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C10G-E054

Solutions for

Biopharmaceutical

Application Notebook

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Application Notebook

Index

Bioanalysis

LCMS Bioanalysis of Antibody Drugs Using Fab-Selective Proteolysis nSMOL - Trastuzumab analysis -

nSMOL, which enables selective proteolysis of the Fab region of monoclonal antibodies, was applied to Trastuzumab bioanalysis.

LCMS Bioanalysis of Antibody Drugs Using Fab-Selective Proteolysis nSMOL- Part 2 - Bevacizumab analysis -

nSMOL bioanalysis for Bevacizumab fulfilled the full validation criteria, same as Trastuzumab analysis.

LCMS Bioanalysis of Antibody Drugs Using Fab-Selective Proteolysis nSMOL - Part 3 - Nivolumab analysis -

nSMOL analysis applied to analytical validation of Nivolumab for the pharmacokinetic monitoring into early clinical implementations.

LCMS Bioanalysis of Antibody Drugs Using Fab-Selective Proteolysis nSMOL - Part 4 - Multiplex Analysis -

By utilizing the nSMOL technique, it is possible to detect many antibody drugs and create calibration curves of multiple antibody drugs in a single analysis.

nSMOL Antibody BA Kit [Brochure]

LC/MS/MS Sample Prep Kit for Quantitative Analysis of Monoclonal Antibodies.

Development of MRM Methods for Monoclonal Antibodies Using Skyline

LC/MS/MS, simple integration with Skyline software accelerates optimized method development and MRM analysis for antibody drugs.

Amino Acid and Media Analysis

Simultaneous Analysis of Culture Supernatant of Mammalian Cells Using Triple Quadrupole LC/MS/MS

To analyze media components, Cell Culture Profiling method package was applied to optimized analytical condition.

LC/MS/MS Method Package for Cell Culture Profiling [Brochure]

It provides simultaneous analysis conditions for 95 components.

N-Terminal Amino Acid Sequencing of IgG Antibodies

This article introduces an example of amino acid sequencing of mouse antibody IgG using the PPSQ51A/53A Protein Sequencer.

LC/MS/MS Method Package for D/L Amino Acids [Brochure]

With this method package, high-sensitivity analysis can be performed in a short period of time, bringing efficiency to the chiral separations laboratory.

Peptide Analysis

Peptide Mapping of Antibody Drugs by Nexera-i

Nexera-i was applied and produced reproducible result, which is important for the analysis of tryptic digest.

Glycan Analysis

A Study on a Method for Evaluating Glycans in Biopharmaceuticals

The results of studying a method for releasing O-glycans chemically in which the peeling reaction is suppressed, based on a PMP labeling method were reported.

High-Sensitivity Analysis of 2-AB Glycans by RF-20Axs Florescence Detector

2-aminobenzamide-labelled glycan (2-AB glycan) was analyzed using fluorescence detection, which provides selective and sensitive analysis result.

Software Platform for Glycan Quantification and Qualification by LCMS-8060/8050 - Erexim Application Suite [Brochure]

Erexim Application Suite is designed to facilitate the analysis of site-specific glycan heterogeneity by providing customizable ready-to-use methods and automated data analysis.

Protein Analysis and Aggregation Evaluation

Detection of High-mass Proteins Using a Benchtop MALDI-TOF Mass Spectrometer

A benchtop MALDI-TOF mass spectrometer was applied to perform high-throughput protein detection with high sensitivity.

Detection of Protein Aggregates: Detection of Multimeric Proteins Using MALDI-TOF MS with a High Mass Detector

In this article, we describe the detection of protein aggregates using MALDI-TOF MS that is capable of detecting high mass molecules.

Additives Analysis

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Application News

No. C145B

nSMOL™ Antibody BA Kit

LCMS Bioanalysis of Antibody Drugs Using Fab-Selective Proteolysis nSMOL - Trastuzumab analysis -

nSMOL™ Antibody BA Kit Features

nSMOL is Shimadzu's completely new and breakthrough technology that enables selective proteolysis of the Fab region of monoclonal antibodies. This technique facilitates method development independent of a variety of antibody drugs and achieves a paradigm shift in the bioanalysis of antibody drugs.

Furthermore, this is the only method with respect to antibody drugs that has fulfilled the criteria of "Guideline on Bioanalytical Method Validation in Pharmaceutical Development" for low MW drug compounds issued by the Japanese Ministry of Health, Labour and Welfare. Shimadzu also offers optimization methods and protocols, and nSMOL can be applied to clinical research at various institutions.

LCMS Bioanalysis Solved with the nSMOL Method

Pharmacokinetic information provides some of the most fundamental indicators. The effective drug discovery is supported by the overall pharmacokinetic profile such as drug efficacy and toxicity.

While the enzyme-linked immunosorvent assay (ELISA) has been the current way to measure blood concentration until now, there are essential issues due to the effects of cross-reactivity and inhibitory substances. On the other hand, mass spectrometry may be able to solve these issues because of structure-indicated analysis.

Nevertheless, mass spectrometry has several issues. In particular, direct quantitation analysis (top-down proteomics) of complex matrices, such as plasma, is not suitable for repeat analysis because the ESI interface cannot be maintained due to the large excess analytes.

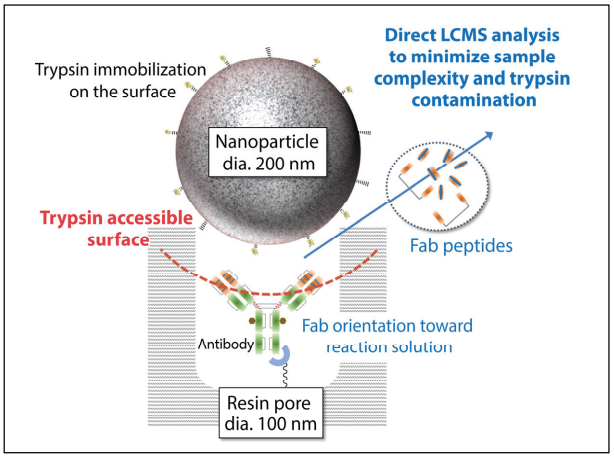
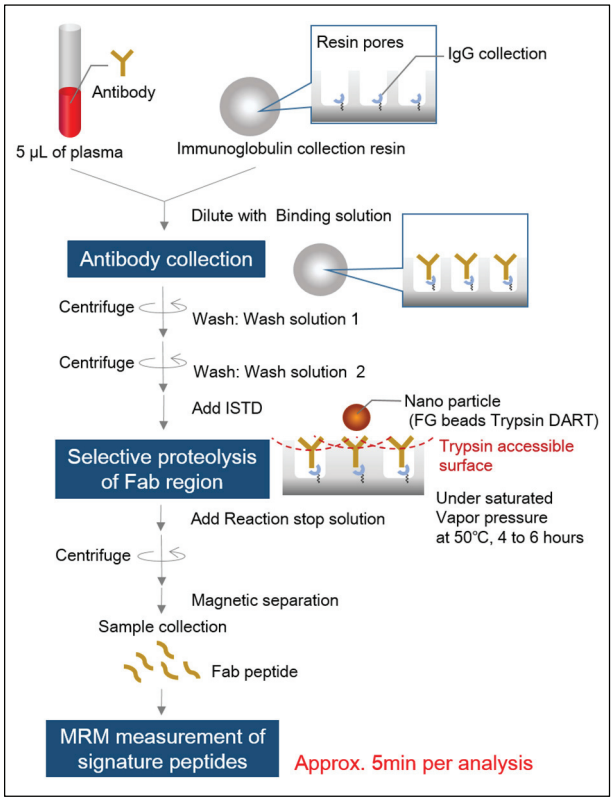


Fig. 1 Principle of the nSMOL Technique

Sample Processing Protocol and Analysis Conditions for Trastuzumab Using the nSMOL

<Sample Processing Protocol>

In the nSMOL protocol, the same sample processing protocol can be applied to all antibody drugs. The procedure is described below.



<LCMS Analysis Conditions>

[LC] NexeraX2 System

Column	: Shim-pack GISS C18 (50 mm × 2.1 mm)
Column oven	: 50 °C
Solvent A	: 0.1 % formic acid/water
Solvent B	: 0.1 % formic acid/acetonitrile
Gradient	: 1 %B (1.5 min)/1-25 %B (3.5 min)/ 95 %B (1 min)/1 %B (1 min)
Flow rate	: 0.4 mL/min
Injection	: 10 µL

[MS] LCMS-8050, 8060

Ionization	: ESI Positive
DL	: 250 °C
Heat Block	: 400 °C
Interface	: 300 °C
Nebulizer gas	: 3 L/min
Drying gas	: 10 L/min
Heating gas	: 10 L/min

Quantitation Peptides of Trastuzumab

Peptide	MRM transition	Purpose
$P_{14}R$	512.1>292.3 (b3+)	For quantitation (IS)
	512.1>389.3 (b4+)	For structure confirmation
	512.1>660.4 (b6+)	For structure confirmation
IYPTNGYTR	542.8>404.7 (y7++)	For quantitation
	542.8>808.4 (y7+)	For structure confirmation
	542.8>610.3 (y5+)	For structure confirmation

* Quantitation range in human plasma : 0.0610 to 250 µg/ml
Averaged accuracy : 100.7 %

The quantitation peptide (signature peptide) is selected from tryptic peptides that contain a complementarity-determining region (CDR) with antibody specificity. However, there is not necessary that the CDR-containing peptide does not have the same amino acid sequence in the endogenous IgGs. For this reason, it should be confirmed that there is no competition with the signature peptide in the biological matrix.

Furthermore, in principle, mass spectrometry can only access the *m/z* and signal intensity. Accordingly, Shimadzu recommends the setting of structure confirmation MRM transition in addition to quantitative MRM in each bioanalysis. This ensures reliable and high quality analysis.

MRM Chromatograms and Calibration Curves

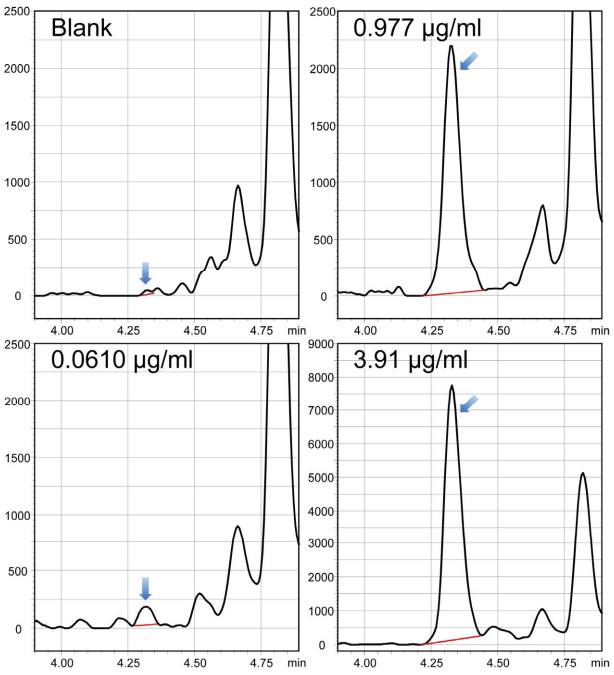


Fig. 2 MRM Chromatogram of Trastuzumab IYPTNGYTR in Human Plasma

Full Validation Results for Trastuzumab

<Precision and accuracy>

Set Concentration [µg/ml]	Data Average (N = 15)	Accuracy (%)	CV (%)
2.93	2.58	88.1	8.2
200	211	106	5.6

<Freeze-thaw test>

Set Concentration [µg/ml]	Data Average (N = 5)	Accuracy (%)	Temperature (°C)
2.93	2.87	98.1	-20
200	199	99.7	-20

<Long-term stability test>

Set Concentration [µg/ml]	Data Average (N = 5)	Accuracy (%)	Temperature (°C)
2.93	3.03	104	-20
200	203	101	-20

<Processed sample stability for 48 h>

Set Concentration [µg/ml]	Data Average (N = 5)	Accuracy (%)	Temperature (°C)
2.93	3.67	91.2	5
200	211	106	5

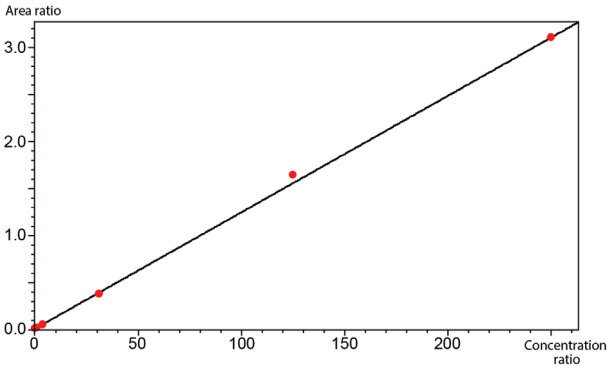


Fig. 3 Calibration Curve of Trastuzumab

Observations, Conclusions, and References

The nSMOL fulfills the guideline criteria for small molecule drug compounds and enabled quantitative analysis of Trastuzumab in human plasma.

The lower limit of quantitation is 0.06 µg/ml and the same assay method can be used from preclinical to clinical testing.

The nSMOL assay described here succeeded in shortening the analysis time by significantly decreasing the noise matrix.

<References>
Iwamoto N et al. *Analyst*, 2014, DOI:10.1039/c3an02104a
Iwamoto N et al., *Anal Methods*, 2015, DOI:10.1039/c5ay01588j
<Chief Scientists>
Noriko Iwamoto, Ph.D. and Takashi Shimada, Ph.D., Technology Research Laboratory

Notes: The product described in this document has not been approved or certified as a medical device under the Pharmaceutical and Medical Device Act of Japan.
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Application News

No. C146A

nSMOL™ Antibody BA Kit

LCMS Bioanalysis of Antibody Drugs Using Fab-Selective Proteolysis nSMOL- Part 2 - Bevacizumab analysis -

nSMOL™ Antibody BA Kit Features

nSMOL is Shimadzu's completely new and breakthrough technology that enables selective proteolysis of the Fab region of monoclonal antibodies. This technique facilitates method development independent of a variety of antibody drugs and achieves a paradigm shift in the bioanalysis of antibody drugs.

Furthermore, this is the only method with respect to antibody drugs that has fulfilled the criteria of "Guideline on Bioanalytical Method Validation in Pharmaceutical Development" for low MW drug compounds issued by the Japanese Ministry of Health, Labour and Welfare. Shimadzu also offers optimization methods and protocols, and nSMOL can be applied to clinical research at various institutions.

Antibody Drug Classification and Selection of Quantitation Peptides

Monoclonal antibodies are produced from the hybridoma with mouse spleen lymphocyte and myeloma cells. While mice are predominantly used as hosts, in recent years a variety of hosts are now available to produce monoclonal antibodies.

Furthermore, production of the variable Fv by phage display technology and high-throughput screening of affinity sequence has become alternative standard procedure.

Antibody drugs are classified into four classes according to specific structure.

The complementarity-determining region (CDR) of antibody specificity against human IgGs becomes smaller according to the "mouse" → "chimeric" → "humanized" → "fully human" antibody. More precise selection of quantitation peptides becomes particularly important in the nSMOL, which is used to perform structure specificity-indicated analysis.

The nSMOL enables selective proteolysis in variable regions. This allows selection of quantitation peptides that reflect the structural characteristics of antibodies. Antibodies have three CDRs on each heavy and light chain, CDR2 is known as the region that makes first contact with an antigen. The signature peptide by nSMOL are mainly from CDR2 containing peptides.

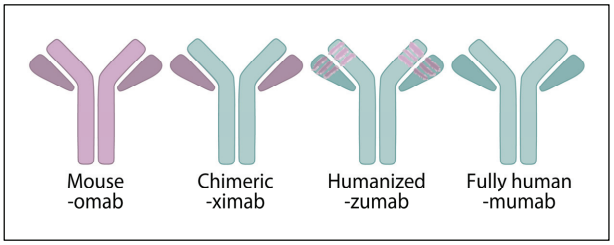

Fig. 1 Antibody Drug Classification

Analysis Conditions for Bevacizumab Using the nSMOL

<Sample Processing Protocol>

With the nSMOL technique, the same sample processing protocol can be applied to all antibody drugs. For details, refer to Shimadzu Application News (Trastuzumab analysis).

<LCMS Analysis Conditions>

[LC] NexeraX2 System

Column	: Shim-pack GISS C18 (50 mm × 2.1 mm)
Column oven	: 50 °C
Solvent A	: 0.1 % formic acid/water
Solvent B	: 0.1 % formic acid/acetonitrile
Gradient	: 1 %B (1.5 min)/1-35 %B (3.5 min)/ 95 %B (1 min)/1 %B (1 min)
Flow rate	: 0.4 mL/min
Injection	: 10 µL

[MS] LCMS-8050, 8060

Ionization	: ESI Positive
DL	: 250 °C
Heat Block	: 400 °C
Interface	: 300 °C
Nebulizer gas	: 3 L/min
Drying gas	: 10 L/min
Heating gas	: 10 L/min

Bevacizumab Quantitation Peptides

Peptide	MRM transition	Purpose
$P_{14}R$	512.1>292.3 (b3+)	For quantitation (IS)
	512.1>389.3 (b4+)	For structure confirmation
	512.1>660.4 (b6+)	For structure confirmation
FTFSLDTSK	523.3>797.4 (y7+)	For quantitation
	523.3>898.5 (y8+)	For structure confirmation
	523.3>650.3 (y6+)	For structure confirmation
STAYYLQMN SLR	642.3>748.4 (y6+)	For quantitation
	642.3>861.5 (y7+)	For structure confirmation
	642.3>620.3 (y5+)	For structure confirmation
VLIYFTSSLH SGVPSR	588.3>775.9 (y14++)	For quantitation
	588.3>602.3 (y6+)	For structure confirmation
	588.3>939.5 (y9+)	For structure confirmation

* Quantitation range in human plasma : 0.15 to 300 µg/ml
Averaged accuracy : 101.3 %

MRM Chromatograms

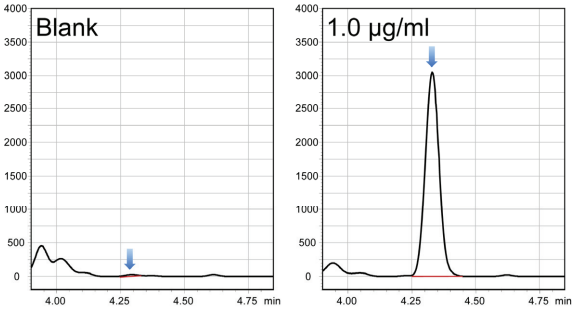

Fig. 2 MRM Chromatograms of FTFSLDTSK (in Human Plasma)

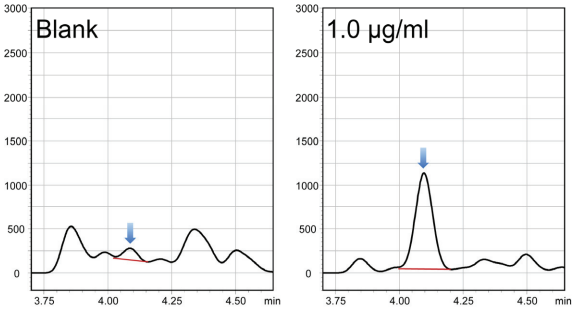

Fig. 3 MRM Chromatograms of STAYYLQMNSLR (in Human Plasma)

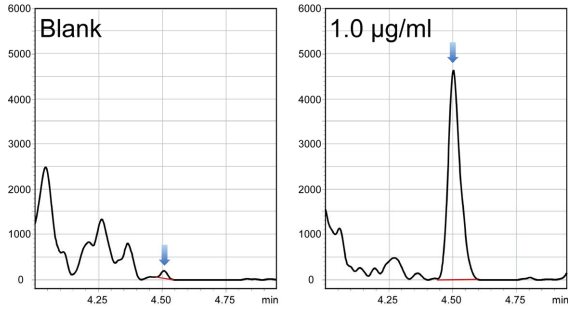

Fig. 4 MRM Chromatograms of VLIYFTSSLHSGVPSR (in Human Plasma)

Full Validation Results for Bevacizumab

<Precision and accuracy>

Set Concentration [µg/ml]	Data Average (N = 15)	Accuracy (%)	CV (%)
0.439	0.464	106	11.7
240	235	98.1	4.45

<Freeze-thaw test>

Set Concentration [µg/ml]	Data Average (N = 5)	Accuracy (%)	Temperature (°C)
0.439	0.395	89.9	-20
240	253	106	-20

<Long-term stability test>

Set Concentration [µg/ml]	Data Average (N = 5)	Accuracy (%)	Temperature (°C)
0.439	0.477	109	-20
240	223	93.1	-20

<Processed sample stability for 48 h>

Set Concentration [µg/ml]	Data Average (N = 5)	Accuracy (%)	Temperature (°C)
0.439	0.445	101	5
240	245	102	5

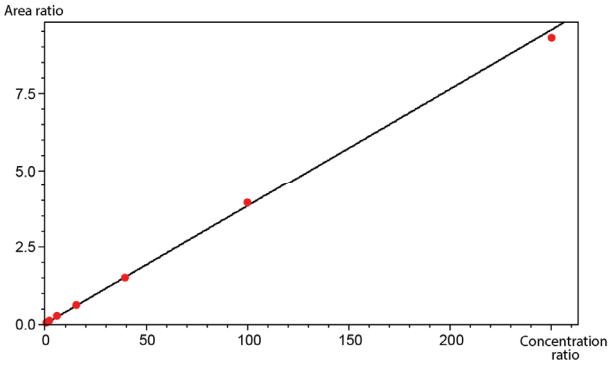

Fig. 5 Calibration Curve of Bevacizumab

Observations, Conclusions, and References

Although Bevacizumab quantitation peptide using nSMOL was obtained from the same region of Trastuzumab, the optimal peptide sequences for bioanalysis will depend on the interference with endogenous IgGs.

With respect to multiplexed quantitation of three sequences, nSMOL bioanalysis for Bevacizumab fulfilled the full validation criteria.

The lower limit of quantitation is 0.15 µg/ml and the same assay method can be used from preclinical to clinical trials.

<References>
Iwamoto N et al. *Analyst*, 2014, DOI:10.1039/c3an02104a
Iwamoto N et al., *Drug Metab Pharmacokinet*., 2016, DOI:10.1016/j.dmpk.2015.11.004
<Chief Scientists>
Noriko Iwamoto, Ph.D. and Takashi Shimada, Ph.D., Technology Research Laboratory

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Application News

No. C147A

nSMOL™ Antibody BA Kit

LCMS Bioanalysis of Antibody Drugs Using Fab-Selective Proteolysis nSMOL - Part 3 - Nivolumab analysis -

nSMOL™ Antibody BA Kit Features

nSMOL is Shimadzu's completely new and breakthrough technology that enables selective proteolysis of the Fab region of monoclonal antibodies. This technique facilitates method development independent of a variety of antibody drugs and achieves a paradigm shift in the bioanalysis of antibody drugs.

Furthermore, this is the only method with respect to antibody drugs that has fulfilled the criteria of "Guideline on Bioanalytical Method Validation in Pharmaceutical Development" for low MW drug compounds issued by the Japanese Ministry of Health, Labour and Welfare. Shimadzu also offers optimization methods and protocols, and nSMOL can be applied to clinical research at various institutions.

Method Validation for Nivolumab Bioanalysis

Cancer cells have been found to evade immune surveillance mechanism through the expression of immunosuppressive ligands, and avoid cytotoxicity from immune cells.

Nivolumab was developed by Dr. Honjo et al. as a breakthrough medicine that activate immune cells by blocking PD-1 mediated inhibitory signals*. Innovative drugs that apply these immunological mechanisms are named as immune checkpoint inhibitors, and many drug discovery for this field now continue to progress around the world.

These medicines are used in a cancer chemotherapy which act on advanced and complex immunological mechanisms. Therefore, it is important to progress integrative clinical trials in order to develop more efficient treatments by using many clinical indexes and biomarkers.

Shimadzu has applied the nSMOL and performed analytical validation of Nivolumab for the pharmacokinetic monitoring into early clinical implementations.

Quantitation Peptides of Nivolumab

Peptide	MRM transition	Purpose
$P_{14}R$	512.1>292.3 (b3+)	For quantitation (IS)
	512.1>389.3 (b4+)	For structure confirmation
	512.1>660.4 (b6+)	For structure confirmation
ASGITFSNSG MHWVR	550.8>661.5 (y11++)	For quantitation
	550.8>746.4 (y13++)	For structure confirmation
	550.8>785.4 (y6 +)	For structure confirmation

* Quantitation range in human plasma : 0.15 to 300 µg/ml
Averaged accuracy : 100.4 %

Analysis Conditions for Nivolumab Using the nSMOL

<Sample Processing Protocol>

With the nSMOL, the same sample processing protocol can be applied to all antibody drugs. For details, refer to Shimadzu Application News (Trastuzumab analysis).

<LCMS Analysis Conditions>

[LC] NexeraX2 System

Column	: Shim-pack GISS C18 (50 mm × 2.1 mm)
Column oven	: 50 °C
Solvent A	: 0.1 % formic acid/water
Solvent B	: 0.1 % formic acid/acetonitrile
Gradient	: 1 %B (1.5 min)/1-40 %B (3 min)/ 95 %B (1 min)/1 %B (1 min)
Flow rate	: 0.4 mL/min
Injection	: 10 µL

[MS] LCMS-8050, 8060

Ionization	: ESI Positive
DL	: 250 °C
Heat Block	: 400 °C
Interface	: 300 °C
Nebulizer gas	: 3 L/min
Drying gas	: 10 L/min
Heating gas	: 10 L/min

Structure Configuration of Nivolumab Candidate Signature Peptides Identified in nSMOL Reactions

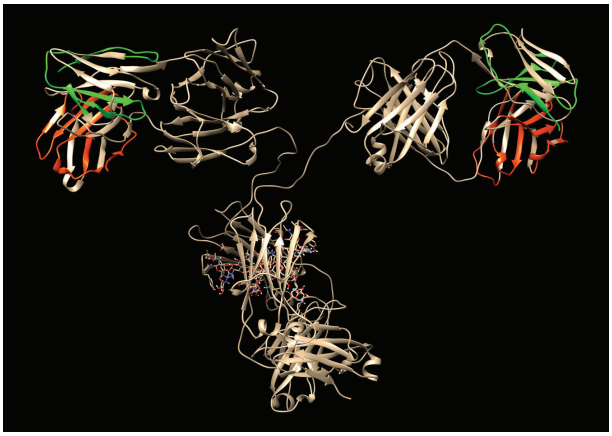

Fig. 1 Structure Configuration of Nivolumab Tryptic Peptides

Detected peptides are indicated in red (heavy chain) and green (light chain). Fv-selective proteolysis has been progressing by nSMOL.

MRM Chromatograms

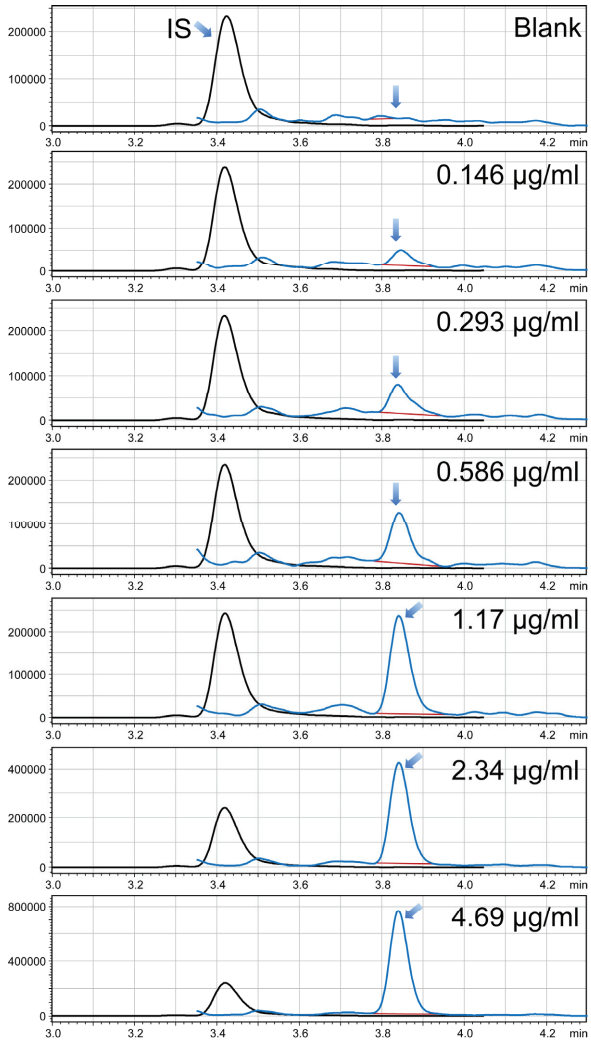

Fig. 2 MRM Chromatograms of ASGITFSNSGMHWVR (Blue), and $P_{14}R$ Internal Standard (Black) (in Human Plasma)

Full Validation Results for Nivolumab

<Precision and accuracy>

Set Concentration [µg/ml]	Data Average (N = 15)	Accuracy (%)	CV (%)
2.93	2.97	101	7.51
200	202	101	6.75

<Freeze-thaw test>

Set Concentration [µg/ml]	Data Average (N = 5)	Accuracy (%)	Temperature (°C)
2.93	2.73	95.6	-20
200	183	96.1	-20

<Long-term stability test>

Set Concentration [µg/ml]	Data Average (N = 5)	Accuracy (%)	Temperature (°C)
2.93	3.03	104	-20
200	213	107	-20

<Processed sample stability for 48 h>

Set Concentration [µg/ml]	Data Average (N = 5)	Accuracy (%)	Temperature (°C)
2.93	3.08	105	5
200	195	97.6	5

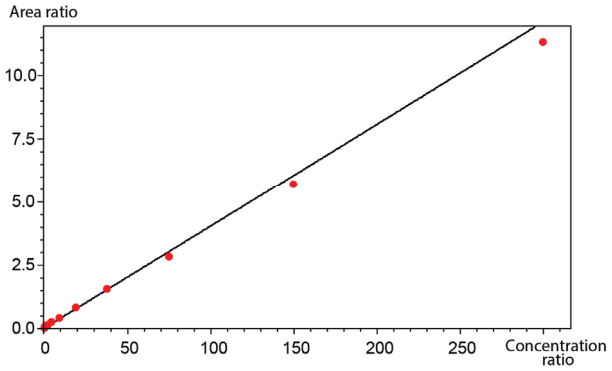

Fig. 3 Nivolumab Calibration Curve

Observations, Conclusions, and References

Although eight candidate signature peptides including CDRs were obtained using nSMOL, only the peptide ASGITFSNSGMHWVR indicated a positive correlation to drug concentration. This indicates that the sequence homology of fully human antibodies and endogenous IgGs is extremely similar.

In order to set suitable bioanalysis conditions, peptide candidates with structural specificity must be strictly selected. By utilizing Fv-selective reactions, Shimadzu nSMOL greatly facilitates the development of assay methods. The lower limit of quantitation is 0.15 µg/ml and the same assay method can be used from preclinical to clinical trials.

<References>
* Ishida Y, Agata Y, Shibahara K, and Honjo T., *EMBO J*, 1992, 11(11):3887
Iwamoto N et al. *Analyst*, 2014, DOI:10.1039/c3an02104a
Iwamoto N et al., *J. Chromatogr. B*, 2016, DOI:10.1016/j.jchromb.2016.04.038
<Chief Scientists>
Noriko Iwamoto, Ph.D. and Takashi Shimada, Ph.D., Technology Research Laboratory

Notes: The product described in this document has not been approved or certified as a medical device under the Pharmaceutical and Medical Device Act of Japan.
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Application News

No. C148A

nSMOL™ Antibody BA Kit

LCMS Bioanalysis of Antibody Drugs Using Fab-Selective Proteolysis nSMOL - Part 4 - Multiplex Analysis -

nSMOL™ Antibody BA Kit Features

nSMOL is Shimadzu's completely new and breakthrough technology that enables selective proteolysis of the Fab region of monoclonal antibodies. This technique facilitates method development independent of a variety of antibody drugs and achieves a paradigm shift in the bioanalysis of antibody drugs.

Furthermore, this is the only method with respect to antibody drugs that has fulfilled the criteria of "Guideline on Bioanalytical Method Validation in Pharmaceutical Development" for low MW drug compounds issued by the Japanese Ministry of Health, Labour and Welfare. Shimadzu also offers optimization methods and protocols, and nSMOL can be applied to clinical research at various institutions.

Antibody Drug Classification and Quantitation Peptide Selection

The development of antibody drugs, which are molecular target drugs, has progressed dramatically in recent years and most Fc regions has a structure derived from human IgG. The nSMOL enables collection of IgG fractions in plasma via Fc regions, and selective proteolysis on Fv of antibody drugs using trypsin immobilized on the surface of nanoparticles. This reaction field allows selection of quantitation peptides that reflect the structural characteristics of antibodies. Antibodies have three CDRs respectively on each heavy and light chain, and the collected peptides using the nSMOL are mainly peptides including CDRs.

In recent years, development of technology capable of quantifying many items of antibodies in a single analysis is required for due to the variety of antibody drugs and combination therapy. The nSMOL supports multiplex analysis and can quantify many antibodies in a single analysis with high precision because subject molecules of nSMOL are all IgGs in plasma. This indicates that the nSMOL can be applied in antibody pharmacokinetics for combination therapy.

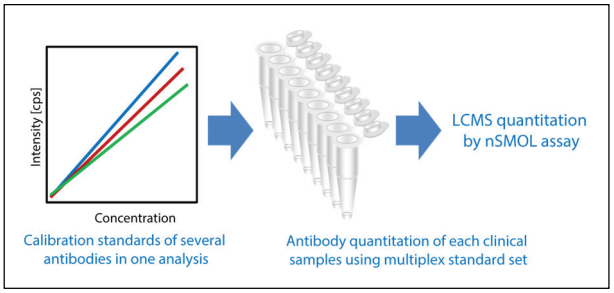

Fig. 1 Multiplex Analysis Using nSMOL

Conditions of Multiplex Analysis Using the nSMOL

<Sample Processing Protocol>

With the nSMOL, the same sample processing protocol can be applied to all antibody drugs. For details, refer to Shimadzu Application News (Analysis Example of Trastuzumab).

<LCMS Analysis Conditions>

[LC] NexeraX2 System

Column	: Shim-pack GISS C18 (50 mm × 2.1 mm)
Column oven	: 50 °C
Solvent A	: 0.1 % formic acid/water
Solvent B	: 0.1 % formic acid/acetonitrile
Gradient	: 1 %B (1.5 min)/1-42 %B (4 min)/ 95 %B (1 min)/1 %B (1 min)
Flow rate	: 0.4 mL/min
Injection	: 10 µL

[MS] LCMS-8050, 8060

Ionization	: ESI Positive
DL	: 250 °C
Heat Block	: 400 °C
Interface	: 300 °C
Nebulizer gas	: 3 L/min
Drying gas	: 10 L/min
Heating gas	: 10 L/min

Quantitation Peptides for Multiplex Analysis

Peptide	MRM transition	Purpose
$P_{14}R$	512.1>292.3 (b3+) 512.1>389.3 (b4+) 512.1>660.4 (b6+)	For quantitation (IS) For structure confirmation For structure confirmation
Brentuximab vedotin (VLIYAASNLE SGIPAR)	837.5>343.1 (y3+) 837.5>600.3 (y6+) 837.5>213.1 (b2+)	For quantitation For structure confirmation For structure confirmation
Rituximab (ASGYTFTSY NMHWVK)	598.1>817.5 (y13++) 598.1>707.5 (y11++) 598.1>657.1 (y10++)	For quantitation (IS) For structure confirmation For structure confirmation
Cetuximab (SQVFFK)	378.2>540.3 (y4+) 378.2>441.2 (y3+) 378.2>294.2 (y2+)	For quantitation For structure confirmation For structure confirmation

*	Quantitation range in human plasma	: 0.58 to 300 µg/ml
	Accuracy	: 91.5 to 115 %
	Precision	: 4.4 to 11.8 %

MRM Chromatograms

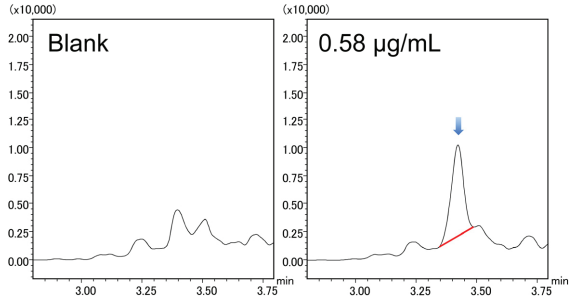

Fig. 2 Brentuximab MRM Chromatograms (in Human Plasma)

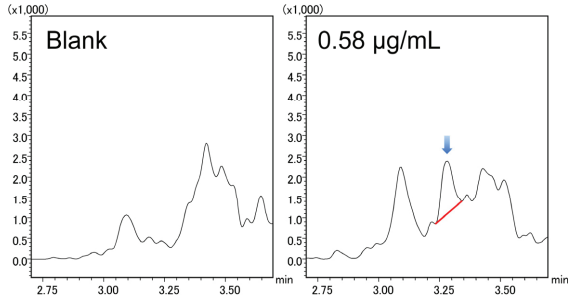

Fig. 3 Rituximab MRM Chromatograms (in Human Plasma)

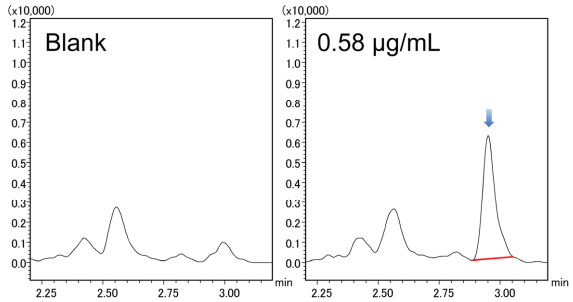

Fig. 4 Cetuximab MRM Chromatograms (in Human Plasma)

Precision of Multiplex Analysis

○ Brentuximab vedotin

Set Concentration [µg/ml]	Data Average (N = 9)	CV (%)	Accuracy (%)
1.76	1.74	7.60	99.2
240	249	7.12	104

○ Rituximab

Set Concentration [µg/ml]	Data Average (N = 9)	CV (%)	Accuracy (%)
1.76	1.77	5.44	101
240	247	9.65	104

○ Cetuximab

Set Concentration [µg/ml]	Data Average (N = 9)	CV (%)	Accuracy (%)
1.76	1.71	8.48	97.5
240	255	5.52	106

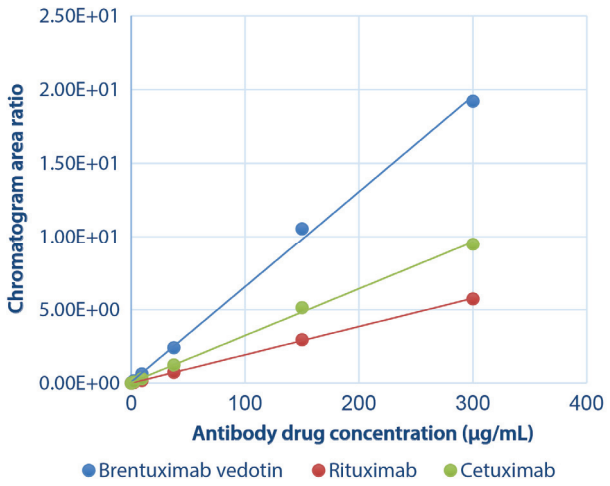

Fig. 5 Multiplex Calibration Curve

Observations, Conclusions, and References

By utilizing the nSMOL technique, it is possible to detect many antibody drugs in a single analysis. Therefore, it is also possible to create calibration curves of multiple antibody drugs in a single analysis by mixed multiple antibody drugs into plasma. Using the three antibodies presented this experimental model here, the precision and accuracy of quantitative values fulfilled the validation criteria of the guidelines issued by the FDA.

The lower limit of quantitation is 0.58 µg/ml and the same assay method can be used from preclinical to clinical trials.

<References>
Iwamoto N et al. *Analyst*, 2014, DOI:10.1039/c3an02104a
Iwamoto N et al., *Clinical Pharmacology & Biopharmaceutics*, 2016, DOI:10.4172/2167-065X.1000164
< Chief Scientists >
Noriko Iwamoto, Ph.D. and Takashi Shimada, Ph.D., Technology Research Laboratory

Notes: The product described in this document has not been approved or certified as a medical device under the Pharmaceutical and Medical Device Act of Japan.
It cannot be used for the purpose of medical examination, treatment or related procedures.

First Edition: Mar. 2017

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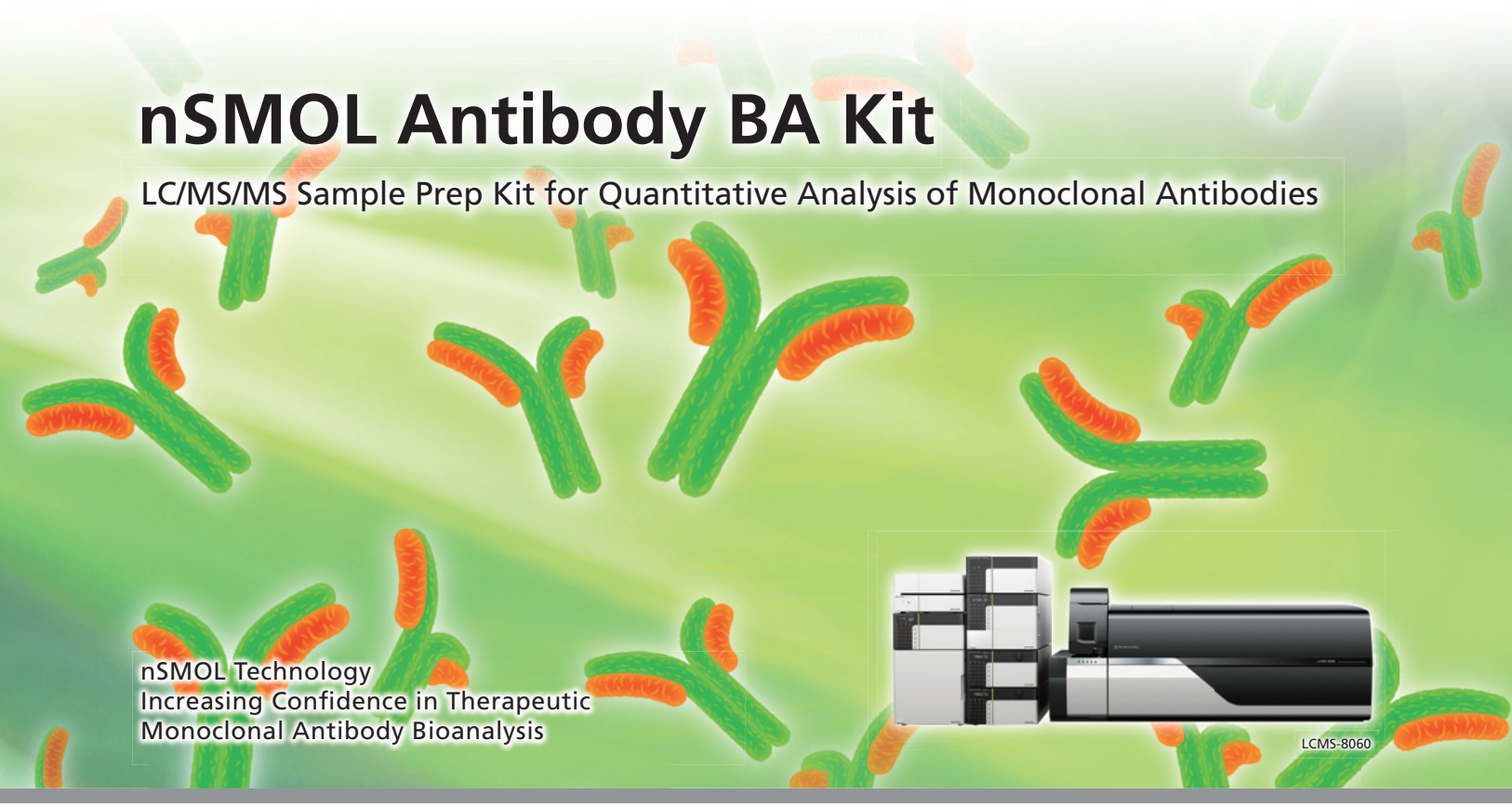
nSMOL Antibody BA Kit

LC/MS/MS Sample Prep Kit for Quantitative Analysis of Monoclonal Antibodies



nSMOL (nano-surface and molecular orientation limited proteolysis) is Shimadzu's proprietary, innovative technique that enables selective proteolysis of the Fab region of monoclonal antibodies. The nSMOL Antibody BA Kit is a ready-to-use reagent kit for collecting monoclonal antibodies from blood or other biological samples using immunoglobulin collection resin, and then performing selective proteolysis of the Fab region of these antibodies via trypsin-immobilized nanoparticles. Variable region-derived peptides produced by limited proteolysis can then be quantified via MRM measurements utilizing a high-performance LCMS-8050/8060 triple quadrupole liquid chromatograph mass spectrometer. An unparalleled convenient and rapid workflow provided by the nSMOL Antibody BA Kit dramatically improves the productivity and robustness of LCMS mAb bioanalysis.

• Faster, Less Expensive Method Development:

Dramatically improves response and quantitative repeatability.
No capture antibodies or ligands required.

• nSMOL Proteolysis:

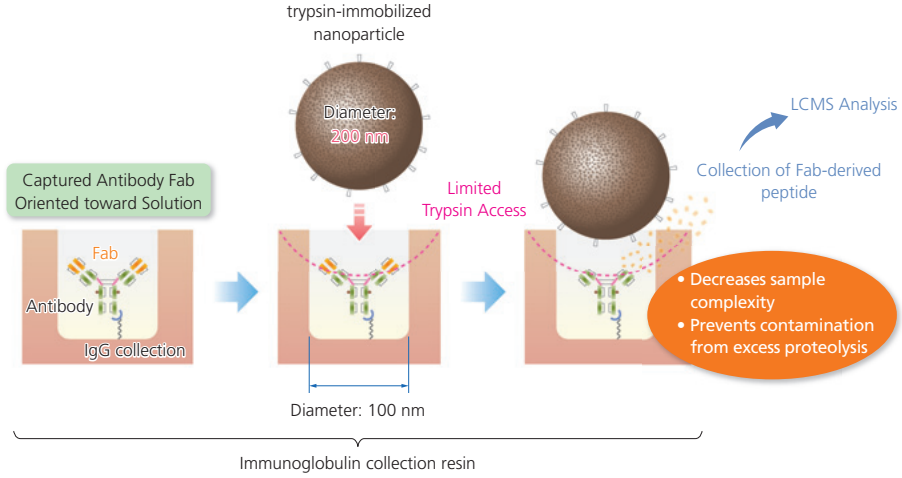
Selective collection of Fab peptides.
Limits contamination from excessive peptides or trypsin.

• Performance:

Highly sensitive and accurate assays are possible for a variety of antibodies.

• Highly Versatile:

Applicable to a wide variety of pharmaceutical antibodies.



nSMOL Antibody BA Kit

LC/MS/MS Sample Prep Kit for Quantitative Analysis of Monoclonal Antibodies

Kit Contents

The nSMOL Antibody BA Kit provides prepared reagents and protocols for sample prep via the nSMOL method.

Reagent	Quantity	Capacity	Storage Temperature
Immunoglobulin collection resin	2	1.3 mL/each	4 °C
Wash solution 1 (Binding solution)	1	80 mL	4 °C
Wash solution 2	1	80 mL	4 °C
Reaction solution	1	10 mL	4 °C
Enhanced reaction solution	1	Freeze-dried	4 °C
Reaction stop solution	1	1 mL	4 °C
FG beads Trypsin DART	1	1.1 mL	-20 °C *1

*1: If it is being stored for a month or longer, store it at -80 °C.

Note: The reagent kit is transported at refrigeration temperatures (2 to 8 °C).

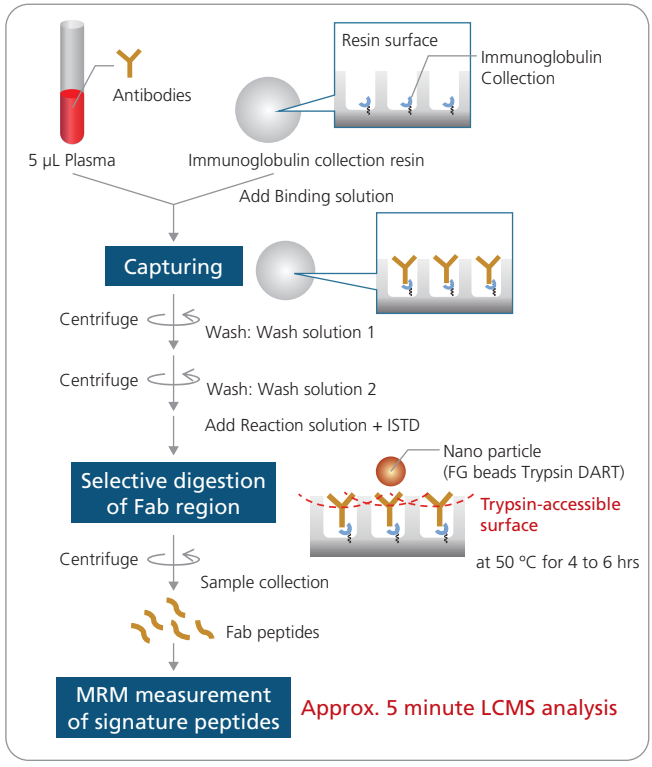
The following consumables are purchased separately.

Specified Consumables	Manufacturer	P/N
nSMOL reaction socket tube	SHIMADZU	225-32260-91
		225-32260-92
Ultrafree-MC, GV 0.22 µm	Merck Millipore	UFC30GV00
		UFC30GVNB
Micro tube 2mL, PP	SARSTEDT	72.708

Note: For other equipment required, please refer to instruction manual.

Simple Workflow

This kit enables highly reproducible data and avoids the troublesome steps of denaturing, reduction, and alkyation normally associated with protein digestion. There is also no need for solid phase extraction after reaction. After nSMOL preparation, samples can be injected directly onto the LCMS.



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Application News

No.C130

Liquid Chromatography Mass Spectrometry

Development of MRM Methods for Monoclonal Antibodies Using Skyline

Monoclonal antibodies, or mAbs, have been used for over a decade in the treatment of a number of diseases but predominantly in the treatment of cancer and autoimmune diseases. Quantifying therapeutic mAbs in biological samples has been traditionally addressed by ligand binding assays (LBA's), however, there are major limitation in terms of extended method development times, reagent procurement, and matrix effects.

LC-MS/MS methods are emerging as an alternative approach to LBA's and this paper describes the application of MRM methods to the quantitation of peptide fragments containing β-amyloid antibody (6E10) CDR's (complementarity determining regions). CDR's are targeted by LC-MS/MS as antibodies share a conserved sequence and are only differentiated by the three loops (CDR1-3) which are diverse and contain antibody specific peptides. By selectivity detecting CDR1-3 by LC-MS/MS helps to characterize and quantify disease specific antibodies isolated from patient sera or plasma and can be used to identify differing therapeutic agents.

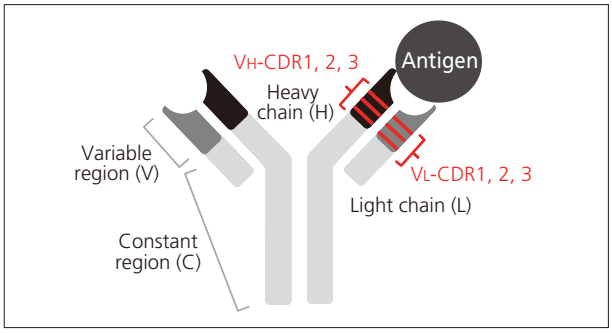


Fig. 1 Structure of Immunoglobulin and Complementarity Determining Regions (CDRs)

Development of MRM Method for Tryptic Fragment Containing CDR Using Skyline Software

Shimadzu LCMS-8050/8060 systems were used to identify CDR1-3 peptide fragments following a tryptic digest and Skyline software optimization[1]. The MRM method was set up to specifically detect peptide fragments containing β-amyloid antibody (6E10) CDRs.

Skyline was used to predict the peptide sequence for LC-MS/MS detection and also to optimize response for each precursor ion and product ion generated. The Skyline optimized MRM analysis method for CDR1-3 was then used to acquire highly specific and sensitive LC-MS/MS data.

1) Skyline is software developed by the MacCoss Lab of Biological Mass Spectrometry at the University of Washington.

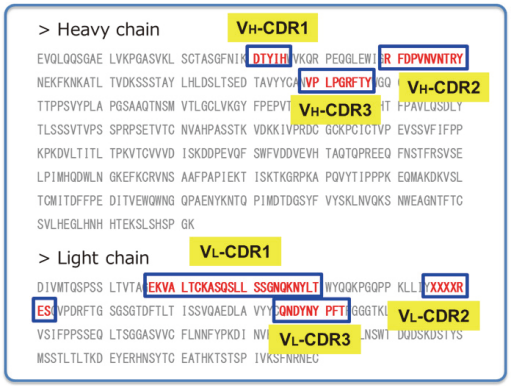


Fig. 2 Amino Acid Sequences and CDRs in β-Amyloid Antibodies (6E10)

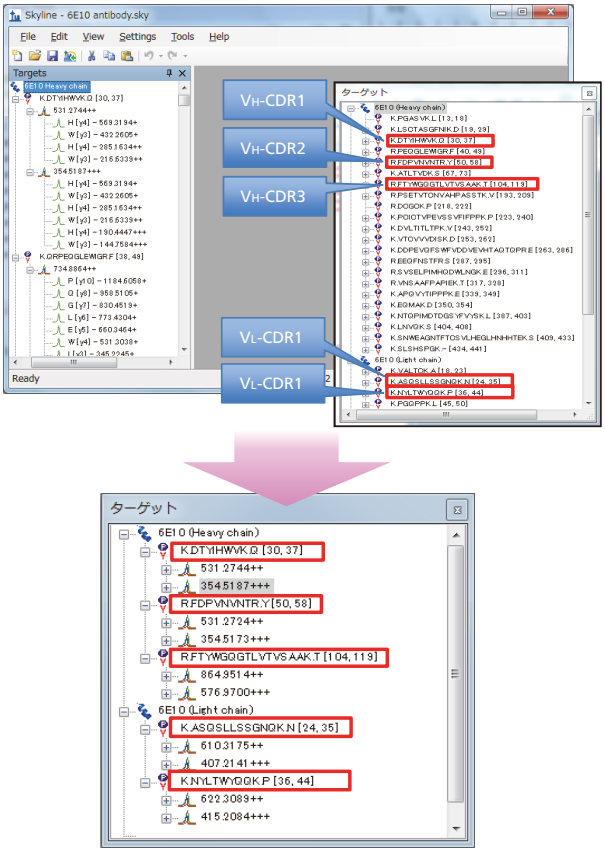


Fig. 3 Creating Analysis Methods Using Skyline (Selecting Trypsin Digestion Fragments Containing CDRs)

Amino acid sequence information of full-length monoclonal antibody (FASTA file) was imported to Skyline, and the peptide fragments produced by enzyme digestion were predicted. Further, peptide fragments containing CDRs were selected, and all the transitions and collision energies predicted were output as a LabSolutions LCMS analysis method.

Optimization of Analytical Method Using a Combination of the LCMS-8050/8060 and Skyline Software

Skyline targeted proteomics software (MacCoss et al., University of Washington, Seattle Washington) was used to predict precursor and product ions as well as collision energies and retention times using Shimadzu-specific models.

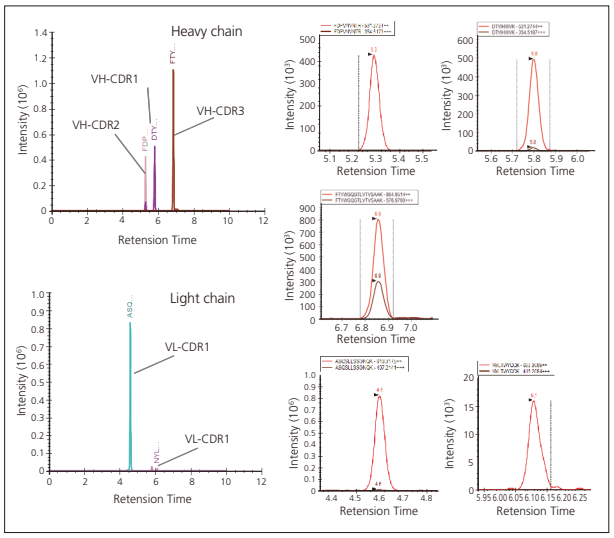


Fig. 4 Detection of Trypsin Fragments Containing CDR1-3 Using Standard Monoclonal Antibodies (6E10) Subjected to Trypsin Digestion (Data Analysis by Skyline)

Specific and sensitive tryptic peptide MRM's were selected and quantitative data was acquired using a calibration range from 6.67-6670 ng/mL.

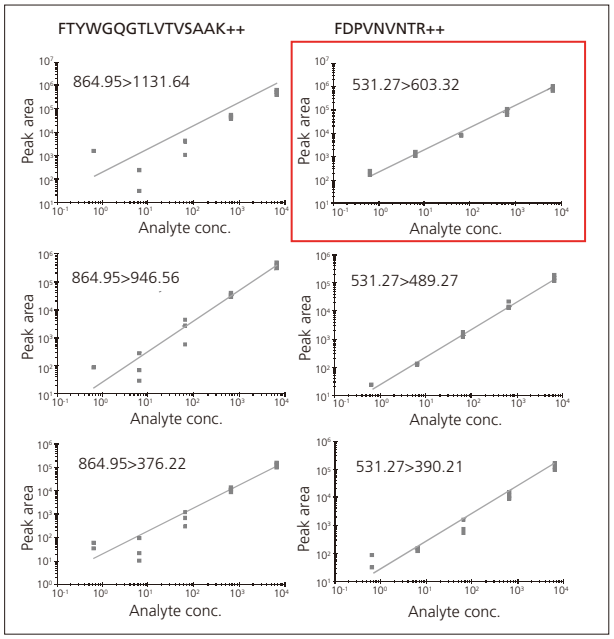


Fig. 5 Calibration Curve for Selected Peptides with Three Transitions.

The transition 531.27 > 603.32 was selected for quantitation as the dynamic range is 4 orders of magnitude. (The calibration curves are plotted on logarithmic scales).

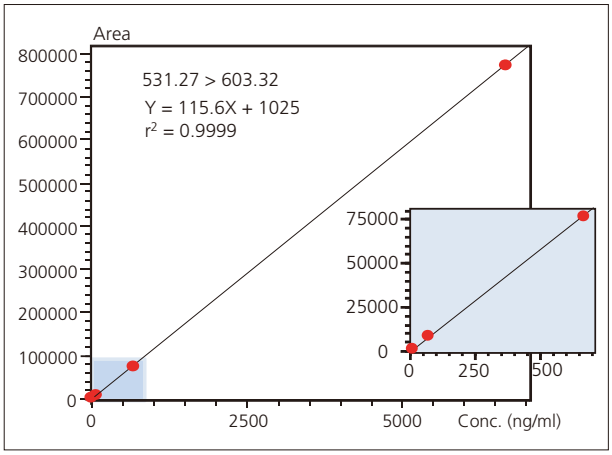
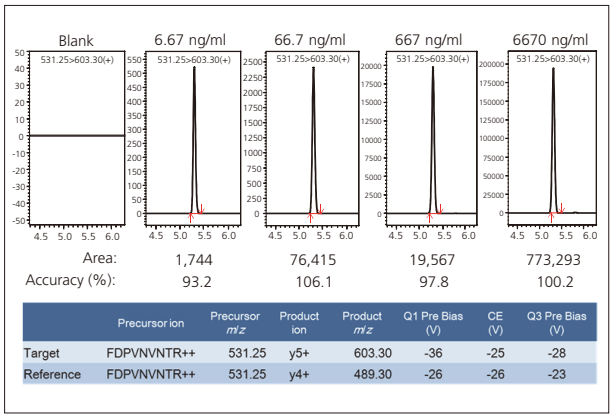


Fig. 6 Calibration Data for the Transition 531.27 > 603.32 on a Linear Scaling.

The LC-MS/MS method generates a linear response (r^2 = 0.999) for a calibration range over 4 orders of magnitude.



	Precursor ion	Precursor m/z	Product ion	Product m/z	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
Target	FDPVNVNTR++	531.25	y5+	603.30	-36	-25	-28
Reference	FDPVNVNTR++	531.25	y4+	489.30	-26	-26	-23

Fig. 7 MRM Chromatograms for the FDPVNVNTR++ Peptide Transition 531.27 > 603.32.

The LC-MS/MS method acquired calibration data within an accuracy range of 93-106 % over the entire calibration range.

Conclusions

- The Shimadzu LCMS-8050/8060 system delivers an excellent solution for the quantitation of CDR peptide fragments.
- Wide dynamic range with a linear response of 4 orders of magnitude.
- High selectivity and sensitivity with high speed scanning capabilities reduces sample consumption even for very low amounts of therapeutic antibodies.
- Simple integration with Skyline software to help accelerate optimized method development and MRM analysis.

Notes

- The products mentioned in this article have not been approved/ certified as medical devices according to the Pharmaceutical and Medical Device Act in Japan.
- The analytical methods mentioned in this article cannot be used for diagnostic purposes.

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Application News

No.C106A

Liquid Chromatography Mass Spectrometry

Simultaneous Analysis of Culture Supernatant of Mammalian Cells Using Triple Quadrupole LC/MS/MS

Industrial fermentation for the production of biofuels or biopharmaceutics requires routine monitoring of medium conditions such as pH, dissolved gas, carbon source (glucose) and nitrogen source (glutamine) for optimization and control of the fermentation process. However, culture media also consist of various other biologically important compounds such as vitamins, nucleic acids and other primary metabolites, which would lead to more detailed understanding of the bioprocess if monitored altogether.
To meet the demand for comprehensive analysis of medium component, we optimized the analytical conditions and developed this "Method Package for Cell Culture Profiling" that can monitor relative abundance of 95 compounds listed herein. Using this Method Package, we demonstrated the change in abundance of culture medium components associated with hybridoma growth over a period of 5 days.

List of Compounds

No.	Compound Name	Class.
1	2-Isopropylmalic acid	IS
2	Gluconic acid	Carbohydrate
3	Glucosamine	Carbohydrate
4	Hexose (Glucose)	Carbohydrate
5	Sucrose	Carbohydrate
6	Threonic acid	Carbohydrate
7	2-Aminoadipic acid	Amino acid
8	4-Aminobutyric acid	Amino acid
9	4-Hydroxyproline	Amino acid
10	5-Glutamylcysteine	Amino acid
11	5-Oxoproline	Amino acid
12	Alanine	Amino acid
13	Alanyl-glutamine	Amino acid
14	Arginine	Amino acid
15	Asparagine	Amino acid
16	Aspartic acid	Amino acid
17	Citrulline	Amino acid
18	Cystathionine	Amino acid
19	Cysteine	Amino acid
20	Cystine	Amino acid
21	Glutamic acid	Amino acid
22	Glutamine	Amino acid
23	Glutathione	Amino acid
24	Glycine	Amino acid
25	Glycyl-glutamine	Amino acid
26	Histidine	Amino acid
27	Isoleucine	Amino acid
28	Kynurenine	Amino acid
29	Leucine	Amino acid
30	Lysine	Amino acid
31	Methionine	Amino acid
32	Methionine sulfoxide	Amino acid
33	N-Acetylaspartic acid	Amino acid
34	N-Acetylcysteine	Amino acid
35	Ornithine	Amino acid
36	Oxidized glutathione	Amino acid
37	Phenylalanine	Amino acid
38	Pipecolic acid	Amino acid
39	Proline	Amino acid
40	Serine	Amino acid
41	Threonine	Amino acid
42	Tryptophan	Amino acid
43	Tyrosine	Amino acid
44	Valine	Amino acid
45	4-Aminobenzoic acid	Vitamin
46	Ascorbic acid	Vitamin
47	Ascorbic acid 2-phosphate	Vitamin
48	Biotin	Vitamin
49	Choline	Vitamin
50	Cyanocobalamin	Vitamin
51	Ergocalciferol	Vitamin
52	Folic acid	Vitamin
53	Folinic acid	Vitamin
54	Lipoic acid	Vitamin
55	Niacinamide	Vitamin
56	Nicotinic acid	Vitamin
57	Pantothenic acid	Vitamin
58	Pyridoxal	Vitamin
59	Pyridoxine	Vitamin
60	Riboflavin	Vitamin
61	Tocopherol acetate	Vitamin
62	Adenine	Nucleic acid
63	Adenosine	Nucleic acid
64	Adenosine monophosphate	Nucleic acid
65	Cytidine	Nucleic acid
66	Cytidine monophosphate	Nucleic acid
67	Deoxycytidine	Nucleic acid
68	Guanine	Nucleic acid
69	Guanosine	Nucleic acid
70	Guanosine monophosphate	Nucleic acid
71	Hypoxanthine	Nucleic acid
72	Inosine	Nucleic acid
73	Thymidine	Nucleic acid
74	Thymine	Nucleic acid
75	Uracil	Nucleic acid
76	Uric acid	Nucleic acid
77	Uridine	Nucleic acid
78	Xanthine	Nucleic acid
79	Xanthosine	Nucleic acid
80	Penicillin G	Antibiotics
81	2-Aminoethanol	Other
82	2-Ketoisovaleric acid	Other
83	3-Methyl-2-oxovaleric acid	Other
84	4-Hydroxyphenyllactic acid	Other
85	Citric acid	Other
86	Ethylenediamine	Other
87	Fumaric acid	Other
88	Glyceric acid	Other
89	Histamine	Other
90	Isocitric acid	Other
91	Lactic acid	Other
92	Malic acid	Other
93	O-Phosphoethanolamine	Other
94	Putrescine	Other
95	Pyruvic acid	Other
96	Succinic acid	Other

HPLC Conditions

Column	: RP Column
Mobile Phase A	: 0.1 % Formic Acid aq.
Mobile Phase B	: 0.1 % Formic Acid in Acetonitrile
Mode	: Gradient elution
Flowrate	: 0.35 mL/min.

MS Conditions (LCMS-8050)

Ionization	: ESI (Positive / Negative)
Nebulizer Gas Flow	: 3.0 L/min.
Drying Gas Flow	: 10.0 L/min.
Heating Gas Flow	: 10.0 L/min.
DL Temp.	: 250 °C
Block Heater Temp.	: 400 °C
Interface Temp.	: 300 °C

A murine hybridoma cell line was cultured in DMEM (see Table 1 for conditions) and its culture supernatant was sampled every 24 hours for 5 days after inoculation. LCMS sample was prepared by adding an internal standard to the sample and then removing proteins by taking supernatant after mixing with acetonitrile, which was further diluted with ultrapure water prior to injection. 1 µL was injected to LCMS for simultaneous MRM quantitation of all 96 compounds. Fig. 1 shows a growth curve and viability plot of the cell line, and Fig. 2 shows the quantitative value (ratio of peak area with respect to internal standard) of representative compounds over 5 days.

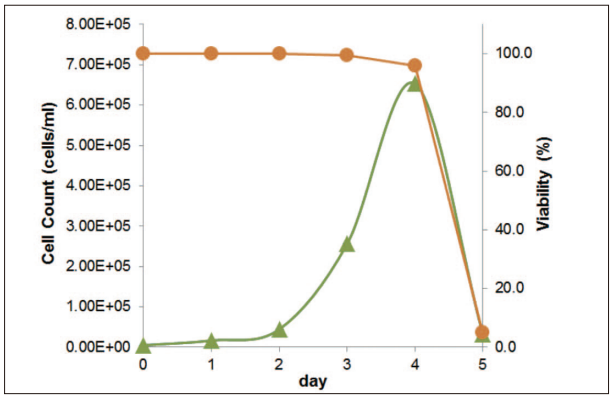

Fig. 1 Growth Curve and Viability of Cell Culture

Table 1 Cell Culture Conditions

Cell line	: SJK-287-38 (ATCC® CRL-1644™)
Medium	: DMEM (Low Glucose) + 10 % FBS + Gln, $NaHCO_3$)
Condition	: 37 °C, 5 % CO_2, cells pelleted at 120 rpm
Scale	: 24 mL (n = 4)

The culture supernatant sample and Fig. 1 was courteously provided by Kyokuto Pharmaceutical Industrial Co., Ltd.

Representative results are shown below. (A) Glucose, glutamine and few other amino acids, which are the primary sources of carbon and nitrogen, have decreased in abundance with growing cell number. (B) In contrast, lactic acid increased in abundance over time as a result of glucose consumption for anaerobic respiration. Similar pattern of increase was observed for a few other compounds. (C) No change in relative abundance was observed for essential amino acids and some vitamins.

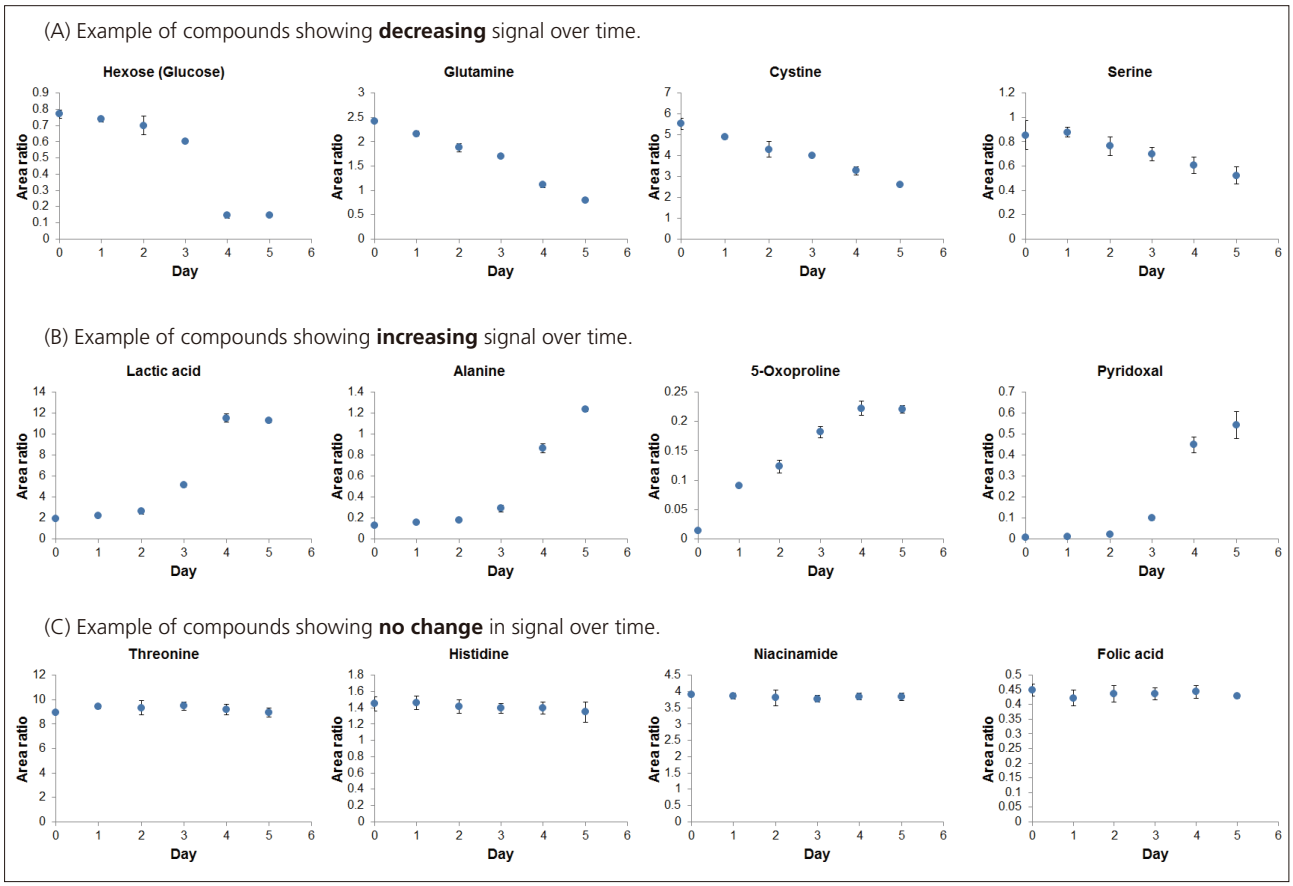

Fig. 2 Monitoring the Change in Culture Supernatant Components with Culture Time

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Second Edition: Jul. 2016

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LC/MS/MS Method Package for Cell Culture Profiling

For LabSolutions Version 5

LCMS-8050

Provides Simultaneous Analysis Conditions for 95 Components

These methods target culture medium components and metabolites secreted by cells. This culture medium analysis platform enables the simultaneous analysis of up to 95 components, the highest number of target analysis components* that can be analyzed by such a platform. Such features make it possible to acquire detailed data concerning cell culture profiles.

* Per survey results as of January 2015

Enables Simultaneous Analysis in 17 minutes

Compounds such as amino acids and vitamins are commonly analyzed by each compound group, which makes analysis of a culture medium time-consuming. By providing conditions for efficient and simultaneous multi-component analysis, this method package enables simultaneous analysis in 17 minutes.

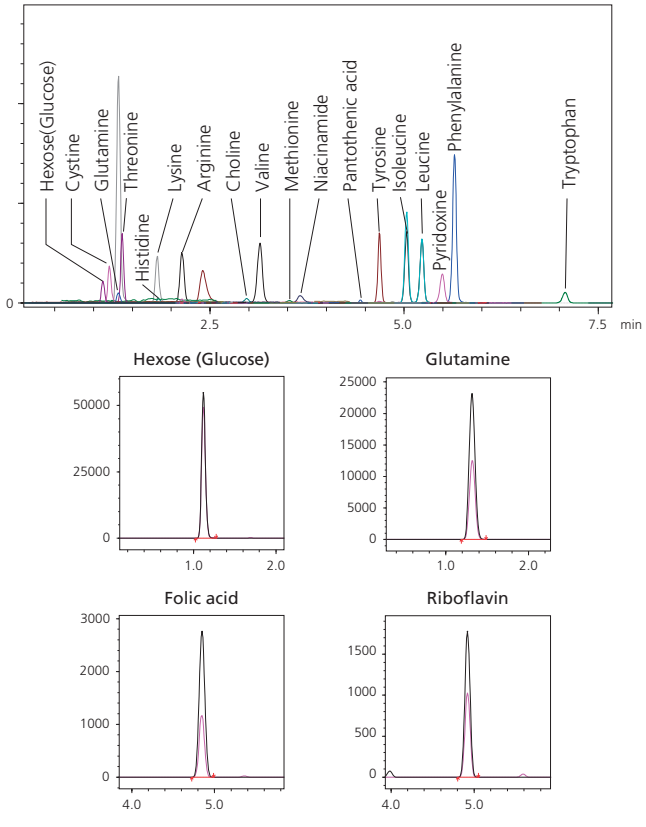
Optimized Methods for the Analysis of a Culture Medium

Pre-set analysis conditions fully utilize the capabilities of LC-MS/MS for analyzing trace components such as vitamins. In addition, since there is no saturation of the signal with high-concentration components, such as glucose or amino acids, it is possible to measure a variety of culture medium components using the same vial.

Note: In order to gain even more accurate quantitative results, a dilution series needs to be created.

Ready-to-Use Methods Provided

Shimadzu Method Packages deliver conditions for efficient and simultaneous multi-component analysis. They enable the user to quickly and easily implement complex methods without costly and laborious method development by providing sample preparation protocols, LC separation conditions, and MS acquisition parameters.



Example Showing the Analysis of DMEM (500 times dilution using ultrapure water, 1 µL analyzed)

LC/MS/MS Method Package for Cell Culture Profiling

For LabSolutions Version 5

List of Registered Compounds

Internal Standard

- 2-Isopropylmalic acid

Sugars

- Gluconic acid
- Glucosamine
- Hexose (Glucose)
- Sucrose
- Threonic acid

Nucleic Acid Associated Compounds

- Adenine
- Adenosine
- Adenosine monophosphate
- Cytidine
- Cytidine monophosphate
- Deoxycytidine
- Guanine
- Guanosine
- Guanosine monophosphate
- Hypoxanthine
- Inosine
- Thymidine
- Thymine
- Uracil
- Uric acid
- Uridine
- Xanthine
- Xanthosine

Antibiotics

- Penicillin G

Amino Acids and Derivatives

- 2-Aminoadipic acid
- 4-Aminobutyric acid
- 4-Hydroxyproline
- 5-Glutamylcysteine
- 5-Oxoproline
- Alanine
- Alanyl-glutamine
- Arginine
- Asparagine
- Aspartic acid
- Citrulline
- Cystathionine
- Cysteine
- Cystine
- Glutamic acid
- Glutamine
- Glutathione
- Glycine
- Glycyl-glutamine
- Histidine
- Isoleucine
- Kynurenine
- Leucine
- Lysine
- Methionine
- Methionine sulfoxide
- N-Acetylaspartic acid
- N-Acetylcysteine
- Ornithine
- Oxidized glutathione
- Phenylalanine
- Pipecolic acid
- Proline
- Serine
- Threonine
- Tryptophan
- Tyrosine
- Valine

Vitamins

- 4-Aminobenzoic acid
- Ascorbic acid
- Ascorbic acid 2-phosphate
- Biotin
- Choline
- Cyanocobalamin
- Ergocalciferol
- Folic acid
- Folinic acid
- Lipoic acid
- Niacinamide
- Nicotinic acid
- Pantothenic acid
- Pyridoxal
- Pyridoxine
- Riboflavin
- Tocopherol acetate

Others

- 2-Aminoethanol
- 2-Ketoisovaleric acid
- 3-Methyl-2-oxovaleric acid
- 4-Hydroxyphenyllactic acid
- Citric acid
- Ethylenediamine
- Fumaric acid
- Glyceric acid
- Histamine
- Isocitric acid
- Lactic acid
- Malic acid
- O-Phosphoethanolamine
- Putrescine
- Pyruvic acid
- Succinic acid

Remarks and Precautions

1. For Research Use Only. Not for Use in Diagnostic Procedures.
2. Shimadzu makes no warranty regarding the accuracy of information included in the database or the usefulness of information obtained from using the database.
3. It is the user's responsibility to adopt appropriate quality control tests using standard samples to confirm qualitative and quantitative information obtained with this method package.

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Application News

No. B63

Protein Sequencer

N-Terminal Amino Acid Sequencing of IgG Antibodies

Foreword

Recently, the term "biomedicine" is often heard in the field of pharmaceuticals. While also called biopharmaceuticals, they refer to proteinaceous drugs and antibody drugs developed and manufactured using biotechnologies including genetic recombination, cell fusion, and cell culture. In contrast, conventional medicines are referred to as "low molecular drugs" and are produced through chemical synthesis. While both types are chemical compounds, compared to chemical synthesized low molecular drugs, biomedicines characteristically have a much higher molecular weight. Of the top 10 drugs in all pharmaceuticals sold worldwide in 2015, seven were biomedicines.

Biomedicines are highly effective, low in side effects, and can be used to treat a wide range of illnesses. Unfortunately, unlike low molecular drugs that until now have been mainstream, mass production of biomedicines is not possible in the same way as chemical synthesized products. Biomedicine production comprises multiple processes including manufacture, refinement, dosage form design, and storage. In order to guarantee the quality of biomedicines, influences originating from raw materials and manufacturing processes need to be taken into consideration in addition to performing qualification testing of products. This means that management of manufacturing and quality of drugs requires a different approach compared to chemical synthesized low molecular drug products. Guidelines currently exist for evaluating the quality of biomedicines. These guidelines require that characteristic analysis is performed and one type of characteristic analysis is N-terminal amino acid sequencing. This analysis is performed to compare and verify the N-terminal amino acid sequence presumed from the gene sequence with the N-terminal amino acid sequence of the biomedicine product. The analysis technique used is the Edman method. This technique determines amino sequences by cleaving amino acids sequentially from the N-terminus of proteins and obtains very reliable amino acid sequences. The PPSQ-51A/53A Protein Sequencer is a system that automates this technique. This system facilitates identification of amino sequences from the N-terminus of target proteins and peptides.

This article introduces an example of amino acid sequencing of mouse antibody IgG using the PPSQ-51A/53A Protein Sequencer as an instance of N-terminal amino acid sequencing of biomedicines.

T. Kuriki

Method

The basic structure of antibodies comprises two H chains (heavy chains with higher molecular weight) and two L chains (light chains with lower molecular weight).
Since intact antibodies have high molecular weight (approx. 150 kDa), performing N-terminal amino acid sequencing using the Protein Sequencer in this state proves to be very difficult. In this analysis example, 20 pmol of mouse monoclonal antibody IgG was separated into H chains and L chains by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and these chains were then electroblotted onto a PVDF membrane. The PVDF membranes of CBB-stained L chains and H chains were analyzed using the PPSQ-53A.

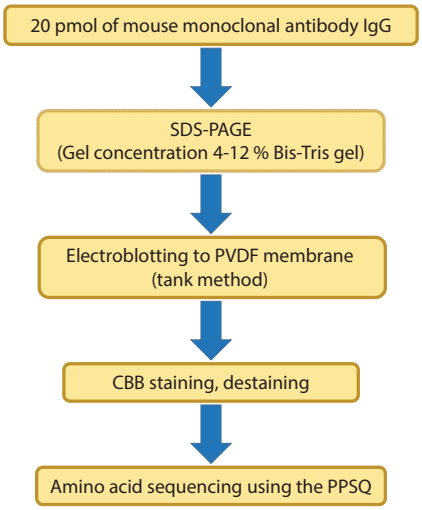

Fig. 1 Protocol for N-Terminal Amino Acid Sequencing

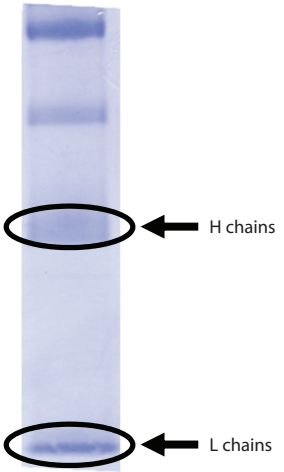

Fig. 2 PVDF Membrane After Electroblotting

Results of N-Terminal Amino Acid Sequencing

Fig. 3 and Fig. 4 show the results of N-terminal amino acid sequencing of L chains and H chains from the mouse monoclonal antibody IgG. Each figure shows chromatograms from cycle 1 to 5 for each sample (cycle 1 is the raw chromatogram and others are subtracted chromatograms). In Fig. 3, cycle 1 in the L-chain results identified the amino acid residue of the N-terminus as asparagine (Asp) and cycle 2 identified the second amino acid residue of the N-terminus as isoleucine (Ile). After performing analysis to the 20th residue, the sequence from the N-terminus was identified as Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Thr-Leu-Ser-Ala-Ser-Val-Gly-Asp-Arg-Val-Thr. Searching the database revealed this to be kappa light chain IgG1.

Likewise, Fig. 4 shows the H-chain results up to cycle 5. The sequence from the N-terminus was identified as Glu-Val-Gln-Leu-Gln-Glu-Ser-Gly-Pro-Glu-Leu-Val-Lys-Pro-Gly and a database search revealed this to be immunoglobulin heavy chain. As shown in this example, performing analysis with the Protein Sequencer enables easy and accurate identification of N-terminal sequences and demonstrates that the Protein Sequencer is an effective system for managing the quality of biomedicines.

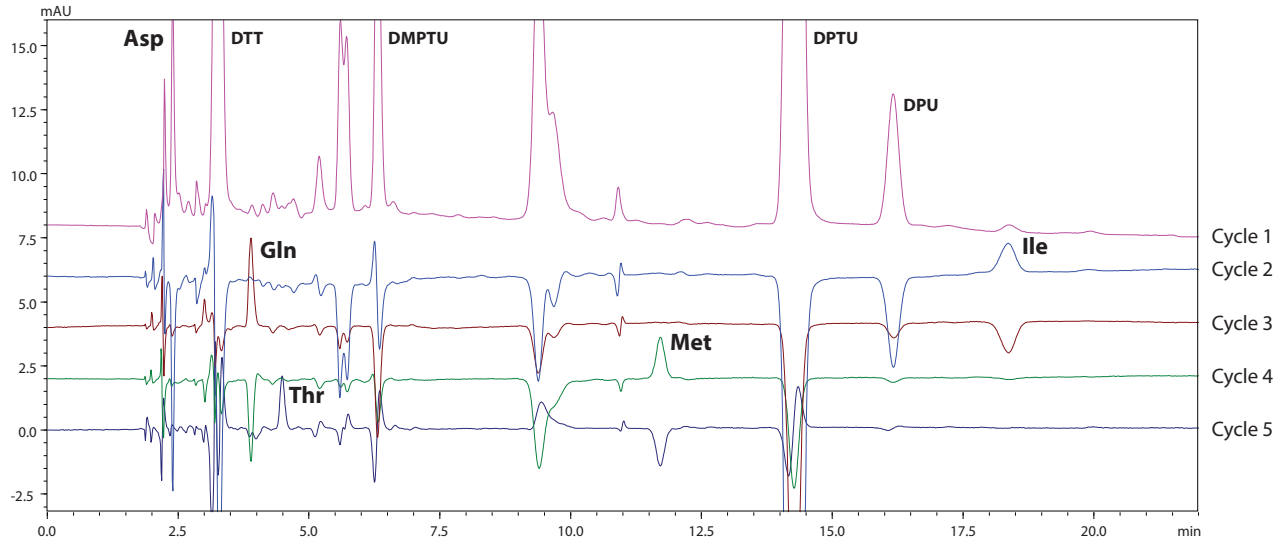

Fig. 3 L-Chain Chromatograms (Cycles 1 to 5)

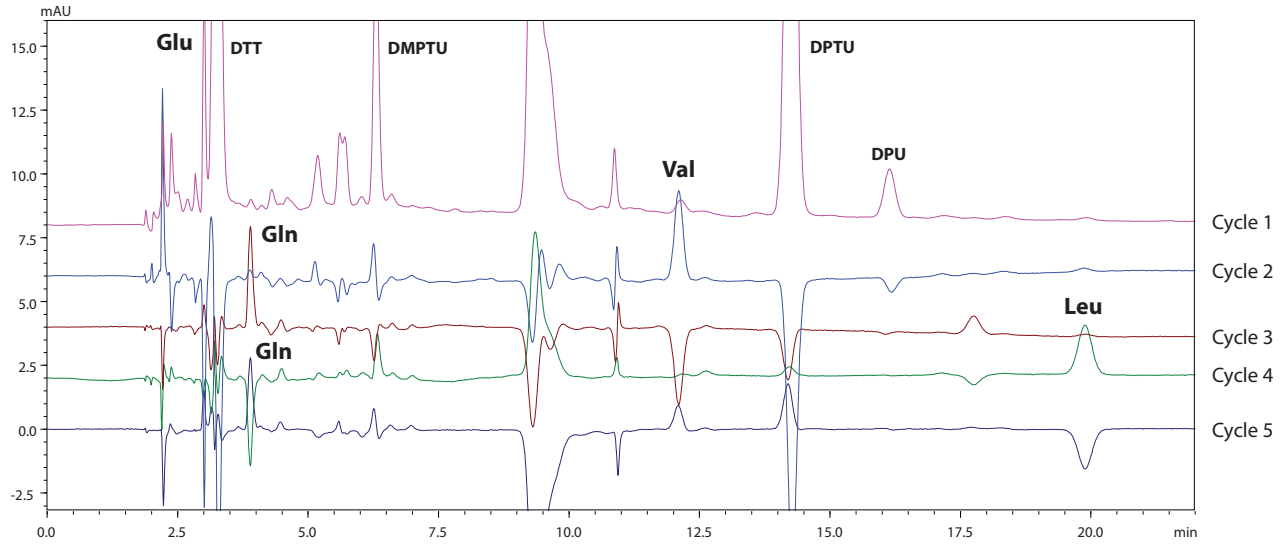

Fig. 4 H-Chain Chromatograms (Cycles 1 to 5)

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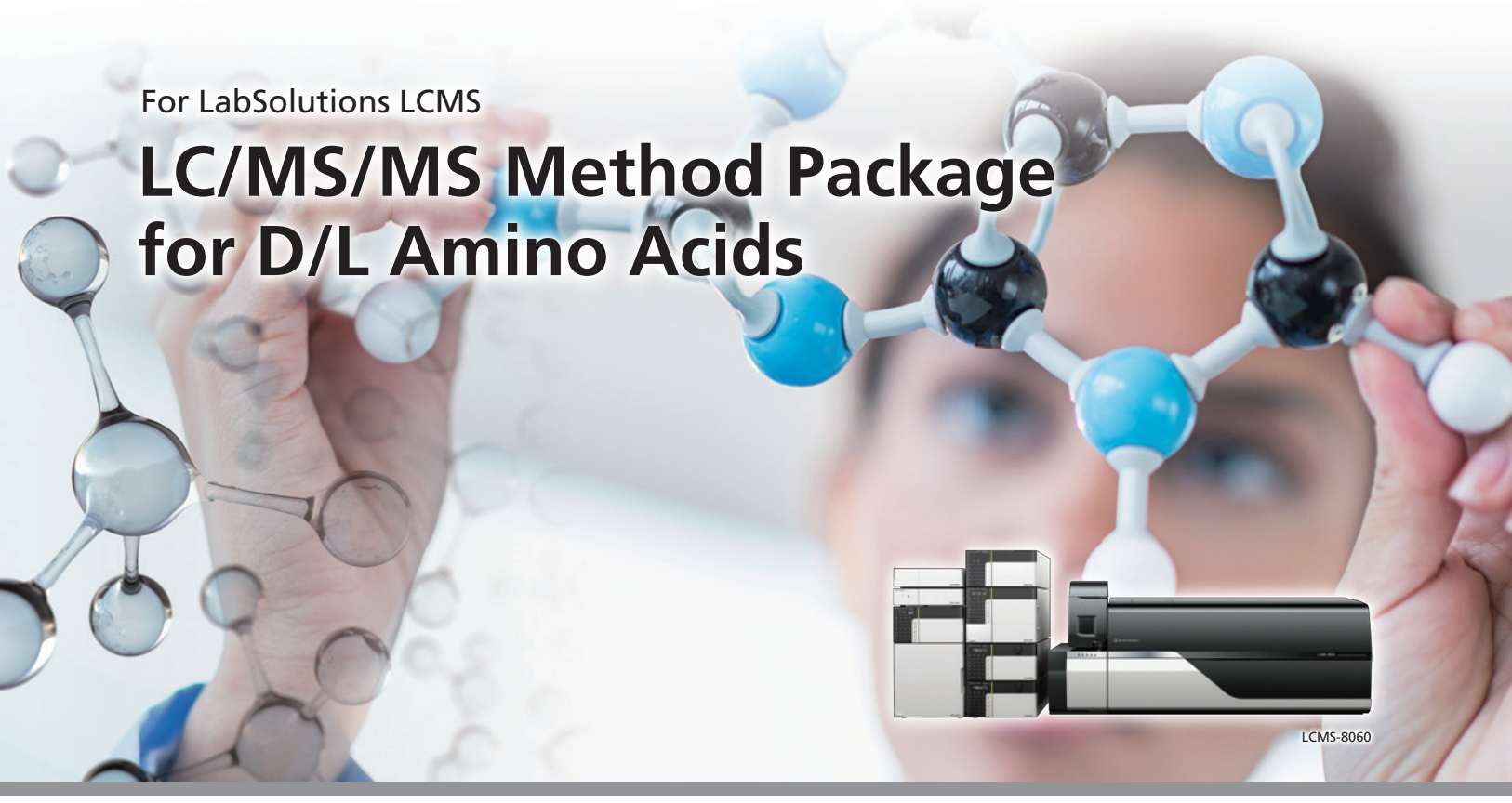


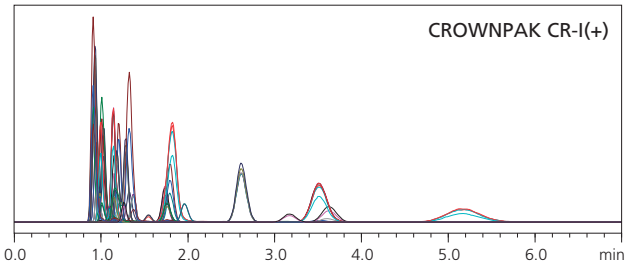
For LabSolutions LCMS

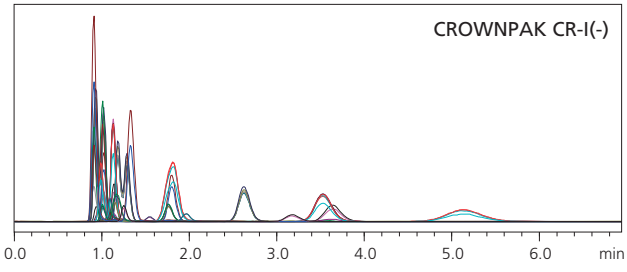
LC/MS/MS Method Package for D/L Amino Acids

Most important amino acids exist as stereoisomers. D- and L- forms of mirror image isomers, or enantiomers, are named according to their activity on polarized light. By using CROWNPAK CR-I(+) and CR-I(-) columns with chiral stationary phases, the D- and L-forms of amino acids can be analyzed separately. With CR-I(+) elution order is from D- to L-, and with CR-I(-) the elution order is reversed.

In Just Ten Minutes, Chiral Amino Acids Can Be Analyzed Simultaneously

With conventional chiral amino acid analysis, it is necessary to perform derivatization or use very long run times. With this method package, derivatization is not necessary, and high-sensitivity analysis can be performed in a short period of time, bringing efficiency to the chiral separations laboratory.





HPLC Conditions

Column	: CROWNPAK CR-I(+)/(-) (3 mmI.D. × 150 mmL., 5 µm)
Mobile Phase	: Acetonitrile/Ethanol/Water/TFA = 80/15/5/0.5
Flowrate	: 0.6 mL/min
Injection Volume	: 1 µL
Column Temp.	: 25 °C

MS Conditions

Nebulizer Gas Flowrate	: 3.0 L/min
Drying Gas Flowrate	: 15.0 L/min
Heating Gas Flowrate	: 5.0 L/min
Interface Temp.	: 250 °C
DL Temp.	: 250 °C
Heat Block Temp	: 300 °C

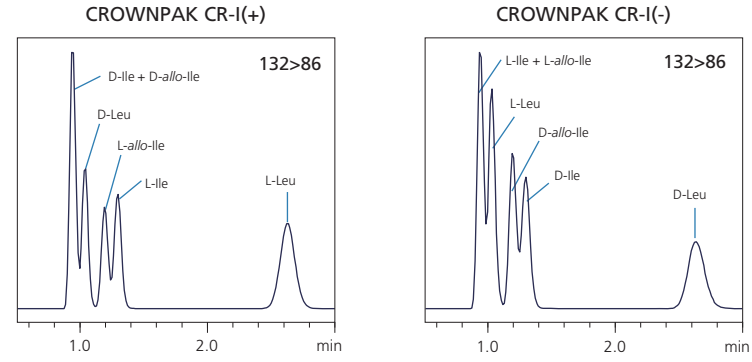
For LabSolutions LCMS

LC/MS/MS Method Package for D/L Amino Acids

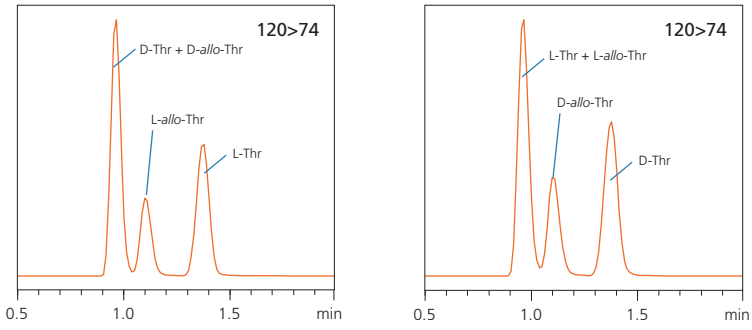
All D/L Amino Acids Can Be Quantified by Column Switching

The physicochemical properties of Glutamine and Lysine, Isoleucine and allo-Isoleucine, Threonine and allo-Threonine are extremely similar. They have virtually the same MS/MS fragmentation patterns, and share many of their MRM transitions. Chromatographic separation is therefore required to analyze them individually by LC-MS/MS. Even if there is coelution on the CR-I(+) column, confirmation can be made by automated switching to a secondary CR-I(-) column.

Analysis of D/L-Isoleucine, D/L-*allo*-Isoleucine, and D/L-Leucine



Analysis of D/L-Threonine, and D/L-*allo*-Threonine



List of Registered Amino Acids

D/L-Alanine	D/L-Cysteine	D/L-Histidine	D/L-Lysine	D/L-Serine	D/L-Tyrosine
D/L-Arginine	D/L-Glutamine	D/L-Isoleucine	D/L-Methionine	D/L-Threonine	D/L-Valine
D/L-Asparagine	D/L-Glutamic acid	D/L-*allo*-Isoleucine	D/L-Phenylalanine	D/L-*allo*-Threonine	
D/L-Aspartic acid	Glycine	D/L-Leucine	DL-Proline	D/L-Tryptophane	

Precautions

1. DL-Proline is a secondary amine, so it cannot be separated with these analysis conditions.
2. LabSolutions LCMS Ver. 5.86 or later is required.

CROWNPAK CR-I(+) and CR-I(-) are products of Daicel Corporation.

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Application News

No.L488

High Performance Liquid Chromatography

Peptide Mapping of Antibody Drugs by Nexera-i

Peptide mapping by HPLC is one of the important quality assurance tests used for verifying the primary structure of antibody drugs. Typically, following enzymatic digestion of the antibodies, separation is conducted using a traditional reversed phase column. Due to the large number of peaks that require separation, the use of small-particle columns and core shell columns for peptide analysis has spread in recent years.
In order to compare elution profiles for identity and mutation confirmation, a highly repeatable system is required. The Nexera-i integrated UHPLC is the ideal system for such an analysis. Here, the Nexera-i is used in the analysis of IgG (human immunoglobulin G) tryptic digest.

Analysis of IgG Tryptic Digest

For this investigation, after reduction and alkylation of IgG, tryptic enzyme digestion was used as shown in Fig. 1 for sample preparation.

Table 1 shows the analytical conditions. Here, the Aeris 1.7 µm PEPTIDE XB-C18 100 Å small-particle core-shell column and the Nexera-i integrated UHPLC system was used. Mobile phase A was 0.1 % trifluoroacetic acid (TFA) in water and mobile phase B was 0.08 % TFA in acetonitrile. To ensure proper gradient performance with TFA, an optional 300 µL mixer was used.
Fig. 2 shows the chromatogram of IgG tryptic digest, in which an extremely large number of peaks are clearly separated.

Table 1 Analytical Conditions

Column	: Aeris 1.7 µm PEPTIDE XB-C18 100 Å (150 mm L. × 2.0 mm I.D., 1.7 µm)
Mobile Phase	: A: 0.1 % trifluoroacetic acid in water B: 0.08 % trifluoroacetic acid in acetonitrile
Time Program	: B.Conc. 0 % (0 min) → 45 % (90 min) → 100 % (90.01 - 95 min) → 0 % (95.01 - 110 min)
Flowrate	: 0.2 mL/min
Column Temp.	: 60 ˚C
Injection Vol.	: 10 µL
Detection	: LC-2040C 3D at 215 nm
Flow Cell	: High-speed high-sensitivity cell

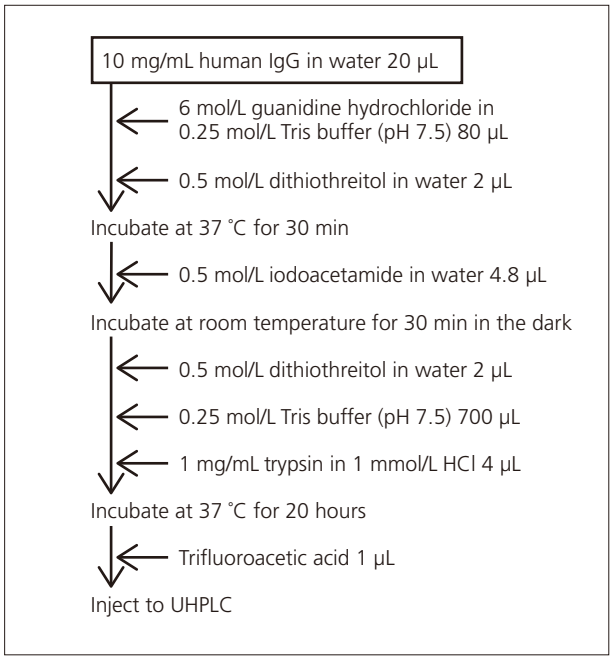

Fig. 1 Sample Preparation

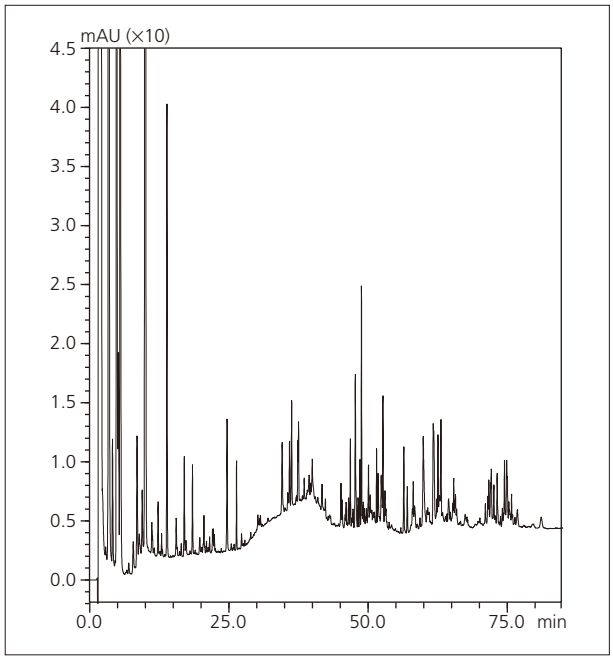

Fig. 2 Chromatogram of IgG Tryptic Digest

Repeatability

Due to the large number of peaks that must be separated when conducting peptide mapping, a gradient with a long shallow slope is required. In this analysis, the mobile phase B percentage changes from 0 % to 45 % over 90 minutes, resulting in a slope of 0.5 %/min. The optimized low-pressure gradient valve of the Nexera-i and mixer selection for use with TFA will provide repeatable delivery even with a shallow gradient.

Tables 2 and 3 show the intra-day and inter-day repeatability of retention time, respectively. Fig. 3 shows the IgG tryptic digest chromatogram intra-day repeatability. Selecting the principal peaks from the chromatogram (peaks labeled a to f), we checked their repeatability. We calculated the intra-day repeatability based on the results of six repeat analyses. The inter-day repeatability was calculated based on average of three analyses per day over a period of six days. In peptide mapping using the Nexera-i, it is clear that good intra-day and inter-day repeatability is obtained.

Table 2 Intra-day Repeatability of Retention Time (n=6)

Peak	Avg. R.T. (min)	Std. Dev. (min)	%RSD (%)
Peak a	9.929	0.027	0.271
Peak b	24.669	0.047	0.192
Peak c	36.299	0.042	0.117
Peak d	48.815	0.033	0.068
Peak e	59.864	0.032	0.054
Peak f	74.535	0.043	0.057

Table 3 Inter-day Repeatability of Retention Time (n=6)

Peak	Avg. R.T. (min)	Std. Dev. (min)	%RSD (%)
Peak a	9.907	0.016	0.159
Peak b	24.708	0.033	0.132
Peak c	36.355	0.034	0.093
Peak d	48.877	0.034	0.069
Peak e	59.901	0.027	0.046
Peak f	74.555	0.036	0.049

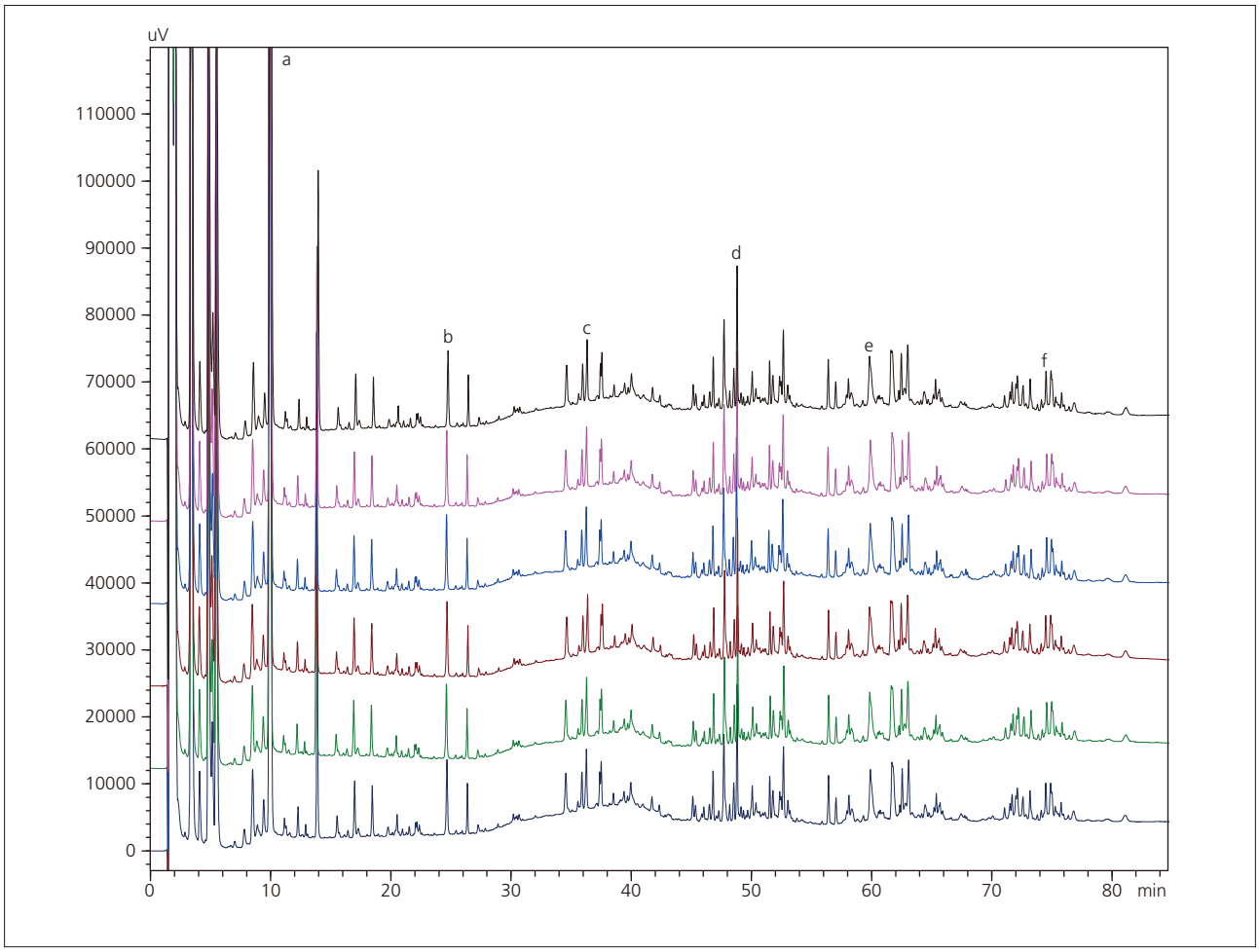

Fig. 3 Intra-day Repeatability of Chromatogram of IgG Tryptic Digest

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Application News

No. B64

MALDI-TOF Mass Spectrometry

A Study on a Method for Evaluating Glycans in Biopharmaceuticals

- Suppressing Peeling Reactions in Pretreatment for *O*-glycan Analysis --

Many protein-based biopharmaceutical products, typified by antibody drugs, are synthesized in cultured cells derived from eukaryotes such as CHO (Chinese hamster ovary) cells. For this reason, there are inevitably many post-translational modifications to the biosynthesized proteins. Among these, modifications of glycans have gained attention as items for evaluating the quality of biopharmaceuticals since they are associated with the adjustment of protein functions, as well as with the unwanted development of antigenicity depending on their structure. However, there are various technical challenges in evaluating glycans. With *O*-linked glycans (*O*-glycan), it is particularly difficult to comprehensively release glycans from protein using enzymes, leading to the use of mainly the following two chemical methods: hydrazinolysis and β-elimination. However, these methods have issues that need to be improved. Hydrazinolysis requires great care since an explosive reagent is handled and therefore is not easy to implement. With the β-elimination method, a peeling reaction where glycans gradually decompose due to a continuous elimination reaction occurs. Conventionally, in analysis of *O*-glycans using β-elimination, glycans are released so as not to cause a continuous elimination reaction by using the reductive β-elimination method, which involves simultaneous releasing of glycans under alkaline conditions and reducing the root portion of the glycan with a reducing agent. However, this method completely reduces the root portion of the glycan and therefore does not allow labeling such as with fluorescent reagents after releasing glycans, thus limiting the available analysis methods. Also, in analysis by mass spectrometry of glycans obtained in this way, the high sensitivity analysis is not possible because the ionization efficiency of the glycan itself is not high. To address this, a non-reductive β-elimination/fluorescent labeling method is being examined as a method to bind a fluorescent labeling reagent such as 2-AB or PA by not reducing the root of the glycan, but this has not succeeded in significantly suppressing the continuous elimination reaction. Even so, in academic researches where *O*-glycan was the object of analysis, the existence of by-products due to this peeling reaction has not been problematic enough to hinder researches. However, glycans have to be evaluated for quality control in drugs which are to be administered to the human body, such as biopharmaceuticals, and the question of how to handle the existence of by-products during this evaluation is a major issue.

In this article, we report the results of studying a method for releasing *O*-glycans chemically in which the peeling reaction is suppressed, based on a PMP labeling method[*1].

S. Nakaya

One-pod-PMP Labeling with Modified Alkali Reagents

For the release of glycans by an alkali catalyst and PMP labeling of the released glycans, we used a method based on the One-Pod method of Wang et al[*2], which uses 28 % ammonia. We used ammonium carbamate as the alkali catalyst, anticipating an inhibitory effect on the peeling reaction. For the sample, we used fetuin (Fetuin from fetal bovine serum: Sigma-Aldrich F3004-25MG), and released *O*-glycans and accomplished labeling by the following method.

10 mg of fetuin was dissolved in 1 mL of water. The solution was then dispensed 10 µL each into 1.5 mL micro tubes with a screw cap, and dried by centrifugal evaporator at room temperature. After drying, the following two types of reagent solution were prepared as the glycan releasing/labeling reagent solution.

First, an "ammonia PMP labeling solution" (final concentration of PMP: 0.5 M, final concentration of ammonia: 28 %) was made by adding 200 µL of 28 % ammonia (Sigma-Aldrich) to 17.4 mg of PMP (1-Phenyl-3-methyl-5-pyrazolone, Sigma-Aldrich). Next, an "ammonium carbamate PMP labeling solution" (final concentration of PMP: 0.5 M, final concentration of ammonium carbamate: 2.5 M) was made by mixing equal amounts of the following two solutions: 34.8 mg of PMP with 200 µL of methanol added, and 390 mg of ammonium carbamate added to 1 mL of water.

50 µL of each of these solutions was added to dried fetuin and left to react for 16 hours at 50 °C, to simultaneously release glycans from the protein and accomplish the PMP labeling. The reacted solution was transferred to a glass micro tube, and after adding 500 µL of water and stirring well, it was dried by centrifugal evaporator at room temperature. A 1 % acetic acid solution (500 µL) and chloroform (500 µL) were added to the dried reaction sample, stirred strongly, then after separation into the water layer (upper layer) and the chloroform layer (lower layer) by centrifugation, the water layer was transferred into a new glass micro tube. After repeating the process of adding chloroform to the transferred water layer and performing liquid/liquid separation two times, the water layer containing the PMP-labeled glycans was collected in a 1.5 mL tube.

The collected solution was dried by centrifugal evaporator at room temperature then re-dissolved in 1 mL of water, and then injected into a C18 SPE cartridge (SupelClean LC-18 SPE Tube 1 mL, Supelco) that had been washed with 2 mL of acetonitrile and 2 mL of water. After washing with 3 mL of 3 % acetonitrile solution, the PMP-labeled glycans were eluted with 1 mL of a 30 % acetonitrile solution. The eluted solution was dried by centrifugal evaporator at room temperature, then re-dissolved in 50 µL of a 50 % acetonitrile solution and used for mass spectrometry and liquid chromatograph analysis.

MADLI-TOF MS Analysis of PMP-labeled *O*-glycan

A 0.1 % trifluoroacetic acid (TFA) solution (10 µL) was added to a PMP-labeled *O*-glycan sample (2 µL) and stirred well, then adsorbed into NuTip Carbon (glygen) that had been washed with 1 M sodium hydroxide (10 µL × 3), water (10 µL × 5), 50 % acetonitrile/0.1 % TFA solution (10 µL × 3), and 0.1 % TFA solution (10 µL × 5). After washing with 0.1 % TFA solution, the entire volume of the solution eluted with 50 % acetonitrile/0.1 % TFA solution (approx. 3 µL) was placed on the MALDI target plate. After the loaded sample solution dried, 0.5 µL of the matrix solution for MALDI was dried on top of this sample, then the result was recrystallized with 0.2 µL of ethanol and then analyzed. The matrix for MALDI was prepared by dissolving 5 mg of DHB (2,5-dihydroxybenzoic acid, Shimadzu GLC) in 50 % acetonitrile/0.05 % TFA solution (500 µL), and a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-7090, Shimadzu/Kratos) was used for the analysis.

The analysis result is shown in Fig. 1. The detected signal intensity of peeling reaction products was weaker when using ammonium carbamate with a final concentration of 2.5 M compared to when using 28 % ammonia.

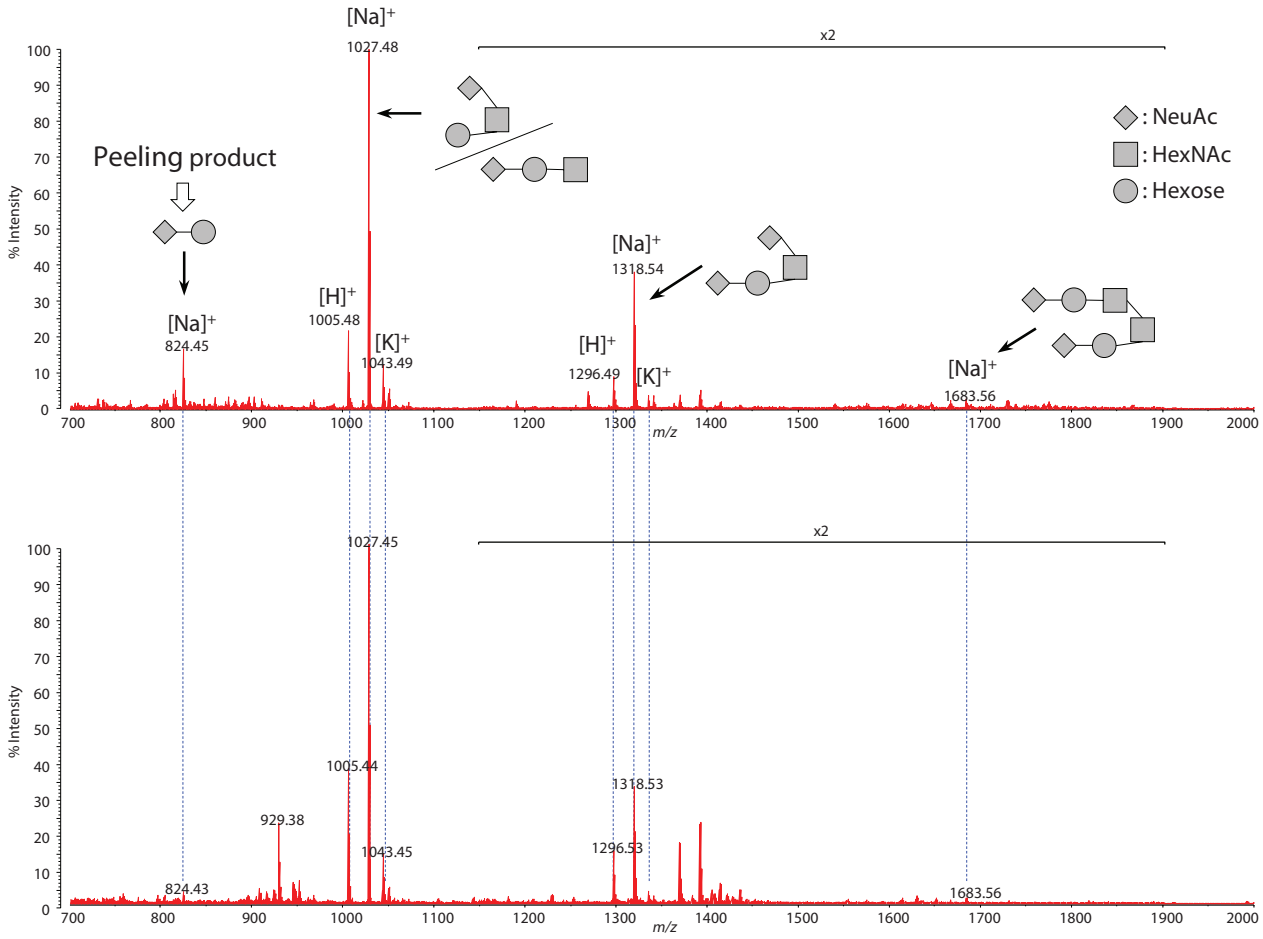

Fig. 1 MALDI-TOF Mass Spectra of the PMP-labeled *O*-glycan of Fetuin
Top: Reaction with Ammonia with a Final Concentration of 28 %,
Bottom: Reaction with Ammonium Carbamate with a Final Concentration of 2.5 M

LC Analysis of PMP-labeled *O*-glycan

The prepared PMP-labeled *O*-glycan samples were subjected to LC analysis under the conditions shown in Table 1. The samples were prepared twice each using pretreatments with different alkali catalysts.

With the sample using 28 % ammonia, 20 µL was injected and with the sample using ammonium carbamate, 40 µL was injected and then analyzed.

Table 1 LC Analysis Conditions for PMP-labeled *O*-glycan

Instrument	:	Nexera
Column	:	GlycanPac AXH-1, Analytical 1.9 µm 150 mm L 2.1 mm I.D. (Thermo Scientific)
Mobile phase A	:	100 mM Ammonium formate (pH 4.5)
Mobile phase B		Acetonitrile
Total flow rate	:	0.25 mL/min
Concentration of mobile phase B	:	0 to 15 min: 90 %, 15 to 40 min: 90 → 45 %, 40 to 45 min: 30 %, 45 to 50 min: 90 %
Column temp.	:	40 °C
Detection	:	UV absorption 245 nm (SPD-M30A)

As shown in Fig. 2, analysis results indicate that a peak derived from peeling reaction products was detected in the case of pretreatment using 28 % ammonia, whereas it was hardly detectable in the case of pretreatment using ammonium carbamate with a final concentration of 2.5 M. Table 2 summarizes the peak areas for peeling reaction products and main *O*-glycans. Peeling reaction products constituted about 12% of the total when using 28% ammonia, and about 2% when using ammonium carbamate with a final concentration of 2.5 M. This suggests that the peeling reaction was suppressed by the use of ammonium carbamate. However, it was also found that the efficiency of the *O*-glycan release/labeling reaction was lower than when using ammonia.

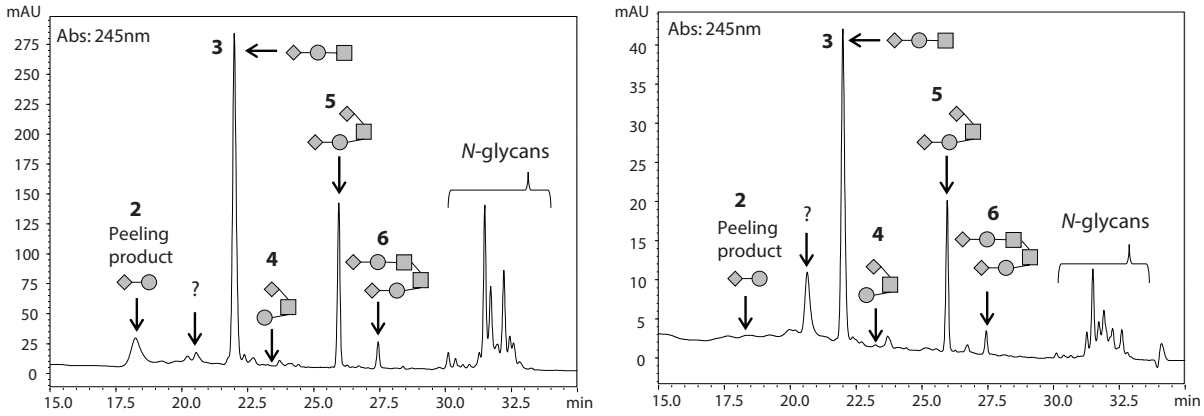

Fig. 2 Example LC Chromatograms for PMP-labeled *O*-glycans
Left: Reaction with Ammonia with a Final Concentration of 28%,
Right: Reaction with Ammonium Carbamate with a Final Concentration of 2.5 M

Table 2 LC Profiling of PMP-labeled *O*-glycans (n = 2)

28 % Ammonia

	Retention Time			Area			% Area			Height		
Peak No.	Average	SD	RSD	Average	SD	RSD	Average	SD	RSD	Average	SD	RSD
2	18.2	0.01	0.04	470403.0	174277.78	37.05	12.53	1.35	10.78	17485.5	6695.59	38.29
3	22.0	0.01	0.03	2334391.0	543258.83	23.27	63.39	2.31	3.64	239416.0	52457.42	21.91
4	23.3	0.01	0.02	2882.0	87.68	3.04	0.08	0.02	23.85	424.5	16.26	3.83
5	26.0	0.01	0.02	766212.0	241747.08	31.55	20.57	1.02	4.96	113022.0	34481.36	30.51
6	27.5	0	0.01	126936.0	32437.82	25.55	3.44	0.04	1.29	18832.0	4775.8	25.36

2.5 M Ammonium carbamate

	Retention Time			Area			% Area			Height		
Peak No.	Average	SD	RSD	Average	SD	RSD	Average	SD	RSD	Average	SD	RSD
2	18.3	0.04	0.24	13180.0	10096.07	76.6	2.21	1.48	67.07	436.5	355.67	81.48
3	22.0	0.01	0.05	401757.0	53148.97	13.23	70.18	0.29	0.41	43209.5	4999.95	11.57
4	23.2	0.00	0.01	2356.5	259.51	11.01	0.41	0.01	1.82	265.0	29.70	11.21
5	26.0	0.00	0.01	134460.0	7737.16	5.75	23.6	1.67	7.1	20017.5	979.34	4.89
6	27.4	0.01	0.03	20582.5	2150.31	10.45	3.6	0.09	2.39	3207.0	294.16	9.17

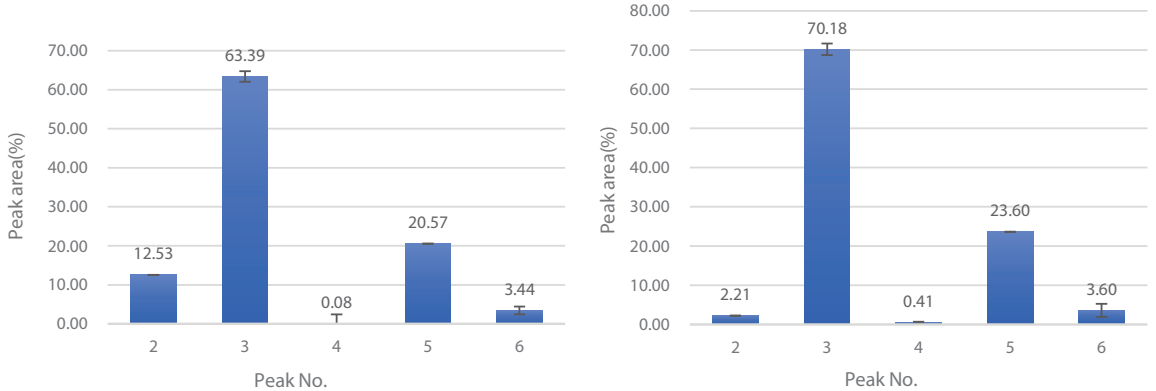

Fig. 3 LC Profiling Graphs for PMP-labeled *O*-glycans (n = 2)
Left: Reaction with Ammonia with a Final Concentration of 28%,
Right: Reaction with Ammonium Carbamate with a Final Concentration of 2.5 M

Observations

The results of this experiment confirm that the generation of peeling reaction products, which are an artefact that is problematic when attempting to perform accurate *O*-glycan profiling, can be suppressed to a few percent of the total *O*-glycans by carrying out One-pod PMP labeling using ammonium carbamate. Although there are restrictions such as the amount of samples required, this method can be used for *O*-glycan profiling.

However, the reagent and reaction conditions that were used in this study gave a glycan release efficiency and labeling efficiency that were not high when compared with other methods. The technique of simultaneously accomplishing glycan release and labeling is effective in terms of suppressing the peeling reaction. For PMP labeling, however, detection with LC is based on UV absorbance, and the detection sensitivity is low in comparison with fluorescence detection such as for PA labeling and 2-AB labeling, and there is susceptibility to background effects due to contaminant reagents. In order to eliminate these effects, it is necessary to investigate reagents with good reaction efficiency that can simultaneously perform releasing and labeling, and to conduct further studies on the reaction conditions.

References

*1 S Honda. et al., High-performance liquid chromatography of reducing carbohydrates as strongly ultraviolet-absorbing and eletrochemically sensitive 1-phenyl-3-methyl-5-pyrazolone derivatives. *Anal. Biochem.* 1989, 180, 351–357.

*2 C Wang. et al., One-pot nonreductive *O*-glycan release and labeling with 1-phenyl-3-methyl-5-pyrazolone followed by ESI-MS analysis. *Proteomics*. 2011, 11, 4229–4242

Acknowledgments

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First Edition: Jun. 2017

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Application News

No.L483

High Performance Liquid Chromatography

High-Sensitivity Analysis of 2-AB Glycans by RF-20Axs Florescence Detector

Glycans, present in antibody-drug products have an effect on their safety and efficacy, therefore requiring that the types and quantities of the glycans present be investigated. Due to the culture conditions, the diversity and heterogeneity of the glycan structures cannot be avoided so their management must be implemented during the production process.
In Application News No. L452, the analysis of a pyridylamino (PA)-glycan using a fluorescence detector was introduced. Here, the analysis of a 2-aminobenzamide-labelled glycan (2-AB glycan) is introduced. As in Application News No. L452, the world's highest sensitivity fluorescence detector, the RF-20Axs, was used for detection.

Analysis of Low Concentration Standard Solution

In this study, the fluorescent-labeled glycans that were used include 2-AB Man-5, 2-AB G2, and 2-AB G2FS1 (Prozyme). Their structures are shown in Fig. 1.
The analytical conditions that were used are shown in Table 1. The glycans were separated using hydrophilic interaction chromatography (HILIC). Fig. 2 shows the results of analysis of a 0.5 nmol/L standard solution using a 2 µL (1 fmol) injection. As can be confirmed from the obtained data, sufficient sensitivity is achieved even using an ultralow amount injection. The limits of detection (S/N=10) and quantitation (S/N=3.3), respectively, are shown in Table 2.

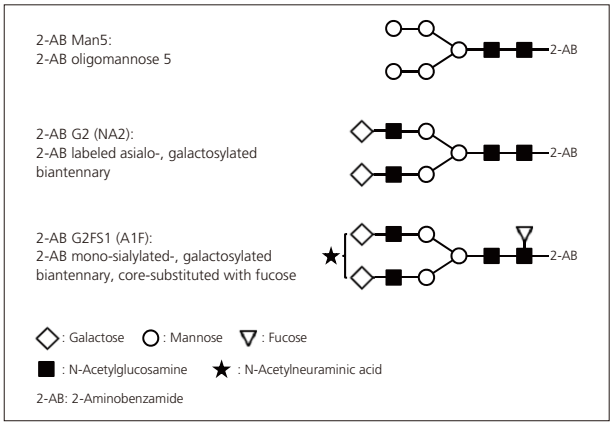

Fig. 1 Structures of 2-AB Glycans Used in This Study

Table 1 Analytical Conditions

System	: Prominence
Column	: TSKgel Amide-80 (150 mm L. × 2.0 mm I.D., 3 µm)
Mobile Phase	: A: 50 mmol/L Ammonium formate pH 4.4 B: Acetonitrile
Time Program	: B.Conc. 73 % (0 min) → 60 % (48 min) → 0 % (49 - 53 min) → 73 % (54 - 80 min)
Flowrate	: 0.4 mL/min (0 - 48 min, 58.01 - 80 min) 0.2 mL/min (48.01 - 58 min)
Column Temp.	: 40 °C
Injection Vol.	: 2 µL
Detection	: Ex 330 nm, Em 420 nm
Flow Cell	: Conventional cell

*Preparation of Mobile Phase A
After dissolving 3.15 g (50 mmol) ammonium formate in 1 L distilled water, about 340 µL formic acid was added to obtain a pH of 4.4.

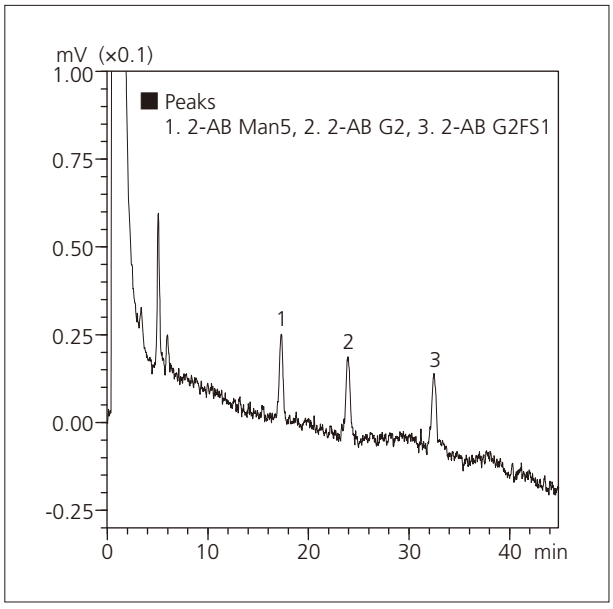

Fig. 2 Chromatogram of 1 fmol Each of 2-AB-Labeled Glycans (0.5 nmol/L each, 2 µL injection)

Table 2 Limits of Detection and Quantitation

Glycan standard	LOD (fmol)	LOQ (fmol)
2-AB Man5	0.44	1.33
2-AB G2	0.45	1.36
2-AB G2FS1	0.50	1.48

Repeatability and Linearity

Fig. 3 shows the results of six repeat measurements of a 20 nmol/L standard solution, and Table 3 shows the respective retention time and peak area repeatability values obtained (n=6). As indicated by the results, good repeatability was obtained. Fig. 4 shows the results of linearity evaluation using standard solution concentrations from 1 nmol/L to 100 nmol/L. Excellent linearity was obtained with a coefficient of determination (R^2) value greater than 0.999.

Analysis of 2-AB Glycan Mixture

We conducted analysis of 2-AB Human IgG N-Linked Glycan Library (Prozyme) as a mixed glycan sample. Fig. 5 shows the results of analysis in which 2 µL (160 fmol) of 80 nmol/L 2-AB Human IgG N-Linked Glycan Library was injected.

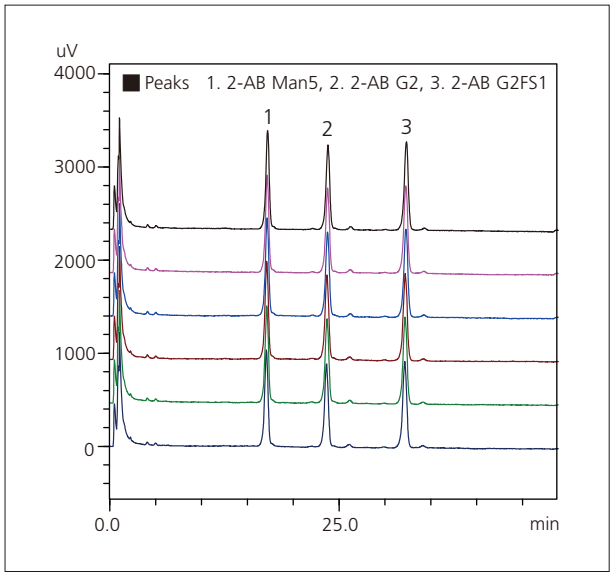

Fig. 3 Chromatogram of 40 fmol Each of 2-AB-Labeled Glycans (20 nmol/L each, 2 µL injection)

Table 3 Repeatability

Glycan standard	R.T. %RSD	Area %RSD
2-AB Man5	0.273	0.743
2-AB G2	0.245	0.684
2-AB G2FS1	0.196	0.589

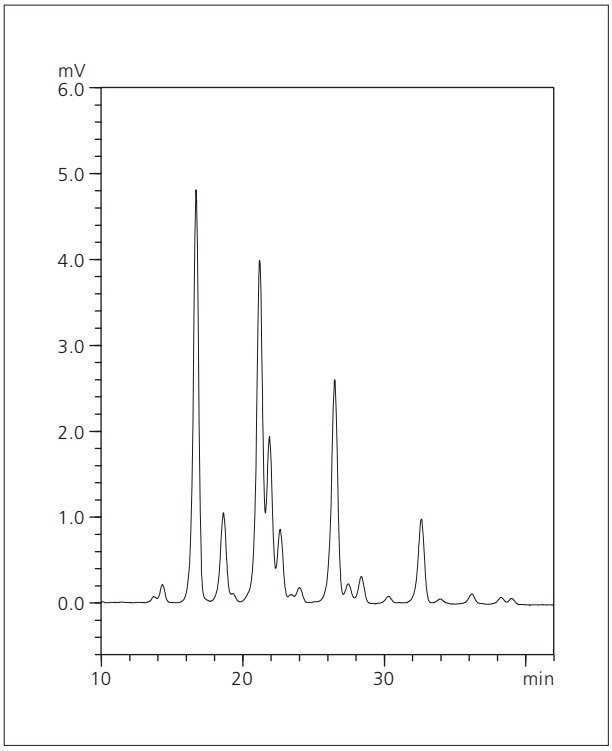

Fig. 5 Chromatogram of 160 fmol of 2-AB Human IgG N-Linked Glycan Library (80 nmol/L, 2 µL injection)

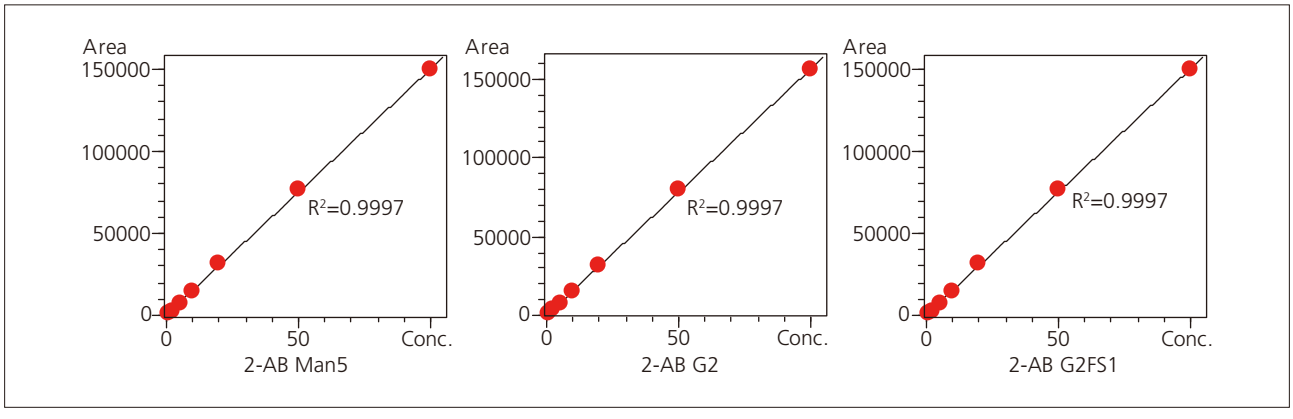

Fig. 4 Linearity from 2 to 200 fmol (1 to 100 nmol/L, 2 µL injection)

First Edition: Feb. 2015

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C146-E294

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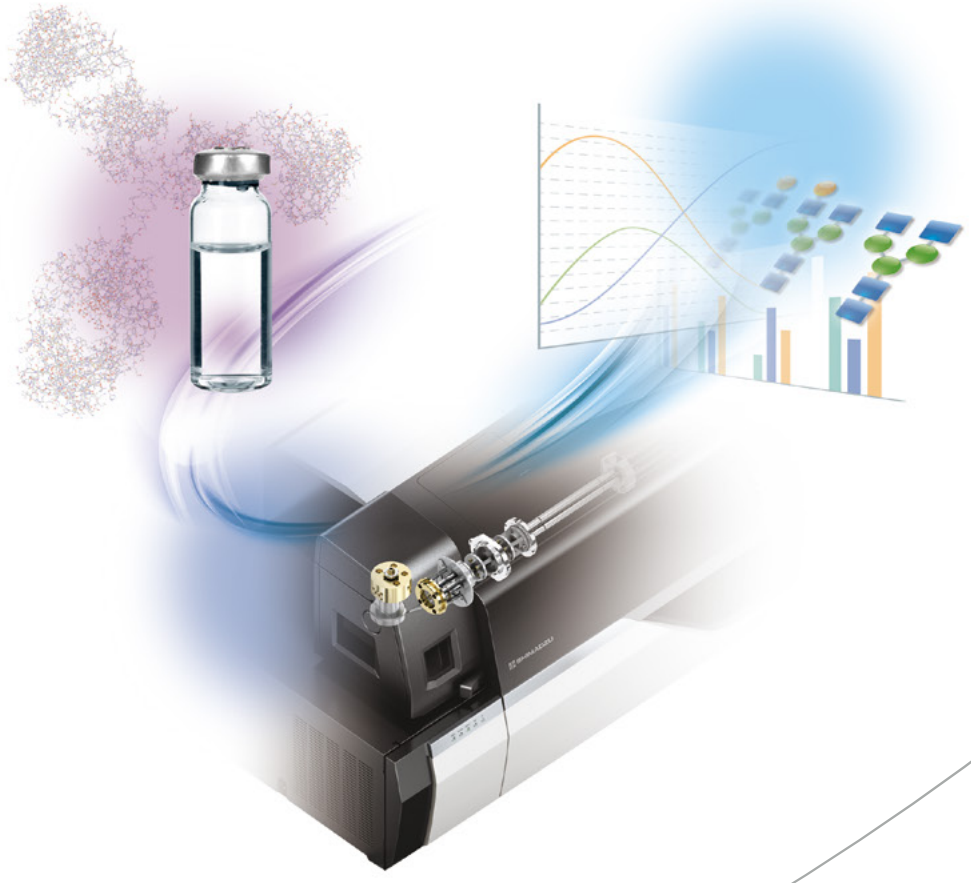
Software Platform for Glycan Quantification and Qualification by LCMS-8060/8050

Erexim Application Suite



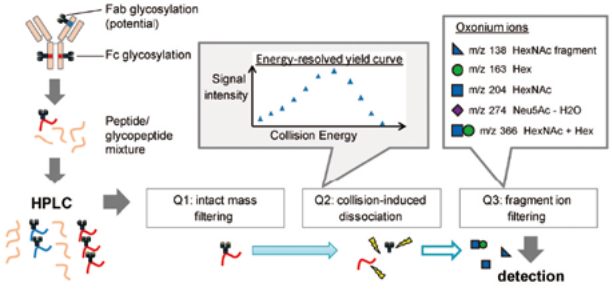
Simplifies Glycan Heterogeneity Analysis at Individual Glycosylation Sites

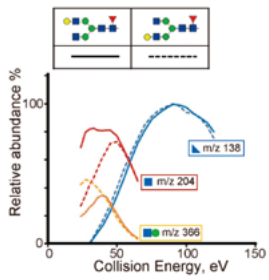
Analysis of N-linked glycans are most frequently performed by first detaching the glycan from the protein. Although this approach is accurate in both quantitative and qualitative respects, the result given is an averaged picture of glycans derived from all possible glycoproteins and glycosylation sites. In order to focus on the glycan heterogeneity occurring at a specific glycosylation site of interest, analysis needs to be performed at the glycopeptide level using enzymatic protein digests. However, since glycopeptides have unique masses, data analysis requires labor-intensive informatics and manual data manipulation. Erexim Application Suite is designed to facilitate the analysis of site-specific glycan heterogeneity by providing customizable ready-to-use methods and automated data analysis.

- Supports all glycan structures with a customizable database
- MRM method file generated with minimal user input
- Visualization of quantitative and qualitative results

What is Erexim™ (Energy-resolved oxonium ion monitoring)?

When analyzing glycans or glycan-containing molecules by MS/MS, the product ions generated by fragmentation include a high abundance of glycan-derived low *m/z* ions called the oxonium ions. Although the species and relative abundance of oxonium ions reflect the glycan structure of origin, conventional MS/MS provides insufficient features to differentiate between glycan structures. Energy-resolved oxonium ion monitoring, abbreviated as Erexim, adds another dimension to MS/MS data by acquiring data at a series of collision energies (CE) of fragmentation. A plot of the change in oxonium ion abundances with respect to CE, the Erexim profile, now contains the resolving power to differentiate between similar glycan structures. Erexim requires triple quadrupole mass spectrometry for its ultrafast scan speed to acquire a multitude of data points and for its quantitative ability to acquire reproducible profiles. Moreover, one of the product ions targeted in Erexim is specific to the N-glycan core structure and is an ideal reporter ion for relative quantitation of glycan structures.

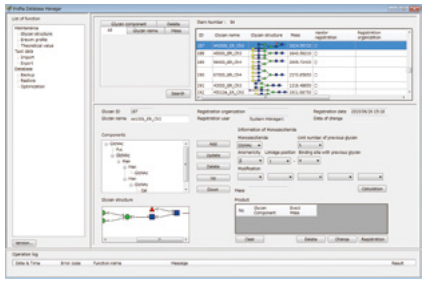




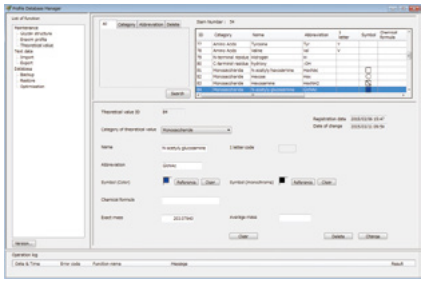
Reference: A. Toyama *et al.*, *Anal. Chem.* 2012, 84, 9655–9662

Customizable glycan structure database **Profile Database Manager**

The database of Erexim Application Suite contains 45 entries of N-glycan structures. Each entry contains monosaccharide composition, linkage information, amino acid sequence (if glycopeptide) and the reference Erexim profile. Entries may be added by customers to keep updated with research progress.



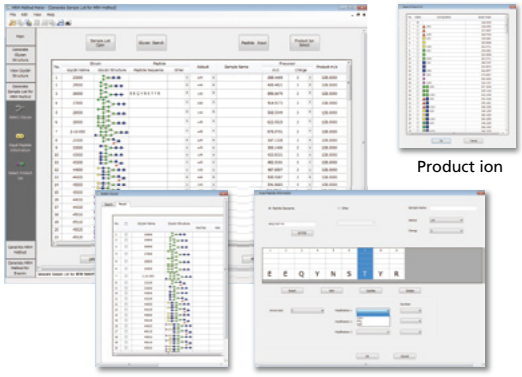
Glycan structure manipulation



Detailed definition of components

Compile input into a "ready-to-use" method **MRM Method Maker**

For detailed quantitative analysis, as well as for Erexim profile data acquisition, the number of MRM transitions may be hundreds and it is extremely labor-intensive to design them correctly. MRM Method Maker automatically produces MRM transitions according to the selection of glycan structures of interest, peptide sequence, ion adduct type and other inputs. MS acquisition parameters such as CE, dwell time, etc. can also be assigned collectively.

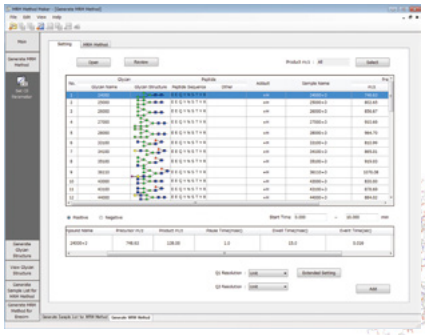


Product ion

Glycan structure

Peptide (label) and adducts

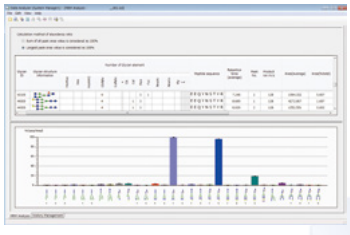
Selection of analysis targets



Setting MS Acqusition Parameters

Automated graphical representation of data **Data Analyzer**

Because glycans and glycopeptides are detected at multiple charge states in LC/MS, their quantitation requires complex data manipulation to correctly combine all ions derived from each molecular species. This process is automated by the Data Analyzer, and the result will be presented graphically, either as a bar chart of relative abundance or as an Erexim profile plot.

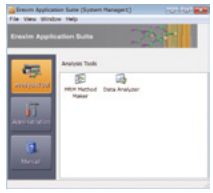


Bar chart of relative abundance

Erexim profile plot

Erexim™ Application Suite — from glycan analysis to data presentation

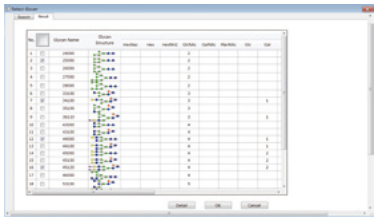
Each application is executed from the main page of LabSolutions.



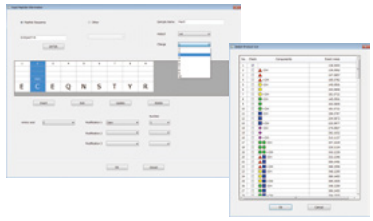
1st Step: Analysis of glycan heterogeneity (Quantitative Analysis)

MRM analysis workflow

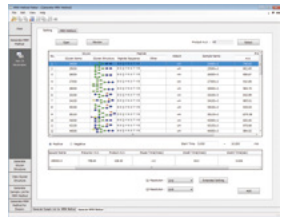
Launch MRM Method Maker



Select glycan structures of interest

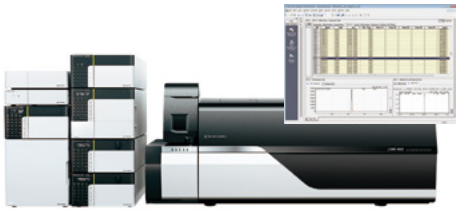


Enter peptide information, charge states, adducts, product ion species



Select parameters for MS Acquisition

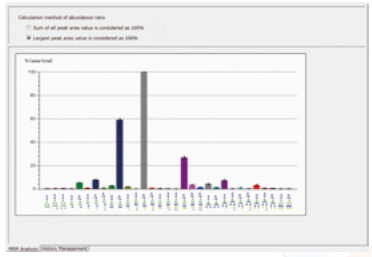
Method File generated



LCMS-8060/8050
Analysis and peak detection

Launch Data Analyzer

Load raw data

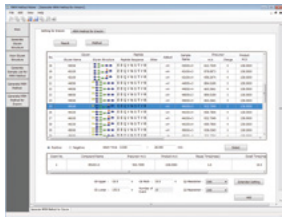


Graph of relative abundance of glycans

Reporting

2nd Step: Erexim Profile Acquisition (Qualitative Analysis)

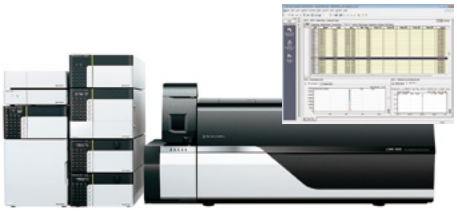
Erexim profiling workflow



Launch MRM Method Maker

Load MRM analysis data; Select glycan structure for Erexim profiling

Parameter setting for Erexim

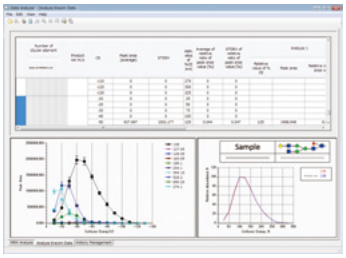


LCMS-8060/8050
Analysis and peak detection

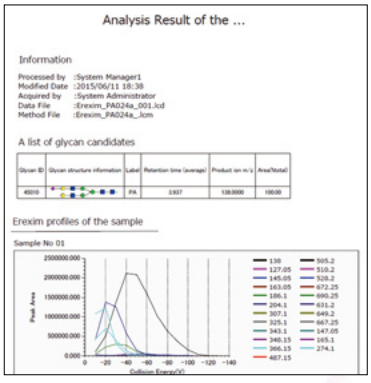
Method File generated

Launch Data Analyzer

Load raw data



Erexim profile plot
(register to database)



Reporting

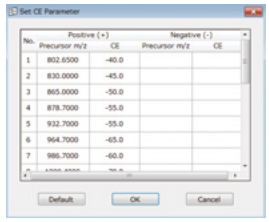
Analysis of a commercially available IgG glycopeptide

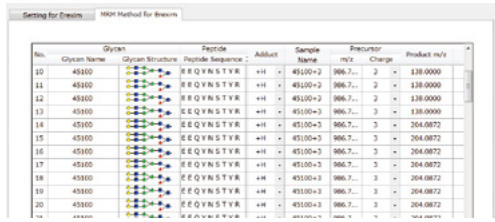
Here we show an example of N-glycan heterogeneity analysis by Erexim Application Suite, targeted specifically for the glycosylation site in the Fc region of a commercially available monoclonal antibody. The sample was prepared by digesting the antibody solution (50 µg) with trypsin for 2 hours, then removing hydrophobic peptides and residual trypsin by passing the reaction mixture through a Supel-Select HLB SPE cartridge. The flow-through fraction is rich in Fc region glycopeptides.

1 Using the MRM Method Maker, glycan structures of interest were selected to generate the list of target glycopeptides.

Glycan ID	Structure	Glycan ID	Structure	Glycan ID	Structure	Glycan ID	Structure	Glycan ID	Structure
26000		44000		45110		53000		54110	
27000		44100		45020		53100		55010	
28000		45000		45120		54000		55110	
23100		45100		34000		54100		56000	
33000		44010		33100		55000		56100	
43000		44110		34100		55100			
43100		45010		34110		54010			

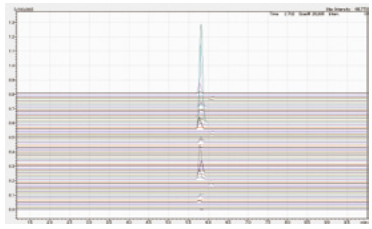
2 MS acquisition parameters such as Dwell Time, Pause Time, CE were entered, which converts the compound list to MRM transitions. CE values can be filled automatically with empirically derived optimum values.





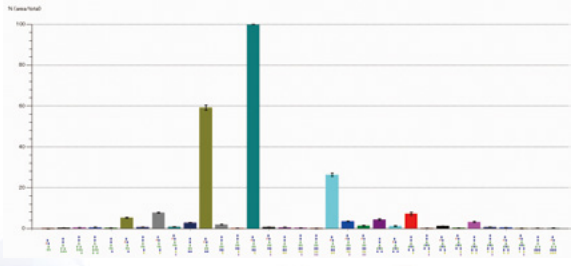
3 The MRM transition list generated in step (2) was saved as a method file, which was downloaded to the LCMS-8060 to perform the analysis. Three replicate measurements were performed.

Column: Aeris Peptide XB-C18 2.1 × 150 mm (Phenomenex)
Mobile Phase A: 0.1% Formic Acid
Mobile Phase B: 90% Acetonitrile / 0.1% Formic Acid
Gradient: 2%B (0–2 min) – 30%B (10 min) – 98%B (11–12 min) – 2%B (12–15 min)
Flow Rate: 0.3 mL/min
Injection Volume: 10 µL



MRM chromatogram

4 After performing peak integration with LabSolutions, the saved data was loaded into Data Analyzer. The results shown below were automatically generated.



Ratio graph of N-linked glycans binding to the IgG Fc region
(Amino acid sequence of the Fc region: EEQYNSTYR)

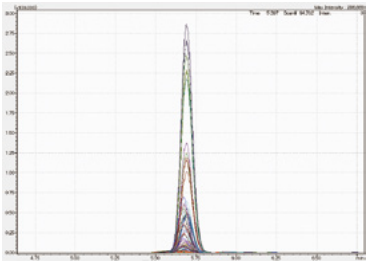
Glycan ID	%Area	STDEV	Glycan ID	%Area	STDEV	Glycan ID	%Area	STDEV
23100	0.009	0.003	44010	0.075	0.019	54010	0.079	0.007
26000	0.111	0.036	44100	43.191	0.62	54100	0.482	0.056
27000	0.19	0.052	44110	0.281	0.021	54110	0.114	0.053
28000	0.241	0.037	45000	0.229	0.061	55000	1.382	0.251
33000	0.113	0.007	45010	0.12	0.05	55010	0.264	0.035
33100	2.278	0.104	45020	0.027	0.025	55100	0.17	0.055
34000	0.291	0.039	45100	11.383	0.585	55110	0.014	0.012
34100	3.339	0.049	45110	1.511	0.142	56000	0.034	0.022
34110	0.37	0.043	45120	0.571	0.135	56100	0.084	0.046
43000	1.228	0.127	53000	1.871	0.321			
43100	25.506	1.055	53100	0.521	0.129	Total	100	
44000	0.837	0.102	54000	3.084	0.601			

Ratio of N-linked glycans

5 Using MRM Method Maker, Glycan Structure ID 45100 and 44100 was selected the target for Erexim profile acquisition. A Collision Energy (CE) range of –10 ~ –130 V at 10 V intervals was selected.

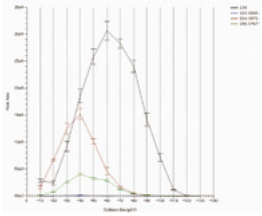
6 The information generated in Step (5) was saved as the LabSolutions Method File, which was downloaded to the LCMS-8060 to perform analysis. Three replicate measurements were performed.

Column: Aeris Peptide XB-C18 2.1 × 150 mm (Phenomenex)
Mobile Phase A: 0.1% Formic Acid
Mobile Phase B: 90% Acetonitrile / 0.1% Formic Acid
Gradient: 2%B (0–2 min) – 30%B (10 min) – 98%B (11–12 min) – 2%B (12–15 min)
Flow Rate: 0.3 mL/min
Injection Volume: 10 µL

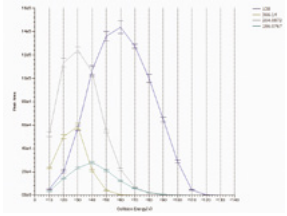


MRM Chromatogram

7 After performing peak integration in LabSolutions, the saved data was loaded into Data Analyzer. The results shown on the right are the Erexim profile plots generated.

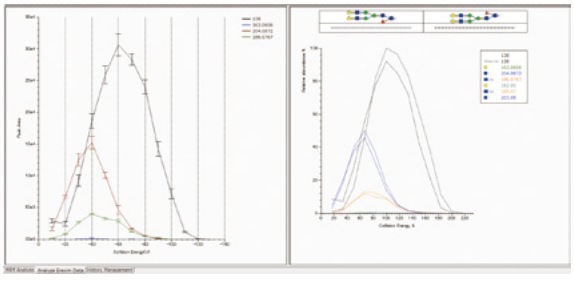


Erexim profile plot for Glycan ID 45100

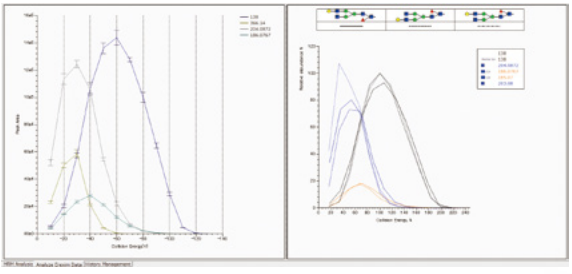


Erexim profile plot for Glycan ID 44100

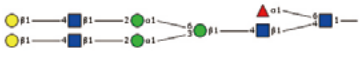
8 Referring to the Erexim plots registered in the database revealed that the newly acquired data appeared similar to the reference data of the same glycan mass, giving an indication that what was detected from the sample had the same structure as Glycan ID 45100_ER_Ch3. In contrast, the acquired data for Glycan ID 44100 was different from two of the reference profiles having the same glycan masses, 44100a_ER_Ch3 and 44100b_ER_Ch3. The curve for product ion *m/z* 204 in the acquired data fell in between the two reference profiles, providing the researchers important information regarding the composition of the sample.



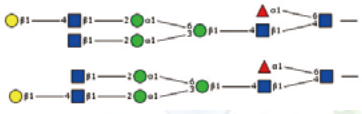
Comparison of Erexim profile plots of Glycan ID 45100 (Left panel: acquired data, Right panel: overlay of acquired data onto reference plot)



Similarly, acquired data (left) and overlay of two reference plots (right)



Structure of 45100_ER_Ch3



Structures of 44100a_ER_Ch3 (top) and 44100b_ER_Ch3 (bottom)

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Application News

No. B74

MALDI-TOF Mass Spectrometry

Detection of High-mass Proteins Using a Benchtop MALDI-TOF Mass Spectrometer

The applicability of MALDI-TOF mass spectrometry to perform protein detection is well recognized in the life science field. In this field, SDS polyacrylamide gel electrophoresis and size exclusion chromatography have been historically used, however, they have drawbacks such as being time-consuming or lacking accuracy in molecular weight determination. Due to its ability to provide more accurate molecular weight information, MALDI-TOF mass spectrometry has become the primary tool for the analysis of protein primary structures. Moreover, in recent years, the analysis of proteins at the femtomole and subfemtomole levels is often required, which is increasing the demand for higher sensitivity measurements with MALDI-TOF mass spectrometry.

The mass range of MALDI-TOF mass spectrometry is potentially unlimited and gives full access to low- to high-mass molecules, such as antibodies. Monoclonal antibodies (see Fig. 1) are utilized for diagnostic and therapeutic purposes. In their development and quality control processes, it is very important to use fast and reliable analytical methods.

Here, we demonstrate the capability of a benchtop MALDI-TOF mass spectrometer (MALDI-8020, Fig. 2)to perform high-throughput protein detection with high sensitivity.

S. Salivo (Y. Yamazaki)

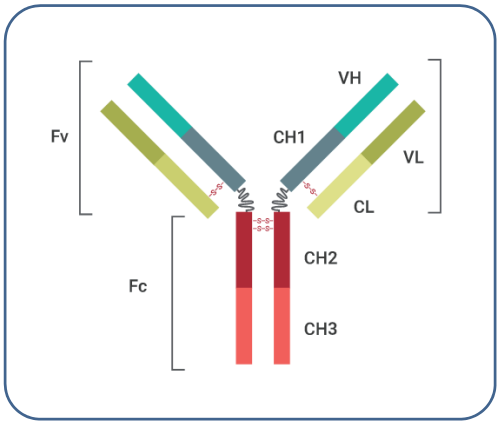

Fig. 1 Generalized Structure of an Antibody

Sample Preparation and Measurement Conditions

The samples - bovine serum albumin (BSA) and immunoglobulin A (IgA) - were purchased from Sigma-Aldrich. These were prepared at a concentration of 500 fmol/µL and 20 pmol/µL, respectively. Fifty shots were accumulated per profile (200 profiles per spectrum). The mass spectra were recorded using the average masses.

Tuning	Linear
Polarity	Positive
Laser rep. rate	200 Hz
Accumulation rate (shots/profile)	50
Profiles	200

Fig. 2 Benchtop MALDI-TOF MS: MALDI-8020

Results

To demonstrate the MALDI-8020 sensitivity, Fig. 3 shows the mass spectrum of BSA. The singly- (approx. 66 kDa), doubly- (approx. 33 kDa) and triply-charged (approx. 22 kDa) ions were observed with good signal-to-noise ratio which compared favourably with the linear mode performance of a conventional MALDI-TOF instrument.

Fig. 4 shows the mass spectrum of IgA. The singly-charged mass expected at approx. 160 kDa was observed along with the doubly-charged ion (approx. 80 kDa). The signal detected at approx. 54 kDa is consistent with the mass of the heavy chain (approx. 55 kDa expected).

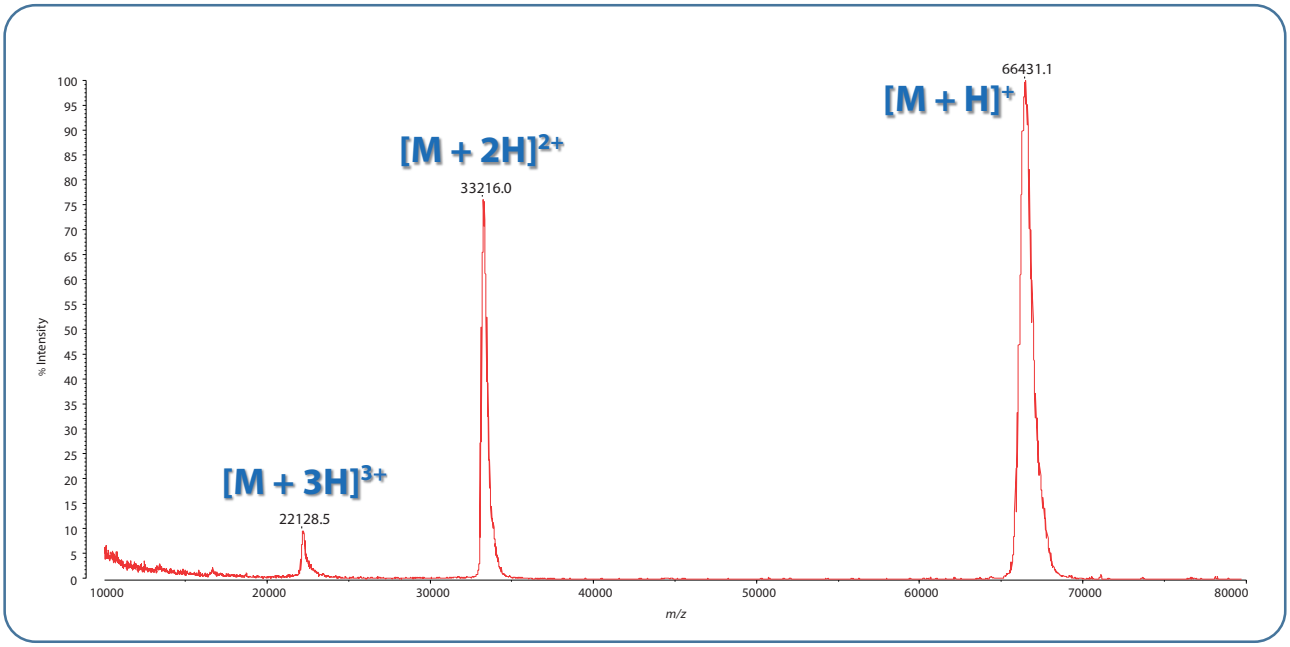

Fig. 3 Mass Spectrum of BSA (500 fmol/µL; 250 fmol on-target)

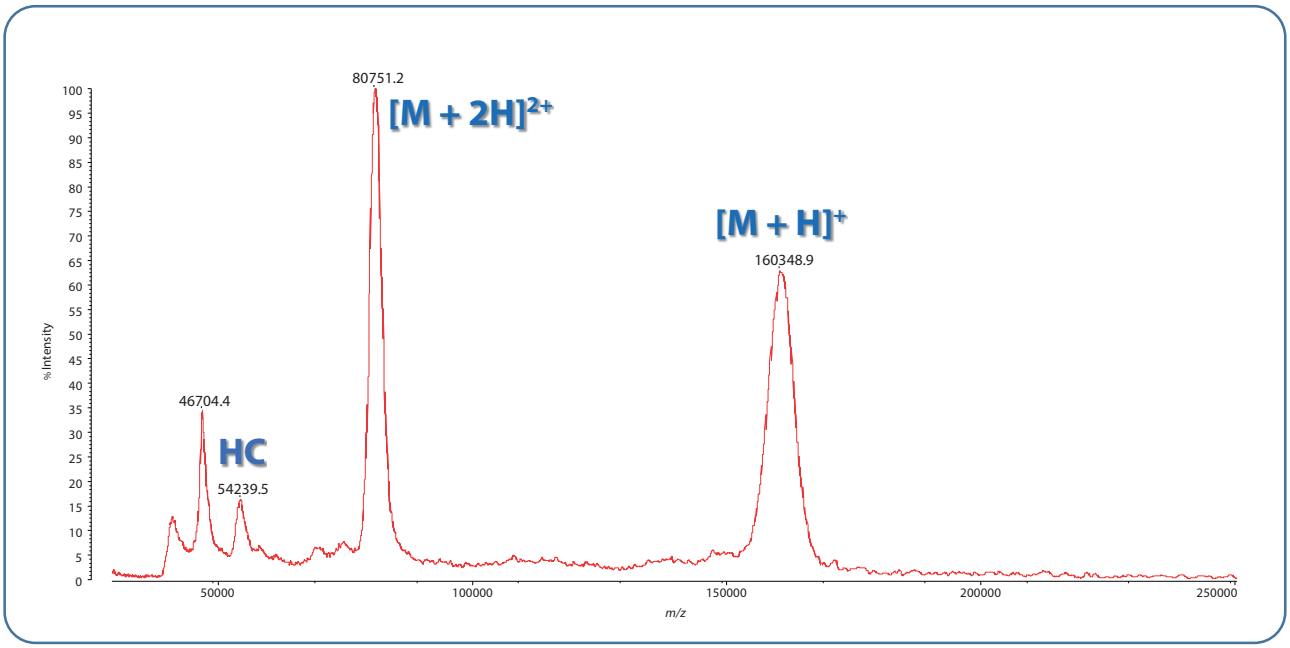

Fig. 4 Mass Spectrum of IgA (20 pmol/µL; 10 pmol on-target). HC = heavy chain

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Application News

No.B60

MALDI-TOF Mass Spectrometry

Detection of Protein Aggregates: Detection of Multimeric Proteins Using MALDI-TOF MS with a High Mass Detector

The unintended formation for protein aggregates may cause antigenicity and impair protein function in protein drug development and during the various types of research that use proteins. Consequently, monitoring the formation of protein aggregates in different environments is a very important matter both for acquiring consistent biochemical data and for the quality control of drugs.
In this article, we will describe the detection of protein aggregates using MALDI-TOF MS that is capable of detecting high mass molecules.

Detection of Multimeric Proteins Generated by Freeze-Thaw and Heat Treatment

Mouse antibody samples (1 µM, 10 µL) either subjected to two cycles of freeze-thaw treatment (-80 ˚C/room temperature) or held at 50 ˚C for 3 hours were mixed with K200 stabilizer (CovalX), a protein cross-linking reagent, and reacted for 1 hour under room temperature conditions.
After the reaction, sample solutions were mixed with a MALDI matrix (sinapinic acid, 10 mg/mL, 50 % acetonitrile aqueous solution/0.1 % trifluoroacetic acid aqueous solution) and analyzed using MALDI-TOF MS with a high mass detector. A sample of untreated antibodies reacted with K200 stabilizer at room temperature was also analyzed.

Analysis of the untreated commercially available antibodies by MALDI-TOF MS with a high mass detector revealed a strong antibody-derived signal at around 148 kDa as well as a weak antibody dimer signal (Fig. 1).

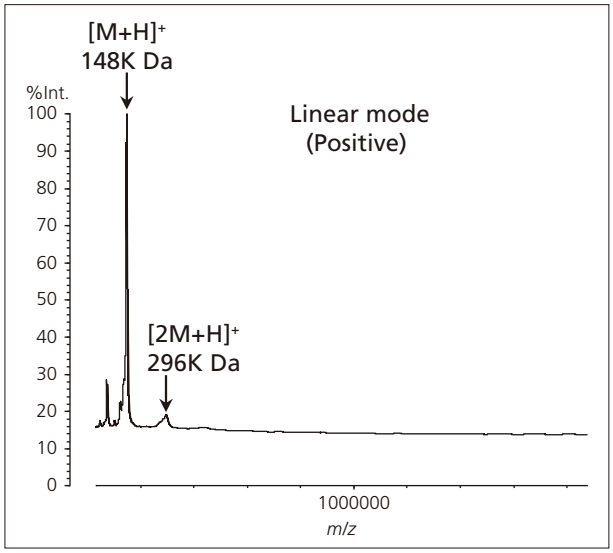

Fig. 1 Mass Spectrum of Untreated Sample

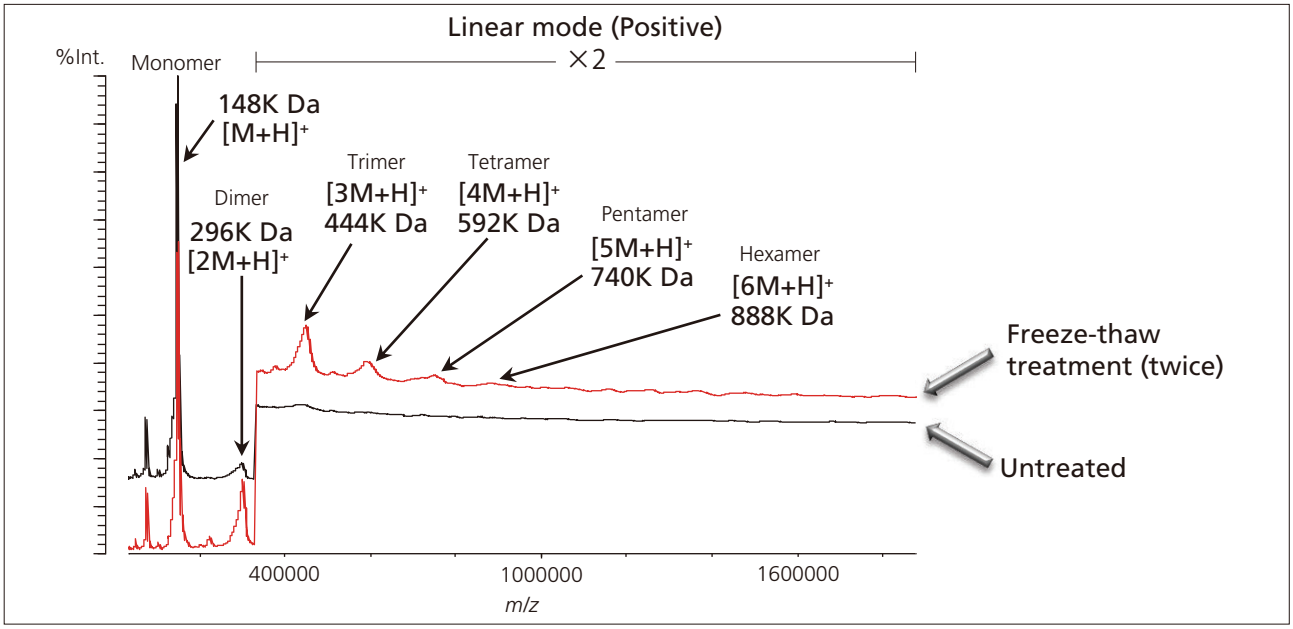

Fig. 2 Comparison of Freeze-Thaw Sample and Untreated Sample Mass Spectra

Compared against the untreated antibody sample, analysis of the sample subjected to repeated freeze-thaw treatment revealed a stronger signal derived from an antibody dimer, along with signals corresponding to an antibody trimer, tetramer, pentamer, and hexamer (Fig. 2). Similar to the sample subjected to freeze-thaw treatment, analysis of the sample held at high temperature also revealed clear signals derived from multimeric antibodies (Fig. 3).

Analysis of multimer-forming proteins using MALDI-TOF MS capable of detecting high mass molecules while utilizing a linking reagent to create cross-links allows us to understand how specific environments change protein aggregation.

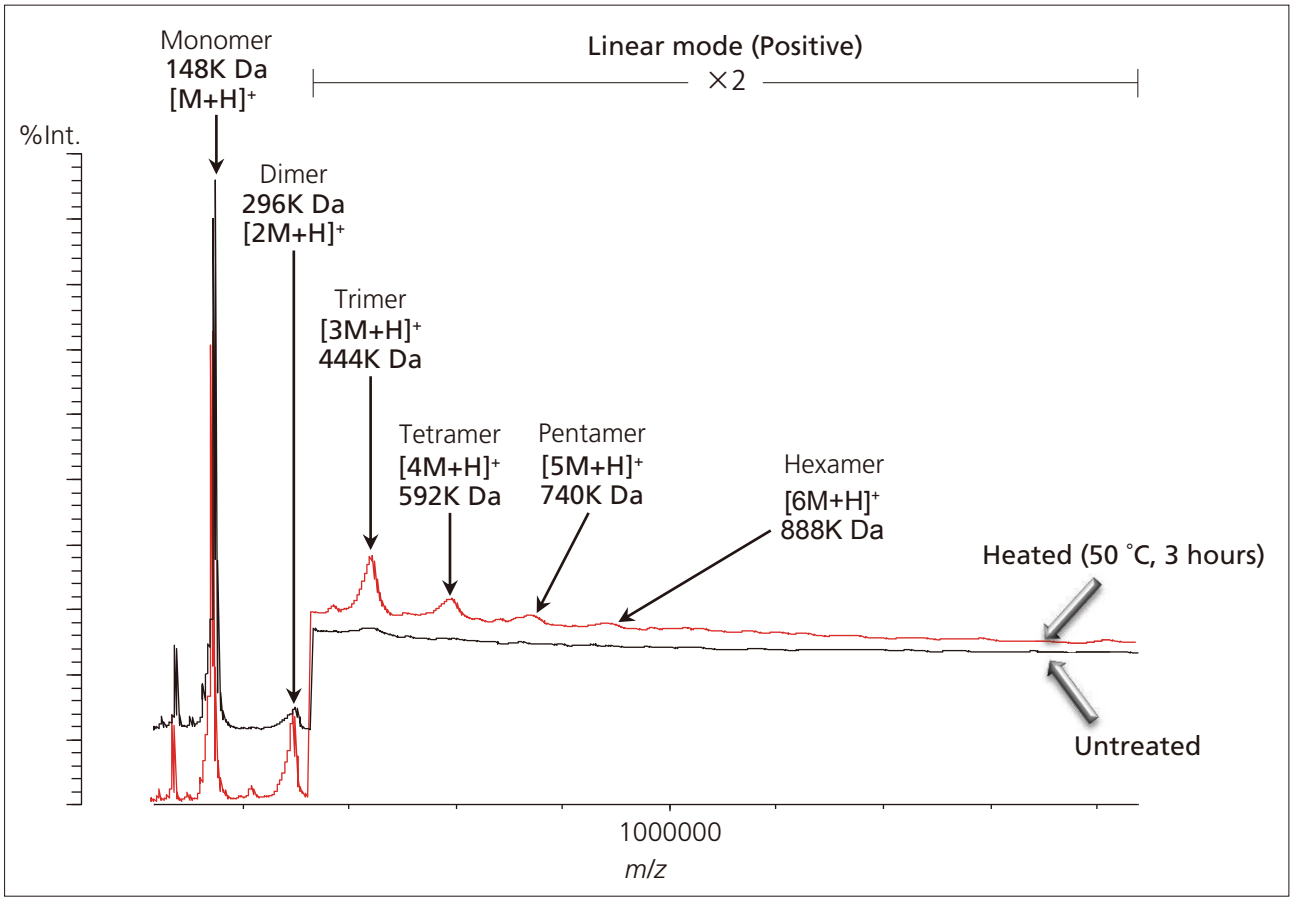

Fig. 3 Comparison of Heated Sample (50 ˚C, 3 hours) and Untreated Sample Mass Spectra

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Application News

No.Q114

Powder Property Analysis

Evaluation of Protein Aggregation Under Various Stress Conditions Using the Aggregates Sizer

Biopharmaceuticals have recently gained attention for their specificity in attacking pathogens, relative lack of side effects, and potent effect. However, compared with low molecular weight pharmaceuticals, biopharmaceuticals are more susceptible to stress, and more likely to aggregate. When a biopharmaceutical aggregates due to stress, this results in a decrease or disappearance of its pharmacological effect, along with the potential for causing serious side effects such as shock symptoms from an immunological reaction. Consequently, a framework is being established that evaluates the stability of biopharmaceuticals in terms of their susceptibility to likely stresses (heat and physical stresses during transport, storage, and at use).
Protein preparations are a type of biopharmaceutical and aggregate to form sub visible particles (SVP) in the size range of 0.2 to 10 µm. Problems with conventional methods of evaluating protein aggregates have been the inability to analyze the SVP size range in a single measurement, inability to take measurements while stress is applied, inability to recover samples after measurement, and inability to perform quantitative measurements. The Aggregates Sizer biopharmaceutical aggregation analysis system (Fig. 1) was developed to overcome these problems.
This article describes how we applied heat and physical stress to intravenous immunoglobulin (IVIG), then evaluated aggregate formation using the Aggregates Sizer. We show how different aggregate formation processes and speeds occur based on stress type and stirrer bar material by quantifying aggregates in the SVP size range.

Materials and Methods

IVIG was used as the sample. A freeze-dried sample was dialyzed with pH 7.4 phosphate buffer saline (PBS) as the external solution, and used as the stock solution (stored at 4 °C). The above stock solution was diluted with PBS (pH 7.4) to 0.87 mg/mL and used as the solution for measurement.
Heat stress was applied by placing 1 mL of IVIG solution (0.87 mg/mL) in a 1.5 mL tube, incubating at 70 °C for 5, 7 and 9 minutes, then taking measurements with a small volume cell (0.4 mL). Stirring stress was evaluated using three different stirrer bars, made of glass, stainless steel and PEEK. The batch cell (Fig. 2) provided with the Aggregates Sizer was filled with 5 mL of IVIG solution (0.87 mg/mL), and measurements were taken while stirring for 8 hours at 190 strokes/min at room temperature.
Particle size distribution and quantitative measurements in the SVP size range were performed by quantitative laser diffraction (qLD) method using the Aggregates Sizer. The Aggregates Sizer is a particle size analyzer that uses a 405 nm semiconductor laser and laser diffraction/scattering to detect the intensity of light scattered by particles between 0.04° and 160°. According to the Mie theory of light scattering, spherical particles of a specific diameter and concentration give rise to a certain scattering pattern and intensity. The particle size distribution can be obtained for the absolute concentration by applying this theory to the scattering pattern of a sample. Quantitative laser diffraction method using the Mie theory requires the refractive index and density of the sample substance. In this experiment, the refractive index was experimentally determined at 1.46-0.10i using a sucrose concentration gradient, and the density used was 1.37 g/cm^3.

Fig. 1 Aggregates Sizer Aggregation Analysis System for Biopharmaceuticals

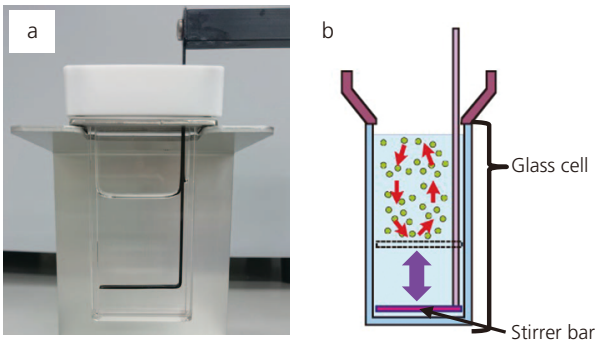

Fig. 2 Batch Cell Structure
(a) Actual batch cell (b) Diagram
The stirrer bar applies physical stress by shaking in a vertical direction.

Results and Discussion

As a representative example, the particle size distribution and amounts of the IVIG aggregates arising from heat stress and stirring stress caused by a glass bar are shown in Fig. 3. Exposure to heat stress only caused an increase in aggregates of around 0.2 µm in size, and no aggregates of 1 µm or larger were formed. Exposure to stirring stress resulted in an increase in aggregates in the region of 0.2 to 10 µm in size over time. The FDA suggests that particles in the SVP size range be evaluated by dividing into two size ranges of 0.2 to 2 µm and 2 to 10 µm, and the Aggregates Sizer is capable of measuring changes in aggregate amounts in both these size ranges simultaneously in a single measurement.

The Aggregates Sizer can also be used to calculate aggregate amounts based on a numerical conversion as well as a mass conversion. In light of these results, we consider quantitative laser diffraction method to be an effective means of evaluating the effect of heat and stirring on proteins in manufacturing and purification operations.

Reference

This article was summarized in part in "Quantitative Laser Diffraction Method for the Assessment of Protein Subvisible Particles" (Totoki et al, 2014, J. Pharm. Sci. 104 (2): 618-626), an article published based on joint research with Susumu Uchiyama et al. of Osaka University. Please refer to the original article for details.

http://onlinelibrary.wiley.com/doi/10.1002/jps.24288/references (open access)

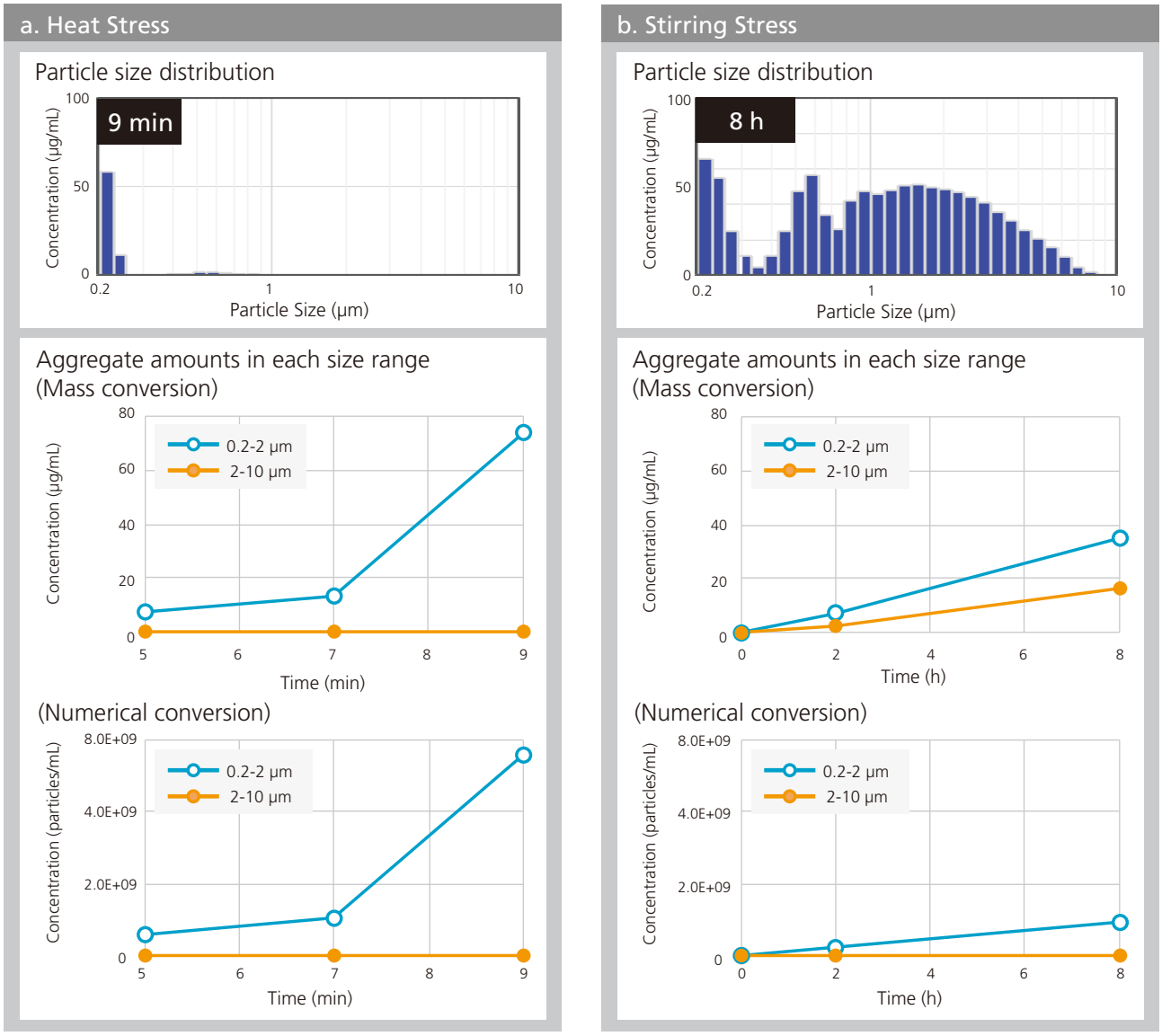

Fig. 3 Particle Size Distributions and Amounts of IVIG Aggregates Formed Under Heat or Stirring Stress

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Application News

No.Q115

Powder Property Analysis

Accelerated Testing of Protein Stability Using the Aggregates Sizer TC (With Temperature Control)

Biopharmaceuticals contact a variety of materials during their production, storage, and transport that include metal, plastic and glass. Protein stability differs depending on the materials it comes into contact with. Although investigations must be performed into materials appropriate for contact with biopharmaceuticals, analyzing the many materials a biopharmaceutical contacts during processing increases costs. Furthermore, several months or more are needed to perform a long-term evaluations of storage stability. Accelerated stability testing of contact materials performed in advance would improve the efficiency of investigations into production processes for biopharmaceuticals.
We conducted accelerated stability testing of proteins via the monitoring of aggregate formation during application of physical stress at constant temperature. Three agitator plate materials (PEEK, stainless steel, and glass) attached to the "Aggregates Sizer TC (with temperature control)", Aggregation Analysis System for Biopharmaceuticals was used for testing. The results we obtained indicated the importance of temperature control for stability testing and suggested different materials have different effects on aggregation.

Materials and Methods

A solution of freeze dried bovine-derived γ-globulin adjusted to 1 mg/mL with PBS (pH 7.4) was used as the sample.
Measurements were taken while stirring 5 mL of the sample solution in the batch cell (with temperature control, Fig. 1 (c)) for 40 minutes at 190 strokes/minute. Experiments were performed using three different materials for the stirrer rod: PEEK, stainless steel (SUS316), and glass. During accelerated testing, measurements were taken while maintaining a set temperature of either 23 ˚C, 30 ˚C, or 42 ˚C using a temperature controlled circulator.
Particle size distribution and quantitative measurements were made by the quantitative laser diffraction method (qLD method) using the Aggregates Sizer. A refractive index of 1.46-0.10i and density of 1.37 g/cm^3 were used.

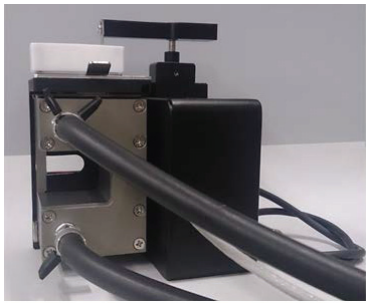
Results and Discussion

The change in particle size distribution over time at 42 ˚C using a PEEK stirrer plate is shown in Fig. 2 as a representative example set of results. Fig. 2 shows that aggregates formed over time. Aggregate formation over 40 minutes at 23 ˚C is shown in Fig. 3 based on the proposed aggregate analysis criteria of aggregate diameter ranges of 0.2 to 2 µm and 2 to 10 µm. Comparing aggregate formation in each size range, particles in the 2 to 10 µm range were most common when using the PEEK stirrer, and particles in the 0.2 to 2 µm range were most common when using the stainless steel stirrer. Results also show the lowest number of aggregates were present in either size range when a glass stirrer plate was used. Aggregated formation at each temperature is compared for PEEK in Fig. 4. This results show that during accelerated testing, aggregate formation increased dependent on temperature. Based on the above findings, temperature control is important for an appropriate analysis of aggregate formation during accelerated testing.

a

b

c



d

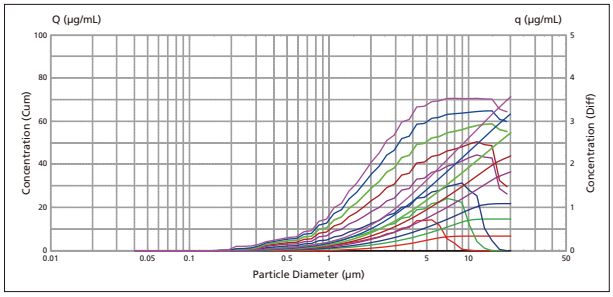


Fig. 1 Aggregation Analysis System for Biopharmaceuticals, "Aggregates Sizer TC (with Temperature Control) "
(a) Main unit, (b) temperature controlled circulator, (c) batch cell (with temperature control), and (d) monitoring screen

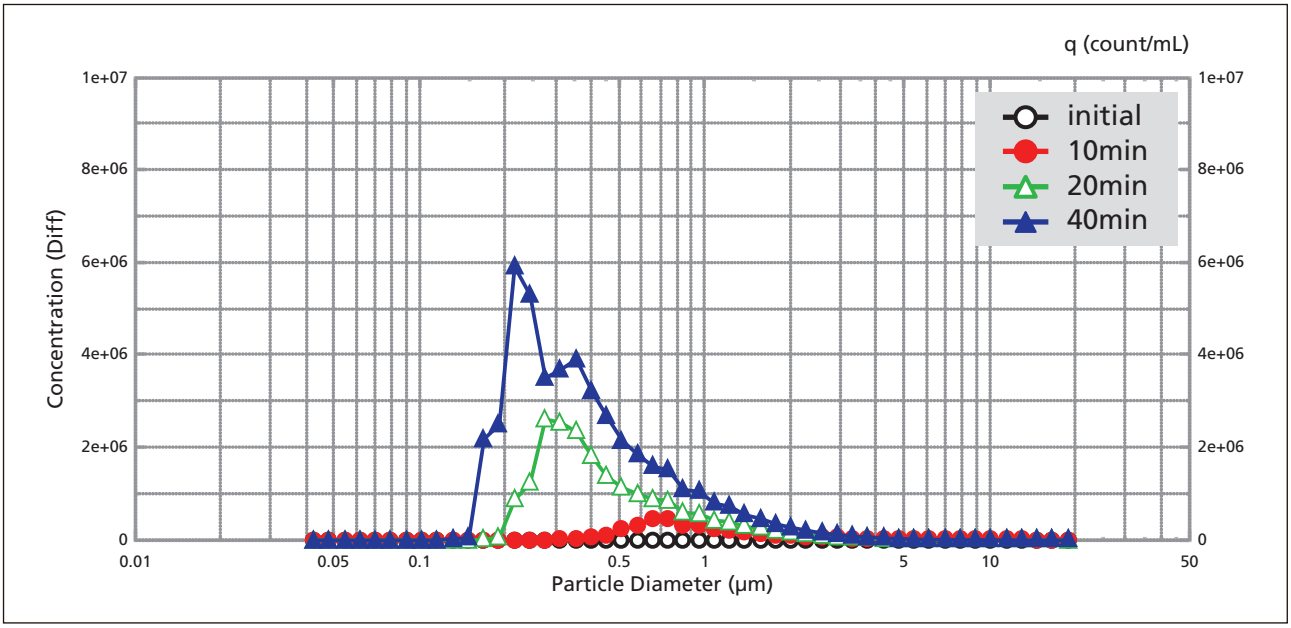


Fig. 2 Aggregate Formation over Time During Accelerated Testing (PEEK at 42 ˚C)

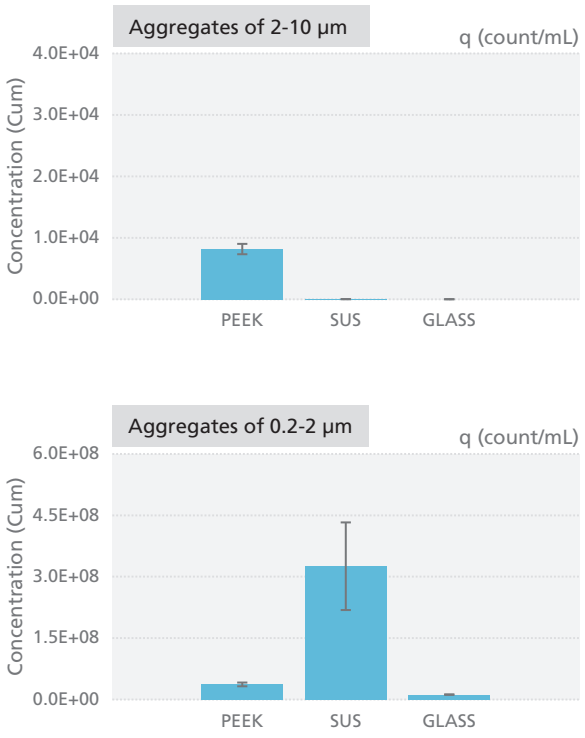


Fig. 3 Comparison of Aggregate Formation in Each Size Range for Each Stirrer Material (After 40 Minutes at 23 ˚C)

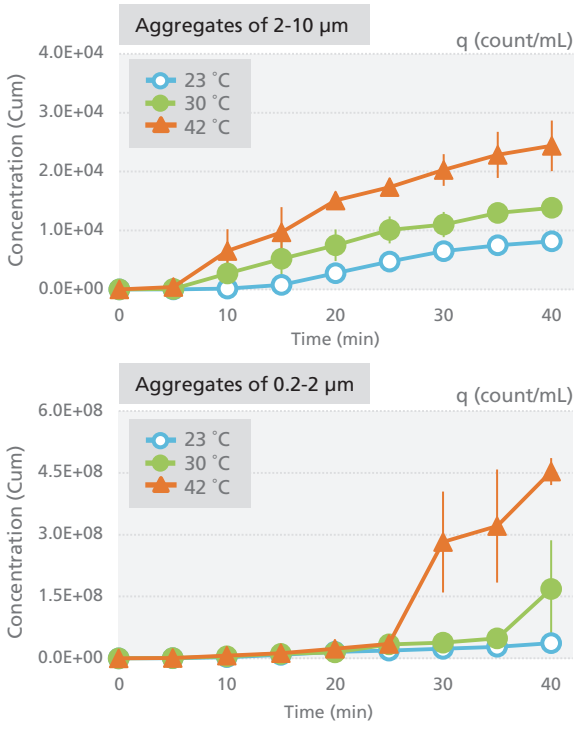


Fig. 4 Aggregate Formation over Time at Each Temperature (PEEK Stirrer)

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Application News

No. Q117

Particle Property Analysis

Aggregates Sizer Enables Evaluation of Biopharmaceutical Additives to Inhibit Protein Aggregation

When performing formulation studies for biopharmaceuticals, investigation into the conditions for inhibiting aggregate formation is required because it is known that aggregate formation in biopharmaceuticals can cause serious side effects in the human body such as anaphylaxis. Solution composition is one such condition, yet it in itself comprises a wide range of conditions which require investigation because protein stability depends on combinations of pH, additive types, and concentrations. Therefore, evaluation that can be performed in a short time is desirable when evaluating stability.

The Aggregates Sizer Aggregation Analysis System for Biopharmaceuticals (referred to as Aggregates Sizer hereafter) is useful for improving efficiency of formulation studies because a large number of analytes can be processed in the time taken to perform a single measurement, which is only a few seconds. We here introduce a study in which we confirmed the differences in the ability of each additive to inhibit protein aggregation, by using Aggregates Sizer to measure the concentrations of aggregate formation in protein solutions containing different additives that have repeatedly been frozen and thawed.

H. Maeda

Samples and Measurement Method

Freeze-dried human gamma globulin was used. A total of seven conditions were examined. Solutions of phosphate-buffered saline (PBS; pH 7.4) and PBS with pH changed to 5.8, 6.8, and 7.8 comprised four conditions. Solutions of PBS containing an additive of either 0.1% of polysorbate 20, 100 mM of L-arginine, or 100 mM of D-sorbitol comprised the remaining three conditions. Each of these seven solutions was used to prepare sample solutions with protein concentrations of 1 mg/ml.

In order to induce aggregate formation, sample solutions were frozen in a freezer at -80 °C and thawed in a water bath at room temperature (this process is referred to as the FT cycle hereafter) repeatedly 16 times. Concentrations of aggregate formation were measured after FT cycle 0 (prior to initial freezing), 1, 2, 4, 8, and 16.

Measurements of the particle size distributions and quantitative values were performed by the quantitative laser diffraction method (qLD method) using Aggregate Sizer. Micro cells, as shown in Fig. 1, were used. The refractive index of 1.46-0.10i and density of 1.37 g/cm^3 were used for the calculation parameters.

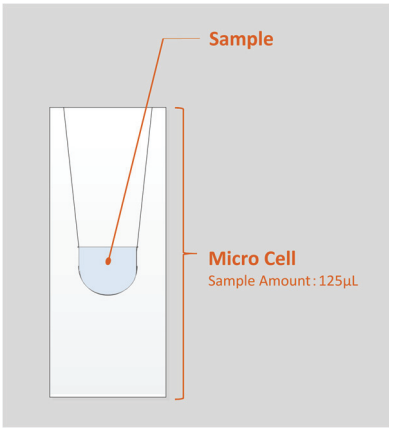

Fig. 1 Micro Cell

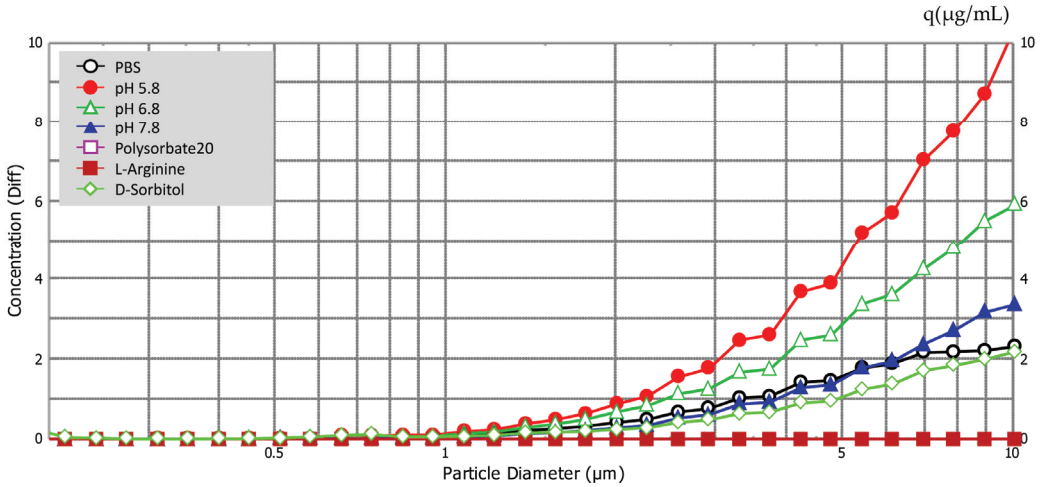

Fig. 2 Particle Size Distribution After the 16th FT Cycle

Results and Observations

Fig. 2 shows the particle size distribution of aggregates for each condition after the 16th FT cycle. We can see that aggregate formation is occurring in the 0.2 µm to 10 µm range.

Fig. 3 shows the concentration of aggregate formation in the 0.2 µm to 2 µm range and the 2 µm to 10 µm range for each condition in Table 1. Regarding pH, we can see that lower values correspond to higher concentrations of aggregates, indicating that changing the PBS to a higher acidity increases aggregate concentration. Regarding additives, for polysorbate 20 and L-arginine additives the intensity of scattered light originating from aggregates was less than the detection sensitivity and almost no aggregates were formed. For D-sorbitol, while aggregate formation was observed, it was inhibited to approximately half compared to PBS only.

Fig. 4 shows the transition in the concentration of aggregate formation for each FT cycle with respect to the pH 5.8 condition. We can confirm that the concentration of aggregate formation increases with the number of FT cycles.

The above results show that Aggregates Sizer is effective for solution composition studies.

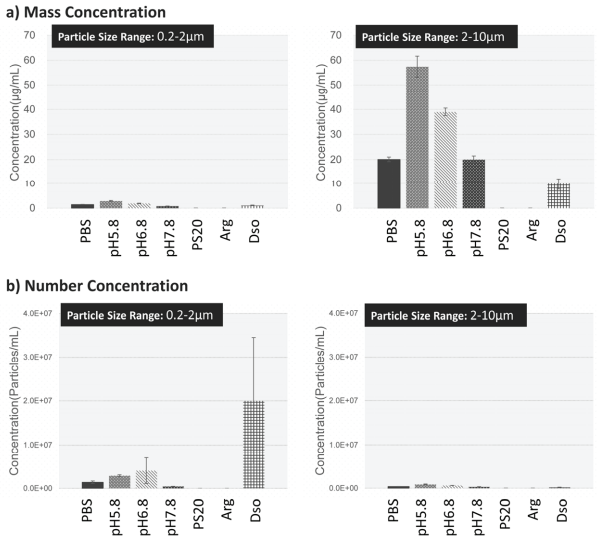

Fig. 3 Concentration of Aggregate Formation After the 16th FT Cycle for Each Condition

Table 1 Concentration of Aggregate Formation After the 16th FT Cycle for Each Condition

	Concentration of aggregate formation (µg/mL)			
	0.2-2 µm		2-10 µm	
	Avg.	SE	Avg.	SE
PBS	1.44	0.04	20.10	0.95
pH5.8	2.85	0.20	57.40	4.25
pH6.8	1.89	0.10	38.99	1.49
pH7.8	0.82	0.01	20.07	1.37
PS20	-	-	-	-
Arg	-	-	-	-
Dso	1.07	0.12	9.93	2.26

** Measured with n = 3

** Abbreviations in the table have the following meanings:
PBS: Phosphate-buffered saline (pH 7.4)
pH 5.8 /pH 6.8 /pH 7.8: PBS with pH changed to the indicated values
PS20: Polysorbate 20
Arg: L-arginine
Dso: D-sorbitol

** Particles were not detected for PS20 and Arg because the scattered light intensity was less than the quantitative lower limit.

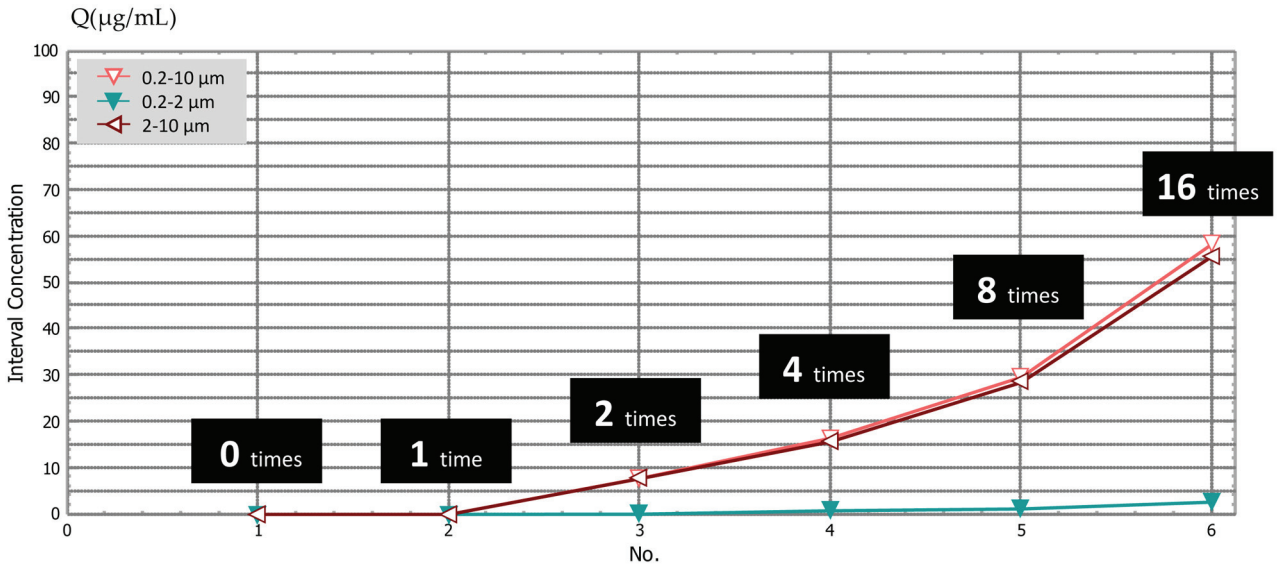

Fig. 4 Transition in the Concentration of Aggregate Formation for the Number of FT Cycles with Respect to the pH 5.8 Condition

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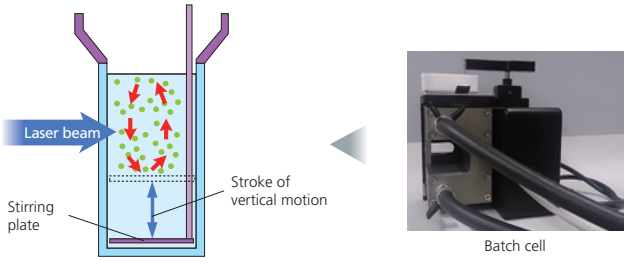
Aggregation Analysis System for Biopharmaceuticals (with Temperature Control Function)

Aggregates Sizer TC

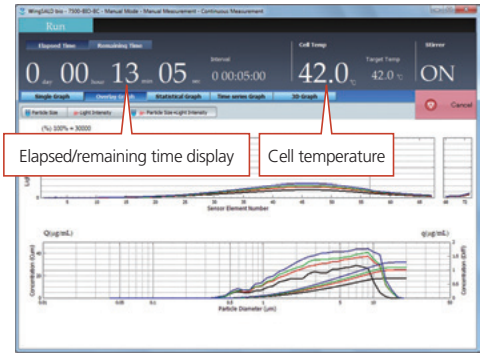
The Aggregates Sizer aggregation analysis system for biopharmaceuticals now includes a temperature controlled cell unit. Consequently, aggregates can be measured without temperature variations affecting the formation of aggregates, even for mechanical stress testing, which takes longer than regular measurements due to the batch cell stirring function involved. In addition, the micro cell size was reduced from 400 µL to 125 µL so that smaller sample quantities can be measured.

Feature 1 Aggregate Formation Process Can Be Determined More Quickly with an Improved Continuous Measurement Function

With the continuous measurement function, up to 200 data points can be measured successively (changes over time) at intervals as short as 30 seconds. During measurements, the system can display a real-time particle size distribution from current measurement data, in addition to the elapsed time and remaining time, and start processing the data measured thus far. This results in a quicker understanding of the aggregate formation process.

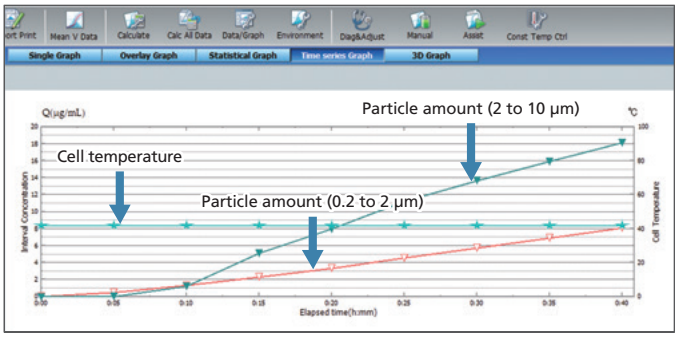


Batch cell



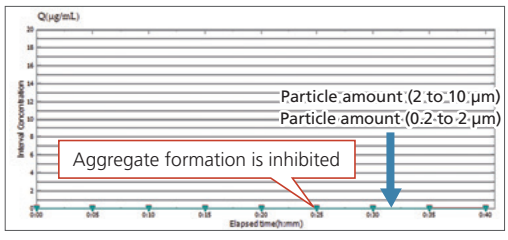
The system includes a refrigerated/heated circulator that can be used to keep the sample at a constant computer-controlled temperature within the 20 to 42°C range (with a temperature accuracy of ±1°C). Consequently, aggregates can be evaluated under constant-temperature conditions, so that aggregate formation is not affected by any temperature changes.

Example: Comparison of aggregate formation in protein solution depending on whether or not polysorbate is added



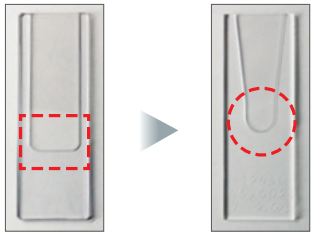
Polysorbate not added

Sample : Bovine gamma globulin
Solution : Phosphate buffer (pH 7.4, with 150 mM NaCl) + polysorbate-20
Sample Concentration : 1 mg/mL
Conditions : Stirred with stainless steel stirring rod (at 5 minute intervals for 40 minutes at constant 25°C temperature)



Polysorbate added

Feature 2 Cell Volume Reduced to 125 µL



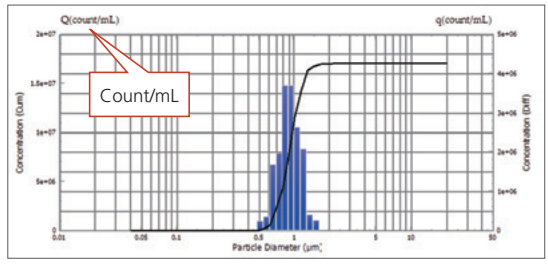
Old Cell　　New Cell

The amount of sample required was reduced from 400 µL to a low 125 µL by eliminating as much of the sample that does not affect measurements as possible. In addition, the micro cell material was completely changed to quartz glass, so that the cells can be cleaned chemically (alkaline) or ultrasonically.

Feature 3 Additional Software Functions Can Be Used to Obtain Necessary Information Quickly

Display Concentration in Terms of Count (count/mL)

In addition to the mass concentration values displayed on previous models (µg/mL), concentration can now also be displayed in terms of number of aggregates per milliliter (count/mL).



Evaluate Aggregate Quantities within Any Specified Particle Size Range

Data can be displayed for the same particle size intervals as used for current techniques (such as for particle size counters). It also can be used to evaluate the particle quantities in specific particle size intervals required by regulations and standards expected to be established in the future.

Specifications

Aggregates Sizer with Temperature Control Function	
Batch Cell Unit (with Temperature Control Function)	
Cell Material	Quartz glass, PTFE (funnel/cap)
Required Liquid Volume	Approx. 5 mL
Stirrer Mechanism	By vertical motion of stirring plate
Stirring Plate Material*	Stainless steel, glass, PEEK
Micro Cell Unit (with Temperature Control Function)	
Cell Material	Quartz glass, PTFE (cap)
Required Liquid Volume	125 µL
Temperature Range	20 to 42°C, constant temperature
Temperature Control Accuracy	±1 °C
Operating Environment	Temperature: 10 to 30 °C, Humidity: 20 to 80 %

Refrigerated/Heating Circulator	
Manufacturer	JULABO
Model	F25-ME
Computer Control	RS-232C connection
Dimensions and Weight	W230 mm × D420 mm × H610 mm, 31 kg
Required Power Supply	100 V AC, 13 A, 50/60 Hz

*The system includes three types of batch cell stirring plates made of three different materials (stainless steel, glass, and PEEK) commonly used for preparing or storing biopharmaceuticals.

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Application News

No.L486

High Performance Liquid Chromatography

Analysis of Polysorbate 80 in IgG Aqueous Solution by Online SPE Using Shim-pack MAYI Column – Part 1

Various analyses are required in the evaluation of drugs. When analyzing samples that contain macromolecular proteins at high concentrations by reversed phase HPLC, degradation of the column packing is a concern when using a typical ODS column, therefore requiring prior removal of proteins. As the Shim-pack MAYI series, with its packing pore outer surface coated with a hydrophilic polymer, offers a line-up of online SPE columns that can quickly eliminate proteins, when combined with a column switching HPLC, a variety of components can be analyzed in a seamless flow from deproteinization to analysis.
Examples of applications related to analysis of drugs in plasma and serum using the MAYI series have previously been reported in Application News No. L285, 286, 293, 305, 307, 315 and 327. By using the Co-Sense for BA bio-sample analysis system, even higher sensitivity, higher precision measurement can be achieved.
Here, we introduce an example of analysis of the polysorbate 80 surfactant, widely used as an additive to prevent protein aggregation and adsorption, and to increase protein solubility in a protein formulation.

Principle of Shim-pack MAYI Column

Fig. 1 shows the structure of the packing used in the Shim-pack MAYI column. While macromolecular proteins are blocked and cannot enter the pores, smaller molecules infiltrate the chemically modified pores to be retained on the column.

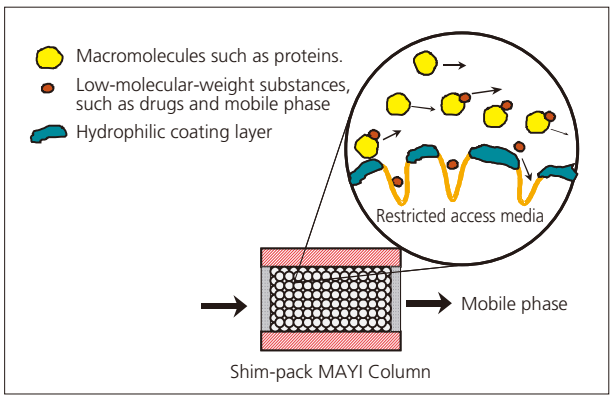

Fig. 1 Principle of Deproteinization with Shim-pack MAYI Column

By incorporating this column in the column switching HPLC flow line shown in Fig. 2, proteins introduced into the pretreatment column from the autosampler are directly discharged out of the system after passing through the column.

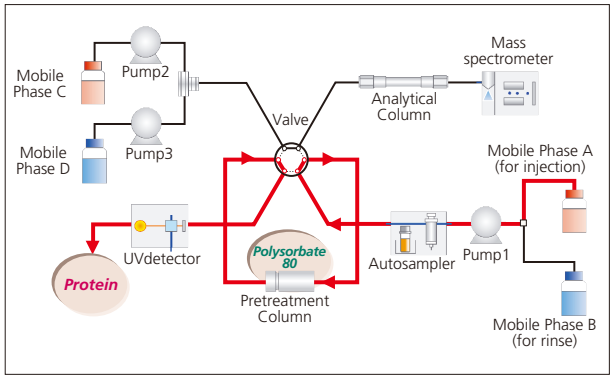

Fig. 2 Flow Diagram

By using a UV detector (wavelength 280 nm) to monitor an IgG model sample (described below), a chromatogram such as that shown in Fig. 3 is obtained, confirming that protein (IgG) is rapidly discharged.

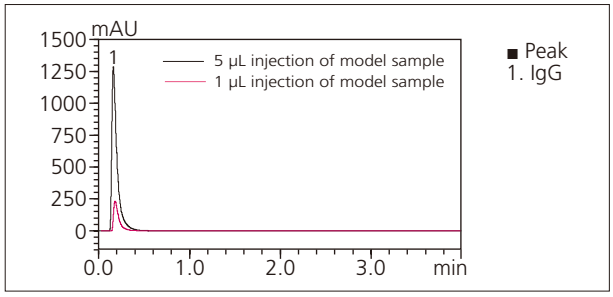

Fig. 3 Confirmation of Protein Elution from Shim-pack MAYI Column

Table 1 Analytical Conditions (Sample Loading)

Column	: Shim-pack MAYI-ODS (5 mm L. × 2.0 mm I.D., 50 µm)
Mobile Phase	: A: 10 mmol/L Ammonium Formate in Water B: 2-Propanol
Time Program	: Solvent switching A (0 - 1.5 min) → B (1.5 - 3.5 min) → A (3.5 - 9 min)
Flowrate	: 0.6 mL/min
Extraction Time	: 1 min
Injection Vol.	: 1 µL
Column Temp.	: 40 °C
Detection	: UV280 nm (Semi-micro cell)

On the other hand, a Shim-pack MAYI-ODS column, with packing pores that are chemically modified with C18 (octadecyl group), was used to extract polysorbate 80 on the pretreatment column side. After discharging the protein (in this case, 1 minute later), the valve is switched to direct the pretreatment column to the analysis channel, while the sample introduction flow line is rinsed to prepare for the next analysis, all operations that were programmed beforehand for automated execution.

Analysis of Standard Solution

The structural formula for polysorbate 80 (polyoxyethylene sorbitan monooleate) is shown in Fig. 4.

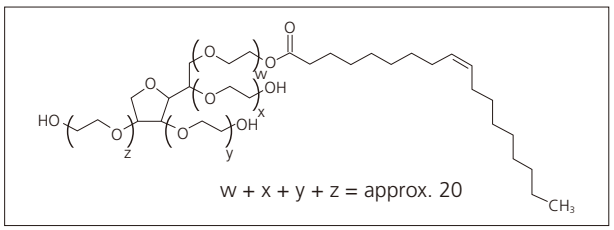

Fig. 4 Typical Structure of Polysorbate 80

Due to the weak UV absorption of polysorbate, a mass spectrometer was used for detection in the analytical flow line. The analytical conditions are shown in Table 2, and the TIC chromatogram of a standard sample (100 µg/mL) is shown in Fig. 5. Generally, polysorbate includes a large number of by-products, and because some of these are very strongly retained, 2-propanol was used as the final mobile phase.

Table 2 Analytical Conditions

Column	: Kinetex 5u C18 100 Å (50 mm L. × 2.1 mm I.D., 5 µm)
Mobile Phase	: C: 10 mmol/L Ammonium Formate in Water D: 2-Propanol
Time Program	: D.Conc. 5 % (0 - 1 min) → 100 % (6 - 7 min) → 5 % (7.01 - 9 min)
Flowrate	: 0.3 mL/min
Column Temp.	: 40 °C
Detection	: LCMS-2020
Ionization Mode	: ESI Positive
Applied Voltage	: 4.5 kV
Nebulizer Gas Flow	: 1.5 mL/min
Drying Gas Flow	: 15 L/min
DL Temp.	: 250 °C
Block Heater Temp.	: 400 °C
Scan Range	: *m/z* 300 - 2000

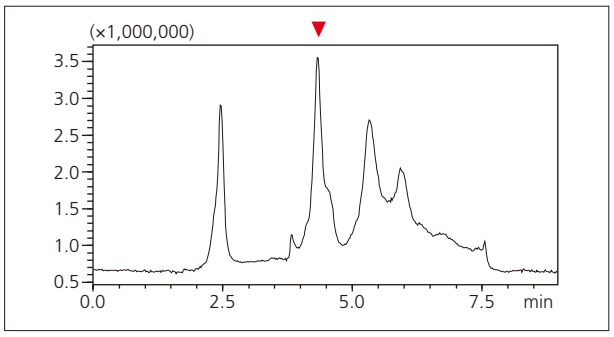

Fig. 5 TIC Chromatogram of 100 µg/mL Polysorbate 80 Standard

The mass spectrum of the peak in the retention time vicinity of 4.4 minutes is shown in Fig. 6. Many peaks are observed because of the included polyoxyethylene, which displays different degrees of polymerization. However, we conducted SIM measurement using the ion at *m/z* 783 as a marker for detection, which is attributable to the 2NH4+ adduct of polyoxyethylene sorbitan monooleate, containing 25 polyoxyethylene groups. (Fig. 7)

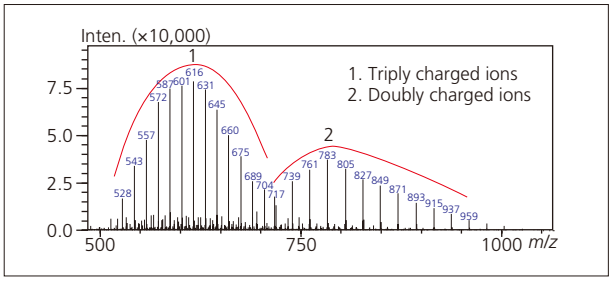

Fig. 6 Mass Spectrum of the Peak at 4.4 min in Fig. 5

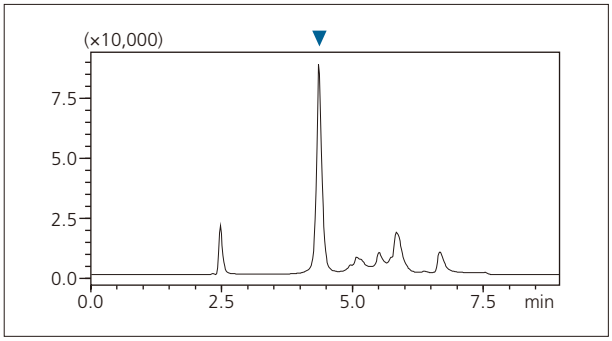

Fig. 7 SIM Chromatogram of 100 µg/mL Polysorbate 80 Standard

The results indicated a coefficient of determination (R^2) greater than 0.999 over a concentration range of 10 to 200 µg/mL, demonstrating excellent linearity. Following this, these conditions were applied to a protein-containing model sample.

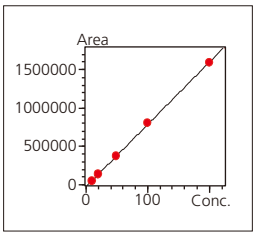

Fig. 8 Linearity (10-200 µg/mL)

Analysis of Antibody Model Sample

Polysorbate 80 was added to 10 mmol/L phosphate buffer solution (pH 6.8) that included 20 mg/mL of IgG, to obtain a concentration of 100 µg/mL, and this was injected into the HPLC as the sample. Utilizing online auto deproteinization, the polysorbate 80 recovery rate was 99 %, demonstrating measurement with excellent repeatability (retention time: 0.034 % RSD, peak area: 1.11 % RSD).

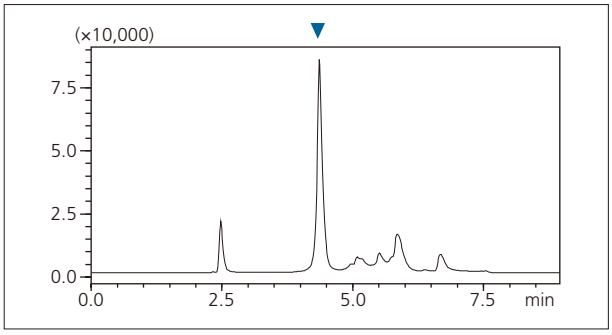

Fig. 9 SIM Chromatogram of Antibody Model Sample

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Application News

No.L487

High Performance Liquid Chromatography

Analysis of Polysorbate 80 in IgG Aqueous Solution by Online SPE Using a Shim-pack MAYI Column – Part 2

Polysorbates play a role in maintaining the stability of proteins to prevent their denaturation, so they are often added to protein formulations. Therefore, evaluation of the quality and stability of these formulations also requires evaluation of the quality of the polysorbate. A polysorbate is not just a mixture of polyoxyethylene sorbitan fatty acid esters with polyoxyethylene chains of different degrees of polymerization. It has a complex composition which includes a variety of by-products as impurities, and those are said to affect the stability of protein formulations. Thus, it is believed that utilizing the high resolution offered with an HPLC coupled with a mass spectrometer can provide effective monitoring of quality.

In Application News No. L486, a method in which automated deproteinization can be conducted followed by quantitative analysis of polysorbate 80 in an antibody model sample was presented. Here, using this system for higher resolution analysis, we conducted detection and mass spectral measurement of possible by-product components of polysorbate 80.

Analysis of Antibody Model Sample

In accordance with the analytical conditions of Table 1, a model sample (20 mg/mL IgG) spiked with 100 µg/mL of polysorbate 80 was injected.

Table 1 Analytical Conditions (Sample Loading)

Column	: Shim-pack MAYI-ODS (5 mm L. × 2.0 mm I.D., 50 µm)
Mobile Phase	: A: 10 mmol/L Ammonium Formate in Water B: 2-Propanol
Time Program	: Solvent switching A (0 - 3.5 min) → B (3.5 - 5 min) → A (5 - 110 min)
Flowrate	: 0.6 mL/min (0 - 5 min, 95.01 - 110 min) 0.1 mL/min (5.01 - 95 min)
Extraction Time	: 3 min
Injection Vol.	: 5 µL
Column Temp.	: 40 ˚C
Detection	: UV280 nm (Semi-micro cell)

After about 3 minutes, switching was made to include the pretreatment column in the analytical flow line, and polysorbate 80 began to elute from that column. In this case, separation with a more gradual gradient elution was achieved using an analytical column longer than that used in quantitative analysis (Application News No. L486). For detection, the LCMS-8050 was used (Fig. 1, Table 2). Fig. 2 shows the TIC chromatogram in which elution is achieved with an especially gradual slope and retention times up to 35 minutes.

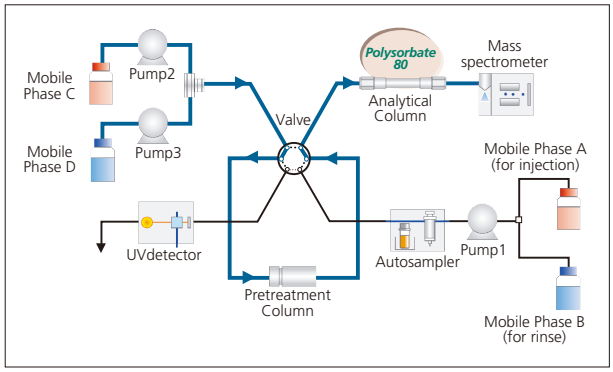

Fig. 1 Flow Diagram

Table 2 Analytical Conditions

Column	: Kinetex 5 µm C18 (100 mm L. × 2.1 mm I.D., 5 µm)
Mobile Phase	: C: 10 mmol/L Ammonium Formate in Water D: 2-Propanol
Time Program	: D.Conc. 3 % (0 - 3 min) → 15 % (35 min) → 100 % (100 min) → 3 % (100.01 - 110 min)
Flowrate	: 0.2 mL/min
Column Temp.	: 40 ˚C
Detection	: LCMS-8050
Ionization Mode	: ESI Positive
Applied Voltage	: 4.5 kV
Nebulizer Gas Flow	: 2 mL/min
Drying Gas Flow	: 10 L/min
Heating Gas Flow	: 10 L/min
Interface Temp.	: 300 ˚C
DL Temp.	: 250 ˚C
Block Heater Temp.	: 400 ˚C
Scan Range	: *m/z* 300 - 2000

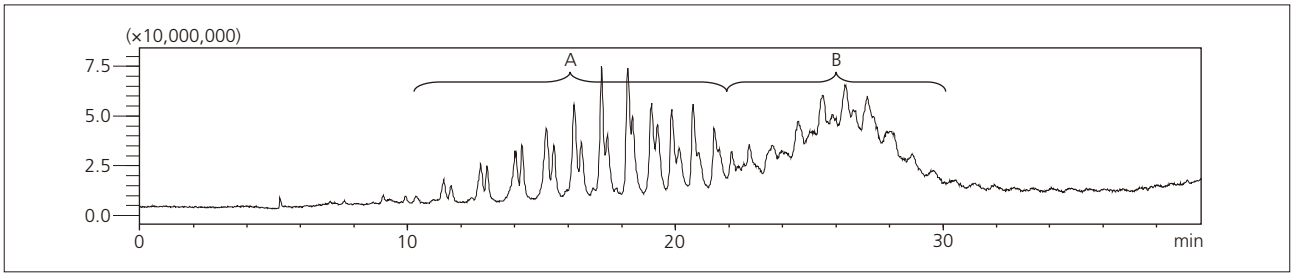

Fig. 2 TIC Chromatogram of Model Sample (0 – 35 min)

Confirmation of Mass Spectra

The TIC chromatogram is shown in Fig. 5, and the mass spectra are shown in Figs. 3, 4 and 6. Peak D, observed as the principle component in the TIC chromatogram, matched the quantitation target substance discussed in Application News No. L486, as well as the spectrum.
Regarding peak A, peaks with mass differences of 44 and 22 are singly and doubly charged ions, and from the literature, it is presumed that they are derived from polyoxyethylene isosorbide and polyoxyethylene. Peak E eluting in the second half showed a similar spectrum, and is assumed to be an ester of this substance.

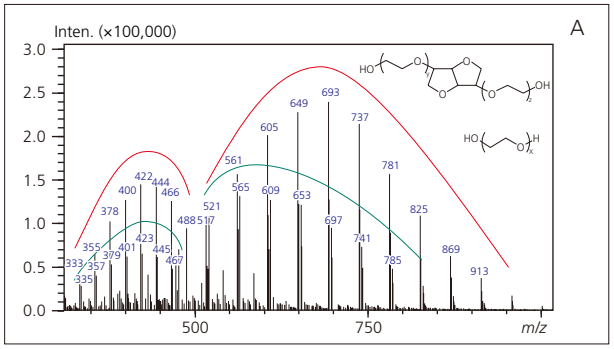

Fig. 3 Mass Spectrum of the Peaks (A)

As for peak B, it is detected as a divalent and trivalent peak similar to the principal component peak D, and is presumed to be polyoxyethylene sorbitan derived from hydrolysis of the ester according to the literature.
Regarding the mass spectra of peaks C, F, and G associated with components eluted in the latter half, this series has the same features, in which the oleic acid bond number and fatty acid type are different from those of the principal component.

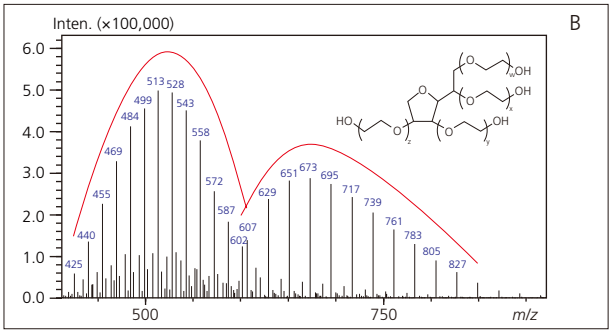

Fig. 4 Mass Spectrum of the Peaks (B)

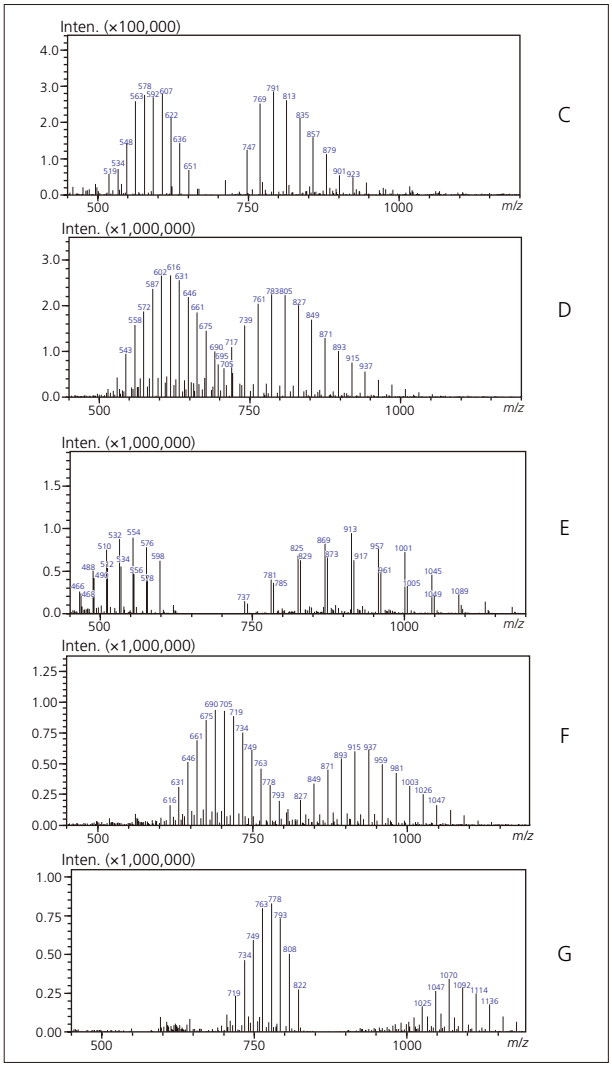

Fig. 6 Mass Spectra of the Peaks (C - G) in Fig. 5

The results showed that the online SPE system combined with high-resolution analysis allows identification and quantitation of each by-product, and that this system can be further applied to monitor degradation due to oxidization or hydrolysis.

[Reference]

E. Hvattum, W.L. Yip, D. Grace, K. Dyrstad, Characterization of polysorbate 80 with liquid chromatography mass spectrometry and nuclear magnetic resonance spectroscopy: Specific determination of oxidation products of thermally oxidized polysorbate 80, J Pharm Biomed Anal 62, (2012) 7-16

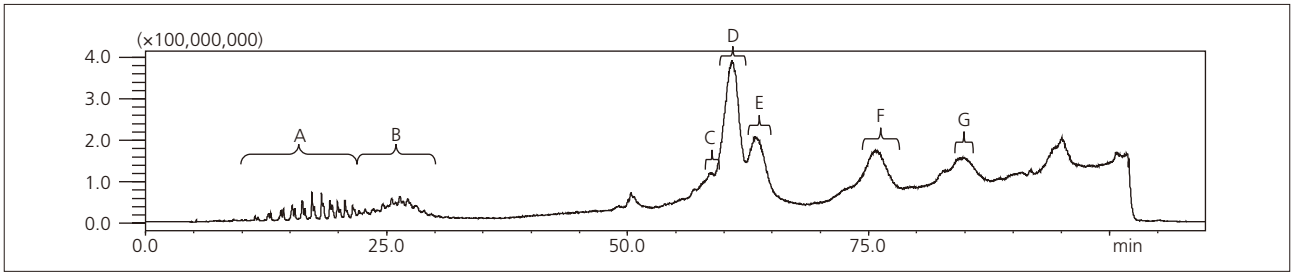

Fig. 5 TIC Chromatogram of Model Sample

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