Guide to Biopharmaceutical Solutions
—From Cell Line Optimization to Pharmacokinetics—
Quantitation of Nucleic Acids

Quantitation of Double-Stranded DNA
- Trace Measurement Using TrayCell and Nano Stick -

Cell Line Optimization

Operating Principle and Features
The UV-1900i UV/VIS spectrophotometer features a space-saving and ergonomic hardware design. The user interface (UI) is displayed on a color touch panel to ensure the system status and operating procedures can be determined easily with a single glance. The Biomethod mode includes six types of built-in measurement conditions: 1. Nucleic acid quantitation, 2. Lowry method, 3. BCA method, 4. CBB (Bradford method), 5. BCA method, and 6. UV method. These methods can be used to measure samples easily for given analytical objectives. The operation panel screenshot function can be used to easily extract measurement results without connecting to a computer. A 10 mm square cell requires a sample volume of approx. 4 mL, but the use of a TrayCell or Nano Stick cell enables measurement of micro sample quantities of approx. 2 to 4 μL.

Measurement Method
Double-Stranded DNA Measurement Method Using a TrayCell
Double-stranded DNA was prepared to create 27.5, 55, 110, 220, and 440 ng/μL standard samples (diluted with ultrapure water). Actual samples were prepared by ethanol precipitation of the same DNA. With the TrayCell, the optical path length can be changed to either 1.0 mm or 0.2 mm by switching between two types of caps. In this example, a cap with a 1.0 mm optical path length was used to measure 4 μL of dripped sample based on the conditions listed in Table 1 (Fig. 1).

Double-Stranded DNA Measurement Method Using a Nano Stick Accessory
Standard samples and actual samples of double-stranded DNA were prepared using the same method as described for the TrayCell above. The same measurement conditions were also used, as listed in Table 1. 3 μL sample volumes were measured using the 0.5 mm optical path length of the Nano Stick (Fig. 2).

Results
Calibration curves and UV spectral results from measurements using the TrayCell and Nano Stick are shown in Fig. 3 and Fig. 4. Both resulted in calibration curves with high linearity and good measurement accuracy, confirmed by correlation and CV values calculated from 10 repeated measurements of a 440 ng/μL sample.

Conclusion
The TrayCell and Nano Stick accessories were used with a UV-1900i UV/VIS spectrophotometer to confirm that micro sample quantities on the order of several microliters can be measured accurately and easily.

Application Examples
- Evaluating DNA purity based on absorbance ratio
- Measuring DNA concentration
- Measuring protein concentration

Table 1   Measurement Conditions

<table>
<thead>
<tr>
<th>Sample Volume</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 μL</td>
<td>260, 320</td>
</tr>
<tr>
<td>3 μL</td>
<td>260, 320</td>
</tr>
</tbody>
</table>

UV-1900i

Benefits
- Spectra can be acquired at ultra-fast scan speeds up to 29,000 nm/min.
- Sample volume of as low as 0.7 μL can be measured using a TrayCell or Nano Stick cell.
- Nucleic acid concentration can be easily determined using the built-in Biomethod mode.

Specifications

<table>
<thead>
<tr>
<th>Instrument</th>
<th>UV-1900i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample volume</td>
<td>10 mm standard cell = 2.5 to 4.0 mL</td>
</tr>
<tr>
<td>TrayCell = 0.7 to 10 μL, Nano Stick = 2 μL.</td>
<td></td>
</tr>
<tr>
<td>Wavelength range</td>
<td>190 to 1,100 nm</td>
</tr>
<tr>
<td>Spectral bandwidth</td>
<td>1 nm</td>
</tr>
<tr>
<td>Light source</td>
<td>20 W halogen lamp and deuterium lamp</td>
</tr>
<tr>
<td>Monochromator</td>
<td>LO-RAY-LIGHT grade blazed holographic grating in Czerny-Turner mounting</td>
</tr>
<tr>
<td>Detector</td>
<td>Silicon photodiode</td>
</tr>
<tr>
<td>Sample compartment</td>
<td>Internal dimensions: W 110 x D 250 x H 115 mm</td>
</tr>
<tr>
<td>Distance between light beams</td>
<td>100 mm</td>
</tr>
<tr>
<td>Dimensions</td>
<td>W 450 x D 501 x H 244 mm</td>
</tr>
<tr>
<td>Weight</td>
<td>16.6 kg</td>
</tr>
<tr>
<td>Output device</td>
<td>USB memory (optional)</td>
</tr>
<tr>
<td>Display</td>
<td>24-bit color touch screen</td>
</tr>
</tbody>
</table>

Others
- Touch panel protective sheet (optional)
- Touch pen (standard included)
- Data files saved in text format or UVPC format*
Quantitation of Nucleic Acids

Quantitation of Double-Stranded DNA Using BioSpec-nano

Operating Principle and Features

The BioSpec-nano has less available optical path length, 0.2 mm and 0.7 mm, which enable quantitation of nucleic acids in very low sample volumes of 1 or 2 μL. Samples can also be measured using an optional cell with a 5 mm optical path length for 2 mL volumes of diluted samples.

An automatic wiping function enables wiping the samples between measurements, eliminating the need to manually clean the sample stage and reducing cross contamination between samples.

Measurement Method

The sample consisted of purified dsDNA dissolved in Tris-EDTA (TE) buffer solution. The individual samples were prepared in the concentration ranges listed in Table 1 for each pathlength. Next, 10 successive measurements were conducted using each of the pathlengths and concentrations using the BioSpec-nano, and the OD (Optical Density, absorbance corresponding to the 10 mm pathlength) at 260 nm was determined. The Y-axis value (Corrected OD260) in Fig. 1, 2, and 3 correspond to BioSpec-nano measurement values. The standard value (Corrected OD260, X-axis in each figure) for determining the accuracy was obtained using the Shimadzu UV-1700 spectrophotometer, an appropriately diluted sample and a 1 mm pathlength cell. The linearities of the standard values, and the deviation from each of the straight lines correspond to OD error.

Results

Analysis Results with 0.2 mm Pathlength

The correlation coefficient of 0.999 for OD260 was obtained with respect to the standard value (Fig. 1). When the OD value was greater than 5 (250 ng/μL dsDNA), the measurement repeatability as CV (%) was less than 1.4 %, and the OD error (%) was from -0.6 % to 2.8 %.

The data are shown in Fig. 1.

Analysis Results with 0.7 mm Pathlength

The correlation coefficient of 0.999 for OD260 was obtained with respect to the standard value (Fig. 2). When the OD value was greater than 1.4 (70 ng/μL dsDNA), the measurement repeatability as CV (%) was less than 1.4 %, and the OD error (%) was from -0.6 % to 4.4 %.

The data are shown in Fig. 2.

Analysis Results with 5 mm Pathlength Cell

The correlation coefficient of 0.999 for OD260 was obtained with respect to the standard value (Fig. 3). When the OD value was greater than 0.2 (70 ng/μL dsDNA), the measurement repeatability as CV (%) was less than 0.8 %, and the OD error (%) was from -1.6 % to 3.6 %.

The data are shown in Fig. 3.

Performance of Automatic Wiping in Nucleic Acid Quantitation

We alternated measurement of purified dsDNA (31.7 OD, 578 ng/μL) and TE buffer solution using 0.7 mm pathlength, 3 μL sample volume, and 1 wipe operation between measurements. Carryover (%) of dsDNA to the TE buffer solution was used as an index of the automatic wiping performance.

Carryover (%)

≤ 0.01% (Nucleic acid concentration in TE measurement) ≤ 0.002% (Nucleic acid concentration in dsDNA measurement)

Application Examples

• Measuring single-strand DNA concentration
• Measuring RNA concentrations
• Measuring protein concentration (refer to p. 34)

Given the steps involved in one set, including measuring double-stranded DNA → wiping → adding TE buffer → wiping, repeating that set 60 times resulted in carryover (%) that remained 0.3 % or less, which confirmed that sample carryover in the sample area when using automatic wiping is extremely low.

Table 1   Analytical Conditions

<table>
<thead>
<tr>
<th>Pathlength</th>
<th>Sample concentration</th>
<th>for 5 mm Pathlength</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 mm</td>
<td>Sample concentration</td>
<td>for 5 mm Pathlength</td>
</tr>
<tr>
<td>0.7 mm</td>
<td>Sample concentration</td>
<td>for 5 mm Pathlength</td>
</tr>
<tr>
<td>5 mm Pathlength Cell</td>
<td>Sample concentration</td>
<td>for 5 mm Pathlength</td>
</tr>
</tbody>
</table>

Instrument   BioSpec-nano
Wavelength range 220 to 800 nm
Spectrum bandwidth 3 nm
Monochromator Holographic grating
Detector Photo diode array
Auto wiping function Provided
Spectrum measuring time 3 sec
Quantitation range Pathlength 0.2 mm, 0 to 3700 ng/μL
Pathlength 0.7 mm, 3 to 100 ng/μL
Dimensions W 210 mm x D 214 mm x H 417 mm
Weight 7 kg

Note: The droplet formation status will affect analysis results. Measure quantities that are large enough to enable proper droplet formation.

BioSpec-nano

• Measure the concentration or check the purity of double-stranded DNA extracts.
• Measure sample quantities as small as 1 μL.
• Automatic wiping function enables a low-carryover system.

With the automatic wiping function, never forget to wipe off samples.
Electrophoresis for DNA/RNA Analysis

Checking for Genome Editing Mutations by Heteroduplex Mobility Assay

Operating Principle and Features

MultiNA is an automatic electrophoresis system that uses a microchip to measure the size of DNA or RNA. It automates all steps, such as creating the gel for agarose gel electrophoresis, applying the sample, electrophoresing, staining, detecting, and mixing. MultiNA uses dedicated reagents, fluorescent dyes, and microchips to fully automate analysis and achieve quick, easy, and high-sensitivity electrophoresis (Fig. 1).

Application

When Transcription Activator-Like Effector Nuclease (TALEN) or a CRISPR/Cas system is used to break a genome at any particular point in a sequence, the cell will repair the double-stranded DNA break. Genome editing is a technology that uses the repair errors that occur during repairing to modify genomes by inserting or deleting code in the original sequence. One technique used to verify whether the genome editing process successfully introduced the intended genetic modification is the heteroduplex mobility assay (HMA). It uses electrophoresis mobility to discriminate between homoduplex and heteroduplex DNA, which have different size structures.

Measurement Method and Result

After the mutation has been induced in an individual, PCR is conducted for the area in the vicinity of the deletion/insertion. The PCR product is denatured, then reannealed to form a heteroduplex product. Then, by checking the migration pattern of the sample using the MultiNA, the presence of short deletions can be verified by means of the structural change, which would be difficult to determine solely by comparing differences in chain length (Fig. 2).


display analysis results in the MultiNA Viewer

Application Examples

- Verify mutations created by genome editing
- Check libraries of next-generation sequencers
- Genotyping or detecting microorganisms or viruses
- Check presence and size of DNA/RNA with good reproducibility

Fig. 1

Conclusion

The MultiNA automatic electrophoresis platform solves previous shortcomings of agarose electrophoresis. It provides an easy way to check the presence and size of DNA/RNA with good reproducibility.

Fig. 2

MCE-202 MultiNA

- Reduces the cost and time involved in analysis
- Enables fully automatic batch analysis of up to 108 samples
- Achieves high sensitivity, high resolution, and high reproducibility

Specifications

- Instrument: MCE-202 MultiNA
- Sample rack: 96-well PCR plate (100 aluminumsheet can be applied to prevent sample evaporation) and 7.5×7.5cm PCR tube (Shimadzu recommended product)
- Microchip: 23 mm separation channel length, on-chip electrodes (insert up to four microchips)
- Pretreatment: Automatic sample injection, automatic separation buffer replenishing, automatic chip cleaning
- Electrophoresis voltage: Max. rated voltage: 1.5 kV, max. current: 250 μA
- Detection method: LED excited fluorescence detector (Wavelength excitation wavelength): 485 nm, emission wavelength: 528 nm
- Loaded samples: Up to 108 samples
- Separation size range: 25 to 500 bp (DNA-100 Kit), 100 to 1000 bp (DNA-1000 Kit), 1000 to 2500 bp (DNA-2500 Kit), 2500 to 6000 bp (DNA-6000 Kit)
- Microchip mixing: Chip mixing kit RA
- Sample volume: 5 μl
- Quantitation range: DNA analysis: 0.5 to 50 ng/μl (0.1 mM Tris-HCl, containing 1 mM EDTA), RNA analysis: 25 to 500 ng/μl (0.1 mM Tris-HCl, containing 1 mM EDTA)
- External dimensions: W: 415 mm x D: 545 mm x H: 508 mm
- Weight: 43 kg
- Power supply: 100 to 120 V, 220 to 240 (CE Marking) 300 VA max.
- Controller: Creating analysis schedules, real-time control, automatic analysis pretreatment, automatic analysis post-treatment, automatic error processing, analysis log management, analysis performance checks
- Data processing: Batch display/analysis results of gel images/peaks (automatic quantification and size prediction by size markers, data searching, data import/export, manual editing and re-analysis)
- Reports: Multidigit display, true display of sample files, RNA structural comparison, analysis performance check results, analysis log

Note: MCE-202 MultiNA is currently not available in US, EU and UK.
Cell Line Optimization

Cell Colony Picking

Cell Colony Picking Method Used to Automate Picking Operations for Cell Genome Editing

Operating Principle and Features

CELL PICKER has a technology to aspirate and discharge liquids. After visually deciding the target cell colony, a button is pressed to automatically attach a pipette tip to the end of the nozzle and reliably move the tip close to the target cell colony. When the tip scrapes off the cell colony, the measuring pump simultaneously activates to aspirate and then discharge the cell colony and a small amount of the medium. Using a tablet computer for observations and operations can reduce the amount of work involved in operations.

Procedure and Cultivation Parameters

A 6-well plate was seeded with 1×10^4 to 1×10^6 cells/well of human colon cancer cells (HCT116 adherent cell line). After cultivating the cells for six days, the CELL PICKER was used to pick cells and seed a 96-well plate. Then, the same cultivation parameters were used to cultivate the seeded cells for six days, after which the cell adhesion and proliferation were checked. The operation process flow is illustrated in Fig. 1.

Results

Cell colonies before and after picking are shown in Fig. 2. The picked cells after cell proliferation are shown in Fig. 3. 100% of the seeded wells produced adherent cell cultures.

Application Examples

- **Picking for establishing iPS cells**
  This confirmed that iPS cell colonies can be cultivated continuously while maintaining their undifferentiated state.
- **Collecting floating cell clusters (Spheroids)**
  The system can also be used for the purpose of collecting spheroids created by 3D cell culturing.
  It can pick a single spheroid from among multiple spheroids in an HEK293 cell line (400 to 500 μm) (Fig. 4).

Table 1  Adherent Cell Cultivation Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>HCT116 cell line (human colon cancer)</td>
</tr>
<tr>
<td>Culture medium</td>
<td>McCoy’s 5A medium with 10% FBS, 2 mM glutamine added</td>
</tr>
<tr>
<td>Coating</td>
<td>Gelatin solution</td>
</tr>
<tr>
<td>Cultivation parameters</td>
<td>Let stand at 37 °C and 5% CO2</td>
</tr>
</tbody>
</table>

Fig. 2   Cell colonies before and after picking

Fig. 3   Cell proliferation after picking

Before picking After picking

Day 1 Day 4

Note: These colonies are different than shown in “Cell colonies before and after picking.”

Scale bar: 200 μm

The white dashed circles in the images are not shown in the software.

Note: The indicated operations require special operating software.

Fig. 4   Before and after picking spheroids

Before picking After picking

Note: These pictures are not shown in the software.

Table 2  Spheroid Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spheroid formation</td>
<td>Pick 5/10/15 μL, Removal 5 μL</td>
</tr>
<tr>
<td>Dimensions</td>
<td>W 280 mm × D 350 mm × H 400 mm (not included microscope)</td>
</tr>
<tr>
<td>Weight</td>
<td>Appr. 8.5 kg</td>
</tr>
<tr>
<td>Power supply</td>
<td>100 to 240 V AC, Frequency: 50/60 Hz, Power consumption: 75 VA</td>
</tr>
<tr>
<td>Operating environment</td>
<td>Temperature: 10 to 35 °C, Humidity: 20 to 85% RH</td>
</tr>
</tbody>
</table>

Note: These pictures are not shown in the software.

Reduces work by enabling cell observations and instrument operations via a tablet computer!

Hand shakes

Button-operated tip

improves reliability!

improves reliability!

benefits

- Automation of manual steps enables reliable picking operations.
- Operations can be recorded easily.
- The compact space-saving design is ideal for installation in cramped laboratories.
Metal Elements Easily Quantified during Culturing

Monitoring of Metal Elements in Cell Culture Supernatant using Atomic Absorption Spectrometry

Operating Principle and Features

Atomic absorption spectrometry involves atomizing elements at high temperatures to quantitate elemental concentrations based on the absorption of specific light wavelengths during atomization. There are two main atomization methods: (1) the electric thermal method, which involves generating heat with an electrical current (high sensitivity), or (2) the flame method, which involves heating with a flammable gas flame. (Table 1 shows a comparison.) Either method can be used in AA-7000 systems, which include an auto-atmosphere changer (AAC) that can be used to automatically switch between the methods for measurements.

Measurement Method and Conditions

The high concentrations of Mg and Zn were measured using the flame method and trace elements (Cu, Mn, Co, and Fe) using the electric thermal method, based on the analytical conditions indicated in Tables 2 and 3. CHO cells were inoculated in a 125 mL flask and cultivated by shaking for four days. Every 24 hours, from immediately after starting cultivation, 1 mL of the cell culture fluid was sampled, removed cells by centrifugation, and then the supernatant was collected. Samples were diluted by 20 times for Cu, Mn, and Zn, 40 times for Co and Fe, and 500 times for Mg before analysis. The nitric acid concentration was 0.5 v/v%. Standard solutions for each element were prepared by diluting the standard solution for atomic absorption spectrometry (1000 mg/L). The nitric acid concentration was prepared to 0.5 v/v%. The calibration curve method was used for all analyses.

Results

The calibration curve coefficient of correlation was r = 0.999 or higher for all components. A spike-and-recovery test was performed for each element by adding a standard solution with a fixed concentration. (The addition recovery rate equals the concentration difference between spiked and unspiked samples divided by the addition concentration.) Test results were roughly within 100 ± 10 %, which is an excellent addition recovery rate. The electric thermal method and flame method were also used to monitor time-series changes in culture supernatant concentrations for each sample. Results of peak profiles and time-series concentration changes in the culture supernatant obtained by the two methods are shown in Figs. 1 and 2.

Conclusion

The concentration of metal elements in a cell culture supernatant were measured using an AA-7000 atomic absorption spectrophotometer, which can measure samples using two types of atomic absorption spectrometry methods, electric thermal and flame.

Application Example (Shimadzu Application News No.)

Analysis of metal elements in cell culture medium (AA-7000)

<table>
<thead>
<tr>
<th>Metal Element</th>
<th>Conc. (µg/L)</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>50.1</td>
<td>48.0</td>
<td>47.3</td>
<td>47.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>11.9</td>
<td>9.84</td>
<td>11.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Measured by reading treatment rate in one-dilution to test solution of cell culture supernatant

Fig. 1: Peak profile

Fig. 2: Time course of Mn and Mg concentration in culture supernatant

Metal Elements in Culture

Metal elements in culture media can be analyzed without any complicated pretreatment steps.

Multiple trace metal elements can be quantified inexpensively and easily.

The system supports the electric thermal method, flame method, or automatically switching between the methods.

Table 1: Comparison of Atomization Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Atomization Efficiency (%)</th>
<th>Detection Limit</th>
<th>Sensitivity (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electric thermal</td>
<td>99.5</td>
<td>0.1</td>
<td>1 ppb</td>
</tr>
<tr>
<td>Flame</td>
<td>99.2</td>
<td>1.0</td>
<td>10 ppb</td>
</tr>
</tbody>
</table>

Table 2: Analytical conditions of the electric thermal method

<table>
<thead>
<tr>
<th>Method</th>
<th>Atomization Efficiency (%)</th>
<th>Detection Limit</th>
<th>Sensitivity (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electric thermal</td>
<td>99.5</td>
<td>0.1</td>
<td>1 ppb</td>
</tr>
<tr>
<td>Flame</td>
<td>99.2</td>
<td>1.0</td>
<td>10 ppb</td>
</tr>
</tbody>
</table>

Table 3: Analytical conditions of the flame method

<table>
<thead>
<tr>
<th>Method</th>
<th>Atomization Efficiency (%)</th>
<th>Detection Limit</th>
<th>Sensitivity (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electric thermal</td>
<td>99.5</td>
<td>0.1</td>
<td>1 ppb</td>
</tr>
<tr>
<td>Flame</td>
<td>99.2</td>
<td>1.0</td>
<td>10 ppb</td>
</tr>
</tbody>
</table>

Table 4: Comparison of Measurement Method and Conditions

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Measurement Method</th>
<th>Wavelength range</th>
<th>Bandwidth (nm)</th>
<th>Background correction method</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA-7000F/AAC</td>
<td>BGC-D2</td>
<td>185.0 to 900.0</td>
<td>0.2</td>
<td>BGC-SR (SR)</td>
</tr>
</tbody>
</table>

Table 5: Specifications

<table>
<thead>
<tr>
<th>Specifications</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimensions and weight</td>
<td>W 700 × D 588 × H 714 mm, 76 kg (Autosampler is not included.)</td>
</tr>
<tr>
<td>Maximum reagent / sample positions</td>
<td>8 positions, Samples: 60 positions (when using an autosampler)</td>
</tr>
<tr>
<td>Maximum sample positions</td>
<td>120 positions (when using an autosampler)</td>
</tr>
<tr>
<td>Lamp mode</td>
<td>EMISSION, NON-BGC, BGC-D2, BGC-SR</td>
</tr>
<tr>
<td>Instrument</td>
<td>AA-7000F/AAC</td>
</tr>
<tr>
<td>Operating Principle</td>
<td>Atomic absorption/spectroscopy with automatic gas switching</td>
</tr>
<tr>
<td>features</td>
<td></td>
</tr>
<tr>
<td>Benefits</td>
<td></td>
</tr>
<tr>
<td>Metal elements in culture media can be analyzed without any complicated pretreatment steps.</td>
<td></td>
</tr>
<tr>
<td>Multiple trace metal elements can be quantified inexpensively and easily.</td>
<td></td>
</tr>
<tr>
<td>The system supports the electric thermal method, flame method, or automatically switching between the methods.</td>
<td></td>
</tr>
</tbody>
</table>
Operating Principle and Features

Triple Quad LC-MS-8060 systems feature UF technology that enables both high sensitivity and high speed. Due to high-speed scanning and high-speed positive-negative ionization switching capability, the system can simultaneously analyze multiple metabolites with a wide variety of chemical properties. The cell culture profiling method package is an analysis method optimized for analyzing multiple components in culture supernatant solutions. It enables analyzing up to 125 compounds (refer to product specifications) in 20 minutes or less (Fig. 1). This example describes monitoring the components in CHO cell culture supernatant over time.

Measurement Method and Results

CHO cell culture solutions were sampled every 24 hours and supernatants obtained by centrifugation. The supernatant was deproteinized and diluted with ultrapure water before analysis. Analytical results over a time course are graphically represented in Fig. 2. Important cell culture factors such as consumption of nutrients during cell proliferation and metabolic secretion can be monitored.

Conclusion

Rapid and comprehensive cell culture media analysis for the determination of nutrients and metabolites is possible.

Application Examples

- Spent media analysis
- Qualitative and quantitative analysis of culture media, bovine serum, and other samples (requires calibration curve preparation)
- Metabolomic analysis of culture supernatant and body fluids

Benefits

- Simultaneous analysis of up to 125 amino acids, vitamins, nucleic acids, or other compounds contained in the culture supernatant within 20 minutes.
- Sensitivity levels have been specified based on the concentration of target components being measured, which can reduce the work involved in creating a series of dilutions.
- Time-series monitoring of multiple components provides powerful support for optimizing cultivation parameters.

Specifications

Software
LC/MS/MS Method Package for Cell Culture Profiling Ver.2

LC unit
Nexera X3 (SCL-40, LC-40BX3, CTO-40S, SIL-40C X3, MR20 µL mixer)

MS unit
LCMS-8045/-8050/-8060

Analysis cycle
Less than 20 minutes per sample

Registered compounds
125 compounds + internal standard substance (2-cyclopentanone acid)

Amino acids and metabolites
60 compounds

Nucleic acids and metabolites
31 compounds

Vitamins
15 compounds

Sugars
4 compounds

Other (organic acids, etc.)
15 compounds

Separation mode
Reverse

Detection mode
MRM (positive/negative)
Cell Culture Media Analysis Platform

Automates Processes from Pretreatment to LC/MS/MS Measurement for Culture Supernatant Analysis

Operating Principle and Features

The C2MAP-2030 is an automatic pretreatment system for removing proteins from culture supernatants by suction filtering the proteins precipitated by adding an organic solvent. Deproteinized culture supernatant samples are automatically transferred to the HPLC autosampler, where they are dispensed onto a microtiter plate (MTP) for storage. These samples are automatically supplied for LC/MS/MS measurement, where 95 components are analyzed simultaneously using the Cell Culture Profiling Method (equivalent to Ver. 1). After peak integration, time-series data for each component can be visualized easily (Fig. 1) by loading the data file into the C2MAP-TRENDS software included with the C2MAP-2030 system. By connecting the C2MAP automatic pretreatment system to the LC-MS/MS system, samples can be analyzed seamlessly. Because sample information is linked to measurement data files, the C2MAP system can also reduce human error, such as from loading the wrong sample. Manual methods can cause variations in data quality, but automated equipment helps ensure any operator can acquire data with good repeatability (Fig. 2). Automation can also reduce operational hours (Fig. 3).

TRENDS software included with the C2MAP-2030 system.

- Organic solvent precipitation and suction filtration
- Sample transferring
- Automatic sample dilution
- Dispensing onto MTP plates
- LC/MS/MS measurement
- Peak integration
- Visualization of temporal changes in each component

Automated process steps

Conclusion

The C2MAP system can automate the deproteinization process for up to 65 culture supernatant samples. By linking it to an LC-MS system, the entire process from pretreatment to LC/MS/MS measurement can be executed seamlessly.

C2MAP Cell Culture Media Analysis Platform System

- Automating culture supernatant analysis processes from pretreatment to LC/MS measurement ensures anyone can acquire high-quality data.
- Dedicated control software makes it easy to link samples to measurement results.
- The system can be operated using only the modules necessary for automatic pretreatment, while the LC-MS/MS system is also used for a separate analysis.

Specifications

- Instrument: C2MAP-2030
- LC unit: Nexera X2 (CBM-20A, LC-30AD, CTO-20AC, SIL-30AC, MR 20 μL mixer, and other units)
- MS unit: LCMS-8050/-8060
- Required sample quantities: 400 to 500 μL (or 100 to 250 μL using optional rack)
- Processing time: 10.8 min per sample (17 min per sample for LC/MS measurements)
- Pretreatment processes: Add internal standard, sample, and organic solvent, mix, filter by suction filtration, and transfer sample after processing
- Pretreatment methods: Batch mode, Sequential mode
- Number of samples: 65 (or 64 using the sequential mode)
- External dimensions: W 670 x D 700 x H 1,190 mm (C2MAP-2030 only)
- Weight: 185 kg (C2MAP-2030 only)
Automating Steps from Preparative Purification to Product Evaluation

Seamless Analysis from Purification of IgG in Human Blood Plasma to SEC Evaluation

Operating Principle and Features

Sodium chloride and other halogen ions essential for biopharmaceutical analysis are highly corrosive to metals. To abate this concern, the Prominence inert LC system uses PEEK or other polymer materials for all parts in contact with liquids, thereby ensuring worry-free operation. The Liquid handler (LH-40) is an integrated autosampler and fraction collector. That means samples acquired during the first analysis can be injected directly into the second analysis without having to transfer them from a fraction collector. For example, with this system, the target protein is purified by an affinity column and fractionated at the first step, and the fractionated protein is reinjected for SEC analysis at the second step. These two steps can be done by just specifying the method and fraction.

Measurement Method and Conditions

5 mL of commercial human plasma was diluted 5-fold with mobile phase in a 15 mL tube and the tube was placed in the LH-40 rack. This sample was purified by affinity chromatography with an IgG purification column following the conditions in Table 1. The elution fractions were collected on the 96-deep-well plate set in the liquid handler. Then, 100 µL of the fraction involving the peak’s top point was analyzed by size exclusion chromatography (SEC) following the conditions in Table 2.

Results

The IgG peak obtained from affinity purification in step 1 and the peak obtained by SEC analysis in step 2 (Fig. 1) were evaluated by performing SDS-PAGE. That resulted in detecting H and L-chain bands for the target IgG (Fig. 2).

Conclusion

By simply setting a sample to the liquid handler (LH-40), the system can not only purify the sample, but also seamlessly further analyze fractions from the sample. For routine work with a prespecified target, it is possible to analyze only the target fraction. By adding a column switching valve and increasing the number of columns, the system can also be used to screen purification parameters or purify samples in multiple steps. After fractions are collected in a 96-deep-well plate, they can be used directly for SDS-PAGE, or various other analytical methods.

Application Examples

• Discovering and checking the quality of proteins in cell cultures
• Optimizing cultivation parameters
• Evaluating proteins in blood

Table 1: Analytical Conditions of Affinity Purification

<table>
<thead>
<tr>
<th>Column</th>
<th>Shim-pack Bio Diol-300 (30 mm × 8.0 mm I.D., 5 µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase A</td>
<td>10 mmol/L (sodium) phosphate buffer pH 6.9</td>
</tr>
<tr>
<td>Mobile phase B</td>
<td>1 M ammonium acetate in 10 mmol/L (sodium) phosphate buffer pH 6.9</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.5 mL/min</td>
</tr>
<tr>
<td>Flow cell</td>
<td>Inert flow cell</td>
</tr>
<tr>
<td>Detection</td>
<td>SPD-20A (280 nm)</td>
</tr>
<tr>
<td>Column Temp.</td>
<td>15 °C</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>100 µL</td>
</tr>
<tr>
<td>Time Program (A. Conc.):</td>
<td>0% (0 – 10 min)</td>
</tr>
<tr>
<td></td>
<td>100% (10.01 - 20 min)</td>
</tr>
<tr>
<td>Column:</td>
<td>Shim-pack Bio Diol-300</td>
</tr>
</tbody>
</table>

Table 2: Analytical Conditions of SEC analysis

<table>
<thead>
<tr>
<th>Column</th>
<th>Shim-Pack Bio Diol-300 (300 mm × 4.6 mm I.D., 5 μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase A</td>
<td>10 mmol/L (sodium) phosphate buffer pH 6.9</td>
</tr>
<tr>
<td>Mobile phase B</td>
<td>Sodium chloride in 10 mmol/L (sodium) phosphate buffer pH 6.9</td>
</tr>
<tr>
<td>Mobile phase C</td>
<td>Sodium chloride in 10 mmol/L (sodium) phosphate buffer pH 6.9</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 mL/min</td>
</tr>
<tr>
<td>Flow cell</td>
<td>Inert flow cell</td>
</tr>
<tr>
<td>Detection</td>
<td>SPD-20A (280 nm)</td>
</tr>
<tr>
<td>Column Temp.</td>
<td>15 °C</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>100 µL</td>
</tr>
<tr>
<td>Time Program (B. Conc.):</td>
<td>0% (20.01 – 35 min)</td>
</tr>
<tr>
<td></td>
<td>100% (35.01 - 40 min)</td>
</tr>
<tr>
<td>Column:</td>
<td>HiTrap rProtein A FF (1 mL)</td>
</tr>
<tr>
<td>Guard Column:</td>
<td>Shim-Pack Bio Diol-300 (G)</td>
</tr>
</tbody>
</table>

Fig. 1: Chromatogram of SEC analysis

Fig. 2: SDS-PAGE (Reducing) Results

Prominence Inert LC System + LH-40 Liquid Handler

**benefits**

- By selecting the target peak after fractionation, fractions can be automatically reinjected for analysis.
- A column switching valve allows automatic switching between columns for purification or analysis.
- Useful for optimizing cultivation parameters or other scenarios that involve comparing large numbers of samples.

Compatible with 96-well plates and a wide variety of test tubes and other containers

**Specifications**

- **System controller**: CBM-20A
- **Solvent delivery unit**: Two LC-20A units
- **Depositing unit**: DGU-20A5R
- **Column oven**: CTO-20AC
- **Mixing system**: PEEK mixer
- **UV-VIS detector**: SPD-20A
- **High-pressure flow channel switching valve**: FCV-12ARI
- **VP option box**: 228-65512-58
- **LH-40 liquid handler, main unit**: 228-65506-41
- **LH valve kit, preparative**: 228-7500E-42
- **Syringe kit, 20 mL**: 228-64173-44
- **Sample coil, 5 mL**: 228-69385-94
- **Analysis kit**: 228-75064-41
- **Sample rack**: 228-75258-41
- **Rack kit, D16**: 228-75064-49
- **Rack kit, MTP**: 228-75064-40

**Examples of SEC analytical column**

- Shim-Pack Bio Diol-300 (300 mm × 4.6 mm I.D., 5 µm) 228-3100-04
- Shim-Pack Bio Diol-300 (G) (30 mm × 8.0 mm I.D., 5 µm) 228-3100-06
**Characterization**

---

**Protein Primary Structure Analysis**

---

### Operating Principle and Features

The PPSQ protein sequencer automates the Edman degradation process. Although using Edman degradation to determine amino acid sequences is very time-consuming, the reliability of the resulting amino acid sequences is very high, making it especially useful for protein amino acid sequencing when no database has been built. PTH-amino acids obtained by Edman degradation are analyzed by isocratic or gradient elution.

### Measurement Method and Conditions

To operate the sequencer, proteins or peptides to be analyzed are applied to a glass filter treated with polybrene or transferred to a PVDF membrane. After electrophoresis, they are stained and placed in the reactor with an excised protein spot. After that, they can be analyzed automatically. In the example, samples were prepared by reducing 2 pmol of IgG from mouse serum, separating into H and L-chains by SDS-PAGE, transferring the chains onto a PVDF membrane, staining, destaining, and then excising the resulting bands (Fig. 1 and 2). The IgG is reduced and separated into H and L-chains. The H and L-chains were separated and purified based on conditions indicated in Table 1 and then their amino acid sequences were analyzed (Fig. 3).

### Results

The amino acids in L-chains can be identified to 13 residues from the N-terminal, as Asp-Ile-Gln-Met-Thr-Gln-Ala-Ser-Leu-Ser-Ala(Val). A database search confirms that the sequence is for an immunoglobulin kappa light chain (Fig. 2).

### Conclusion

PPSQ-51A/53A systems can determine N-terminal sequences easily and accurately. The gradient system can detect peaks with approx. 5 to 5 times higher overall peak height than when using isocratic elution, which means amino acid sequences can be determined for even trace quantities of protein.

### Application Examples

- Identifying the primary sequence of peptides
- Identifying the presence and position of S-S bonds
- Identifying post-translational modifications

### PPSQ-51A / 53A

- Amino acids can be sequenced accurately with extremely high data reliability.
- Ile and Leu residues with identical masses can be differentiated and the presence and position of S-S bonds can also be determined.
- Proteins can be analyzed directly, which makes operations extremely easy.
**PPSQ-51A / 53A + MALDI-8020**

**Benefits**
- Obtain complete sequence coverage using PPSQ and MALDI-TOF MS systems in combination.
- Enables more reliable and accurate amino acid sequencing.
- MALDI-TOF MS enables direct analysis of cyclic peptides or peptides with blocked N-terminals.

---

### Operating Principle and Features

Using the PPSQ sequencer to analyze an amino acid sequence using Edman degradation, as described on the previous page (p. 20), involves analyzing each amino acid one at a time, starting at the N-terminal. That eliminates mass or database dependence and other problems, but Edman degradation is not well suited to processing information for long sequences due to decreased reaction efficiency. To achieve more accurate and reliable N-terminal amino acid sequence information, combine Edman degradation data with In Source Decay (ISD) results obtained using a MALDI-TOF MS system. Amino acid sequencing by mass spectrometry involves the differences between fragment ion masses to determine the amino acid sequence of peptides. ISD increases the laser output to destabilize the differences between fragment ion masses to determine the amino acid sequence. ISD is a technique for increasing the laser output to destabilize the N-terminals. The PPSQ and ISD results are obtained using the MALDI-TOF MS system, which can provide more accurate and reliable data for the analysis of long amino acid sequences.

**Measurement Method**

The PPSQ sequencer requires reduction and alkylation, but MALDI-TOF MS enables direct analysis because samples can be reduced on the plate.

**Results and Conclusion**

Large amounts of information about peptides and proteins can be obtained from molecular weight data measured using a MALDI-TOF MS system. The molecular weight data is useful for quickly judging incorrect amino acid compositions and the presence of potential degradations or modifications. The accurate average molecular weight of peptides can be determined easily by selecting an appropriate matrix. Even when using the MALDI-TOF MS system, the sample is directly deposited on the plate, mass is detected precisely within 20 ppm of the theoretical molecular weight.

As shown in Table 2, N-terminal amino acid sequencing by either MALDI-TOF MS or Edman degradation provides a significant benefit for identifying amino acid sequences. Of all the methods currently available, N-terminal amino acid sequencing by Edman degradation remains the best method for determining the actual N-terminals of proteins and peptides. ISD also provides a reliable means of obtaining sequence information, but matrix interference generally prevents it from being used to observe low-mass fragments relevant to N-terminals. Fig. 2 shows results from BNP analysis using a combination of PPSQ and MALDI-TOF MS systems. Only a portion of the sequence can be determined using either one of these methods, but accurate sequence information can be obtained for the entire length by using both in a complementary way.

---

### Table 1: Theoretical and Measured Masses for BNP

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Theoretical Mass</th>
<th>Measured Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP</td>
<td>5038.59</td>
<td>5038.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+20 ppm</td>
</tr>
</tbody>
</table>

**Specifications**

- **Instrument:** MALDI-8020
- **Mass range:** m/z 1 to 500,000
- **Mass resolution:** > 5,000 FWHM
- **Sensitivity:** > 250 amol
- **Mass accuracy:** < 20 ppm with internal calibration, < 150 ppm with external calibration
- **Acceleration voltage:** 15 kV
- **Laser:** Solid-state laser
- **Wavelength:** 355 nm
- **Repetition frequency:** 50, 100, or 200 Hz (variable)
- **Flight distance:** 850 mm
- **Detector:** Electron Multiplier
- **Ion source cleaning:** Includes automatic cleaning functionality (depending on built-in solid-state laser)
Analysis of Product Peptide Fragments

Using Integrated UHPLC System with High Repeatability for Mapping Peptides in Antibody Drugs

Operating Principle and Features
Using HPLC for peptide mapping requires a system with high repeatability, because the analysis involves comparing elution profiles to confirm whether peptides are identical or have mutations. LC-2060 series integrated UHPLC systems are ideal for such analysis.

Measurement Method and Conditions
Samples were prepared by reduction, alkylation, and then trypsin enzyme digestion of human immunoglobulin G (IgG) (Fig. 1) and analyzed according to the analytical conditions in Table 1.

Results
The chromatogram from the trypsin-digested IgG shows that an extremely large number of peaks are detected and separated (Fig. 2). For peptide mapping, an extremely long gradual gradient is used to separate the many peaks. Consequently, results tend to have poor repeatability, especially if using a low-pressure gradient system. Therefore, the intra-day and inter-day repeatability were also checked. Key peaks (a to f) were selected from the chromatogram: intra-day repeatability was calculated from six consecutive analysis results. Inter-day repeatability was calculated from the daily average values of three analyses on each of six days (Tables 2 and 3). Both the intra-day and inter-day repeatability values indicated good reproducibility.

Conclusion
LC-2060 series systems provide data with excellent repeatability even when using analytical conditions prone to cause poor repeatability in low-pressure gradient systems. They can also be connected to a mass spectrometer for peptide mapping.

Application Examples
• High-sensitivity analysis using a fluorescence detector
• Various UHPLC analyses

Specifications

Instrument
LC-2060 series

Degassing unit
Five Lines: Mobile phase 4 + Rinse solution 1

Pumping method
Parallel-type double plunger

Pulsation
Max. 0.1 MPa (1 mL/min, 10 MPa, Water)

Flowrate setting range
0.0001 to 10 mL/min

Configuration
Four-solvent low-pressure gradient

Gradient settings
0 to 100 %, in 0.1 % steps

Maximum pressure
70 MPa

System delay volume
460 µL

Autosampler
Injection method
Total-volume sample injection

Injection volume setting range
0.1 to 1,000 µL (Specimen: 0.1 to 100 µL, 1 to 500 µL, 1 to 2,000 µL)

Injection cycle time
Min. 14 sec (Specified condition)

Samples for processing
538 vials (1 mL), 216 vials (1.5 mL), 112 vials (4 mL), 4 sample plates

Sample cooler
4 to 45 °C

Column oven
Heating and cooling method
Forced air circulation method

Containable column size
6 columns 10 cm long and 3 columns 30 cm long

Temperature control range
Room temperature – 12 to 90 °C, Setting range 4 to 90 °C

Flowrate switching valve
Max. 1 pc
Operating Principle and Features

Multiple absorption peaks from C=O stretching vibration of peptide bonds overlap to appear as a broad peak near 1650 cm\(^{-1}\) (amide I band). Analyzing the peaks can provide information about the protein secondary structures. Each absorption band in the overlapping group of absorption bands can be determined by a curve-fitting process that optimizes peak information (position, intensity, and FWHM) for the curve being fit to each absorption band, so that the difference between the calculated and measured spectra is minimized. The calculated spectra are commonly based on the Lorenz or Gaussian curve fitting.

The following describes the process for observing the secondary structural changes that occur due to thermal denaturation of proteins based on the second-derivative spectrum and peak separation.

Measurement Method

Egg white was used for the sample because it consists primarily of proteins. 60 μl samples were measured using a MicroMIR measurement accessory with a heatable three-reflection ATR prism (diamond/ZnSe) installed. Since egg white hardens when heated, the three-reflection ATR prism was used because it can also be used to measure solid samples. Due to overlapping between amide I and water vapor peaks, the optical system was purged with dry air. Given the measurement conditions in Table 1, a temperature controller was used to increase the prism temperature from 40 to 100 °C in 10 °C steps, with each temperature setting held for two minutes after placing drops of egg white to ensure adequate heat transfer before measuring. To eliminate the effects of moisture in the egg white, analysis was based on difference spectra calculated by subtracting the spectrum for water at each temperature.

Analysis Using Second-Derivative Spectra

Evaluating second-derivative spectra can be helpful when investigating variations in the secondary structure of proteins (α-helix, β-sheet, β-turn, and random coil structures). The second-derivative spectrum (Fig. 2) determined from Fig. 1 confirmed that thermal denaturation was causing an increase in β-sheet structures near 1639 cm\(^{-1}\) and 1622 cm\(^{-1}\), and β-sheet structures near 1637 cm\(^{-1}\) and α-helix structures near 1655 cm\(^{-1}\) to increase. The peak shift due to thermal denaturation suggests the status of hydrogen bonds may have changed.

Results and Discussion

The difference spectra between egg white and water at each temperature showed an increase in prominent peaks near 1625 cm\(^{-1}\) and 1675 cm\(^{-1}\) at 60 °C or higher temperatures (Fig. 1). This is an enlargement of the 1700 to 1600 cm\(^{-1}\) area. This confirmed its correlation with thermal denaturation.

Conclusion

An FTIR spectrophotometer makes it easy to predict the changes in secondary structures due to thermal denaturation of proteins. It can contribute to protein modification technology, such as improving the thermal properties of proteins with a known structure by heating them to add structural mutations to structures that are prone to unfolding.

Application Examples (Shimadzu Application News No.)

- Predicting secondary structures in proteins
- Predicting the locations of mutations in proteins
- Evaluation of amyloid β aggregation (Aβ10)

Specifications

- **Instrument**: IRTracer-100
- **Interferometer**: Michelson interferometer (30° incident angle) Equipped with Advanced Dynamic Alignment system Sealed interferometer with Automatic Dehumidifier
- **Optical system**: Single-beam optics
- **Beam splitter**: Germanium-coated KBr for Middle IR (Standard) Germanium-coated CsI for Middle/Far IR (Optional)
- **Detector**: DLATGS detector with temperature control for Middle/Far IR (Standard) MCT (HgCdTe) with liquid nitrogen cooling (Middle/Far IR) (Optional)
- **Wavenumber range**: 7,800 to 350 cm\(^{-1}\) (Standard) 12,500 to 240 cm\(^{-1}\) (Optional)
- **Resolution**: 0.25, 0.5, 1, 2, 4, 8, 16 cm\(^{-1}\) (Middle/Far IR) 2, 4, 8, 16 cm\(^{-1}\) (Near IR)
- **Dimensions**: 440 mm x 440 mm x 429 mm
- **Weight**: 47 kg

- By using a heatable three-reflection ATR accessory, infrared spectra can be obtained from proteins in a heated environment.
- Slight variations in infrared spectral shapes can be shown clearly by calculating the second derivative of infrared spectra obtained.
- The secondary structures of proteins can be analyzed by separating amide I band peaks in second-derivative spectra.
Operating Principle and Features

Glycans can affect the safety and efficacy of biopharmaceuticals. One technique used to analyze glycans is to mark them with fluorescence and then analyze them by HPLC using a fluorescence detector. Shimadzu RF-20Axs fluorescence detectors offer low noise and good S/N levels (compared to previous models, as shown in Fig. 1) to provide excellent sensitivity and linearity for glycan analysis. Glycan fluorescent labeling methods include those using pyridylamino (PA)-glycan and 2-aminobenzamide (2-AB)-labeled glycan. Either type of fluorescent-labeled glycan can be analyzed in the same manner.

Measurement Method and Conditions

Glycans in an antibody drug were analyzed by HPLC with detection by the high-sensitivity RF-20Axs fluorescence detector. An Aeris PEPPEX AB-C18 core-shell analytical column was used. The column packing material penetration was optimized for analyzing peptides and other macromolecules, which makes it effective for separating glycans and contaminants in antibody drugs.

Two types of antibody drugs were treated with trypsin and Glycopeptidase F was used to cleave glycans. Then the glycans were fluorescently derivatized by PA and used for analysis (Table 1).

Results

Peak differences noticed between the chromatograms for antibody drugs A and B after about 50 minutes of elution (*) clearly indicated the drugs contained different glycan levels. In addition, many peaks with different response levels were observed (Figs. 2 and 3).

Conclusion

Glycans in antibody drugs can be analyzed using HPLC by fluorescent labeling the glycans after trypsin digestion. RF-20Axs detectors offer high sensitivity and low noise. They can also be connected to an LC-2060 series integrated HPLC system (refer to p. 24).

Application Examples (Shimadzu Application News No.)

- Analysis of 2-AB glycans (L483)
- Quantitative analysis of favipiravir spiked in plasma (L570)

This analysis of glycans in antibody drugs was achieved with help from professor Kenichiro Tadoroki of the Laboratory of Analytical and Bio-Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka.

Table 1   Analytical Conditions

<table>
<thead>
<tr>
<th>Column: Aeris PEPTIDE XB-C18</th>
<th>(150 mm × 2.1 mm I.D, 1.7 μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase A:</td>
<td>20 mmol/L Ammonium Formate</td>
</tr>
<tr>
<td></td>
<td>0.0095 % (v/v) Formic acid-water</td>
</tr>
<tr>
<td>Mobile phase B:</td>
<td>20 mmol/L Ammonium Formate</td>
</tr>
<tr>
<td></td>
<td>0.0095 % (v/v) Formic acid-Methanol</td>
</tr>
<tr>
<td>Time Program (B. Conc.):</td>
<td>0 % (0 min) → 5 % (60 min) → 10 % (70 min) → 100 % (70.01 min - 80 min) → 0 % (95.01 – 110 min)</td>
</tr>
<tr>
<td>Flowrate:</td>
<td>0.4 mL/min</td>
</tr>
<tr>
<td>Column Temp.:</td>
<td>40 °C</td>
</tr>
<tr>
<td>Injection Volume:</td>
<td>3 µL</td>
</tr>
<tr>
<td>Detection: RF-20Axs</td>
<td>(Ex: 320 nm, Em: 400 nm)</td>
</tr>
</tbody>
</table>

Fig. 1   Chromatograms of 10 fmol PA-Glycan

Fig. 2   Chromatogram of PA-Glycans from Antibody Drug A

Fig. 3   Chromatogram of PA-Glycans from Antibody Drug B

RF-20Axs

- The low noise and excellent S/N ratio ensure ample sensitivity for glycan analysis.
- Cell temperature control functionality enables highly reproducible data acquisition.
- Standard, semi-micro, inert, and other cells can be selected based on the given analysis.
Glycan Analysis

Operating Principle and Features

Conventional MS* mass spectrometers are large and require peripheral equipment, but the space-saving MALDmini-1 fits in a space smaller than a piece of A3 size paper. The built-in vacuum pump means the system can be operated anywhere regular 100 V AC power is available. An optional kit is also available for supplying gas from small gas cartridges. Additionally, the MALD ion source and Digital Ion Trap (DIT) technology enable high-sensitivity MS and MS* measurements across a wide mass range, even for trace sample quantities.

Measurement Method

Proteins include many acidic glycans that contain sialic acids, which are analyzed by an HPLC or a mass spectrometer. HPLC generally requires using a reference glycan preparation and can have difficulty discriminating between complex glycans down to sialic acid linkages, for example. Mass spectrometers can have problems with unstable sialic acid residues being prone to desorption during analysis and an inability to discriminate between forms with different binding isomers. Therefore, mass spectrometers can have problems with unstable sialic acid residues on glycans, including the sialic acid linkage types.

Results

A wide variety of bifurcated, trifurcated, and other major glycan composites were detected from the N-linked glycans derived from serum glycoproteins (Fig. 2). A comparison of two types of MS spectra for trifurcated glycans shows the glycans were detected 28 Da apart, which infers that there are two different glycans (α2,3- and α2,6-linked forms) in the same location. Also, given that MS results show a neutral loss mass equivalent to modified sialic acids, this is the basis for differentiating between sialic acid linkage forms.

Fig. 2   Mass Spectrum of N-Linked Glycan Using MALDmini-1

Conventional MS

1. Conventional MALDI-TOF mass spectrometry was used for detection.
2. Stabilized using the sialic acid linkage specific alkylamidation method (SALSA method)*
3. Stabilized using the sialic acid linkage specific alkylamidation method (SALSA method)*
4. Glycans were analyzed by HPLC or a mass spectrometer. HPLC generally requires using a reference glycan preparation and can have difficulty discriminating between complex glycans down to sialic acid linkages, for example. Mass spectrometers can have problems with unstable sialic acid residues being prone to desorption during analysis and an inability to discriminate between forms with different binding isomers. Therefore, mass spectrometers can have problems with unstable sialic acid residues on glycans, including the sialic acid linkage types.

MALDmini-1

• Compact size and simple configuration allows installation in confined spaces.
• Samples can be measured immediately at the same location they are prepared.
• Suitable for a wide range of applications, from measuring the molecular weights of trace samples to structural analysis of complex molecules.

Fig. 1   Overview of Sialic Acid Linkage Specific Alkylamidation (SALSA) Method

MALDmini-1

- Mass range: m/z 550 to 5,000
- Mass resolution: > 4000 FWHM, [Glu1]-Fibrinopeptide B m/z 1570.68, scan speed 1000 Da/s
- Sensitivity (MS) 1 fmol ([Glu1]-Fibrinopeptide B m/z 1570.68, BSA m/z 66431)
- Sensitivity (MS/MS) 10 fmol ([Glu1]-Fibrinopeptide B m/z 1570.68)
- Mass accuracy: Internal standard: < 200 ppm, External standard: < 200 ppm (m/z 1,000 to 5,000)
- MS*: 1 ≤ n ≤ 3
- Sample plate: Disposable FlexiMass-DS, and stainless steel FlexiMass-DR (26 x 76 mm)
- Gas: Argon and helium (min. 99 % at 40 to 60 kPa)
- Gas cartridge: Regulator, He gas tubing, Ar gas tubing, and gas cartridge holder
- Power supply: AC 100 to 240 V, 50/60 Hz, 960 VA
- Dimensions: W 309 mm x D 385 mm x H 320 mm
- Weight: 25 kg
- Operating environment: Temperature: 18 to 28 °C, Humidity: 40 to 70 % max. (with no condensation)
- Software: Saving data: Database using SQLite
- Export file formats: mzML and mzXML

Specifications

Conclusion

Stabilization of sialic acids by the SALSA method and MS* analysis by the MALDmini-1 system can be used to analyze the structure of glycans, including the sialic acid linkage types.

Application Examples (Shimadzu Application News No.)

- Protein identification
- Structural analysis of glycans and glycopeptides
- Checking the mass of various molecules

* Patent No. 06135710

Fig. 2   Metabolism of N-Linked Glycan Using MALDmini-1

N-Linked Glycan Analysis Using MALDmini-1

Structural Analysis and Identification of Sialyl Linkage Isomers

5 μL of commercial serum were denatured and reduced by SDS and DTT. N-linked glycans were cleaved from glycoproteins by adding PNGaseF (Peptide-N-Glycosidase F) and letting it react for 18 hours at 37 °C. 4 μL of the cleaved N-linked glycans were mixed directly with 20 μL of the MALD-TOF mass reaction solution and left to react for one hour at room temperature. Later, a stabilizer reagent with a lactonic structure was added and mixed, and then the GI-01 Anode (GI Sciences) was used to remove the excess reagent. Also, the reducing terminal of the glycan was labeled with 2-amino-benzenesulfonic. Samples prepared by the process above were dropped onto a 0.5 μL sample plate and 0.5 μL of a matrix (α-cyano-4-hydroxycinnamic acid solution containing sodium chloride) was layered on top and dried. Then the MALDmini-1 was used for MS* analysis.
MALDI-8020

Glycan Analysis

Operating Principle and Features

The MALDI-8020 is a linear-mode MALDI-TOF mass spectrometer with a small installation footprint. It is typically used for quality control or profiling applications for peptides, proteins, polymer or oligonucleic acids, and other substances. Despite the benchtop design, the ion optical system features a large diameter inlet to ensure proper system performance levels are maintained for long periods and to reduce the risk of ion source contamination. The UV laser-based rapid automatic source cleaning function (TrueClean) can clean the ion extractor electrode without releasing the vacuum pressure. The system can manage all data and other information in one location and includes a tool for assisting with strict compliance with FDA 21 CFR Part 11.

Measurement Method

Especially for biopharmaceutical development applications, high-end mass spectrometers are commonly used due to the extreme importance of evaluating the properties of N-glycosylation. However, for batch analysis intended for screening or QAVQC applications, relatively inexpensive and user-friendly commercial systems are preferred. The MALDI-8020 model fills this need, offering more than adequate specifications for general profiling, high throughput, and an excellent value for the price.

The following describes an example of profiling IgG glycan modifications without releasing any glycans. Human IgG1K monoclonal antibodies (NISTmAb), IgG from mouse serum, and myeloma IgG were eluted from the beads with an acidic solution and desalted with ZipTip C18 tips. Sinapic and ferulic acids were respectively dissolved in a 50% acetonitrile solution containing 0.1% TFA to a final concentration of 20 mg/mL. Then the resulting solutions were used as matrices for MS analysis. IdeZ (IgG-degrading enzyme) for two hours at 37 °C. Then Protein A magnetic beads were used to recover Fc sections. The Fc regions were analyzed (Fig. 2). The mass gap between peaks in each spectrum indicates one sugar unit. Furthermore, MS measurements of each Fc region were repeated three times and statistically analyzed using eMSTAT Solution. A score plot was obtained easily by simply making some minimal adjustments to a few parameters (Fig. 3).

Results

In the mass spectrum of IgG full-length, the IgG molecular weight was observed near 170 kDa (Fig. 1). Due to inadequate mass resolution in the m/z range for large molecular weights, the mass spectrum for about 25 kDa of the Fc region modified by a glycan was analyzed (Fig. 2). The mass gap between peaks in each spectrum indicates one sugar unit. Furthermore, MS measurements of each Fc region were repeated three times and statistically analyzed using eMSTAT Solution. A score plot was obtained easily by simply making some minimal adjustments to a few parameters (Fig. 3).

Conclusion

The MALDI-8020 provides ample MS resolution for recognizing three Fc types with different varieties of glycan modifications. In addition, statistical analysis using eMSTAT Solution enabled quick classification of the three Fc types, which could be used for batch analysis, QAVQC, or other applications.

Application Examples

• Quality control of antibody drugs
• Synthesis confirmation of nucleic acid drugs

MALDI-8020

- Enables rapid benchtop glycan profiling with minimal pretreatment.
- Easy maintenance and low running costs
- Ideal for analyzing nucleic acids, proteins, and even polymers.

Specifications

- Instrument: MALDI-8020
- Mass range: m/z 1 to 500,000
- Mass resolution: >5,000 FWHM
- Sensitivity: >250 amol
- Mass accuracy: ±20 ppm with internal calibration, ±150 ppm with external calibration
- Acceleration voltage: 15 kV
- Laser: Solid-state laser wavelength: 355 nm, Repetition frequency: 50, 100, or 200 Hz (variable)
- Flight distance: 850 mm
- Detector: Electron Multiplier
- Ion source cleaning: Includes automatic cleaning functionality (depending on built-in solid-state laser)
- Sample plate: Disposable FlexiMass-DS and stainless steel FlexiMass-SSR
- Operating noise: <55 dB
- Main unit power supply: Single-phase 100 to 240 V AC, 50/60 Hz, 1 kVA
- Dimensions: W 600 mm × D 745 mm × H 1,055 mm (excluding protrusions)
- Weight: 86 kg
- Operating environment: Temperature: 18 to 28 °C, Humidity: Max. 70 % (with no condensation)
- Data analysis software: eMSTAT Solution
- Data analysis functionality:
  - Multivariate analysis: PCA (principal component analysis), PLS-DA
  - Univariate analysis: t-Test, Mann-Whitney U-Test, ANOVA (analysis of variance)
  - Discriminant analysis: Support Vector Machine (SVM), Random Forest
- Other:
  - Dynamic grouping
- Display functionality:
  - Multivariate analysis: Peak Matrix, Box Plot, ROC, AUC, Score/Loading Plot, Dendrogram
  - Discriminant analysis: Discriminant analysis results (Group, Score) superimpose points for unknown samples on a score plot

Input/output data:
- Input: Peak list (ASCII, JCAMP, or mzML format)
- Output: Peak list (list format), data analysis results (xml format), graph screenshot
Quantitation of Proteins

Operating Principle and Features

The BioSpec-nano has two available optical path lengths, 0.2 mm and 0.7 mm, and can quantify proteins and nucleic acids in sample quantities as low as 1 µL. Samples can also be measured using an optional cell with a 5 mm optical path length (for 2 mL sample quantities). That means only a small sample quantity is needed for quick protein concentration measurements or to check spectra.

A wiping mechanism enables automatic cleaning between samples, ensuring extremely low carryover and reducing inconsistencies that may occur with manual cleaning (Fig. 1).

Measurement Method

Measurement samples were prepared by dissolving bovine immunoglobulin (IgG) to 1000 μg/mL with phosphate buffered saline. With the optical path length set to 0.7 mm (Fig. 2), 4 μL of the sample was dripped onto the target to measure the optical density (OD) at 280 nm. The protein concentration is calculated based on the molar absorption coefficient (ε) and molecular weight values entered in the software’s analytical condition selection window (Fig. 3). The 280 με value can also be calculated by the software if the number of tryptophan, tyrosine, and cystine residues in the amino acid sequence is entered.

Conclusion

Using the BioSpec-nano, sample concentrations can be measured from small sample quantities of 1 to 4 μL by simply dripping the sample onto the stage. That eliminates any need for any manual processes, such as raising/lowering an arm, placing a cell in position, or wiping off the sample after each measurement. In addition to concentration determination, OD values can be displayed for any user-specified wavelength.

Application Examples

• Measuring single- or double-stranded DNA concentrations (refer to p. 6)
• Measuring labeled nucleic acid concentrations
• Measuring RNA concentrations
• Measuring protein concentrations

Results

Measurement results are shown in Fig. 4. The results show a concentration of approx. 1000 μg/mL, which is the same as prepared. Sample concentration values and spectra can be confirmed in the detail display window. Results can be output in either CSV or PDF format.

BioSpec-nano

benefits

• Measure the concentration of proteins or check the purity.
• Measure sample quantities as small as 1 µL.
• Achieve low carryover with the automatic wiping function.

Specifications

<table>
<thead>
<tr>
<th>Instrument</th>
<th>BioSpec-nano</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength range</td>
<td>220 to 800 nm</td>
</tr>
<tr>
<td>Spectrum bandwidth</td>
<td>3 nm</td>
</tr>
<tr>
<td>Wavelength accuracy</td>
<td>±1 nm</td>
</tr>
<tr>
<td>Pathlength</td>
<td>0.2 mm, 0.7 mm</td>
</tr>
<tr>
<td>Photometric value unit</td>
<td>OD (Optical Density); absorbance converted with 10 mm pathlength</td>
</tr>
<tr>
<td>Sample volume</td>
<td>1 µL min. (pathlength: 0.2 mm) 2 µL min. (pathlength: 0.7 mm)</td>
</tr>
<tr>
<td>Light source</td>
<td>Xenon flash lamp</td>
</tr>
<tr>
<td>Monochromator</td>
<td>Holographic grating</td>
</tr>
<tr>
<td>Detector</td>
<td>Photo diode array</td>
</tr>
<tr>
<td>Auto wiping function</td>
<td>Provided</td>
</tr>
<tr>
<td>Spectrum measuring time</td>
<td>3 sec</td>
</tr>
<tr>
<td>Quantitation range</td>
<td>Pathlength 0.2 mm: 1 to 75 OD, 50 to 3,700 ng/µL Pathlength 0.7 mm: 0.3 to 21 OD, 15 to 1,000 ng/µL</td>
</tr>
<tr>
<td>Optional 5 mm pathlength cell, 0.04 to 3 OD, 2 to 150 ng/µL</td>
<td></td>
</tr>
<tr>
<td>Dimensions</td>
<td>W 210 mm x D 214 mm x H 417 mm</td>
</tr>
<tr>
<td>Weight</td>
<td>7 kg</td>
</tr>
<tr>
<td>Analysis mode</td>
<td>Simple nucleic acid quantitation, labeled nucleic acid quantitation, protein quantitation, labeled protein quantitation, photometric measurement</td>
</tr>
</tbody>
</table>

Note: The droplet formation status will affect analysis results. Measure samples that are large enough to make proper droplet formation.

Fig. 3   Analytical Condition Selection Window

Fig. 2   Optical Path Length Setting Area

Fig. 1   Automatic Wiping Function

Fig. 4   Analytical Results for 0.7 mm Optical Path Length

Fig. 5   Analytical Results for 0.7 mm Optical Path Length
Characterization of Monoclonal Antibodies

Molecular Weight Analysis of Monoclonal Antibodies Using the LCMS-9030 Quadrupole Time-of-Flight Mass Spectrometer

Operating Principle and Features

The LCMS-9030 is a Q-TOF mass spectrometer that includes both quadrupole and time-of-flight separation systems, two types of mechanisms for separating ions. The system includes unique Shimadzu technologies in a variety of locations for acquiring data with both high sensitivity and high resolution, while also ensuring mass accuracy is always stable. For example, it includes technology for increasing the ion usage rate, machining technologies for manufacturing powerful and finely detailed grating electrodes, technology for precision temperature control, and technology for optimizing the distribution of electric potential.

Analytical Conditions

Human IgG1 monoclonal antibodies (mAb) were dissolved in 50 mmol/L aqueous ammonium hydrogen carbonate solution to prepare a 1 mg/mL standard solution (intact mAb). mAb subunits were prepared by adding 8 mol/L urea and a 50 mmol/L Tris-HCl buffer solution containing 50 mmol/L DL-dithiothreitol to 100 μg of the intact mAb to reduce the antibodies to H and L-chains. Measurement conditions for intact mAb and mAb subunits are indicated in Table 1.

Results

Fig. 1 shows the TIC chromatogram, deconvoluted MS spectrum, and the MS spectrum measured from the intact mAb. A comparison of the mass values measured from the intact mAb to theoretical mass values confirmed that measured values were consistent with theoretical values to a precision level of 25 ppm or less (Table 2). Mass Workflow software was used for the deconvolution of intact mAb and mAb subunits. The MS spectrum was also similarly analyzed for mAb subunit H and L-chains. Results from checking their molecular weights provided good values.

Application Examples

Using the LCMS-9030 Q-TOF mass spectrometer with Protein Metrics software enables evaluation of molecular weights in biopharmaceuticals with high sensitivity and high resolution to achieve consistently high precision.

Conclusion

Using the LCMS-9030 Q-TOF mass spectrometer with Protein Metrics software enables evaluation of molecular weights in biopharmaceuticals with high sensitivity and high resolution to achieve consistently high precision.

LCMS-9030

- Trace quantities of impurities contained in pharmaceutical ingredients can be measured with high sensitivity.
- Accurate mass information can be used to identify impurities in products.
- Deconvoluted spectra can be used to check the molecular weights in antibody drugs.

Specifications

<table>
<thead>
<tr>
<th>Instrument</th>
<th>LCMS-9030</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass range</td>
<td>Quadrupole mass range: m/z 10 to 40,000</td>
</tr>
<tr>
<td></td>
<td>TOF mass range: m/z 10 to 2,000</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>ESI positive</td>
</tr>
<tr>
<td></td>
<td>1 pg reserpine</td>
</tr>
<tr>
<td></td>
<td>S/N &gt; 1,000:1 (XMS in MS mode)</td>
</tr>
<tr>
<td></td>
<td>S/N &gt; 10,000:1 (XMS in MSMS mode)</td>
</tr>
<tr>
<td></td>
<td>ESI negative</td>
</tr>
<tr>
<td></td>
<td>1 pg chloramphenicol</td>
</tr>
<tr>
<td></td>
<td>S/N &gt; 1,000:1 (XMS in MS mode)</td>
</tr>
<tr>
<td></td>
<td>S/N &gt; 10,000:1 (XMS in MSMS mode)</td>
</tr>
<tr>
<td>Resolution</td>
<td>(Quadruopole)</td>
</tr>
<tr>
<td></td>
<td>R &lt; 0.8 u FWHM</td>
</tr>
<tr>
<td>Resolution</td>
<td>(TOF)</td>
</tr>
<tr>
<td></td>
<td>ESI positive</td>
</tr>
<tr>
<td></td>
<td>30,000 FWHM at m/z 1,972</td>
</tr>
<tr>
<td></td>
<td>ESI negative</td>
</tr>
<tr>
<td></td>
<td>30,000 FWHM at m/z 1,626</td>
</tr>
<tr>
<td>Mass accuracy</td>
<td>MS mode</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.7 ppm (peak to peak) at m/z 623,5882</td>
</tr>
<tr>
<td></td>
<td>Nat cluster (internal calibration)</td>
</tr>
<tr>
<td></td>
<td>MSMS mode</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.2 ppm (peak to peak) at m/z 1072,2489 + 472,6719</td>
</tr>
<tr>
<td></td>
<td>Nat cluster (external calibration)</td>
</tr>
<tr>
<td>Mass accuracy temperature stability</td>
<td>1 ppm/24 h, 18 to 28 °C at constant temperature</td>
</tr>
<tr>
<td>Maximum acquisition rate</td>
<td>100 Hz</td>
</tr>
<tr>
<td>Polarity switching time</td>
<td>1 sec</td>
</tr>
<tr>
<td>Interface</td>
<td>Standard: ESI</td>
</tr>
<tr>
<td></td>
<td>Optional: APCI, DUIS, CDS, Nano-ESI</td>
</tr>
</tbody>
</table>

Fig. 1   NIST mAb Intact Results

Table 2   Comparison of Measured and Theoretical Mass Values

<table>
<thead>
<tr>
<th>Name</th>
<th>Expected mass</th>
<th>Measured mass</th>
<th>Mass difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0F/G0F</td>
<td>148037</td>
<td>148039</td>
<td>2.0</td>
</tr>
<tr>
<td>G0F/G0F+Lys</td>
<td>148165</td>
<td>148164</td>
<td>-1.5</td>
</tr>
<tr>
<td>G0F/G0F-GlcNAc</td>
<td>147631</td>
<td>147631</td>
<td>0.0</td>
</tr>
<tr>
<td>G0F/G0F+Lys-GlcNAc</td>
<td>147834</td>
<td>147837</td>
<td>3.2</td>
</tr>
<tr>
<td>G1F/G1F</td>
<td>148328</td>
<td>148326</td>
<td>-1.4</td>
</tr>
<tr>
<td>G1F/G1F+Lys</td>
<td>148490</td>
<td>148489</td>
<td>-0.9</td>
</tr>
<tr>
<td>G1F/G2F</td>
<td>148524</td>
<td>148525</td>
<td>1.0</td>
</tr>
<tr>
<td>G1F/G2F+Lys</td>
<td>148652</td>
<td>148653</td>
<td>1.6</td>
</tr>
<tr>
<td>G2F/G2F</td>
<td>148686</td>
<td>148688</td>
<td>1.9</td>
</tr>
<tr>
<td>G2F/G2F+Hex</td>
<td>148848</td>
<td>148850</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Measuring Protein Aggregates (with temperature control and mixing)

Aggregates Sizer

- With a single system, it is possible to measure all aggregates within the 0.1 to 10 µm range at the same time.
- Real-time measurements can be performed while controlling the temperature and applying stirring stress.
- Micro quantities (125 µL) can be measured using a micro cell.

### Operating Principle and Features

The Aggregates Sizer aggregation analysis system for biopharmaceuticals can measure all aggregates within the previously difficult-to-measure 0.1 to 10 µm range at the same time, measure aggregates while applying a stress, and perform quantitative measurements. The Aggregates Sizer is a particle size analyzer that measures particle diameters based on the laser diffraction method. Normally, using the laser diffraction method, particle size can be determined based on a sample's scattering patterns but concentration cannot be determined. However, the Aggregates Sizer can measure absolute concentration values by calibrating with a standard sample of known particle sizes and concentrations.

### Measurement Method and Conditions

The sample solution was prepared by mixing freeze-dried bovine gamma globulin with PBS (pH 7.4) to a concentration of 1 mg/mL. 5 mL of the sample solution was measured in a temperature-controlled batch cell (Fig. 1) while stirring for 40 minutes at 190 strokes per minute. Stirring rods made of three materials, PEEK, stainless steel, and glass, were used for comparison. A temperature-controlled circulator was used during accelerated testing to maintain the temperature at three different constant temperature levels (23, 30, and 42 °C) for comparison.

### Results

A comparison of aggregate formation during the 40 minutes at 23 °C for the 0.2 to 2 µm range versus the 2 to 10 µm range is shown in Fig. 2. It indicates that aggregate formation was greatest in the 2 to 10 µm range using the PEEK stirrer, and greatest in the 0.2 to 2 µm range using the stainless steel stirrer. The glass stirrer resulted in the least aggregate formation in both ranges. The time-course changes in the particle size distribution for the PEEK stirrer at 42 °C (Fig. 3) show that aggregate formation occurs as a function of time. It also confirms that aggregate formation depends on temperature.

### Conclusion

The Aggregates Sizer is ideal for evaluating protein stability, because it is able to measure aggregate concentrations in real time as stirring stress is applied under temperature-controlled conditions and the materials that contact liquid are varied.

### Specifications

- **Instrument**: Aggregates Sizer
- **Measurement principle**: Laser diffraction method
- **Measurement range**: Particle size range: 100 nm to 10 µm
  - Particle size 10 µm: 10 µg/mL to 180 µg/mL
  - Particle size 1 µm: 0.5 µg/mL to 10 µg/mL
  - Particle size 100 nm: 2 µg/mL to 12 µg/mL
- **Concentration measurement accuracy**: ± 30 or less
- **Concentration range**: Particle size 100 nm: 2 µg/mL to 12 µg/mL
  - Particle size 1 µm: 0.5 µg/mL to 10 µg/mL
  - Particle size 10 µm: 10 µg/mL to 180 µg/mL
- **Batch cell**: Cell material: Quartz glass and PTFE (cell cap with temperature control function)
  - Required liquid volume: Approx. 0.125 mL
  - Temperature range with temperature control function: 20 to 42 °C ± 1 °C, set from PC
  - Operating environment: Temperature: 10 to 30 °C, Humidity: 20 to 80 % (with no condensation)
- **Micro cell**: Material: Quartz glass and PTFE (cell cap with temperature control function)
  - Required liquid volume: Approx. 0.125 mL
  - Temperature range with temperature control function: 20 to 42 °C ± 1 °C, set from PC
  - Operating environment: Temperature: 10 to 30 °C, Humidity: 20 to 80 % (with no condensation)

### Benefits

- Accuracy
- Speed
- User-friendliness
- Data analysis capability

### Quality Control

- Evaluating the stability and reproducibility to stress of biopharmaceuticals
- Considering additives for inhibiting aggregation
- Evaluating the concentration of particles in blood
- Using micro quantities (125 µL) can be measured using a micro cell

### Application Examples (Shimadzu Application News No.)

- Evaluating the stability and reproducibility to stress of biopharmaceuticals
- Considering additives for inhibiting aggregation
- Evaluating the concentration of particles in blood

---

*The concentration range depends on particle characteristics, such as shape.*

*The measurement range depends on particle characteristics, such as shape.*
Evaluating Aggregates in Protein Drug Products

Characterization of Insoluble Subvisible Particles in Biopharmaceuticals Using the Flow Imaging Method

Operating Principle and Features

For protein drug evaluation, the United States Pharmacopeia and Japanese Pharmacopoeia specify the light obscuration (LO) method to evaluate insoluble particles that are 10 μm or larger. Meanwhile, flow imaging (FI), a dynamic image analysis method, offers high sensitivity for particles with high permeability and also the ability to classify particles in images, has been gaining attention as a method for analyzing micrometer-level insoluble subvisible particles. The iSpect DIA-10 dynamic particle image analysis system is used to acquire images of particles in liquid samples for measuring the size distribution, concentration, and shapes of particles based on the flow imaging method. Due to the small sample quantities used (minimum 50 μL for measurements) and the optical system that misses very few particles, it is ideal for evaluating insoluble subvisible particles in biopharmaceuticals.

Measurement Method and Conditions

Sample solutions were prepared using freeze-dried human immunoglobulin. The sample powder was dissolved in a pH 5.0 citrate-phosphate buffer solution (1 mg/mL), which was filtered through a 100 nm filter and the filtrate was used as the sample solution. Half the sample solution was heated for three minutes at 80 °C. The other half was stirred with a PEEK polymer stirring plate for ten minutes. Then the heat-aggregated and the stirring-aggregated samples were measured according to the conditions in Table 1.

Table 1: Measurement Conditions

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample amount</td>
<td>50 μL</td>
</tr>
<tr>
<td>Flowrate</td>
<td>0.1 mL/min</td>
</tr>
<tr>
<td>Frame rate</td>
<td>8 frame/sec</td>
</tr>
<tr>
<td>Sample volume</td>
<td>50 to 1000 μL</td>
</tr>
<tr>
<td>Pump</td>
<td>Syringe pump</td>
</tr>
<tr>
<td>Dimensions</td>
<td>W 223 mm × D 465 mm × H 205 mm, 10 kg</td>
</tr>
<tr>
<td>Weight</td>
<td>10 kg</td>
</tr>
<tr>
<td>Wetted part materials</td>
<td>PEEK resin, fluorine resin, quartz, or fluorine rubber</td>
</tr>
<tr>
<td>Pump unit</td>
<td>W 97 mm × D 190 mm × H 150 mm, 3 kg</td>
</tr>
</tbody>
</table>

Results

The particle size distributions and scatter plots obtained from the measurements are shown in Fig. 1. Particle images are shown in Fig. 2. The particle images can be used to distinguish between aggregates, air bubbles, and oil droplets. Particle concentration measurement results are shown in Table 2 and Fig. 3.

iSpect DIA-10

• The optical system, which overlooks very few particles, can be used to evaluate the particle count concentration of micrometer-level aggregates.
• Sample quantities as small as 50 μL can be measured.
• Simple measurements can be performed in three steps, which minimizes the burden on operators.

Conclusion

iSpect DIA-10 systems can measure very small quantities of samples with excellent imaging efficiency. Even the type of insoluble subvisible particles can be predicted from particle images, making it ideally suited for evaluating the concentration of micrometer-level insoluble subvisible particles contained in biopharmaceuticals.

Table 2: Observed Particle Count and Concentration

<table>
<thead>
<tr>
<th>Area Based Diameter (µm)</th>
<th>Heat-aggregate sample</th>
<th>Stirring-aggregate sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 2 μm</td>
<td>417051 96737</td>
<td>129 4669 456 186</td>
</tr>
<tr>
<td>2 - 10 μm</td>
<td>244421 291246</td>
<td></td>
</tr>
<tr>
<td>10 - 25 μm</td>
<td>298 78</td>
<td></td>
</tr>
<tr>
<td>≥ 25 μm</td>
<td>22 9</td>
<td></td>
</tr>
</tbody>
</table>

Application Examples (Shimadzu Application News No.)

• Evaluating the concentration of insoluble particles in biopharmaceuticals
• Evaluating contaminants, coarse particles, and particle shapes in pharmaceuticals
• Evaluating the size of suspended particles in eye drops (Q122)
Evaluating the Thermal Stability of Proteins

Using a Differential Scanning Calorimeter to Measure the Thermal Stability of Proteins

Operating Principle and Features

A differential scanning calorimeter (DSC) can measure the enthalpy changes in heat energy generated (endothermic) or consumed (exothermic) as a sample is heated or cooled. Sample and reference solutions are placed in separate 6 mm diameter cells, with the cells placed in thermally symmetric positions within the furnace, and then the furnace is heated or cooled at a constant rate. For example, when proteins are heated at a constant rate, denaturation can cause the three-dimensional structure to begin unfolding. DSC systems can measure the thermal changes that occur during that process as endothermic peaks (thermal denaturation temperatures). Due to its superior baseline stability, the DSC-60 Plus can easily measure the thermal changes of samples in solution.

Operating Procedure and Measurement Conditions

Samples were prepared by diluting lysozyme from chicken egg white with a phosphate buffer solution (pH 7.0) to the concentrations indicated in Table 1. Then, 20 μL of the sample was sealed with an aluminum hermetic cell. Using 20 μL of the phosphate buffer solution as a reference sample, the samples were heated from 35 to 105 °C at a rate of 5 °C per minute to measure the thermal denaturation temperatures. To investigate the effect of protein pH, three 0.2 mol/L phosphate buffer solutions with pH 4.20, pH 7.05, and pH 9.10, were used as solvents for preparing and measuring the 10 % lysozyme solutions. The temperature was increased from 40 to 100 °C at a rate of 5 °C per minute.

Table 1   Samples Used

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme in Fig. 1</td>
<td>2.5 % of protein</td>
</tr>
<tr>
<td>Lysozyme in Fig. 2</td>
<td>0.2 % of protein</td>
</tr>
</tbody>
</table>

Results

With 0.2 % or 2.5 % lysozyme, endothermic peaks from thermal denaturation appear near 75 °C, which confirms that protein thermal denaturation temperatures can be measured in dilute 0.2 % solutions (Fig. 1 and 2). It also shows that stability is highest for lysozyme with pH 4.20, which had the highest thermal denaturation temperature (Fig. 3).

Conclusion

DSC systems can easily measure the thermal denaturation temperatures of proteins and can be used for evaluating the thermal stability to provide an index for a variety of other evaluations, such as for evaluating the stability of modified proteins or considering different storage solvents.

Application Examples (Shimadzu Application News No.)

- Evaluating the stability of proteins
- Evaluating crystal polymorphism in pharmaceuticals
- Evaluating the thermal stability of proteins

Benefits

- The thermal stability of proteins can be easily evaluated.
- The stability due to pH or solvent differences can be evaluated.
- With the built-in liquid nitrogen cooling chamber, the system can be used to evaluate the protein effects of freezing.

Specifications

<table>
<thead>
<tr>
<th>Instrument</th>
<th>DSC-60 Plus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>Heat flow</td>
</tr>
<tr>
<td>Measurement range</td>
<td>-140 to 600 °C (when using liquid nitrogen with cooling chamber included standard)</td>
</tr>
<tr>
<td>Heat measurement range</td>
<td>±150 mW</td>
</tr>
<tr>
<td>Baseline noise</td>
<td>0.5 mW max. (RMS value for a blank held at 150 °C)</td>
</tr>
<tr>
<td>Atmosphere</td>
<td>Nitrogen, inert gas, or dry air gas flow</td>
</tr>
<tr>
<td>External dimensions</td>
<td>W: 320 mm × D: 500 mm × H: 290 mm</td>
</tr>
<tr>
<td>Weight</td>
<td>28 kg</td>
</tr>
<tr>
<td>Power requirement</td>
<td>100 / 120 / 220 / 240 V AC, ±10 %, 50/60 Hz, 800 VA</td>
</tr>
<tr>
<td>Optional</td>
<td>STCP-1 sample heater and crimp press Cell compatible with crimp attachment<em>1 Cell compatible with sealing attachment</em>2 Aluminum crimped cell<em>1 Aluminum sealed cell</em>2</td>
</tr>
</tbody>
</table>

*1 Used in Application News T152
*2 Used in Fig. 1 to 3 (p. 42)
Operating Principle and Features
ICP-MS systems are generally considered to offer the highest sensitivity available for elemental analysis. These systems use an inductively coupled argon plasma (ICP) generated at about 10,000 °C to ionize elements in liquid samples and then analyze the ions by mass spectrometry with the ability to detect elements down to the ppt level. Given that elemental impurities in pharmaceuticals must be controlled to very low concentrations, ICP-MS systems have been attracting attention because of their high sensitivity. They also have disadvantages, however, such as high argon gas consumption rates and high running costs. In contrast, ICPMS-2030 enables analysis at about onehalf the cost overall. This is because it features Shimadzu’s unique mini-torch plasma system that successfully reduces argon gas consumption to 2/3 of conventional levels. Furthermore, relatively inexpensive 99.99 % pure industrial-grade argon gas can be used instead of 99.999 % to higher purity argon gas normally used for ICP-MS analysis.

Measurement Method and Conditions
The ICH Q3D Guideline for Elemental Impurities specifies Permitted Daily Exposure (PDE) for 24 elements for which toxicity is a concern. We have verified that ICPMS-2030 can adequately assess whether the guidelines are met. The mini-torch and low-cost industrial-grade argon gas were used. One tablet (maximum daily dose of 0.20 g), 0.5 mL of hydrochloric acid, and 5 mL of nitric acid were placed in a quartz decomposition vessel and decomposed in a microwave sample pretreatment system. After decomposition, 0.1 mL of hydrochloric acid was added and the mixture was made up to 20 mL with pure water to prepare the measurement solution (100-fold dilution). Internal standard elements Sc, Ga, Y, and Te were also added during that process (to a 10 μg/g concentration of the measurement solution). Spike-and-recovery test solutions were prepared by adding measurement elements to the decomposed sample. The calibration curve method with the internal standard method was used to quantitatively analyze and perform a spike-and-recovery test for the 24 elements subject to the ICH Q3D guideline according to the measurement conditions shown in Table 1.

Results
For many of the elements, the concentration measurement value was “N.D.,” but concentration was confirmed down to at least four digits below the permitted concentration. Recovery rates for each added element were also good, which clearly shows that measurements were performed correctly (Table 2).

Conclusion
With the ICPMS-2030 system, the 24 elements subject to the ICH Q3D Guideline can be analyzed quickly and accurately at half the running cost of conventional systems.
### nSMOL Antibody BA Kit

**Pretreatment Kit for LC/MS/MS Quantitative Analysis of Monoclonal Antibodies**

#### Benefits
- Selectively recovers Fab peptides and enables analysis without excessive peptides or trypsin.
- Offers general applicability for any type of antibody drug.
- No antibodies or ligands are needed for capturing, which streamlines method development and helps reduce costs.

### Operating Principle and Features of Kit

The nSMOL Antibody BA kit can be used for all types of antibody drugs and eliminates the need for creating and cross-testing antibodies specifically for detection, which is typically required for detecting monoclonal antibodies. Advantages include a broader dynamic range and much higher selectivity than the ELISA method and the ability to analyze multiple components simultaneously. In terms of operation, performing the pretreatment on filter cups avoids tedious washing operations and samples can be collected easily by centrifuging.

The resin surface, the antibody collection resin, is coated to ensure the Fab fragment side of antibodies is oriented toward the outside. By adding nanoparticles with trypsin solidified on the surface to the antibodies, the trypsin can access the antibodies efficiently for trypsin digestion. The peptides derived from Fab fragments by trypsin digestion can be collected easily by centrifuging.

### Kit Contents

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Quantity</th>
<th>Capacity</th>
<th>Storage Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin collection resin</td>
<td>2</td>
<td>5.0 mL per vial</td>
<td>-80 °C</td>
</tr>
<tr>
<td>Wash solution 1 (Binding solution)</td>
<td>1</td>
<td>80 mL</td>
<td>4 °C</td>
</tr>
<tr>
<td>Wash solution 2</td>
<td>1</td>
<td>80 mL</td>
<td>4 °C</td>
</tr>
<tr>
<td>Reaction solution</td>
<td>1</td>
<td>10 mL</td>
<td>4 °C</td>
</tr>
<tr>
<td>Enhanced reaction solution</td>
<td>1</td>
<td>Freeze-dried</td>
<td>4 °C</td>
</tr>
<tr>
<td>Reaction stop solution</td>
<td>1</td>
<td>1 mL</td>
<td>4 °C</td>
</tr>
<tr>
<td>FG beads Trypsin DART</td>
<td>1</td>
<td>1.1 mL</td>
<td>-20 °C</td>
</tr>
</tbody>
</table>

*Note: The reagent is shipped refrigerated (2 to 8 °C).
*1 For long-term storage of 1 month or more, store at -80 °C.

---

**Fig. 1   Simple Workflow**

1. Add Binding solution
2. Wash: Wash solution 1
3. Wash: Wash solution 2
4. Add Reaction solution + ISTD
5. Selective digestion of Fab region
6. Add reaction solution + ISTD
7. Add binding solution
8. Centrifuge
9. MRM measurement of signature peptides
10. LCMS analysis
11. Collection of Fab-derived peptide
12. Decreases sample complexity
13. Prevents contamination from excess proteolysis

**Fig. 2   Operating Principle of Kit**

- Antibody
- Immunoglobulin collection resin
- Trypsin-immobilized nanoparticle
- Limited Trypsin Access
- Collection of Fab-derived peptide
- MRM measurement of signature peptides
- LCMS analysis
- Centrifuge
- Sample collection
- Fab peptides
- Selective digestion of Fab region
- Wash: Wash solution 1
- Wash: Wash solution 2
- Add Reaction solution + ISTD
- Add binding solution
- Centrifuge
- Fab peptides

---

**Index**
Operating Principle and Features

Shimadzu’s nSMOL method is a revolutionary LC/MS pretreatment method that enables Fab-specific protein decomposition of monoclonal antibodies. It enables the development of methods that do not depend on the type of antibody drug, which represents a paradigm shift for antibody drug analysis. It also satisfies criteria specified in the Guideline on Bioanalytical Method Validation in Pharmaceutical Development (Japanese Ministry of Health, Labour and Welfare). Shimadzu offers methods and protocols optimized for both. This method has been optimized for the Shimadzu LCMS-8050 and LCMS-8060 triple quadrupole mass spectrometers (referred to as “LCMS-8050” and “LCMS-8060 NX” below).

Measurement Method and Conditions

Human blood plasma spiked with trastuzumab was pretreated by the nSMOL method and then Fab-derived peptides were obtained. Then, the content of trastuzumab in the blood plasma was quantitatively analyzed by LC-MS (Table 1). The results were fully validated in accordance with the Japanese Ministry of Health, Labour and Welfare Guideline on Bioanalytical Method Validation in Pharmaceutical Development.

Results

The peptide to be quantified (signature peptide) is selected from trypsin peptide fragments that include complementarity-determining regions (CDRs) that determine antibody specificity. However, even if a peptide contains CDRs, there is no guarantee its sequence is not identical to endogenous IgG. That requires confirming that they do not compete within the biological matrix used. However, given the operating principle of mass spectrometers, they can only obtain basic m/z and signal intensity information. Therefore, a data analysis method able to obtain high-quality and accurate analytical results by simultaneously using quantitative MRM settings for bioanalysis and using MRM monitoring for structural confirmation (Table 2 and Fig. 1) was used.

Conclusion

LC/MS quantitative analysis of antibody drugs in blood plasma 0.0610 to 250 μg/mL lower limit of quantitation) can be performed using the same assay method for everything from preclinical testing to human clinical trials.

Application Examples

• Quantitating monoclonal antibodies in blood serum or blood plasma

LC-MS Bioanalysis of Antibody Drugs by nSMOL Fab-Specific Protein Analysis Method

—Example of Trastuzumab Analysis—

Specifications

<table>
<thead>
<tr>
<th>Model</th>
<th>LCMS-8050</th>
<th>LCMS-8060</th>
<th>LCMS-8060 NX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass range</td>
<td>Mix 2 to 2000</td>
<td>Mix 2 to 2000</td>
<td>Mix 2 to 2000</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>ESI positive</td>
<td>1 pg reserpine, SN = 500,000:1 (RMS)</td>
<td>1 pg reserpine, SN = 1,500,000:1 (RMS)</td>
</tr>
<tr>
<td>ESI negative</td>
<td>1 pg chloramphenicol, SN = 500,000:1 (RMS)</td>
<td>1 pg chloramphenicol, SN = 1,500,000:1 (RMS)</td>
<td></td>
</tr>
<tr>
<td>Resolution</td>
<td>R &lt; 0.7 u (FWHM) and adjustable to 0.5 u</td>
<td>R &lt; 0.7 u (FWHM) and adjustable to 0.5 u</td>
<td>R &lt; 0.7 u (FWHM) and adjustable to 0.5 u</td>
</tr>
<tr>
<td>Mass stability</td>
<td>0.05 u/4 hr</td>
<td>0.05 u/4 hr</td>
<td>0.05 u/4 hr</td>
</tr>
<tr>
<td>Mass accuracy</td>
<td>0.1 u</td>
<td>0.1 u</td>
<td>0.1 u</td>
</tr>
<tr>
<td>Scan speed</td>
<td>Max. 30,000 u/sec</td>
<td>Max. 30,000 u/sec</td>
<td>Max. 30,000 u/sec</td>
</tr>
<tr>
<td>Polarity switching time</td>
<td>5 msec</td>
<td>5 msec</td>
<td>5 msec</td>
</tr>
<tr>
<td>Interface</td>
<td>Standard: ESI</td>
<td>Standard: ESI</td>
<td>Standard: IonFocus (ESI, DUIS)</td>
</tr>
<tr>
<td></td>
<td>Optional: Micro-ESI, APCL DUIS</td>
<td>Optional: Micro-ESI, APCL DUIS</td>
<td>Optional: Micro-ESI, APCL DUIS</td>
</tr>
</tbody>
</table>

Benefits

- UF Technologies provide both maximum sensitivity and maximum speed.
- Due to an ultra-fast 5 msec polarity switching speed, positive and negative ions can be measured simultaneously.
- “Easy Maintenance” features lead to greater uptime.

Characterization

Quality Control
Pharmacokinetics

Evaluation of Antibody Drugs in Blood

LCMS-8050 / 8060 / 8060NX

Table 1 LC-MS Analytical Conditions

<table>
<thead>
<tr>
<th>Flowrate (µL/min)</th>
<th>10 µL</th>
<th>10 µL</th>
<th>10 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column oven</td>
<td>50 °C</td>
<td>50 °C</td>
<td>50 °C</td>
</tr>
<tr>
<td>Column temperature</td>
<td>400 °C</td>
<td>400 °C</td>
<td>400 °C</td>
</tr>
<tr>
<td>Drying gas</td>
<td>10 L/min</td>
<td>10 L/min</td>
<td>10 L/min</td>
</tr>
<tr>
<td>Nebulizer gas</td>
<td>300 °C</td>
<td>300 °C</td>
<td>300 °C</td>
</tr>
<tr>
<td>Interface</td>
<td>DL</td>
<td>DL</td>
<td>DL</td>
</tr>
<tr>
<td>Ionization</td>
<td>ESI Positive</td>
<td>ESI Positive</td>
<td>ESI Positive</td>
</tr>
<tr>
<td>Mass range</td>
<td>m/z 2 to 2000</td>
<td>m/z 2 to 2000</td>
<td>m/z 2 to 2000</td>
</tr>
<tr>
<td>Solvent A</td>
<td>0.1 % formic acid/water</td>
<td>0.1 % formic acid/water</td>
<td>0.1 % formic acid/water</td>
</tr>
<tr>
<td>Solvent B</td>
<td>0.1 % formic acid/acetonitrile</td>
<td>0.1 % formic acid/acetonitrile</td>
<td>0.1 % formic acid/acetonitrile</td>
</tr>
<tr>
<td>Gradient</td>
<td>1.5 min → 95 %</td>
<td>1.5 min → 95 %</td>
<td>1.5 min → 95 %</td>
</tr>
<tr>
<td></td>
<td>4.0 min</td>
<td>4.0 min</td>
<td>4.0 min</td>
</tr>
</tbody>
</table>

Table 2 MRM Transitions of Quantified Peptides in Trastuzumab

<table>
<thead>
<tr>
<th>Peptide MRM transition</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>IYPTNGYTR</td>
<td>For quantitation (IS)</td>
</tr>
<tr>
<td>512.1 &gt; 389.3 (b4+)</td>
<td>For structural confirmation</td>
</tr>
<tr>
<td>542.8 &gt; 404.7 (y7++)</td>
<td>For quantitation</td>
</tr>
<tr>
<td>542.8 &gt; 610.3 (y5+)</td>
<td>For structural confirmation</td>
</tr>
<tr>
<td>542.8 &gt; 808.4 (y7+)</td>
<td>For structural confirmation</td>
</tr>
</tbody>
</table>

Table 3 Full Validation Results

<table>
<thead>
<tr>
<th>Set Concentration (µg/mL)</th>
<th>Data Average (N = 5)</th>
<th>Accuracy (%)</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0610</td>
<td>2.93</td>
<td>98.1</td>
<td>-20</td>
</tr>
<tr>
<td>0.133</td>
<td>2.93</td>
<td>88.1</td>
<td>8.2</td>
</tr>
<tr>
<td>0.510</td>
<td>2.93</td>
<td>104</td>
<td>-20</td>
</tr>
<tr>
<td>0.675</td>
<td>2.93</td>
<td>91.2</td>
<td>5</td>
</tr>
<tr>
<td>2.540</td>
<td>2.93</td>
<td>84.2</td>
<td>200</td>
</tr>
</tbody>
</table>

Fig. 1 MRM Chromatogram of IYPTNGYTR in Human Blood Plasma

![Image]
Evaluating the Concentration of Antibody Drugs in Blood

High-Sensitivity LC/MS Bioanalysis of Trastuzumab by nSMOL

Operating Principle and Features
High-performance liquid chromatograph mass spectrometer (LC-MS) systems enable higher performance analysis by decreasing the flowrate in the LC unit and improving the ionization and ion uptake efficiency in the MS unit. The Shimadzu Nexera Mikros is a micro LC-MS system that reduces the LC flowrate to a micro level (approx. 1 to 10 μL/min). That results in between several times to several tens of times higher sensitivity than the previous semi-micro LC-MS system, while maintaining the same robustness and throughput.

Measurement Method and Conditions
The ELISA ligand-binding assay method was the primary method used to determine the concentration of antibody drugs in the blood. This example describes a quantitative method that is based on using a high-sensitivity LC-MS system. Human blood plasma spiked with a trastuzumab standard and blank blood plasma were analyzed as samples. For all the antibody drugs, Fab-derived peptides were obtained using the nSMOL method, which allowed using the same protocol for all samples. These were analyzed to quantify the concentration of trastuzumab in the blood plasma based on the analytical conditions indicated in Tables 1 to 3.

Conclusion
Using the nSMOL method in combination with the Nexera Mikros system enables high-sensitivity quantitation of antibody drugs in blood without sacrificing throughput.

Table 1  LC Analytical Conditions

Table 2  MS Analytical Conditions

Table 3  MRM Transitions of Quantified Peptides in Trastuzumab

Application Examples
- Analyzing lipid mediators with high sensitivity

Results
Measured concentrations of trastuzumab in blood plasma correlated closely with concentration settings, with an R² value of 0.99 or higher and excellent accuracy and precision. Furthermore, the Nexera Mikros system was also used to confirm that the LLOQ value for trastuzumab in blood plasma is 0.00763 μg/mL. A comparison of MRM chromatograms from the Nexera Mikros system (Fig. 1) and a typical semi-micro LC-MS system (Fig. 2) shows that the Nexera Mikros is able to detect with ample sensitivity concentrations that the semi-micro LC-MS system was not able to detect.

Nexera Mikros

benefits
- Micro-flowrate LC-MS system that offers both high sensitivity and high throughput.
- Easy one-step attachment of analytical columns and connection to the LC-MS ionization interface.

Direct injection system: Small injection volumes make it especially well-suited for analyzing desalted or otherwise pretreated samples.

Trap and elute system: System for increasing sensitivity of semi-micro systems without changing the injection volume or other parameters.

Ionization promotion system: System for increasing negative mode sensitivity by adding an LC-20AD nano unit to each of the two systems above.

Specifications
- Solvent delivery unit: LC-Mikros
- Flowrate range: 1 to 500 μL/min
- System pressure capacity: 80 MPa
- Autosampler: SIL-40C XR
- Injection volume range: 0.1 to 50 μL
- Ionization unit: Micro-ESI 8060 or Micro-ESI 9030
- Column oven: CTO-Mikros
- UF-Link enables connecting/disconnecting columns easily with zero dead volume.

Characterization
Quality Control
Pharmacokinetics

Cell Line Optimization
Culture
Purification
Characterization
Quality Control
Pharmacokinetics

Others
Pharmacokinetics

Operating Principle and Features

Metabolomic analysis using a mass spectrometer generally involves using a gas chromatograph mass spectrometer (GC-MS) or high-performance liquid chromatograph mass spectrometer (LC-MS) to comprehensively analyze all the metabolites (metabolome) contained in a sample. That requires selectively using GC/MS or LC/MS based on the target components being analyzed or the given purpose of analysis, as illustrated in Fig. 1. Using a GC/MS to analyze hydrophilic metabolites such as amino acids, organic acids, or sugars requires a derivatization process, but it often suffers from robustness and can comprehensively analyze hundreds of components in a single analysis. In contrast, an LC/MS can efficiently analyze specific metabolites (up to 100 components) without derivatization, making it well-suited for routine analysis of specific components.

Measurement Method and Conditions

Fresh fecal samples were collected from male C57BL/6J mice raised in a normal environment. 450 μl of a physiological phosphate buffer solution was added to 50 mg of the fecal samples and stirred. Then the supernatant was ultracentrifuged to prepare the supernatant for the metabolite analysis. To analyze the primary metabolites by LC-MS/MS, the filtrate was derivatized to prepare the samples for GC-MS/MS. Meanwhile, to analyze the primary metabolites by LC-MS/MS, the filtrate was diluted ten times with ultrapure water in preparation for LC-MS/MS.

For the GC/MS/MS analysis, 475 components were analyzed simultaneously using an MRM method from the Smart Metabolites Database, which includes MRM information for 475 components, mainly for metabolites included in biological samples. For the LC/MSARMS analysis, a method of ion pair LC-MS/MS and a method of ion pair-free LC-MS/MS were used for analysis in LCMS-8040 and LCMS-8050 systems. The method of ion pair LC-MS/MS is intended for simultaneous analysis of 55 metabolite components important for metabolomic analysis in the life sciences, such as for analyzing the glycolytic system, TCA cycle, pentose phosphate pathway, or amino acids/nucleotides, whereas the method of ion pair-free LC-MS/MS is intended for simultaneous analysis of 97 organic acid and other metabolite components that cannot be analyzed using the method of ion pair LC-MS/MS. Both methods are included in the LCMS/MS Method Package for Primary Metabolites Ver. 2.

Results

The GC/MS/MS analysis detected 100 components, mostly short-chain fatty acids and organic acids. It even detected 17 sugar components that are difficult to analyze by LC or LC/MS/MS (Fig. 2). The ion pair-free method detected 17 components, including mainly amino acids. The ion pair-free method detected 75 components, including amino acids, nucleotides, nucleosides, and organic acids involved in the TCA cycle (Fig. 2). Therefore, it is extremely useful to use both GC/MS/MS and LC/MS/MS for comprehensively analyzing metabolites in fecal samples.

Application Examples

• Simultaneous analysis of metabolites (metabolomics)

Conclusion

Because GC/MS/MS and LC/MS/MS methods target different components, comprehensive analysis of metabolites is enabled by using both methods. Furthermore, by using the MRM database for GC/MS/MS analysis and method packages for LC/MS/MS analysis, comprehensive analysis can be performed by operators who are not very familiar with this analysis. The large amounts of data generated can be interpreted easily by using Shimadzu’s Multi-Omic Data Analysis package to visualize the data. These sample and data analysis methods should be extremely useful not only for researching intestinal flora, but also for metabolomic analysis in a wide variety of other pharmacokinetic applications.

Conclusion

Because GC/MS/MS and LC/MS/MS methods target different components, comprehensive analysis of metabolites is enabled by using both methods. Furthermore, by using the MRM database for GC/MS/MS analysis and method packages for LC/MS/MS analysis, comprehensive analysis can be performed by operators who are not very familiar with this analysis. The large amounts of data generated can be interpreted easily by using Shimadzu’s Multi-Omic Data Analysis package to visualize the data. These sample and data analysis methods should be extremely useful not only for researching intestinal flora, but also for metabolomic analysis in a wide variety of other pharmacokinetic applications.

Application Examples

• Simultaneous analysis of metabolites (metabolomics)

Conclusion

Because GC/MS/MS and LC/MS/MS methods target different components, comprehensive analysis of metabolites is enabled by using both methods. Furthermore, by using the MRM database for GC/MS/MS analysis and method packages for LC/MS/MS analysis, comprehensive analysis can be performed by operators who are not very familiar with this analysis. The large amounts of data generated can be interpreted easily by using Shimadzu’s Multi-Omic Data Analysis package to visualize the data. These sample and data analysis methods should be extremely useful not only for researching intestinal flora, but also for metabolomic analysis in a wide variety of other pharmacokinetic applications.

Application Examples

• Simultaneous analysis of metabolites (metabolomics)
Pharmacokinetics

• High sensitivity analysis of volatile sulfur compounds
• Comprehensive analysis of volatile components

biomarker discovery and other pharmacokinetic applications.

This example confirmed that volatile gases generated from intestinal flora can be analyzed without pretreatment using GC/MS and GC-SCD.

Operating Principle and Features

Microorganisms (flora) in intestines generate a wide variety of volatile substances. Comprehensive analysis of such flora is used for biomarker discovery and other research. Volatile substances can be comprehensively analyzed using a gas chromatograph mass spectrometer in combination with a headspace sampler unit (HS-20 + GCMS-QP2020 NX).

Volatile sulfur compounds can be analyzed with high sensitivity by using the headspace sampler in combination with an SCD detector that detects only sulfur components with high sensitivity (HS-20 + Nexis GC-2030 + SCD-2030).

Measurement Method and Conditions

Fresh fecal samples from both germ-free and flora-intact mice were placed directly into headspace vials. The vials were filled with anaerobic gas, sealed, and left to cultivate for 24 hours. Then the vials were placed in the headspace sampler and the evolved gases were analyzed by GC/MS and GC-SCD. (For detailed analytical conditions, refer to the side link to the title.)

Results

The total ion chromatogram (TIC) from a comprehensive analysis of volatile substances (Fig. 1) and the chromatogram from analyzing sulfur-based volatile substances (Fig. 2) both confirmed that a larger number and quantity of volatile substances were detected from the flora-intact mouse than the germ-free mouse. They also confirmed that GC-SCD analysis can detect and identify sulfur compounds, which can have low peak intensity or overlap with other peaks in GC/MS results. GC/MS data for 121 types of compounds acquired from two samples taken from each of six mice with intact flora was analyzed by principal component analysis using SIMCA 15 multivariate analysis software. In the score plot obtained from the above measurements (Fig. 3), the smallest clusters were formed from samples taken from the same individual. The individual-specific clusters clearly show that the system can detect differences between individuals.

Conclusion

This example confirmed that volatile gases generated from intestinal flora can be analyzed without pretreatment using GC/MS and GC-SCD. GC/MS enables comprehensive analysis, whereas GC-SCD enables analysis of low-concentration sulfur components that are difficult to detect by GC/MS. Using the methods in combination can be useful for biomarker discovery and other pharmacokinetic applications.

Application Examples

• Comprehensive analysis of volatile components
• High sensitivity analysis of volatile sulfur compounds
• Discovery of new biomarkers

HS-20 Trap / GCMS-QP2020 NX
HS-20 / Nexis GC-2030 / SCD-2030

The HS-20 headspace sampler enables high-sensitivity analysis of volatile gases without pretreatment. Using the HS-20 trap mode enables even higher sensitivity.

The GC-SCD system enables selectively analyzing sulfur components with high sensitivity.

**Fig. 1** GCMS Total Ion Chromatogram

**Fig. 2** GC-SCD Total Ion Chromatogram

**Fig. 3** Results (Score Plot) from Multivariate Analysis of Flora-Intact Mouse Analysis Results

**Specifications**

**Instrument**

- HS-20 Trap / GCMS-QP2020 NX
- HS unit: Sample injection methods: Sample loop or adsorbent trap
- GC unit: Oven temperature: Room temperature + 10 to 300 °C (1 °C steps)
- GC-MS: High-speed scan rate: 20,000 u/sec
- Weight: 85 kg for GC-MS main units and 10 kg for auxiliary pump

**HS unit**

- Sample injection methods: Sample loop or adsorbent trap
- Oven temperature: Room temperature + 10 to 300 °C (1 °C steps)
- Weight: 40 kg (HS-20Trap)

**GC unit**

- Oven temperature: Room temperature + 2 to 450 °C
- Carrier gas control: Constant liner velocity, constant pressure, or constant flowrate
- Flow controller pressure: Max. 970 kPa
- Measurement modes: Scan, SIM, and scan/SIM simultaneous measurement
- Weight: 43.5 kg (SPL/FID model)

**GC-MS**

- Mass range: 1.5 to 1090
- Measurement modes: Scan, SIM, and scan/SIM simultaneous measurement
- High-speed scan rate: 20,000 u/sec
- Weight: 27 kg

**Others**

- Flow controller pressure: Max. 970 kPa
- Mass range: 1.5 to 1090
- Measurement modes: Scan, SIM, and scan/SIM simultaneous measurement
- High-speed scan rate: 20,000 u/sec
- Weight: 40 kg (HS-20 Trap)

**Purification/Characterization/Quality Control/Pharmacokinetics**

**References**

- [Reference 1](URL)
- [Reference 2](URL)
- [Reference 3](URL)
Characterization  Quality Control  Pharmacokinetics

Biomarker Discovery

Proﬁling Cancer Cells Using a Benchtop MALDI-TOF MS System

Operating Principle and Features
Compared to quadrupole or magnetic sector mass spectrometers, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometers offer the advantage of both a broad mass measurement range and fast measurement speed. They are especially well-suited for measuring nucleic acids, proteins, and molecules. Through a benchtop system with a compact size, the MALDI-8020 offers world-class resolution and sensitivity levels. In addition, a shorter vacuum evacuation time is achieved by increasing the laser speed and modifying the exhaust system, and a signiﬁcantly shorter measurement time is achieved by increasing stage speed.

Measurement Method
Cancer cells can metastasize throughout the body by means of extracellular vesicles. Therefore, a MALDI-TOF MS system was used to proﬁle differences in the expression level of proteins derived from extracellular vesicles from regular lymph node cells metastasized from colon cancer versus from lymph node cells with elevated chemotherapy resistance. Protein was collected from the extracellular vesicles obtained from cultivated cells and then the MALDI-8020 system was used to obtain a mass spectrum. Alpha-cyano-4-hydroxycinnamic acid (CHCA) was used as a matrix. eMSTAT Solution was used to analyze the resulting mass spectrum by multivariate analysis (Fig. 1).

Results
Components derived from the protein were detected in the m/z 2,000 to 25,000 range of the extracellular vesicle mass spectrum. Multivariate analysis score plot results discriminated between groups with resistance versus groups with sensitivity to the drug. From the peak matrix, peaks that characterized chemotherapy resistance were detected in the m/z 2,000 to 7,000 range (Fig. 2).

Conclusion
Using the MALDI-8020 system in combination with statistical analysis software shows its potential for use in biomarker discovery research. This type of protein proﬁling method can be expected to be useful for less invasive cancer diagnosis or for monitoring chemotherapy.

Application Examples (Shimadzu Application News No.)
- Profiling protein expression in tissue
- Analysis of tumors and cell aggregates (B105)
- Monitoring the primary structures of proteins (B105)
- Can search a wide range of molecular weights for nucleic acids, proteins, and molecules.

MALDI-8020
- Enables rapid and highly sensitive benchtop proﬁling.
- Easy maintenance and low running costs
- Can search a wide range of molecular weights for nucleic acids, proteins, and molecules.

<table>
<thead>
<tr>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Instrument</strong></td>
</tr>
<tr>
<td><strong>Mass range</strong></td>
</tr>
<tr>
<td><strong>Mass resolution</strong></td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
</tr>
<tr>
<td><strong>Mass accuracy</strong></td>
</tr>
<tr>
<td><strong>Acceleration voltage</strong></td>
</tr>
<tr>
<td><strong>Laser</strong></td>
</tr>
<tr>
<td><strong>Flight distance</strong></td>
</tr>
<tr>
<td><strong>Detector</strong></td>
</tr>
<tr>
<td><strong>Ion source cleaning</strong></td>
</tr>
<tr>
<td><strong>Sample plate</strong></td>
</tr>
<tr>
<td><strong>Operating noise</strong></td>
</tr>
<tr>
<td><strong>Main unit power supply</strong></td>
</tr>
<tr>
<td><strong>Dimensions</strong></td>
</tr>
<tr>
<td><strong>Weight</strong></td>
</tr>
<tr>
<td><strong>Operating environment</strong></td>
</tr>
<tr>
<td><strong>Data analysis software</strong></td>
</tr>
</tbody>
</table>

Discriminant analysis results (Group, Score) superimpose points for unknown samples on a score plot.

<table>
<thead>
<tr>
<th>Input/Output data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Input</strong></td>
</tr>
<tr>
<td><strong>Output</strong></td>
</tr>
</tbody>
</table>
TORAST-H Series

Shimadzu original low adsorption glass vial
TORAST-H Glass Vial

- Low adsorption glass vial suitable for long-term storage.
- Minimized adsorption of bases, acids and neutrals
- Superior quality control

Adsorption Test for Long-term Storage

When a sample is stored in a general vial for a long time, the sample may adsorb into the surface of the vial, causing the reproducibility to be poor. The TORAST-H Glass Vial contains low adsorption characteristics that makes it excellent for long-term sample storage.

<table>
<thead>
<tr>
<th>Product</th>
<th>Details</th>
<th>Cap</th>
<th>SN</th>
<th>Volume (μL)</th>
<th>Qty</th>
<th>P/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>TORAST-H Glass Vial</td>
<td>Clear glass with a label (diameter 9-425)</td>
<td>Screw</td>
<td>No</td>
<td>1.5 mL</td>
<td>100</td>
<td>370-04300-01</td>
</tr>
<tr>
<td></td>
<td>Clear glass with a label (diameter 9-425)</td>
<td>Black</td>
<td>Yes</td>
<td>1.5 mL</td>
<td></td>
<td>370-04300-02</td>
</tr>
<tr>
<td>Screw Cap for</td>
<td>Polypropylene vial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>370-04310-01</td>
</tr>
<tr>
<td>TORAST-H Glass Vial</td>
<td>Polypropylene vial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>370-04310-02</td>
</tr>
</tbody>
</table>

Shimadzu original low adsorption polypropylene vial
TORAST-H Bio Vial

- Extremely low adsorption of peptides
- Extremely low adsorption of basic compounds
- User-friendly design

Adsorption Test Using Trypsin Digestion Products of Myoglobin (approx. 1.9 pmol/mL)

The results confirmed the phenomenon that highly polar peptides with retention times detected between approx. 7 and 8 minutes mostly adsorb to glass vials, whereas highly hydrophobic peptides with retention times detected between approx. 12 and 16 minutes mostly adsorb to polypropylene (PP) vials.

Exterior Design that Enables Using Vials Directly for Flash Centrifugation

If using a Shimadzu i-Series LC system, use the special vial detection plate (P/N: 228-51891-03) shown to the right.
Spectrofluorophotometer

**RF-6000**

**benefits**
- Highest S/N Ratio in its class: 1,000 or more (RMS) / 350 or more (P-P)
- High-speed scanning of 60,000 nm/min minimizes scan time.
- 2,000 hour long-life Xenon lamp
- Spectrum-Corrected Excitation and Emission spectra can be scanned.

**Fluorescent Dyes for DNA Detection**

Specified complementary DNA can be detected by using a DNA probe which is marked by fluorochrome. These probes become luminescent when bonded to DNA.

The following shows the results of a 3D measurement of DNA marked by two different kinds of DNA probes. Unique fluorescent peaks and profiles (3D Emission-Excitation matrix, as shown below) can be quickly measured using the high-speed scanning function.

**Analytical Balances**

**AP Series**

**benefits**
- Supports LabSolutions Balance chromatography data integrity.
- Increases productivity with the fastest response performance in its class.
- Using the internal windbreak plate in combination with a STABLO-AP ionizer ensures reliable results.

**Integrated Management of Analytical Data via a Network System Using LabSolutions**

**Using the Internal Windbreak Plate in Combination with a STABLO-AP Ionizer Ensures Reliable Results**

Internal Windbreak Plate

The internal windbreak plate suppresses the influence of convection and airflow within the weighing chamber, improving the stability and response of measurement values.

STABLO-AP Ionizer

Sweeps away static electricity from containers and samples! It can be used as an external stand configuration or installed inside the balance unit.

Minimum display value: 0.1 mg or 0.01 mg
References:

### Culture

p. 12, Monitoring of Metal Elements in Cell-Culture Supernatant using Atomic Absorption Spectrometry
1) Jin H Suk et al., Biotecnology Progress, 30, 429-442 (2016)
2) Paletta et al., Applied Microbiology and Biotechnology, 100, 5999-6009 (2018)
3) Application News A634 “Direct Analysis of Metallic Elements in Cell Culture Medium by Atomic Absorption Spectrophotometry (AAS)”

p. 14, Simultaneous Analysis of Components in CHO Cell Culture Supernatant for Optimization of the Culture Process

### Quality Control


p. 30, N-Linked Glycan Analysis Using MALDI-MSI—Structural Analysis and identification of Sialyl Linkage Isomers

p. 40, Characterization of Insoluble Subvisible Particles in Biopharmaceuticals Using the Rios Imaging Method

p. 44, Analysis by ICP Mass Spectrometry Specified in the ICH-Q3D Guideline for Elemental Impurities
1) Guideline for Elemental Impurities in Drug Products (IP&G Guideline Notification No. 4, September 30, 2015)
2) GUIDELINE FOR ELEMENTAL IMPURITIES Q3D(2011)
Note: Currently, biopharmaceuticals are not subject to ICH Q3D.

### Pharmacokinetics

p. 48, LC/MS Bioanalysis of Antibody Drugs by nSMOL Fab-Specific Protein Analysis Method—Example of Trastuzumab Analysis—
1) Isao Inoue et al., Analyst, DOI: 10.1039/c3an02104a
2) Inoue N et al., Anal Methods, DOI: 10.1039/c3ay50158b

p. 50, High-Sensitivity LC/MS Bioanalysis of Trastuzumab by nSMOL
1) Isao Inoue et al., Analyst, DOI: 10.1039/c3ay50158b
2) Inoue N et al., Anal Methods, DOI: 10.1039/c3ay50158b
3) Application News C145A

p. 52, Comprehensive Analysis of All Metabolites Using GC/MS and LC/MS for Researching Intestinal Bacteria

p. 56, Profiling Cancer Cells Using a Benchtop MALDI-TOF MS System
1) Stäberger, G. et al., Analytical Chemistry, 90, 13178-13182 (2018)