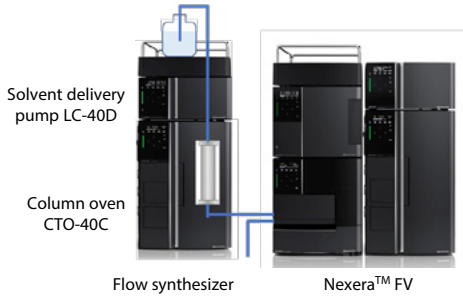


Fig. 4 System setup of flow synthesizer and Nexera FV

■ LabSolutions™ FV for easy creation of analytical batch

LabSolutions FV, a dedicated on-line monitoring software, allows users to easily perform complicated procedures such as creating an analytical batch and entering operational settings during reaction monitoring (Fig. 5). The analytical batch is automatically created by simply entering information such as HPLC conditions and sampling interval, allowing to start on-line HPLC analysis without any difficulties. It also supports input of external signals and start of sampling at a specified time, making it possible to link with other systems.

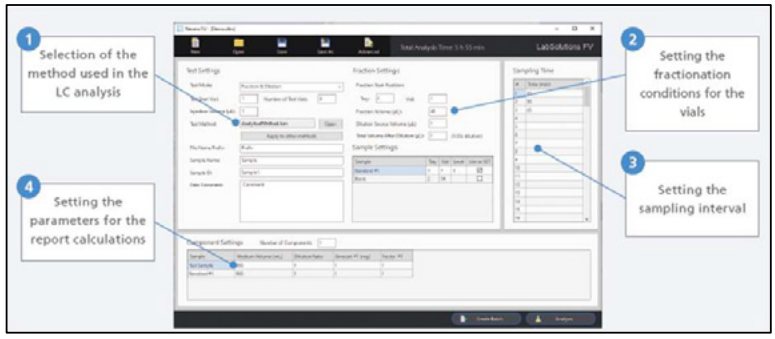


Fig. 5 Setting screen of LabSolution FV

■ Analysis of reaction solution

In this monitoring of the flow synthesis reaction, measurements were performed using the direct injection method, in which the reaction solution was injected directly from the flow through vial into the HPLC. Table 1 shows the HPLC analytical conditions, and Fig, 6(a) shows the chromatogram of the reaction solution (at 0 min) and Fig, 6(b) shows the chromatogram of the reaction solution (at 300 min). Phenylpropionic acid and product peaks were detected at 0.49 min and 1.15 min, respectively. Reaction monitoring was conducted from 20 to 960 minutes after the start of pumping, and it was confirmed that under these conditions, the esterified product was obtained constantly at 95~96% purity after 120 min.

Table1 HPLC conditions

Column	:	Shim-pack Velox™ C18 *1 (50 mm × 2.1 mm I.D., 1.8 µm)
Mobile phase A	:	0.5% Formic acid in Water
Mobile phase B	:	Acetonitrile
Time program (%B)	:	30% (0 min)→95% (0.45-1.40 min)→30% (1.41-3.5 min)
Flow rate	:	0.8 mL/min
Column temp.	:	40 ˚C
Injection vol.	:	1 µL
Detection	:	UV 254 nm

*1 P/N： 227-32001-02

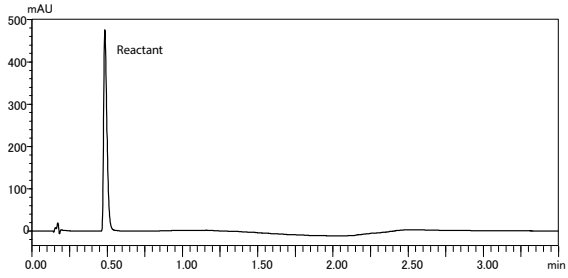


Fig. 6(a) Chromatogram of reaction solution (0 min)

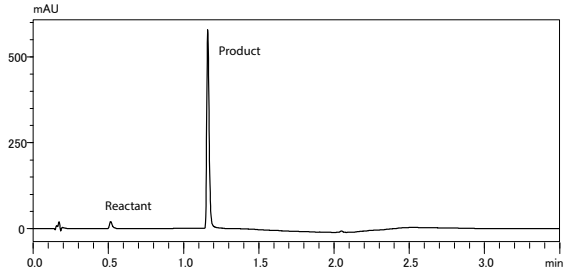


Fig. 6(b) Chromatogram of reaction solution (After 300 min)

■ Reaction product tracking using trend plot

A trend plot of the variations in the peak areas of phenylpropionic acid and the reaction product based on the reaction monitoring results is shown in Fig.7, created using LabSolutions' Multi-Data Report*2 function. Using the multi-data report function, a report (Fig. 8) can be automatically created after all analyses are complete, allowing visualized confirmation of the yield and the variation of intermediate in the synthesis process.

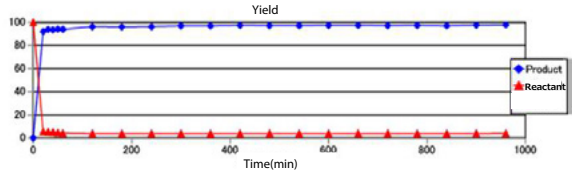


Fig. 7 Trend plot for phenylpropionic acid(reactant) and product

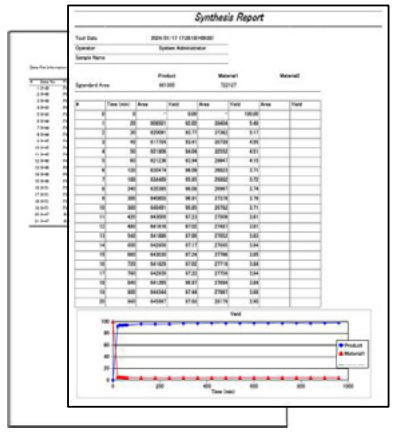


Fig. 8 Multi data report*2

*2 Multi data report is a function equipped in LabSolutions DB/CS

■ Conclusion

On-line monitoring of the esterification reaction of phenylpropionic acid by flow synthesis was performed using Nexera FV. Connecting the reaction system and the flow through vial, sampling and analysis was able to be performed automatically according to the analytical batch. In addition, the multi-data reporting function allowed reaction tracking on reactant and product by automatic generation of trend plot after analysis. Nexera FV and LabSolutions FV simplified analytical batch creation, automated sampling, and reporting. It results in a reduction of working time and increase of efficiency.

01-00779-EN First Edition: Sep. 2024

Application News

On-Line Dissolution Test of Loxoprofen Sodium Tablets

Ayano Tanabe, Kana Matsuoka

User Benefits

- On-line dissolution test can significantly reduce the man-hours required from test solution sampling to HPLC analysis.
- Fraction analysis mode provides sampling of test solution at minimum 5-minute intervals and is expected to save labor and prevent human error.
- Direct injection analysis mode doesn't require fractionation vials, resulting in cost reduction.

■ Introduction

Disintegration tests and dissolution tests are specified by the Japanese Pharmacopoeia (JP) and the U.S. Pharmacopeia (USP). Nexera FV is an on-line HPLC system for automated dissolution testing of drug products. By connecting to a dissolution tester, the system can provide tablet loading, analysis of the test solution at each sampling time, calculation of dissolution rate, and preparation of report output automatically. This system eliminates human error and realizes labor-saving and efficient dissolution testing.

This article reports the results of an on-line dissolution test of loxoprofen sodium tablets using Nexera FV.

■ On-line dissolution testing with Nexera FV

A sampling device for dissolution tester and a filtration device are required to build up an on-line dissolution testing system. Test solution sampled from the dissolution tester is filtered by the filtration device and introduced directly into a flow vial (see Fig. 2) contained in the autosampler of Nexera FV. Analysis is started by injecting the test solution into the HPLC flow path, following the aspiration of it in the flow vial.

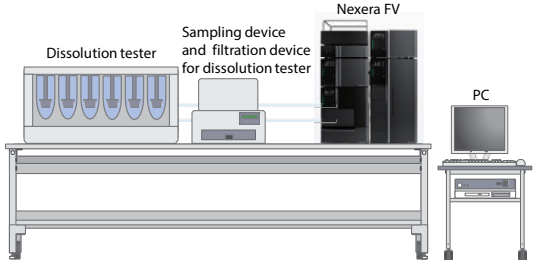


Fig.1 On-line dissolution test system setup using Nexera FV

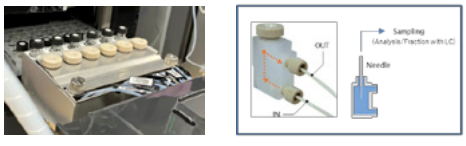


Fig.2 Flow vial

Nexera FV offers two different analysis modes: fraction analysis mode and direct injection analysis mode.

In fraction analysis mode, the test solution from the dissolution tester is once fractionated into vials or microtiter plate, and sampling intervals as short as 5 minutes can be supported. LC analyses can be performed collectively after the dissolution testing is completed or inserted between sampling intervals. An automatic dilution and an automatic addition of internal standard can be supported in this mode as well.

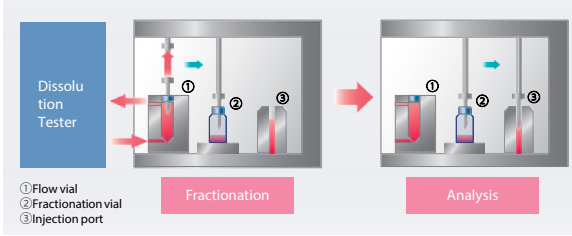


Fig.3 Fraction analysis mode

In direct injection analysis mode, the test solution from the dissolution tester is injected directly from a flow vial into the HPLC. After the test solution in first flow vial has been analyzed, the test solution in the second flow vial is subjected to HPLC analysis. This mode is useful when the sampling interval is large enough.

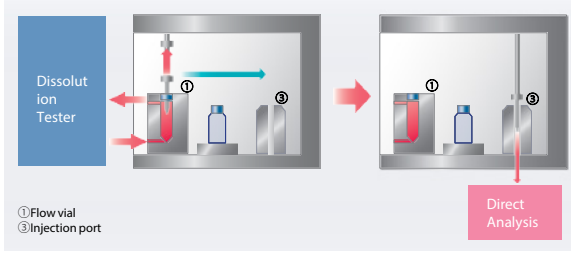


Fig.4 Direct injection analysis mode

■ On-line dissolution test of loxoprofen sodium tablets

On-line dissolution test of loxoprofen sodium tablets was performed. The HPLC conditions were optimized based on those described in the Japanese Pharmacopoeia 18th Edition as determination method for "Loxoprofen Sodium Tablets" to allow ultra high-speed analysis, which provides reductions of the retention time to 1 minute and the analysis time to 1.5 minutes. Table 1 shows the dissolution test conditions and Table 2 shows the HPLC analytical conditions.

Table 1 Dissolution test conditions

System	:	NTR-6600AST (TOYAMA SANGYO CO., LTD.)
Dissolution method	:	Paddle
Dissolution media		Water
Media volume	:	900 mL
Rotation speed	:	50 rpm
Bath temperature	:	37 ℃
Total time	:	30 min
Sampling time	:	5、10、15、20、25 and 30 min

Table 2 HPLC analytical conditions

Column	:	Shim-pack™ XR-ODS Ⅱ *1 (75 mm×3.0 mm I.D., 2.2 μm)
Mobile phase	:	Methanol/Water/Acetic Acid/Triethylamine =600 : 400 : 1 : 1
Flow rate	:	0.8 mL/min
Column temp.	:	40 ℃
Injection vol.	:	2 μL
Detection	:	UV 222 nm

*1 P/N：228-41624-91

●Analysis of standard solution

Using a 1.5 mL ordinary vial, six repeated analyses of a standard solution of loxoprofen sodium (60 mg/L) were performed. Fig. 5 shows the chromatograms and Table 3 shows the results. Good results of around 0.05% RSD for both retention time and peak area values were obtained.

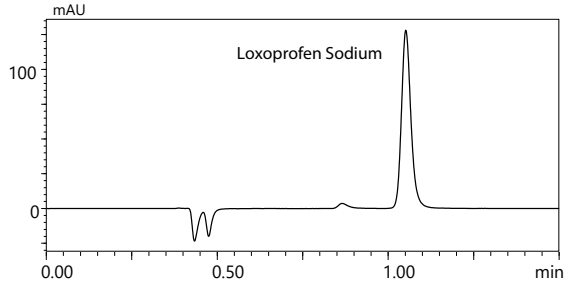


Fig.5 Chromatogram of standard solution of loxoprofen sodium (60 mg/L)

Table 3 Retention Time repeatability and peak area repeatability for a standard solution (60 mg/L、n=6)

	Retention time (min)	Area
1st	1.051	248,209
2nd	1.052	248,265
3rd	1.053	248,129
4th	1.052	248,232
5th	1.052	248,380
6th	1.051	248,038
Averages	1.052	248,209
%RSD	0.07	0.05

●Fraction analysis mode

The test solutions were sampled six times at 5, 10, 15, 20, 25, and 30 minutes. Fig. 6 shows the chromatogram of the test solution loxoprofen sodium tablet (68.1 mg per tablet indicated, dissolution time; 30 minutes).

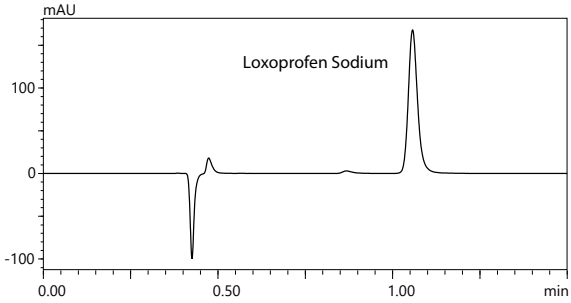


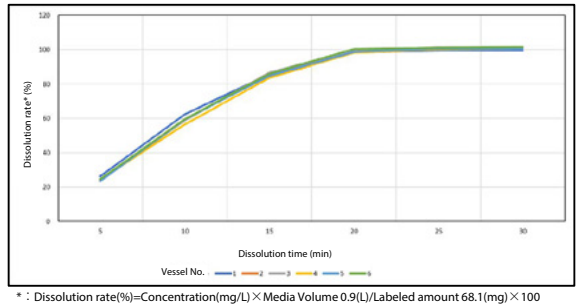
Fig.6 Chromatogram of test solution of loxoprofen sodium tablet (dissolution time; 30 min)

Table 4 shows the time-dependent variation of the dissolution rate in each vessel, and Fig. 7 shows the dissolution curve of loxoprofen sodium tablets.

The Japanese Pharmacopoeia, as ascribed, specifies that the dissolution rate of loxoprofen sodium tablets must be at least 85% in 30 minutes, and the results obtained from this study met this criterion.

Table 4 Time-dependent variation of dissolution rate in each vessel (%)

Time (min) / Vessel No.	5	10	15	20	25	30
1	26.28	62.34	85.67	98.45	99.53	99.49
2	23.88	59.71	84.98	99.07	101.08	100.89
3	23.90	58.78	86.68	99.80	101.27	101.18
4	24.33	56.44	83.55	98.18	100.25	100.64
5	23.54	59.62	84.81	98.77	100.54	100.61
6	24.77	59.54	85.97	100.41	101.15	101.59



*：Dissolution rate(%)=Concentration(mg/L)×Media Volume 0.9(L)/Labeled amount 68.1(mg)×100

Fig.7 Dissolution curves

●Direct injection analysis mode

Then, on-line dissolution test in direct injection analysis mode was performed. The test solutions from six vessels were sampled at 30 minutes only. Table 5 shows the dissolution rate in each vessel. In direct injection analysis mode, each dissolution rate 30 minutes was over 85%, which met the criterion as well.

Table 5 Dissolution rate (%) in each vessel

Time(min) / Vessel No.	30
1	103.49
2	101.67
3	100.40
4	100.29
5	102.42
6	102.32

■ Conclusion

In this article, an on-line dissolution test of loxoprofen sodium tablets using Nexera FV has been reported. The fraction analysis mode allows 5-minute sampling interval. The direct injection analysis mode doesn't require preparing vials for fractionation resulting in cost reduction. Thus, Nexera FV is an on-line HPLC system setup that can provide automated and efficient dissolution testing for drug products.

Application News

Software for Efficient Method Development
Preparative Purification Liquid Chromatograph

Seamless Purification Workflow from Analytical to Preparative Using a Single Quad LC-MS System

Shinichi Fujisaki, Yusuke Masuda

User Benefits

- Scaling-up from analytical column to preparative column and purity/recovery check can be completed in a single system.
- LabSolutions™ MD efficiency optimizes separation conditions by automatically creating analysis schedules.
- Single quadrupole mass spectrometer LCMS-2050 provides *m/z* information for target compounds.

■ Introduction

Preparative LC is utilized in various fields, such as pharmaceuticals, food, and chemical engineering, for purifying target compounds from mixed samples, searching for active ingredients in natural products, and analyzing the structures of impurities and unknown compounds. To achieve high purity and recovery rate for fractionation of target compounds, it is crucial to establish analytical conditions that separate these compounds from other co-existing components. Due to the significant sample and mobile phase consumption associated with preparative LC conditions, the optimization of separation conditions is typically performed on an analytical scale to minimize these consumptions. During this optimization, various HPLC conditions, including gradient profiles, are adjusted to find the optimal separation. This is a time-consuming process for creating each analysis schedule. Also, confirmation of purity and recovery rate after scaling-up is followed by transferring fractions from fraction tubes to an autosampler manually. This article presents an efficient preparative purification workflow (Fig. 1), which includes investigating separation conditions at analytical scale, scaling-up for fractionation, and confirming purity and recovery rate. All of these processes are carried out using single analytical/preparative convertible LC-MS system of Nexera™ Prep.

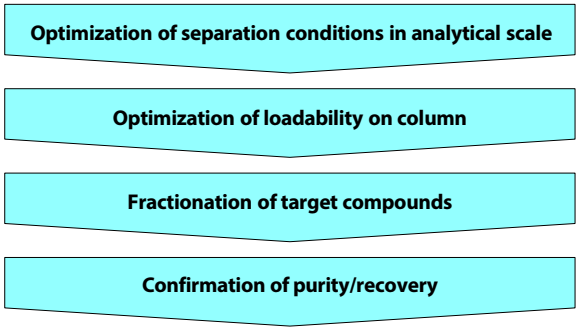


Fig. 1 Workflow of Preparative Purification

■ Overview of LC-MS System

The flow path diagram of analytical/preparative convertible LC-MS system is shown in Fig. 2. The analytical flow path (the upper of Fig. 2) is used to optimize separation conditions, loadability on column, and purity/recovery check, while the preparative flow path (the lower of Fig. 2) is used for the preparative separation of target compounds. The liquid handler (LH-40), which has both analytical and preparative flow paths and can inject fractions from fraction tubes directly into the analytical flow path, allows a complete workflow of preparative purification with this system. In addition, LCMS-2050 provides not only mass information of target compounds when optimizing separation conditions, but also a combination trigger of UV and MS signals during fractionation. Therefore, target compounds can be recovered with high purity. To do so, this system is configured to split the preparative flow path and introduce a portion of the mobile phase into MS with a make-up solvent to achieve both fractionation and MS detection.

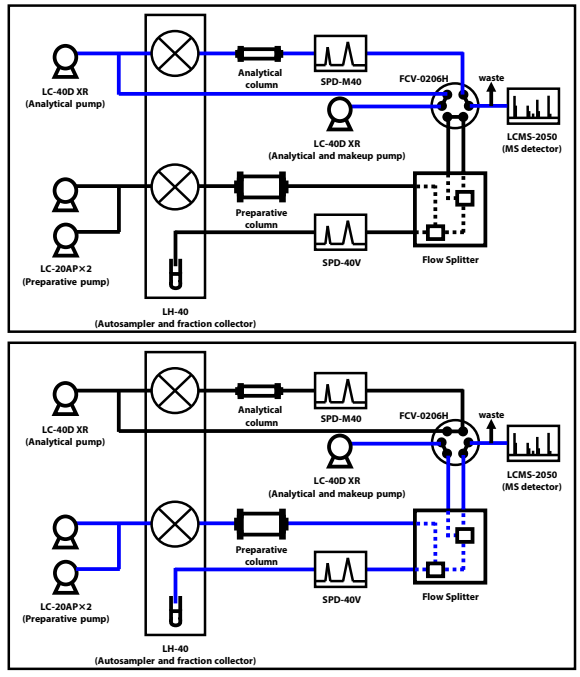


Fig. 2 Analytical Flow Path (Upper), Preparative Flow Path (Lower)
*blue colored flow path is in operation

The following section describes an example of using this analytical/preparative convertible LC-MS system to provide an efficient preparative purification workflow with a mixture of seven pharmaceutical standards (target compound for fractionation : Hydrocortisone) as model compounds.

■ Optimization of Separation Conditions in Analytical Scale

The separation conditions of Hydrocortisone are optimized in analytical scale. LC chromatogram (analytical conditions : Table 1) before optimization is shown in Fig. 3, in which Salicylic acid is eluted very close to Hydrocortisone. Increasing loadability under these conditions may cause further deterioration of the separation of these two compounds and the reduction of the purity at the recovery. Consequently, improvement of the separation is essential. The separation is optimized by varying the gradient conditions in nine different profiles (initial concentration and gradient slope in three different levels each). LabSolutions MD, a dedicated software for supporting method development, was used for automatic analysis schedule generation to improve the efficiency of the optimization (Fig. 4). The obtained chromatograms are shown in Fig. 5. The separation of Hydrocortisone and Salicylic acid was optimized at an initial concentration of 15% and a gradient slope of 20 minutes ((3) in Fig. 5). Then, the next optimization of loadability was conducted based on these conditions. During optimizing separation conditions, mass information (Hydrocortisone : *m/z* 363.3) was simultaneously obtained by LCMS-2050.

Table 1 Analytical Conditions

Mobile Phase	: Pump A : 0.1% formic acid in water
	: Pump B : Acetonitrile
Column	: Shim-pack Scepter™ C18-120
	(150 mm × 4.6 mm I.D., 5 µm)*1
Sample	: (A) Hydrocortisone, (B) Salicylic acid,
	(C) Metoclopramide, (D) Lidocaine,
	(E) Furosemide, (F) Papaverine, (G) Quinidine
Sample Concentration	: 100 mg/L (Hydrocortisone), 10 mg/L (others)
Injection Volume	: 10 µL
LC Conditions	
Time Program	: B Conc. 25%(0 min)→45%(20 min)
	→25%(20.01-25 min)
Column Temp.	: Ambient
Flow rate	: 1 mL/min
Sample loop size	: 500 µL
Syringe size : 500 µL	
Detection (PDA)	: 245 nm (SPD-M40, conventional cell)
MS conditions	
Ionization	: ESI/APCI (DUIS™), positive and negative
Mode	: SCAN (*m/z* 100-500)
Nebulizing Gas Flow	: 2.0 L/min (N2)
Drying Gas Flow	: 5.0 L/min (N2)
Heating Gas Flow	: 7.0 L/min (N2)
DL Temp.	: 200 ℃
Desolvation Temp.	: 450 ℃
Interface Voltage	: 3.0/-2.0 kV (positive/negative)

*1 P/N : 227-31020-05

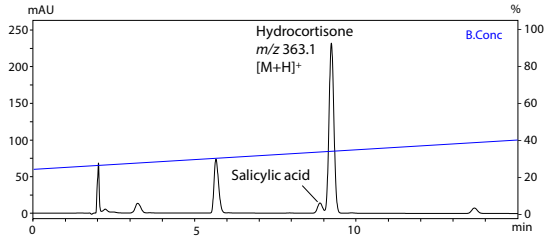


Fig. 3 HPLC Chromatogram before Optimizing the separation

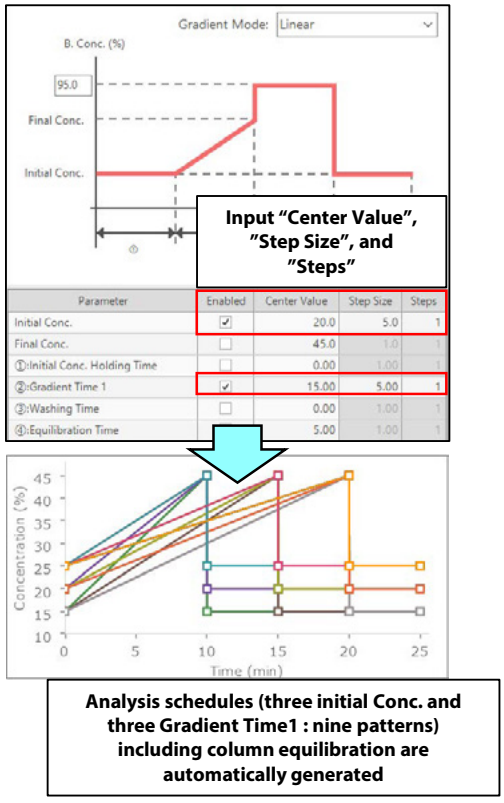


Fig. 4 Automatic Generation of Analysis Schedules by LabSolutions MD

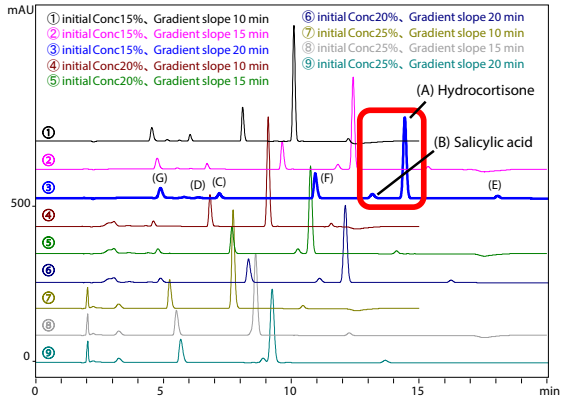


Fig. 5 Result of Optimizing Separation Conditions with LabSolutions MD
*blue colored chromatogram ③ shows the best separation of Hydrocortisone and Salicylic acid

■ Optimization of Loadability on Column

Fig. 6 shows the result of optimization of loadability conducted at injection volumes of 10, 20, 30, 40, 50, and 100 µL using Hydrocortisone (10,000 mg/L) under the optimized conditions at the analytical scale (③ in Fig. 5). At an injection volume of 100 µL, the separation of Hydrocortisone and Salicylic acid was not sufficient (inside the blue oval in Fig. 6). In addition, a small peak (circled in red in Fig. 6), which seemed to be an impurity, was not clearly separated at the base of the peak. Up to an injection volume of 50 µL, Hydrocortisone is well separated from the neighboring peaks. Consequently, scaling-up was implemented using 50 µL injection volume.

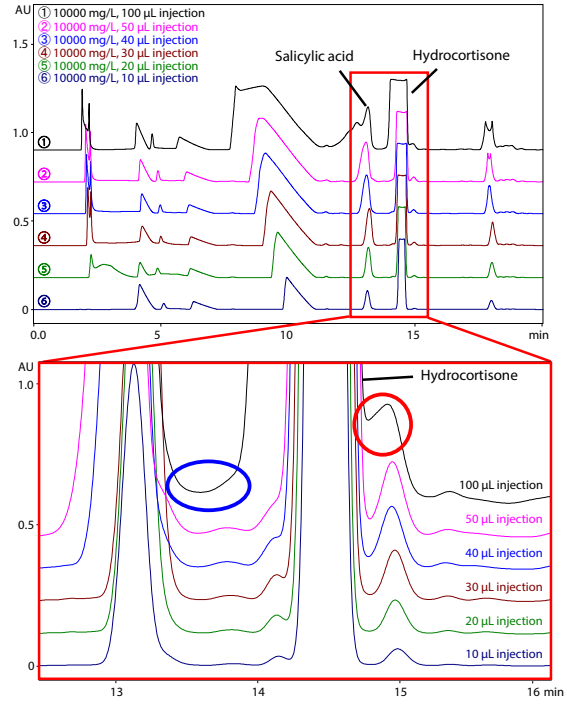


Fig. 6 Result of Optimization of Sample Loadabily

■ Fractionation of Target Compounds

Hydrocortisone was fractionated using a UV trigger. The preparative conditions are presented in Table 2 (only the settings differing from those in Table 1 are listed), and the resulting preparative HPLC chromatogram is shown in Fig. 7 (the blue area represents the fractionation interval). Based on the cross-sectional area ratio (approximately 20-fold) between the preparative column (20 mm I.D.) and the analytical column (4.6 mm I.D.), the flow rate was increased to 20 mL/min (with a constant linear velocity before and after scaling-up), and the injection volume was set at 1 mL. As a result, similar separation profiles were achieved before and after scaling-up, allowing for effective fractionation of Hydrocortisone while maintaining adequate separation from Salicylic acid."

Table 2 Preparative Conditions

Column	: Shim-pack Scepter™ C18-120 (150 mm × 20 mm I.D., 5 µm)*1
Sample Concentration	: 10000 mg/L (Hydrocortisone), 1000 mg/L (others)
Injection Volume	: 1 mL
LC Conditions	
Flow rate (Prep)	: 20 mL/min
Flow rate (Makeup)	: 1.5 mL/min (Methanol)
Sample loop size	: 2 mL
Syringe size : 5 mL	
Detection (UV)	: 245 nm (SPD-40V, preparative cell)
MS conditions	
Desolvation Temp.	: 100 ℃

*1 P/N : 227-31102-03

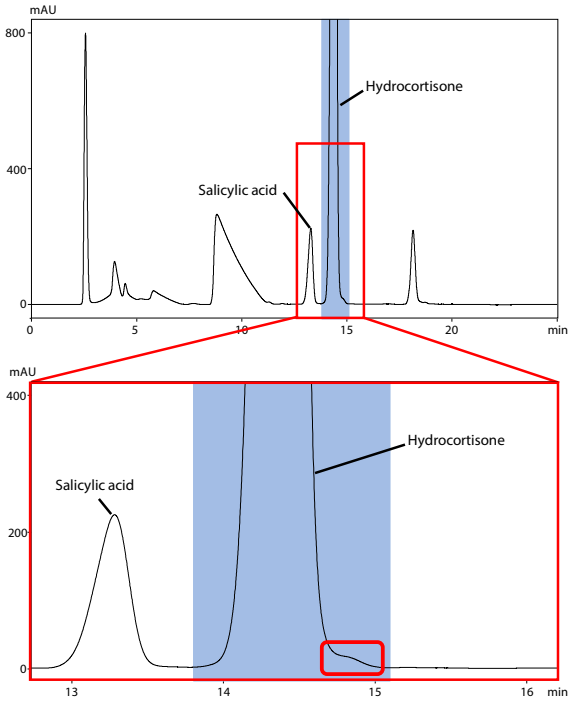


Fig. 7 UV triggered Preparative Chromatogram
*fractionation interval is colored blue

■ Confirmation of Purity/Recovery

Fig. 8 shows the chromatogram obtained when a fractionated Hydrocortisone was re-injected into analytical flow path, as well as the chromatogram of a standard mixture (as a reference for calculating recovery) prepared to be the same concentration as fractionated Hydrocortisone. The purity and recovery rate of Hydrocortisone are shown in Table 3. The purity was more than 99% using area normalization, and the recovery rate was also excellent.

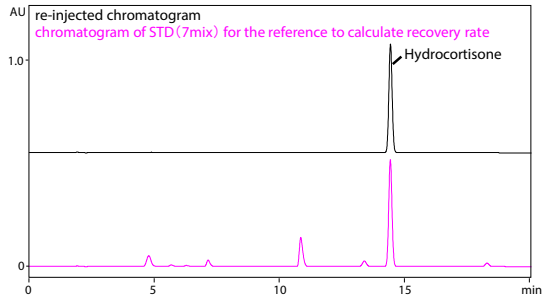


Fig. 8 Re-injected Chromatogram (Upper)
Chromatogram of STD (Lower)

Table 3 Purity and Recovery Rate of Fractionated Hydrocortisone (n=3, average value)

	Purity (Area %)	Recovery Rate (%)
Hydrocortisone	99.7	101.2

In the lower part of Fig. 7, an enlarged image of the interval where Hydrocortisone was eluted is presented. A very small peak, likely an impurity, is observed at the base of the Hydrocortisone (inside the red frame). The fractionation results demonstrate a purity of 99.7% (Table 3) with UV trigger only. However, the preparative LC-MS system also supports MS triggered fractionation, enabling the separation of target compounds with even higher purity by avoiding impurities, thanks to the high identification performance of the MS. More information on the MS triggered preparative LC-MS system can be found in Application News "01-00651-EN".

■ Conclusion

The analytical/preparative convertible LC-MS system is employed to efficiently complete the preparative purification workflow which consists of the optimization of separation conditions at an analytical scale, scaling-up for fractionation, and confirmation of purity and recovery rate. LabSolutions MD, equipped to automatically generate analysis schedules with various HPLC parameters, facilitates the efficient optimization of separation conditions. Also, LCMS-2050 provides mass information simultaneously. Moreover, the liquid handler LH-40 allows for the direct injection of obtained fractions into the analytical flow path, ensuring seamless confirmation of purity and recovery rate without transferring fractions from a fraction tubes to an autosampler manually.

01-00650-EN First Edition: Nov. 2023

index

Automated scale-up system for reversed phase purification - Nexera™ ASAPrep™

Application News

Streamlining of Preparative Purification Work by Nexera ASAPrep

Rintaro Matsuda, Yusuke Masuda, Hidetoshi Terada

User Benefits

- Automatic generation of optimal preparative conditions based on a unique scale-up algorithm streamlines preparative purification operations.
- Intuitive UI design and automatic conditioning function allow easy preparative purification, regardless of operator's experience.
- Automatic determination of difficulty level of sample preparation supports assignment of preparative purification work.

■ Introduction

Since impurities as well as main compound are usually generated during compound synthesis, purification is essential to improve the precision of subsequent processes. Preparative purification liquid chromatography (preparative LC) is often used for purification work, but there is a need to improve the efficiency of this process since this work requires expert users and a considerable amount of time.

Nexera ASAPrep (Automated Scale-up from Analytical to Preparative), an automated scale-up HPLC system for reversed-phase purification, is equipped with a function that automatically determines the difficulty level of sample preparation and automatically generates optimal preparative conditions. Furthermore, the intuitive UI design and automatic conditioning function allow anyone to easily perform preparative purification, regardless of operator experience. Through these features, this system can support the assignment of preparative purification tasks based on the difficulty level of preparation, thereby helping to improve the efficiency of preparative purification process.

This article introduces a preparative purification workflow with Nexera ASAPrep, using three mixtures containing the main compounds and related impurities.

■ Preparative purification workflow using HPLC

In preparative purification using HPLC, ideally, the chromatographic separation is evaluated under generic conditions, followed by optimization of the separation and investigation of the maximum loading amounts on analytical scale before confirming reproducibility and adjusting the loading amounts on preparative scale (Fig. 1 green arrow). However, the process of optimizing the separation especially requires know-how and a lot of work hours on preparative HPLC. Therefore, if the above-mentioned workflow is applied to all synthetic compounds that require purification, the overall throughput of the preparative laboratory will be drastically decreased, including also additional work for HPLC expert users.

In preparative purification using HPLC, a shortcut workflow may be taken for the purpose of improving throughput. After analyses under prefixed initial conditions, preparative HPLC conditions are set based on the retention time of the target compound on analytical scale and preparative purification is directly executed (Fig. 1, red arrows). While this flow can be expected to increase throughput because it skips optimizing the separation, it is unavoidable that the risk that the target compound and impurities may be co-eluted during preparative purification. In addition, it is difficult to eliminate human factors from preparative purification process, since the selection of the workflow requires the judgment by preparative HPLC expert to reduce the risk.

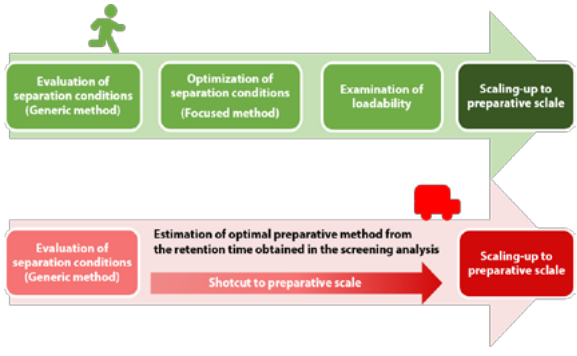


Fig. 1 Ideal preparative purification workflow (green arrow) and shortcut workflow (red arrow)

■ Streamlining of preparative purification workflow using HPLC

Fig. 2 shows a schematic diagram of the automatic preparative scale-up function in Nexera ASAPrep. This system automatically determines the difficulty level of preparative purification based on the results of sample analyses (screening analyses) using two sets of prefixed analytical conditions (analysis time: 2 min) employing acidic and basic mobile phases respectively.

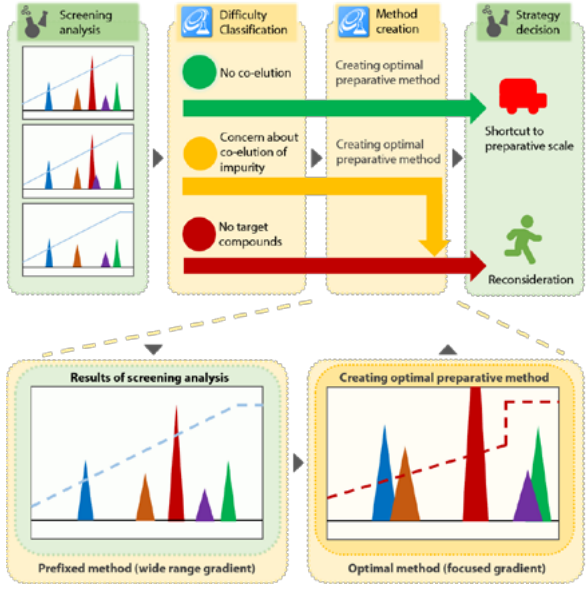


Fig. 2 Autoscaling-up feature of Nexera ASAPrep.

Specifically, there are three levels: when sufficient peak separation of the target compound has been achieved in the screening analyses (green judgment), when there is concern about the presence of impurities during preparative run because sufficient peak separation of the target compound is not achieved in the screening analyses (yellow judgement), and when the target compound is not detected in the screening analyses (red judgement). In the case of green and yellow judgements, the system automatically creates preparative HPLC conditions that optimize the separation near the target peak elution interval based on the results of the screening analyses. Through these functions, the operator only needs to perform screening analyses using the two sets of prefixed conditions provided by the system, then automatic determination of the difficulty level for the preparative separation of the sample and automatic creation of preparative HPLC conditions are performed. For a sample with a green judgment (low difficulty), purification can be performed by shortcutting the process of evaluation of prep conditions on analytical scale without any examination from expert users. For the sample with a red judgement (high difficulty), purification is performed by the preparative HPLC expert from optimizing separation on analytical scale. This allows for easy streamlining of the purification workflow. For a sample with a yellow judgement (difficulty: medium), the automatically generated preparative HPLC conditions can be used as base conditions for further optimization, thus a reduction of workload can be expected.

■ Analytical conditions and target compounds

The screening analytical conditions and target compounds are listed in Table 1 and Table 2, respectively. In this article, screening analyses were performed on a mixed sample containing the main compound and several related substances using the two sets of prefixed analytical conditions, acidic and basic conditions.

Table 1 Screenig analytical conditions

System	: Nexera ASAPrep_analytical system
Column	: Shim-pack Scepter^{TM} C18-120 (50 mm × 3.0 mm I.D., 3 µm[*1])
Sample	: Please see table 2.
Sample Concentration	: Main : 10 mg/L
Mobile Phase (acidic condition)	: Pump A : 0.05 % formic acid in water : Pump B : 0.05 % formic acid in acetonitrile
Mobile Phase (basic condition)	: Pump A : 10 mmol/L ammonium hydrogen carbonate in water/acetonitrile = 90/10 : Pump B : 10 mmol/L ammonium hydrogen carbonate in water/acetonitrile = 10/90
Injection volume	: 2 µL
LC Conditions	
Time program	: B.Conc 5 % (0 min)→5-90 % (0-1 min)→ 90 % (1-2 min)
Column Temp.	: 40 ˚C
Flow rate	: 1.5 mL/min
Detection (PDA)	: 190 - 800 nm (SPD-M40, semi-micro cell[*2])
MS Conditions	
Ionization	: ESI/APCI (DUIS^{TM}), positive and negative
Mode	: SCAN (*m/z* 150-1000)
Nebulizing Gas Flow	: 2.0 L/min (N_2)
Drying Gas Flow	: 5.0 L/min (N_2)
Heating Gas Flow	: 7.0 L/min (N_2)
DL Temp.	: 200 ˚C
Desolvation Temp.	: 450 ˚C
Interface Voltage	: 3.0/-2.0 kV (positive/negative)

*1 P/N : 227-31015-01
*2 P/N : 228-64725-41

Table 2 Main compounds of each mixture

	Mixture A	Mixture B	Mixture C
Main	Vanillin	Indomethacin	Bifonazole

■ Screening analysis

In the screening analyses, after setting the mobile phases and the three mixed samples to HPLC system for screening analyses, the four items of "sample location," "sample name," "injection volume," and "target mass" were set according to the procedure shown in Fig. 3, and the check mark icon was clicked.

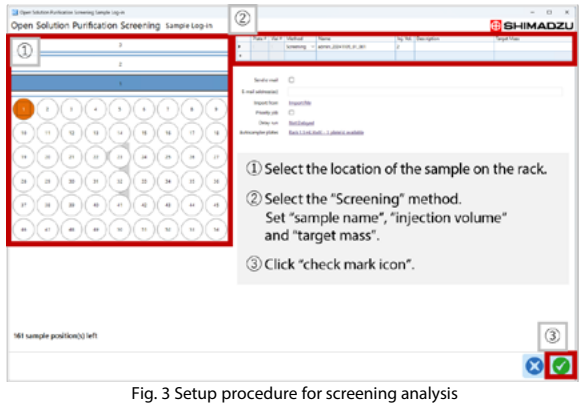


Fig. 3 Setup procedure for screening analysis

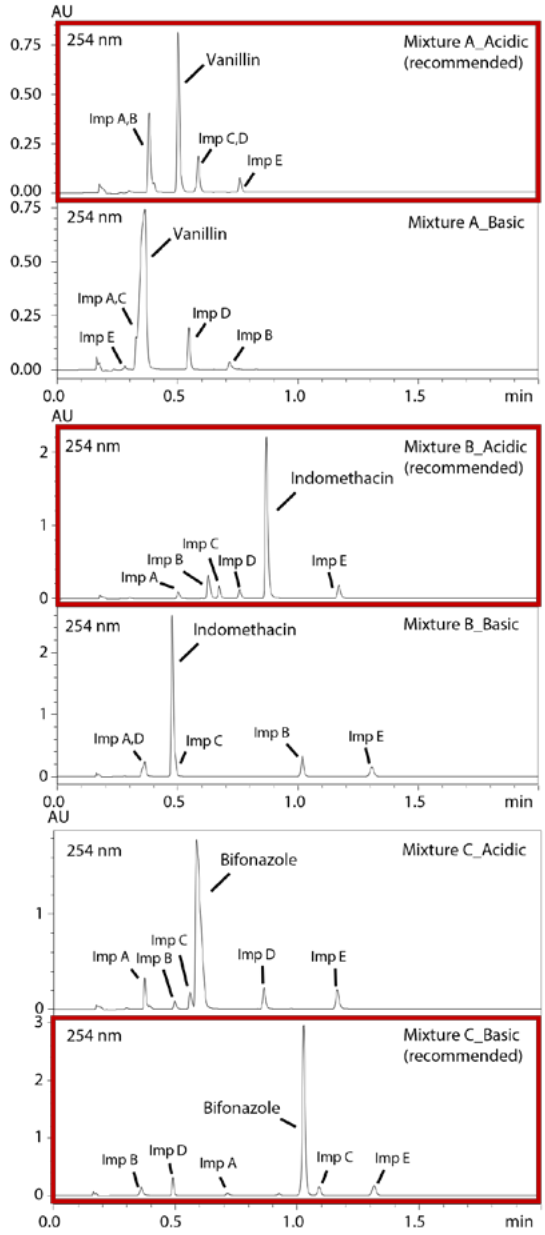


Fig. 4 LC screening chromatograms of each mixed sample
Mixture A (top), Mixture B (middle), Mixture C (bottom)

Sample Name	Descriptio	Mass Found	Masses	Judge	NH4HCO3 RT	NH4HCO3 B.Conc	NH4HCO3 Screening Reason	HCOOH RT	HCOOH B.Conc	HCOOH Screening Reason
C5_Vanillin	sub : C8...	152.1	152.1	HCOOH	0.36	2.4	Below B.Conc threshold	0.5	15.8	Passed
B4_Indomethacin	sub : C4...	357.8	357.8	HCOOH	0.48	12.4	Preferred mobile phase is HCOOH	0.87	47.5	Passed
B16_Bifonazole	sub : C...	310.4	310.4	NH4HC...	1.03	58.2	Passed	0.59	20	Preferred mobile phase is NH4HCO3

Fig. 5 Determination of purification difficulty level based on the screening analyses

By simply setting the above four items and clicking the icon, analysis order is automatically adjusted to minimize the number of conditions witching, and mobile phase replacement and column stabilization are automatically performed when the conditions are switched (automatic conditioning function) as well as consecutive analyses under the two different acidic and basic mobile phase conditions. This makes it easy for the HPLC operators to perform screening analyses.

The HPLC chromatograms obtained from the screening analyses and the results of the determination of preparative difficulty level are shown in Fig. 4 and Fig. 5, respectively. For each sample, the results of the determination of preparative difficulty level and the initial "B. Conc" in the preparative run were obtained by performing the analysis under two different acidic and basic mobile phase conditions in 2 min analysis time. In addition to above-mentioned green, yellow, and red judgments, it is indicated that which mobile phase condition of acidic or basic, should be tried at first, and the reason for the judgment, such as lack of spectral purity of MS peaks. Therefore, even if the result of difficulty determination is only yellow or red, and a further investigation is required, HPLC operator can smoothly proceed with necessary work based on the reasons indicated in the determination results. Determination results and initial "B.Conc" are automatically output as an Excel file.

Table 3 Preparative conditions

System	: Nexera ASAPrep Preparative System
Column	: Shim-pack Scepter C18-120 (150 mm × 20 mm I.D., 5 µm[*3])
Sample	: Please see table 2.
Sample Concentration	: Main : 10 mg/L
Mobile Phase (acidic condition)	: Pump A : 0.1 % formic acid in water : Pump B : 0.1 % formic acid in acetonitrile
Mobile Phase (basic condition)	: Pump A : 10 mmol/L ammonium hydrogen carbonate in water/acetonitrile = 90/10 : Pump B : 10 mmol/L ammonium hydrogen carbonate in water/acetonitrile = 10/90
Injection volume	: 500 µL
LC Conditions	
Time program	: B.Conc x[*4] % (0 min)→x-x+20 % (0-9 min)→100 % (9-12 min)
Column Temp.	: ambient
Flow rate	: 20 mL/min
Detection (PDA)	: 190 - 800 nm (SPD-M40, prep cell[*5])
MS Conditions	
MS makeup	: Methanol
Flow rate (makeup)	: 1.5 mL/min
Desolvation Temp.	: 100 ˚C
Interface Voltage	: 3.0/-2.0 kV (positive/negative)

*3 P/N : 227-31102-03
*4 x : Initial B.Conc. of focused gradient
*5 P/N : 228-64727-41

■ Execution of preparative purification

Since the results of the screening analyses indicated that all the mixtures could be applied to preparative purification, and acidic conditions were recommended for the mixture containing vanillin and indomethacin as major compounds, and basic conditions for the mixture containing bifonazole as a major compound, preparative purifications were carried out for all the mixtures. Under respective recommended conditions. Table 3 shows the preparative HPLC conditions. After setting the mobile phase and the three mixed samples in preparative HPLC system, the Excel file containing the results of the determination of preparative difficulty level output at the screening analyses was imported according to the procedure shown in Fig. 6, and the check mark icon was clicked. The mobile phase replacement and column stabilization are automatically performed when mobile phase changeover is required. This allows HPLC operator to perform preparative isolation easily and efficiently.

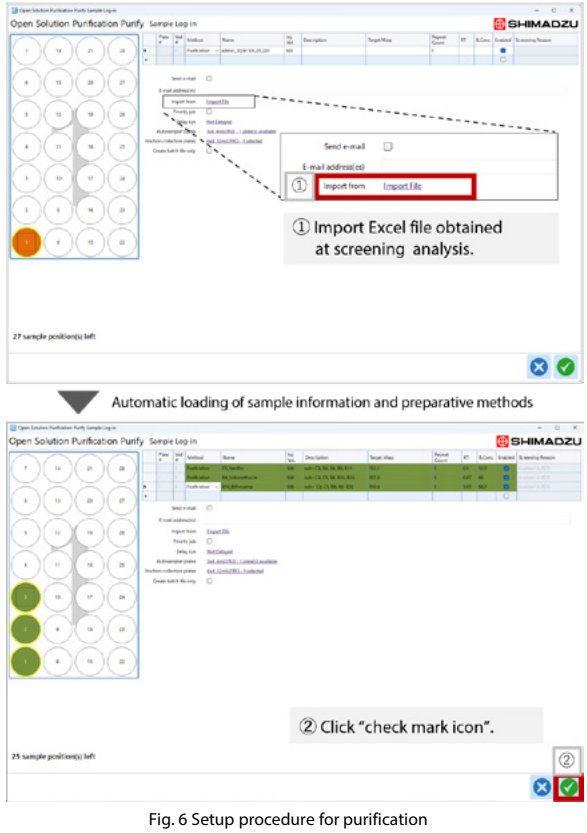


Fig. 6 Setup procedure for purification

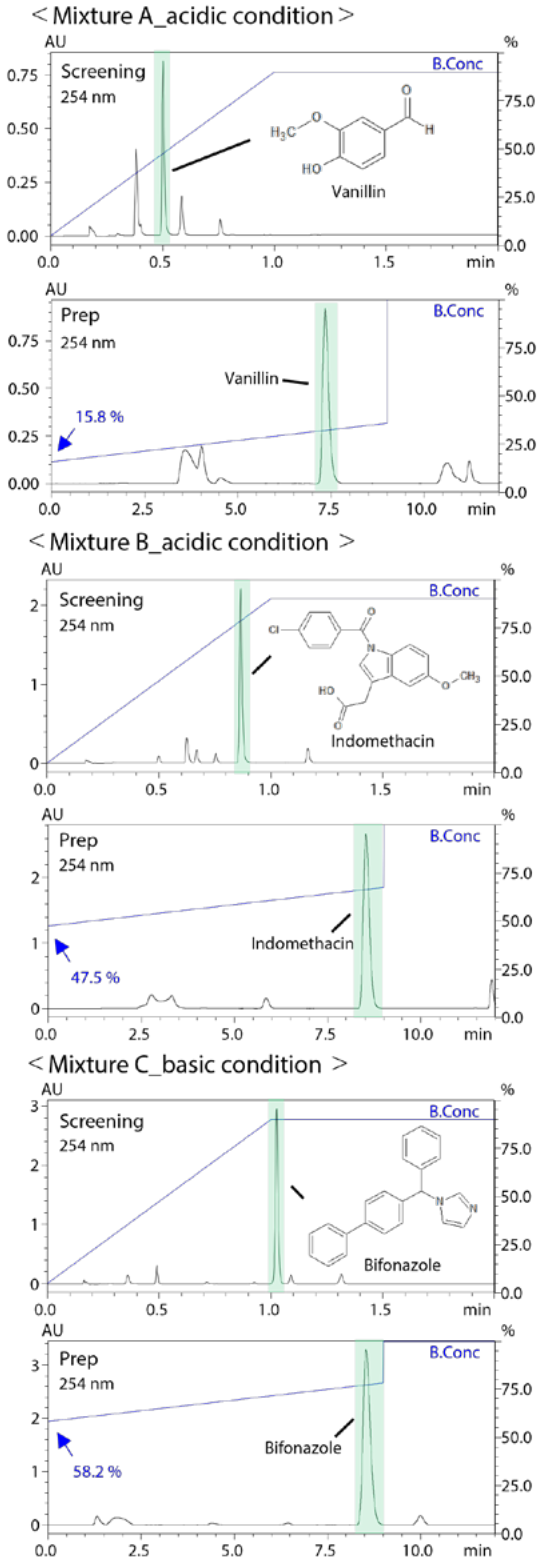


Fig. 7 Comparison of LC screening chromatogram and preparative chromatogram. Mixture A (top), Mixture B (middle), Mixture C (bottom)

Fig. 7 shows the comparison between chromatograms in screening analyses and those in preparative runs of the three samples. The samples with the same concentrations as in the screening analyses were injected to preparative HPLC in 250-fold increased volume, and peaks of the main compounds were separated from the related substances better than those in the screening analyses. Acidic mobile phase condition was recommended for the acidic compounds Vanillin and Indomethacin, and basic mobile phase condition for the basic compound Bifonazole, suggesting that acidic (basic) mobile phase condition would be suitable for the preparative analysis of acidic (basic) compound.

■ Conclusion

A preparative purification workflow using Nexera ASAPrep was introduced. The intuitive UI design, automatic conditioning function, and automatic creation of preparative HPLC conditions provide easy execution of screening analyses and preparative purification, regardless of the experience of HPLC operator. In addition, this system automatically determines the preparative difficulty level of samples based on the screening results, and indicates the risk of co-elution of main compound and related substances on preparative scale as first answer. Through these functions, the system can support the assignment of preparative purification samples according to the preparative difficulty level, thereby helping to improve the efficiency of preparative purification workflow.

01-00829-EN First Edition: Dec. 2024

index

MEMO

Shimadzu Corporation
www.shimadzu.com/an/