MERCK

Analytix Reporter

Special Edition

Pharma Analysis & QC

Supelco®
Analytical Products

Analytix Reporter

Special Edition
Pharma Analysis & QC

Dear Reader,

Over the past several years, biologics have continued to make up an increasing proportion of biopharmaceutical sales and pipeline candidates. Biologics now account for more than half of the top selling drugs by revenue. In fact, 2018 saw a record 17 biologics license application (BLA) approvals and the 5-year annual average has more than doubled over the last decade. This trend is expected to continue for the foreseeable future with many different modalities of biologics in preclinical and clinical development. Currently, most global pharmaceutical companies' pipelines consist of
20-40% biologics; the tipping point to a majority biologics pipeline is expected this decade. But relative to synthetic small molecule therapeutics, the manufacturing process for biologics is far more complicated and requires the use of more intense characterization, from even the earliest stages of development through production and QA/QC to ensure safety and effectiveness. While liquid chromatography/mass spectrometry (LC/MS) has always had an important role in small molecule pre-clinical animal studies and in human clinical studies, expanded use is realized both upstream into target identification and downstream into manufacturing and QC release testing with biologics.

Just some of the characterization of biologics being performed in the LC-MS lab include: intact mass, glycan analysis, peptide mapping, impurity and degradant identity, and bioanalytical assays for DMPK purposes. Beyond the capabilities of the mass spectrometer, consumables suppliers are now introducing novel sample preparation tools as well as isotopically labeled and certified reference standards for the biologics market. Specifically, for preclinical analysis of monoclonal antibodies, the introduction of immunoaffinity capture and release sample preparation kits are becoming popular. Furthermore, chromatography solvents and columns are tailored to meet the needs of large biologics. This trend includes large pore size (1000 A) chromatography columns, smaller particles and smaller internal diameter columns all designed to couple biologics separations with mass spectrometers. Improvements include improved peak shape and faster analysis. Ultra-pure LC-MS solvents have been introduced to minimize unwanted fragmentation or ionization.

My expectation is that as biologics continue towards being a majority modality in the pharmaceutical pipeline, new and unique LC-MS tools will become available for use from discovery through QC.

Sincerely yours,

Wayne Way
Pharma QC Strategic Marketing Mgr

For questions & comments contact us at
Analytix@merckgroup.com

SigmaAldrich.com/Supelco

For your personal copy, sign up at
SigmaAldrich.com/ar-reg

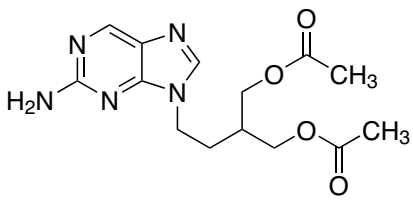
SMALL MOLECULES

Famciclovir Tablets USP Monograph Method Using Purospher™ STAR RP-8 Endcapped HPLC Column and UV Detection

Sonal Shinde, Application Specialist, Analytix@merckgroup.com

Famciclovir is an antiviral drug indicated for the treatment of herpes zoster, herpes simplex virus 2 (genital herpes), herpes labialis (cold sores), etc. It is a guanosine analogue, a prodrug form of penciclovir, and marketed by Novartis under the trade name Famvir. Generics are produced by TEVA and Mylan, among others.

Purospher™ STAR RP-8 endcapped HPLC columns can be used to monitor organic impurities in tablet formulations following the new USP monograph for Famciclovir Tablets. The method suitability requirements are defined by the relative standard deviation (NMT 5.0% for Famciclovir standard solution) and the chromatographic resolution between propionyl famciclovir and 6-chloro famciclovir (NLT 1.2 using the system suitability solution). The method acceptance criteria is defined by the relative retention times for Famciclovir related compound A, Famciclovir related compound B, Famciclovir, 6-Chloro famciclovir, and Propionyl famciclovir and are about 0.2, 0.5, 1.0, 1.32, and 1.35, respectively. This application note illustrates with required analytical data that the method meets USP41-NF36 guidelines.



Experimental Conditions	
Column:	Purospher™ STAR RP-8 endcapped (5 µm) 250x4.6 mm (**1.51454**)
Mobile phase:	[A] 2.72 g/L of monobasic potassium phosphate in water. Adjust with 1 M phosphoric acid to a pH of 4.0 ±0.05. [B] Acetonitrile
Gradient:	See table
Flow rate:	1 mL/min
Pressure drop:	132-147 bar (1914-2131 psi)
Detection:	UV @ 220 nm (analytical flow cell; 10 µL)
Temperatures:	Column: 50 °C; Autosampler: 8 °C
Injection volume:	20 µL
Samples:	
Standard solution:	1 µg/mL of USP Famciclovir RS in Mobile Phase A (**Figure 1**)
SST solution:	0.5 mg/mL of USP Famciclovir System Suitability Mixture RS in Mobile Phase A (**Figure 2**)
Test solution:	Nominally 1 mg/mL of Famciclovir in Mobile Phase A prepared as follows. Transfer an amount equivalent to 250 mg of Famciclovir, from not less than (NLT) 10 finely powdered tablets, to a 250-mL volumetric flask. Add about 125 mL of mobile phase A and sonicate for 30 min with intermittent shaking. Dilute with mobile phase A to volume. (**Figure 3**)
Other samples in monograph method (not shown here)	
Peak ID solution	4 µg/mL of USP Famciclovir Related Compound A RS and 10 µg/mL of USP Famciclovir Related Compound B RS in Mobile Phase A

Gradient		
Time (min)	**A (%)**	**B (%)**
0	95	5
50	75	25
60	75	25
65	95	5
75	95	5

Figure 1. Chromatographic Data – Blank & Standard Solution

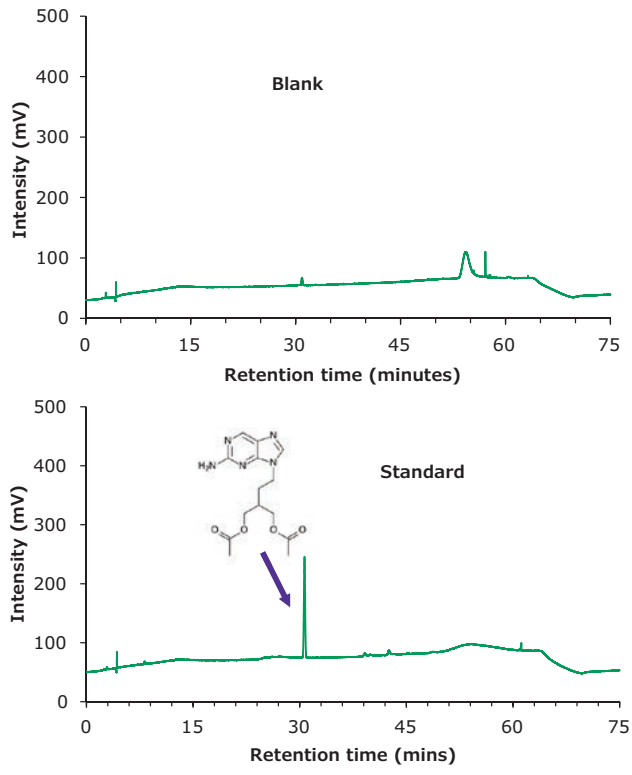
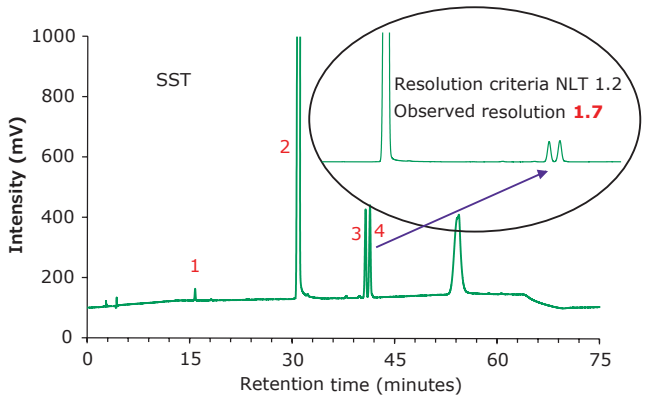
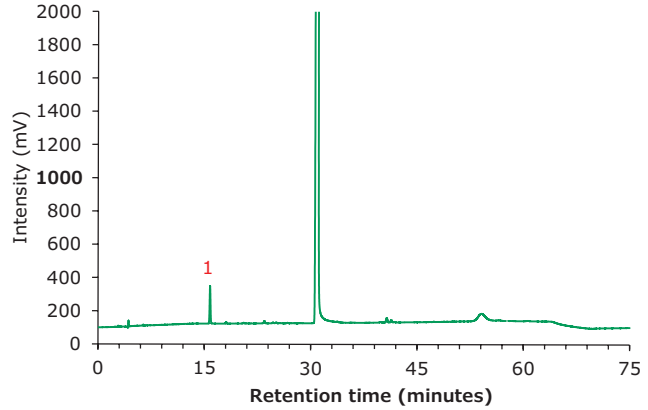


Figure 2. Chromatographic Data - System Suitability Test (SST) Solution



Peaks	Compound	Retention Time (min)	RRT	Resolution	Tailing Factor
1	Famciclovir Related compound B	15.8	0.51	-	0.99
2	Famciclovir	30.8	1.00	47.9	1.02
3	6-Chloro famciclovir	40.7	1.32	25.7	0.98
4	Propionyl famciclovir	41.4	1.34	1.7	0.98

Figure 3. Chromatographic Data - Test Solution



Peaks	Compound	Retention Time (min)	RRT	Resolution	Tailing Factor
1	Famciclovir Related compound B	15.8	0.51	0	0.99
2	Famciclovir	30.8	1.00	47.5	1.02
3	6-Chloro famciclovir	40.7	1.32	8.9	0.98
4	Propionyl famciclovir	41.4	1.34	7.7	0.98

Validation and Verification

1. Specificity: Inject solution and determine the retention time of desired analyte in the presence of other components such as impurities and excipients.

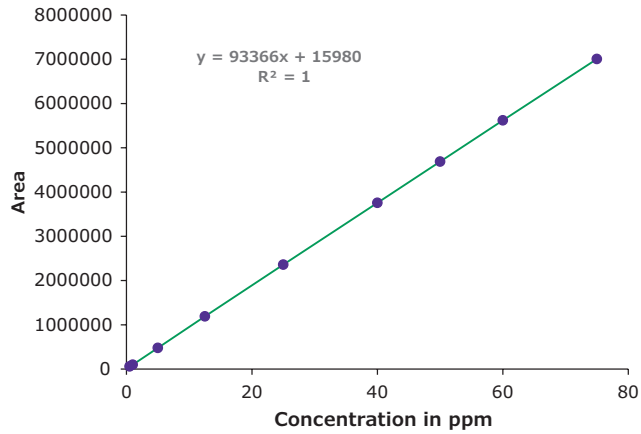
	Compound	RT (min)
1	Famciclovir Related compound B	**15.8**
2	Famciclovir	**30.9**
3	6-Chloro famciclovir	**40.7**
4	Propionyl famciclovir	**41.3**

2. Standard Repeatability (1 ppm)

Sample	Area Units
STD 1	**214,771**
STD 2	**213,539**
STD 3	**214,102**
STD 4	**214,935**
STD 5	**214,216**
Mean	**214,313**
Standard Deviation	**559**
RSD (%)	**0.3**

3. Linearity, LOD & LOQ

Famciclovir Concentration (µg/mL)	Area Units
0.5	**59,951**
1	**98,532**
5	**478,367**
12.5	**1,190,770**
25	**2,360,140**
40	**3,757,032**
50	**4,687,957**
60	**5,618,958**
75	**7,007,616**
LOD (ppm)	**0.3**
LOQ (ppm)	**0.9**



Featured Products

Description	Cat. No.
Purospher™ STAR RP-8 endcapped (5µm) Hibar® 250-4.6 HPLC column, 250 x 4.6 mm	**1.51454**
Acetonitrile gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur.	**1.00030**
ortho-Phosphoric acid 85 % for analysis EMSURE® ACS,ISO,Reag. Ph Eur	**1.00573**
Potassium dihydrogen phosphate for analysis EMSURE® ISO	**1.04873**
Reference Materials	
Famciclovir United States Pharmacopeia (USP) Reference Standard	**1269152**
Famciclovir Related Compound A United States Pharmacopeia (USP) Reference Standard	**1269174**
Famciclovir Related Compound B United States Pharmacopeia (USP) Reference Standard	**1269185**
Famciclovir System Suitability Mixture United States Pharmacopeia (USP) Reference Standard	**1269163**

For our complete listing of pharma reference materials including primary (compendial) standards visit us at **SigmaAldrich.com/standards-pharma**

SMALL MOLECULES

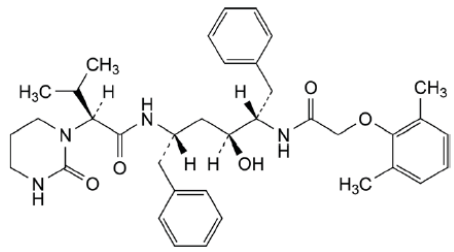
Lopinavir Assay Following European Pharmacopoeia 11 Guidelines Using an Ascentis® Express C18 Column and UV Detection

Anita Piper, Scientist Instrumental Analytics R&D, Analytix@merckgroup.com

Introduction

This paper illustrates the possible setting-up of an assay method for Lopinavir testing following European Pharmacopoeia guidelines (11). The monograph assay method calls for a column with L = 0.25 m, I.D. = 4.6 mm end-capped octadecylsilyl silica gel for chromatography with 4 µm particle size. No particular HPLC column is referenced in the EP knowledge database for the assay method, and the method is of isocratic nature.

This provides an opportunity to replace the monograph column geometry/particle size with a shorter and faster alternative column (up to 70% reduction in length) packed with smaller particles (up to 50% reduction). This can save valuable time and ensure improved separation efficiency, which typically translates into better method performance and sensitivity. In this study, the limit of detection (LOD) is better than 1 ppm using HPLC-UV detection.



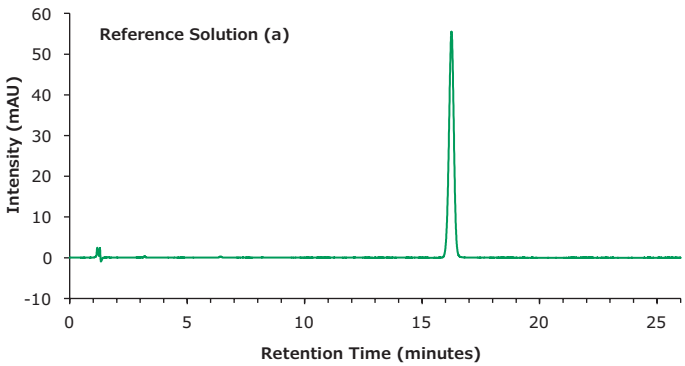
Lopinavir

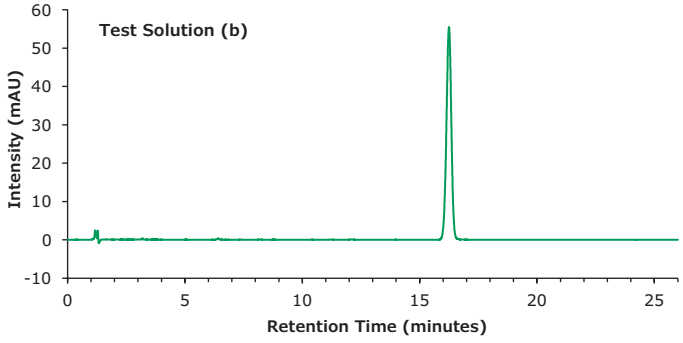
Experimental

Conditions	
column:	Ascentis® Express C18, 15 cm x 4.6 mm, 2.7 um (**53829-U**)
mobile phase:	acetonitrile/phosphate buffer solution 45/55 (v/v)
buffer preparation:	dissolve 0.9 g of dipotassium hydrogen phosphate and 2.7 g of potassium dihydrogen phosphate in 900 mL of water and mix well. Adjust to pH 6.0 with phosphoric acid, dilute to 1000 mL with water and filter.
flow rate:	1.0 mL/min
pressure:	2219 psi (153 bar)
column temp.:	50 ºC
detector:	UV, 215 nm UV = 215 nm (micro flow cell; 1.4 µL/7 mm)
injection:	12 µL
samples:	
solvent mixture:	acetonitrile/water 50/50 (v/v)
test solution (a):	dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 100 mL with the solvent mixture.
test solution (b):	dilute 5.0 mL of the test solution (a) to 100 mL with the solvent mixture.
reference solution (a):	dissolve 50.0 mg of Lopinavir CRS in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 5 mL of this solution to 100 mL with the solvent mixture.

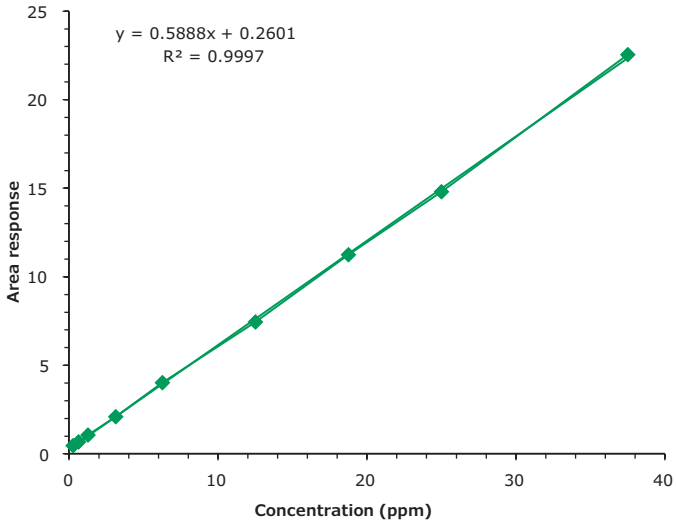
Concentration

No.	Compound	Retention Time (min)	Tailing Factor
1	t_0 void volume	1.1	
2	Lopinavir CRS	16.2	0.97





Chromatographic Data



1. Specificity: Inject Reference Solution (a) and Determine the Retention Time and the Content of Desired Analyte				
		Retention Time (min)	Area (%)	Tailing Factor
1	Lopinavir CRS	16.3	96.3	0.95

2. Standard Repeatability (25 ppm)	
Measurement	**Area (mAU*min)**
STD 1	14.79
STD 2	14.82
STD 3	14.86
STD 4	14.90
STD 5	14.93
Mean	**14.86**
Standard Deviation	**0.06**
(%) RSD	**0.4**

3. LOD & LOQ	
Conc. (ppm)	**Mean Area, n=5 (mAU*min)**
0.25	0.46
0.63	0.67
1.25	1.06
3.13	2.10
6.25	4.02
12.50	7.44
18.75	11.24
25.00	14.86
37.50	22.54
STEYEX	**0.1348**
Slope	**0.5888**
LOD (ppm)	**0.8**
LOQ (ppm)	**2.3**

Conclusion

In this study in reference to European Pharmacopeia 11 Guidelines, a shorter and faster Fused-Core® (Superficially Porous Particle, SPP) column was evaluated for an assay method for Lopinavir, achieving a limit of detection (LOD) of better than 1 ppm using HPLC-UV detection.

Featured Products

Description	Cat. No.
Ascentis® Express C18, 15 cm x 4.6 mm, 2.7 µm	**53829-U**
Acetonitrile gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	**1.00030**
Water for chromatography (LC-MS grade) LiChrosolv® or tap fresh from an appropriate Milli-Q® water purification system	**1.15333**
Millex® syringe filter Durapore® (PVDF), non-sterile, 0.45 µm pore size, 13 mm diameter	**SLHVX13**
Ortho-phosphoric acid, EMSURE® ACS,ISO, Reag. Ph Eur	**1.00573**
Potassium di-hydrogen phosphate, EMSURE® ISO	**1.04873**
Di-Potassium hydrogen phosphate, anhydrous for analysis EMSURE®	**1.05104**
Reference Materials	
Lopinavir, European Pharmacopoeia (EP) Reference Standard	**Y0001498**
Lopinavir for system suitability, European Pharmacopoeia (EP) Reference Standard	**Y0001505**
Lopinavir for peak identification, European Pharmacopoeia (EP) Reference Standard	**Y0001506**

Related Products

Description	Cat. No.
Lopinavir, United States Pharmacopeia (USP) Reference Standard, 350 mg	**1370101**
Lopinavir, Pharmaceutical Secondary Standard; Certified Reference Material	**PHR1927**
Di-Potassium hydrogen phosphate, anhydrous for HPLC, LiChropur®	**5.43839**

Did you know...

…that we also offer a comprehensive portfolio of reference materials for “Dexamethasone”. Use the search function for “Dexamethasone” on

SigmaAldrich.com/standards

… that thin-layer chromatography allows the quantitative determination of Dexamethasone and Dexamethasone sodium hydrogen sulfate in blood and in pharmaceutical preparations. (M. Amin, Fresenius Z Anal Chem (1988) 329: 778- 780).Find our TLC products at

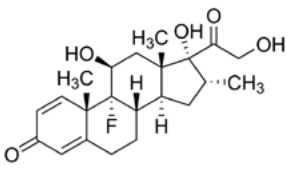
SigmaAldrich.com/TLC

Dexamethasone: An HPLC Assay and Impurity Profiling Following the USP Monograph

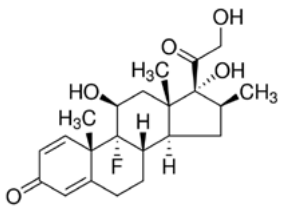
Sophia Kwende, Quality Control Scientist, Analytix@merckgroup.com

A simple, precise, and sensitive reversed-phase high performance liquid chromatography (RP-HPLC) gradient method was adapted for establishing traceability and total chromatographic analysis of dexamethasone. The given experimental conditions follow the USP43-NF38 monograph method for dexamethasone assay and organic impurity profiling. Baseline-resolved peaks were obtained for dexamethasone, betamethasone, dexamethasone acetate, and desoximetasone within 20 minutes with a Titan™ C18 UHPLC column (10 cm x 2.1 mm, 1.9 µm particles). This column has the same phase, length, and ID like in the monograph, but the packing is based on monodisperse particles with a slightly larger average particle size (1.9µm), than referenced in the monograph (1.7 µm). The method was validated following the guidelines in USP General Chapters <621>, <1225>, and <1226>. The use of lower sample concentrations was compensated by a larger injection volume (to maintain mass on column) to improve reproducibility. The chromatographic separation was achieved using a mixture of 3.4 g/L monobasic potassium phosphate solution (pH 3.0) and acetonitrile as the mobile phase with gradient elution and UV detection at 240 nm. Although, comparatively a shorter relative retention time (RRT) was observed for dexamethasone acetate and desoximetasone, both the compounds showed an excellent chromatographic resolution (Rs > 10). Under the applied conditions, system suitability requirements are met, and the method demonstrates good selectivity, reproducibility, sensitivity, and accuracy.

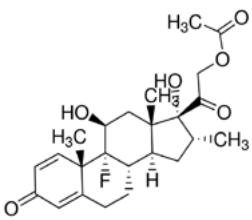
Experimental Conditions			
column:	Titan™ C18 UHPLC Column 10 cm x 2.1 mm, 1.9 µm		
mobile phase:	[A] 3.40 g of potassium dihydrogen phosphate in 1000 mL water and adjust to pH 3.0 with phosphoric acid [B] acetonitrile		
gradient:	**time (min)**	**% A**	**% B**
	0 – 10	76	24
	10 – 15	76 – 45	24 – 55
	15 – 16	45 – 10	55 – 90
	16 – 16.1	10 – 76	90 – 24
	16.1 – 20	76	24
flow rate:	0.4 mL/min		
pressure drop:	320 - 470 bar		
column Temp.:	35 °C		
detector:	UV @ 240 nm (analytical flow cell; 13 µL)		
injection:	5 µL		
Samples			
diluent:	acetonitrile and water (56:44) v/v.		
test solution:	dissolve 0.04 g of Dexamethasone CRS in 25 mL diluent (1.6 mg/mL).		
system suitability solution:	dissolve 12 mg of Dexamethasone and 8 mg of Betamethasone in 100 mL diluent		
standard solution:	dissolve 1.6 mg of Dexamethasone, 2.4 mg of Betamethasone, 4.8 mg of Dexamethasone acetate using 10 mL diluent further take 1.0 mL of this solution and dilute to 100 mL using the diluent.		
Reference solution	dilute 5 mg of EP dexamethasone for system suitability in 5 mL diluent		



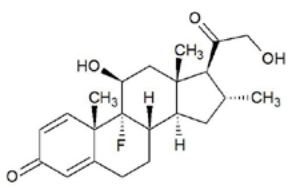
Dexamethasone



Betamethasone

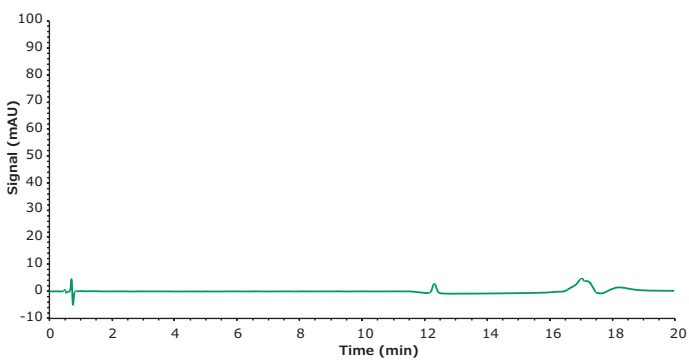


Dexamethasone Acetate

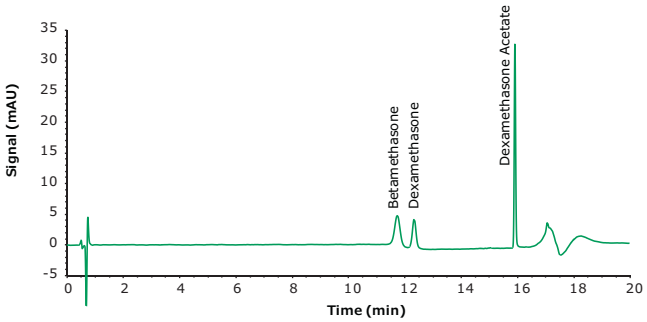


Desoximetasone

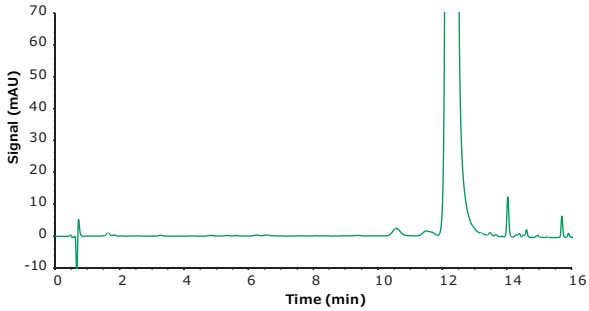
Blank



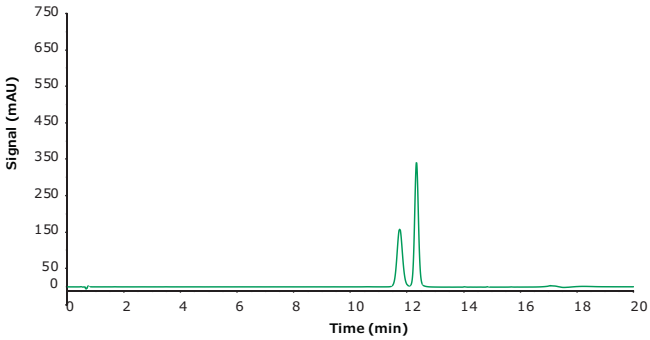
Standard Solution



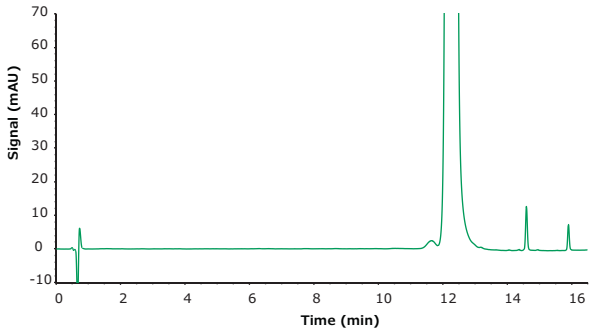
Dexamethasone (Test Solution)



System Suitability Solution



Reference Solution



Chromatographic Data (System Suitability Solution)

Peaks	Compound	Retention Time (min)	RRT	Resolution	Resolution requirement per USP43-NF38	Tailing factor	Tailing requirement per USP43-NF38	Theoretical Plates
1	Betamethasone	11.7	0.95	-		1.03	NMT 2.0	17101
2	Dexamethasone	12.3	1.00	1.99	NLT 1.5	1.0	NMT 2.0	38792

Chromatographic Data (Reference Solution)

Peaks	Compound	Retention Time (min)	RRT	RRT Reference per USP43-NF38	Resolution	Tailing factor	Theoretical Plates
1	Betamethasone	11.7	0.95	0.94	-	1.15	10,612
2	Dexamethasone	12.3	1.00	1.00	1.5	1.03	20,692
3	Desoximetasone	14.6	1.19	1.58	10.2	1.06	340,463
4	Dexamethasone Acetate	15.9	1.29	1.74	13.4	1.02	449,570

Repeatability (Reference Solution)

Peaks	Compound	Area Response (n=3)	Standard deviation	RSD (%)	RSD (%) Reference per USP43-NF-38
1	Betamethasone	39.70	0.48	1.2	5.0
2	Dexamethasone	25,221.73	263.78	1.0	5.0
3	Desoximetasone	50.18	0.50	1.0	5.0
4	Dexamethasone Acetate	27.44	0.34	1.2	5.0

Featured Products

Product list	Cat. No
Titan™ C18 UHPLC Column (100 x 2.1 mm, 1.9 µm)	**577124-U**
Water for chromatography (LC-MS grade) LiChrosolv® or tap fresh from a Milli-Q® ultrapure water system	**1.15333**
Acetonitrile gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	**1.00030**
Potassium phosphate monobasic, , anhydrous, puriss. p.a., ACS reagent, reag. ISO, reag. Ph. Eur., 99.5-100.5%	**60220-M**
Ortho-phosphoric acid EMSURE®	**1.00573**
Millex® syringe filter units, disposable, Durapore® PVDF, pore size 0.22 µm, non-sterile	**SLHVX13NK**
Pharmacopeia Reference Materials	
Dexamethasone United States Pharmacopeia (USP) Reference Standard	**1176007**
Betamethasone United States Pharmacopeia (USP) Reference Standard	**1066009**

Product list	Cat. No
Dexamethasone acetate United States Pharmacopeia (USP) Reference Standard	**1176506**
Desoximetasone United States Pharmacopeia (USP) Reference Standard	**1173508**
Dexamethasone for system suitability European Pharmacopoeia (EP) Reference Standard	**Y0001177**

Related Products

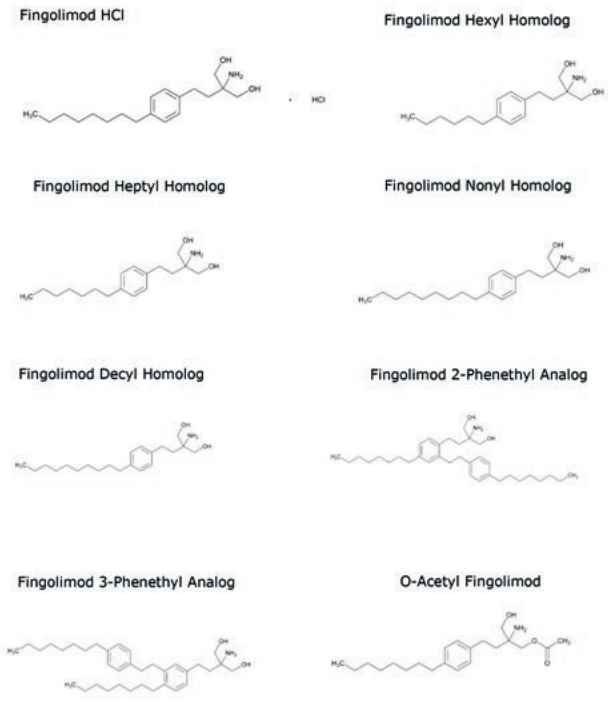
Product list	Cat. No
Dexamethasone, Pharmaceutical Secondary Standard; Certified Reference Material	**PHR1526**
Dexamethasone acetate, Pharmaceutical Secondary Standard; Certified Reference Material	**PHR1527**
Betamethasone, Pharmaceutical Secondary Standard; Certified Reference Material	**PHR1398**

Analysis of Fingolimod Hydrochloride According to USP Monograph Guidelines

Unites States Pharmacopeia (USP43-NF38) Monograph

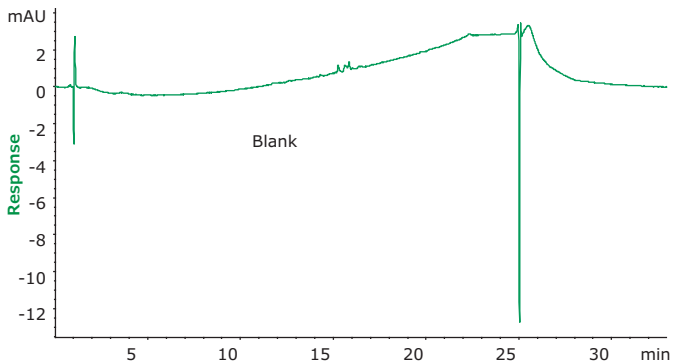
In this article, a rapid, accurate, and simple method is presented for the total chromatographic purity analysis of fingolimod hydrochloride using High Performance Liquid Chromatography equipped with a Diode Array Detector (HPLC-DAD). The experimental conditions follow guidelines, with minor, but allowed modifications from the USP43-NF38 monograph methods for the assay and organic impurity profiling of fingolimod hydrochloride. Using the 33-minute gradient method from USP and a Purospher® STAR RP-18 HPLC column (150 x 3.0 mm, 3 µm), baseline separation of fingolimod and its impurities was achieved. A 0.1% solution of phosphoric acid in water and acetonitrile were used as the mobile phase for the gradient elution.

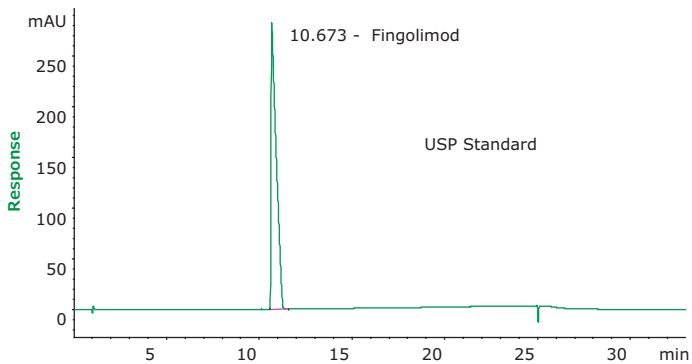
Chemical Structures of Fingolimod and its Impurities

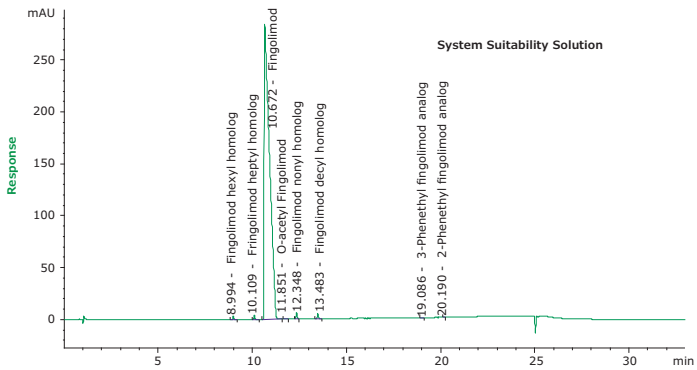


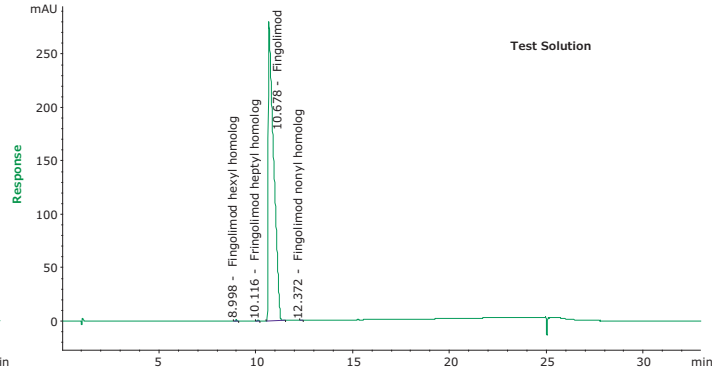
Experimental

Conditions	
column:	Purospher® STAR RP-18 Hibar RT (3µm) 150x3.0 mm
detection:	UV @ 215 nm (DAD)
mobile phase:	[A]: 0.1% phosphoric acid in water [B]: acetonitrile
gradient:	Time (min) / A (%) / B (%) 0 / 80 / 20 20 / 5 / 95 23 / 5 / 95 23.1 / 80 / 20 33 / 80 / 20
flow rate:	0.8 mL/min
pressure:	135-300 bar
temperatures:	column: 40 °C; autosampler: 10 °C
injection volume:	5 µL
Sample	
diluent:	mobile phase A:B (50:50)
test solution:	dissolve 15 mg of USP Fingolimod Hydrochloride RS in 25 mL diluent (0.6 mg/mL).
system suitability solution:	dissolve 15.0 mg of USP Fingolimod for System Suitability using 25 mL diluent (0.6 mg/mL).
standard solution:	dilute 1.0 mL of the test solution to 100.0 mL using mobile phase further dilute 1.0 mL of this solution to 10.0 mL using the mobile phase (0.003 mg/mL)









Specificity (System Suitability Solution)

Peaks	Compound	Retention Time (min)	RRT	Requirement (USP43-NF38)	Resolution	Requirement (USP43-NF38)	Tailing	Requirement (USP43-NF38)
1	Fingolimod Hexyl Homolog	9.0	0.84	0.82	-	-	1.0	-
2	Fingolimod Heptyl Homolog	10.1	0.94	0.93	11.6	-	1.1	-
3	Fingolimod	10.7	1.00	1.00	2.1	-	4.4	Not more than (NMT) 5
4	O-Acetyl Fingolimod	11.8	1.10	-	3.0	-	0.7	-
5	Fingolimod Nonyl Homolog	12.3	1.15	1.13	4.2	1.2 (between peak 4 and 5)	1.2	-
6	Fingolimod Decyl Homolog	13.5	1.26	1.23	9.8	-	1.4	-
7	3-Phenethyl Fingolimod Analog	19.8	1.85	1.97	42.1	-	0.8	-
8	2-Phenethyl Fingolimod Analog	20.2	1.89	2.00	2.8	0.8 (between peak 7 and 8)	1.2	-

Repeatability (System Suitability Solution)

Peaks	Compound	Area Response (N=3)	Standard Deviation	RSD (%)	RSD (%) (USP43-NF38)
3	Fingolimod	5496.1	2.3	0.04	NMT 0.73%

Conclusion

A rapid, accurate, and simple method for the total chromatographic purity analysis of Fingolimod hydrochloride by HPLC-DAD was developed, well within the boundaries of the USP43-NF38 monograph methods. The applied conditions met the system suitability criteria, and the method demonstrated good resolution/selectivity, reproducibility, and sensitivity.

See our complete offering for HPLC at
SigmaAldrich.com/HPLC

Featured Products

Description	Cat. No
Purospher® STAR RP-18 Hibar RT 15 cm x 3.0 mm, 3 µm	**1.50414**
Acetonitrile gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	**1.00030**
Water for chromatography (LC-MS grade) LiChrosolv® or tap fresh from an appropriate Milli-Q® ultrapure water system	**1.15333**
Phosphoric Acid for HPLC, LiChropur™, 85%	**49685**
Fingolimod Hydrochloride United States Pharmacopeia (USP) Reference Standard	**1270480**
Fingolimod System Suitability Mixture United States Pharmacopeia (USP) Reference Standard	**1270526**
Syringe Filter Millex-HV Durapore® (PVDF) membrane, hydrophilic, Non-sterile, 0.45 µm pore size, 13 mm diameter,	**SLHVX13NK**

Stable Isotope-Labeled Amino Acid Mixes

Matthias Nold, Shailly Krishna Rajusth, Product Manager Reference Materials, Analytix@merckgroup.com

Amino acids play a central role in biochemistry being the building blocks of proteins or as precursors in the biosynthesis of secondary metabolites. From being used as food additives (as artificial sweeteners and flavor enhancers) to the synthesis of drugs, biodegradable plastics, and chiral catalysts - amino acids have a range of uses. Amino acid analysis generally involves hydrolysis of the peptide bonds and analysis of the released amino acids by appropriate analytical methods.[1]

Isotopically labeled amino acids can function as internal standards for an amino acid analysis (AAA) using Isobaric-tagged isotope dilution mass spectrometry (IT-IDMS) methods. The IT-IDMS methods offer better accuracy, precision, and sensitivity in comparison to the traditional AAA methods.[2]

We have always strived to provide you with a wide range of high-quality reference materials. Our reference materials portfolio offers more than 20,000 products. View SigmaAldrich.com/standards for the complete range. Recently, two new certified solution mixtures of labeled amino acids have been added to the portfolio. These mixes are suitable for use as an internal standard during a LC/MS or GC/MS quantitation (after derivatisation) of amino acids by isotope dilution methods and other research applications.

Key Features

- *Trace***CERT®** certified reference material, traceable to primary material from NIST
- Isotopically Labeled Amino Acid Mix produced by an established workflow following ISO 17034
- qNMR certified components in the mix (following ISO 17025 accreditation)
- Tested for homogeneity and long-term stability using LC method
- Supplied with a comprehensive certificate including the overall uncertainty

Product Number:	**96378**
Product Name:	Stable Isotope Labeled Amino Acid Mix Solution 1
Grade:	certified reference material, *Trace***CERT®**
Solvent:	HCl (0.1 M)
Concentrations:	1250-2500 nmol/mL
Package Size:	1 mL

Component	CAS No.	Nominal Concentration
L-Alanine-$^{13}C_3$, ^{15}N	202407-38-3	2.5 mmol/L
L-Arginine-$^{13}C_6$	55443-58-8	2.5 mmol/L
L-Aspartic acid-$^{13}C_4$	55443-54-4	2.5 mmol/L
L-Cystine-$^{13}C_6$, $^{15}N_2$	1252803-65-8	1.25 mmol/L
L-Glutamic acid-$^{13}C_5$	55443-55-5	2.5 mmol/L
Glycine-$^{13}C_2$,^{15}N	211057-02-2	2.5 mmol/L
L-Histidine-$^{13}C_6$	55443-59-9	2.5 mmol/L
L-Isoleucine-$^{13}C_6$, ^{15}N	202468-35-7	2.5 mmol/L
L-Leucine-$^{13}C_6$, ^{15}N	202406-52-8	2.5 mmol/L
L-Lysine$^{13}C_6$	55443-57-7	2.5 mmol/L
L-Methionine$^{13}C_5$, ^{15}N	202468-47-1	2.5 mmol/L
L-Phenyl-$^{13}C_6$-alanine	180268-82-0	2.5 mmol/L
L-Proline-$^{13}C_5$	201740-83-2	2.5 mmol/L
L-Serine-$^{13}C_3$, ^{15}N	202407-34-9	2.5 mmol/L
L-Threonine-$^{13}C_4$	55443-53-3	2.5 mmol/L
L-Tyrosine-(phenyl-$^{13}C_6$)	201595-63-3	2.5 mmol/L
L-Valine-$^{13}C_5$	55443-52-2	2.5 mmol/L

Product Number:	**01428**
Product Name:	Stable Isotope Labeled Amino Acid Mix Solution 2
Suffix:	certified reference material, *Trace***CERT®**
Solvent:	HCl (0.1M)
Concentrations:	500-2500 nmol/mL
Package Size:	1 mL

Component	CAS No.	Nominal Concentration
L-Alanine-$^{13}C_3$, ^{15}N	202407-38-3	0.5 mmol/L
L-Arginine-$^{13}C_6$	55443-58-8	0.5 mmol/L
L-Aspartic acid-$^{13}C_4$	55443-54-4	0.5 mmol/L
L-Glutamic acid-$^{13}C_5$	55443-55-5	0.5 mmol/L
Glycine-$^{13}C_2$,^{15}N	211057-02-2	2.5 mmol/L
L-Leucine-$^{13}C_6$, ^{15}N	202406-52-8	0.5 mmol/L
L-Methionine$^{13}C_5$, ^{15}N	202468-47-1	0.5 mmol/L
L-Phenyl-$^{13}C_6$-alanine	180268-82-0	0.5 mmol/L
L-Proline-$^{13}C_5$	201740-83-2	0.5 mmol/L
L-Tyrosine-(phenyl-$^{13}C_6$)	201595-63-3	0.5 mmol/L
L-Valine-$^{13}C_5$	55443-52-2	0.5 mmol/L

Find a complete list of amino acid reference material on **SigmaAldrich.com/aminoacidstandards**

References

1. Odia A.; Zekeri Esezobor O. Therapeutic Uses of Amino Acids. Amino Acid - New Insights and Roles in Plant and Animal, Asao T., Asaduzzaman M., Eds; IntechOpen, 2017 DOI: 10.5772/intechopen.68932
2. Woolfitt A.R., Solano M.I., Williams T.L., James L. Pirkle J. L., Barr J.R., Amino Acid Analysis of Peptides Using Isobaric-Tagged Isotope Dilution LC–MS/MS, Anal. Chem. 2009, 81, 10, 3979–3985, https://pubs.acs.org/doi/10.1021/ac900367q

LARGE MOLECULES

BIOshell™ IgG 1000 Å C4 UHPLC Column for Improved mAb Separations

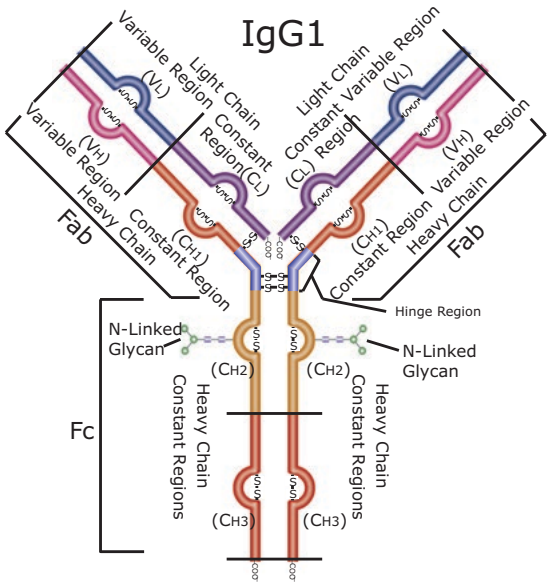
Cory E. Muraco, Senior R&D Scientist, Technology and Workflow R&D, Analytix@merckgroup.com

Introduction

Monoclonal antibodies (mAbs) are widely manufactured by many biopharmaceutical companies to treat a myriad of diseases ranging from Alzheimer's disease, Parkinson's disease, ulcerative colitis, and many types of cancers. Most recombinant therapeutic mAbs belong to the human immunoglobulin G (IgG) category among the immunoglobulin superfamily. A schematic of an IgG antibody is depicted in **Figure 1**.

A general IgG antibody is composed of two light chains (LC) that are tethered to two heavy chains (HC) through disulfide bonds. In addition, due to the fact that the LC and HC are composed of amino acids with reactive side chains, IgG's can be post-translationally modified through phosphorylation, methylation, oxidation, and nitrosylation, among other modifications. These modifications may change the binding affinity of the mAb so that it binds either the wrong antigen, does not bind any antigen, or associates with the wrong cell surface receptor. Biopharmaceutical companies need to develop rigorous methods to assess lot-to-lot reproducibility of their candidate biologic drug, and the above mentioned modifications are known as Critical Quality Attributes (CQAs) that both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) monitor. Due to these stringent requirements from regulatory bodies, much research has been pursued in the past 20 years to develop accurate, robust, and high-throughput methods to assess biopharmaceutical purity and structure.

Figure 1. Graphical Depiction of a Human IgG1 Antibody. Note the Structural Complexity of the Different Domains of the Antibody[1]

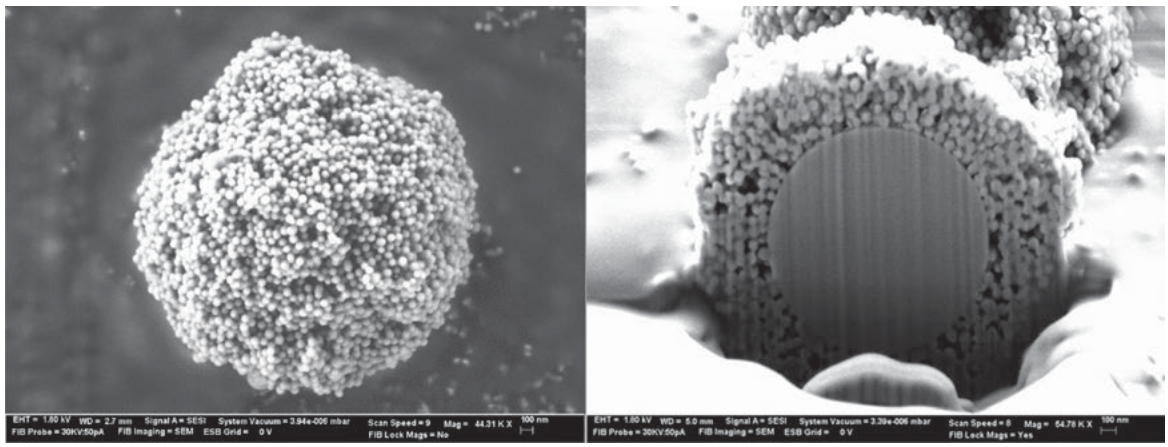


Ultra-high performance liquid chromatography (UHPLC) has emerged as a promising technique to characterize these biomacromolecules. Most biopharmaceutical R&D laboratories, as well as quality control (QC) laboratories, have ready access to this type of instrumentation. Due to the lower system dispersion, lower dead volume, and higher upper pressure limit of these instruments, biopharmaceutical companies have been able to develop methods that not only probe the finest structural details of a candidate drug, but have enabled QC labs to assay hundreds of sample in a single day.

Besides advances in UHPLC instrumentation, there have been many advances in the field of HPLC column and stationary phase development. Two main types of particle morphology are prevalent in the industry today: fully porous particles (FPPs) and superficially porous particles (SPPs, also called core-shell or Fused-Core™ particles). To take advantage of the low dispersion of UHPLC instrumentation, columns with sub-2 µm FPPs with pore sizes of 300 Å have been used to accommodate for the larger hydrodynamic radii of biomacromolecules. These columns have been the industry standard since the mid 2000's. However, these columns suffer limitations when analyzing larger or more complex proteins like mAbs and antibody-drug conjugates (ADCs). The relatively small pore size, in addition to a totally porous architecture, restricts the free diffusion of large molecules through the particle. This architecture concomitantly results in an increase in the mass transfer term of the van Deemter equation, leading to peak tailing, loss of resolution, and low recovery.

In recent years, the use of columns packed with SPPs has been in vogue, especially in the area of biologic characterization. Historically, Horvath and Kirkland pioneered the concept and initial synthetic techniques for producing SPPs in the late 1960's to early 1970's.[2,3] The past 40 years have seen a resurrection and renaissance of these particles' use in UHPLC, and now, advanced versions of these particles are available for several different application areas. These applications include small molecule pharmaceutical separations, pesticide analysis, glycan analysis, chiral separations,

Figure 2. SEM Image of the BIOshell™ IgG 1000 Å C4 SPP Particle. Left Panel Shows the Porous Outer Layer (shell) of the Particle. Right Panel is a Cutaway of the Particle, Revealing the Solid, Non-porous Silica Core. Data Courtesy of Advanced Materials Technology (AMT)



and large molecule separations. **Figure 2** shows a scanning electron microscopy (SEM) image of an SPP. Note the presence of the solid silica core in the SPP.

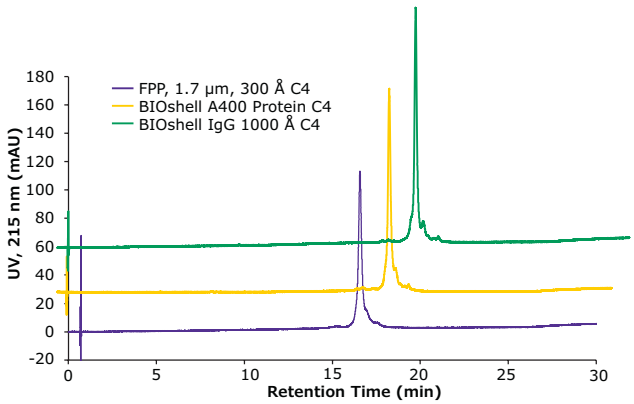
The current BIOshell™ line of columns utilizes SPP technology to provide an alternative to sub-2 µm FPPs for biomolecule separations. Recently, a new addition to the BIOshell™ line of columns has been introduced: the BIOshell™ IgG 1000 Å C4 column. This column is packed with 2.7 µm SPPs that are composed of a 0.5 µm shell thickness and a 1.7 µm solid silica core. The particle is also composed of 1000 Å pores, permitting the unrestricted analysis of mAbs, ADCs, and other, much larger, biomacromolecules. Advantages over columns packed with FPPs are numerous: the SPP shows significant advantage in mass transfer, leading to less band spreading; columns packed with SPPs are more uniformly packed than columns composed of FPPs, leading to a lower Eddy dispersion (A term) in the van Deemter equation; and larger particle sized SPPs have efficiencies similar to or better than sub-2 µm FPPs, leading to the ability of the analyst to run at higher flow rates with less risk of on-column frictional heating due to elevated column backpressure. The remainder of this article will highlight, in more detail, advantages of the BIOshell™ IgG 1000 Å C4 column as compared to commercial FPP columns for large molecule separations.

Efficiency Advantage of the BIOshell™ IgG 1000 Å C4 Column

As noted in the previous paragraph, the efficiency gain observed when comparing SPP and FPP particle architecture is due mostly to the short diffusion path within the SPP architecture, thus enhancing mass transfer. In addition, the BIOshell™ IgG column has 1000 Å pores, therefore minimizing any secondary size-exclusion effects that could lead to band broadening and loss of resolution. These concepts are illustrated in **Figure 3** in which the SigmaMAb monoclonal antibody

Figure 3. Comparisons of Chromatographic Results of the Analysis of SigmaMAb, a Recombinant IgG1 Antibody Standard

Conditions	
Column:	As indicated
Mobile Phase:	[A] 70:30 water (0.1% TFA): acetonitrile (0.1% TFA)
	[B] 50:50 water (0.1% TFA): acetonitrile (0.1% TFA)
Gradient:	0% B to 50% B in 25 min
	50% B to 100% B in 5 min
Flow Rate:	0.4 mL/min
Column Temp.:	75 °C
Detector:	UV, 215 nm
Injection:	1.0 µL
Sample:	SigmaMAb (**MSQC4**), 1 g/L, water (0.05% TFA)



standard is assayed on three columns: BIOshell™ IgG 1000 Å C4, BIOshell™ A400 Protein C4, and a 1.7 µm, 300 Å FPP packed column. Note the lower peak width, at half height, of the main antibody peak as well as improved resolution of minor variants surrounding the main antibody peak, observed on the BIOshell™ IgG column.

Figure 4. Comparison of peak widths, at half height, for a series of five monoclonal antibodies

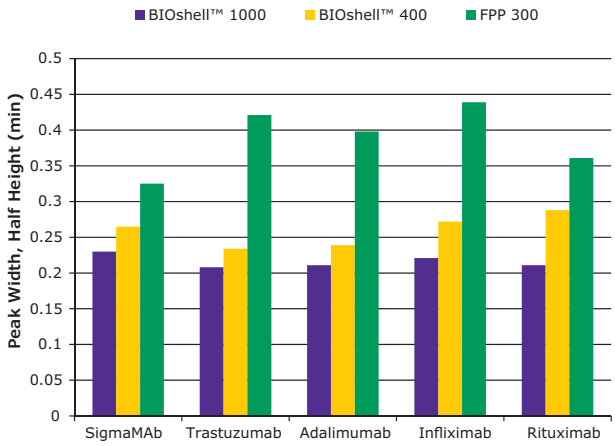


Figure 5. SigmaMAb Peak Volume Versus Flow Rate. Note How the Two Columns Packed With SPPs Have Shallow Slopes Compared to the FPP Column. Conditions Were the Same as Outlined in **Figure 3**, Except in each Case, Gradient Volume Was Held at 10 mL. Peak Volume Was Calculated as Peak Width at Half Height Multiplied by Flow Rate

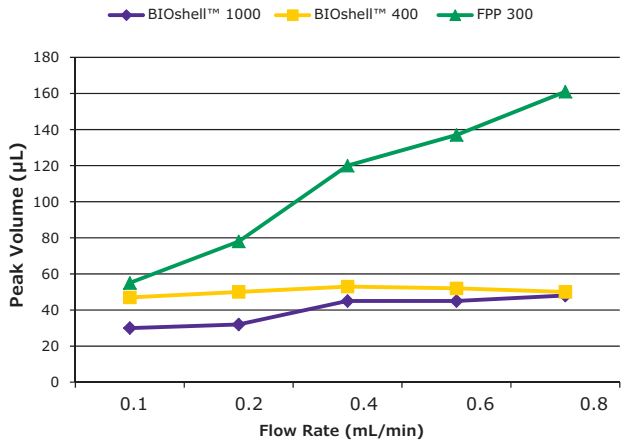
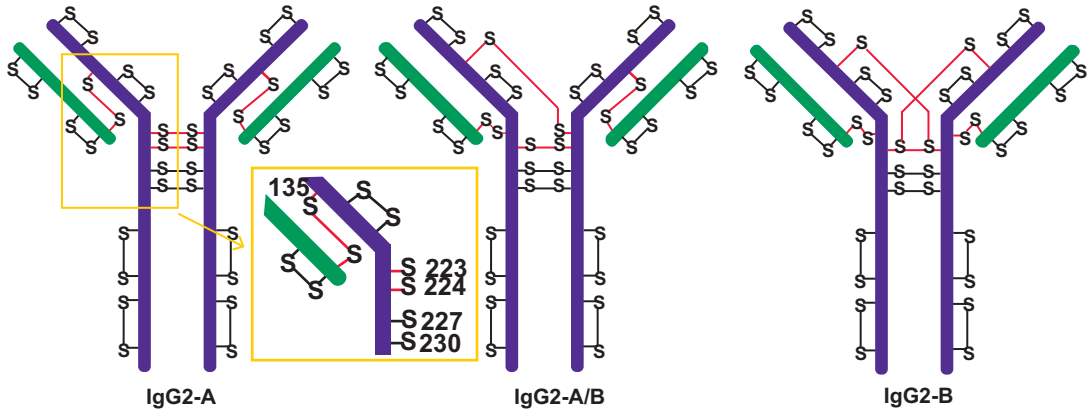


Figure 6. Major Disulfide Isoforms of IgG2 Antibodies. The Inset in the Yellow Square Shows the Amino Acid Numbers of the Cysteines in the Hinge Region. The "S" with the Red Line Connecting it Denotes Cysteines Active in Disulfide Bond Scrambling. Adapted from Reference 4



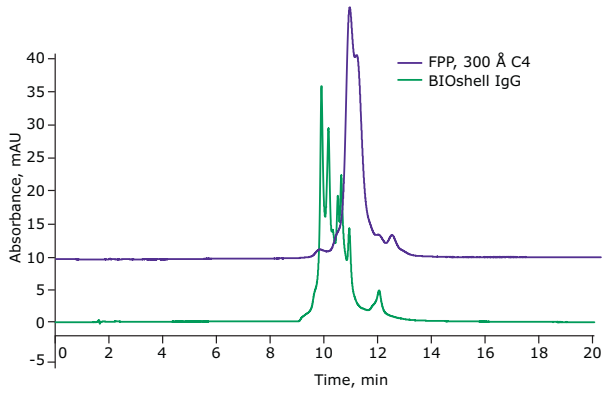
In order to ensure that this trend observed in **Figure 3** was translatable to any mAb, a series of mAbs was assayed on the same columns, using the same chromatographic method, and the peak width at half height, a measure of efficiency, was compared. **Figure 4** summarizes those results. None of the mAbs assayed generated peak widths, at half height, greater than 0.23 min on the BIOshell™ IgG 1000 Å C4 column whereas this value was consistently higher on the other two columns.

Reduced Mass Transfer Effects from the BIOshell™ IgG Column

One method to gauge the efficiency of a column operating at high flow rates is to examine the peak volume of an analyte at varying flow rates. For SPPs, the mass transfer term of the van Deemter equation is relatively unaffected by flow rate. Thus, theoretically, peak volume should show relatively little change with increasing flow rate but should change (i.e., increase) for analytes assayed with FPP-packed columns. Using SigmaMAb as the analytical probe, this investigation was conducted using the BIOshell™ IgG 1000 Å C4 column, BIOshell™ A400 Protein C4, and the FPP, C4 column used previously. **Figure 5** summarizes the results. The two BIOshell™ columns, as expected, show little change in peak volume with increasing flow rate while the FPP 300 column shows a steep increase due to the effect on the C term in the van Deemter equation.

Figure 7. Analysis of Denosumab, an IgG2 Antibody, by RPC

Conditions:	
Column:	As indicated
Mobile Phase:	[A] 88:10:2 water (0.1% DFA): acetonitrile (0.1% DFA): n-propanol (0.1% DFA)
	[B] 10: 20:70 water (0.1% DFA): acetonitrile (0.1% DFA): n-propanol (0.1% DFA)
Gradient:	14% B to 24% B in 20 min
Flow Rate:	0.2 mL/min
Column Temp.:	80 °C
Detector:	UV, 280 nm
Injection:	2.0 µL
Sample:	Denosumab, 2.0 mg/mL, water (0.1% DFA)



Resolving Cysteine Variants of IgG2 Antibodies with the BIOshell™ IgG Column

Because of their reduced effector function activity, IgG2 antibodies are becoming the favored format for some protein therapeutics. All IgG2 biologics, however, are composed of different ratios of IgG2 isoforms that only differ by the pattern of disulfide bonding in the hinge region. **Figure 6** displays some of the possible isoforms of an IgG2 antibody.[4]

Because some of these isoforms may have immunogenic effects on a patient, and to ensure lot-to-lot reproducibility of a biologic, a method is required to resolve these different variants. Recently, an analytical reversed-phase chromatography (RPC) method was developed to resolve these different variants on the BIOshell™ IgG 1000 Å C4 column. **Figure 7** compares the chromatographic results of using a column packed with FPP versus using the BIOshell™ IgG column in resolving different disulfide bond isoforms of denosumab. Notice the drastically improved resolution of disulfide bond isoforms using the BIOshell™ IgG column over the column packed with FPPs. Using this RPC method with the BIOshell™ IgG column, in combination with techniques such as redox amplification, thiol tagging, and mass spectrometry, it would be possible to identify and confirm the peaks in **Figure 7**.

Conclusion:

As the market for new drugs is slowly overtaken by biologics, the challenges in determining the purity of a new drug will be daunting. New column technology, like the BIOshell™ IgG 1000 Å C4 column, will help scientists develop new methodologies to resolve these challenges. The BIOshell™ IgG column, incorporating SPPs, allows for high speed, high efficiency separations without a drastic increase in backpressure. As regulatory agencies require biopharmaceutical companies to add more CQAs in the monitoring of new drugs, the BIOshell™ IgG column can provide a valuable addition to the analyst's tool box.

References

1. C. E. Muraco, *LC/GC North America*, **35**, 734-745 (2017)
2. C. G. Horvath, B. A. Preiss, S. R. Lipsky, *Anal. Chem.*, **39**, 1422-1428 (1967)
3. J. J. Kirland, *Anal. Chem.* **41**, 218-220 (1969)
4. L. M. Jones, W. Cui, H. Zhang, M. L. Gross, *J. Am. Soc. Mass Spectrom.* **24**, 835-842 (2013)

Featured Products

Description	Cat.No.
BIOshell™ IgG 1000 Å C4, 10 cm x 2.1 mm I.D., 2.7 µm	**63288-U**
BIOshell™ A400 Protein C4, 10 cm x 2.1 mm I.D., 3.4 µm	**66825-U**
EXP® Pre-Column Filter	**51163-U**
EXP® Pre-Column Filter Cartridges	**51164-U**
SILu™Lite SigmaMAb Universal Antibody Standard human	**MSQC4**
Water for chromatography (LC-MS Grade) LiChrosolv®	**115333**
Acetonitrile hypergrade for LC-MS LiChrosolv®	**100029**
Trifluoroacetic acid, ≥99%, purified by redistillation, for protein sequencing*	**299537**

*Product available in North America

Related Products

BIOshell™ lgG, C4 2.7 µm Guard Cartridge 5mm x 2.1mm, Pk.3	**63291-U**
Guard Cartridge Holder, for use with Ascentis® Express & BIOshell™ Guard Columns, pk of 1	**53500-U**
Trifluoroacetic acid for protein sequence analysis	**1.08178**

For more information about our BIOshell™ HPLC Columns for Biopolymer Separations please visit **SigmaAldrich.com/bioshell**

LARGE MOLECULES

Analysis of a Bispecific Monoclonal Antibody Using Size Exclusion Chromatography and Mass Spectrometry

Stacy Shollenberger, Senior Product Manager, Food and Environmental Sample Preparation

Cory E. Muraco, Senior R&D Scientist, Technology and Workflow R&D, Analytix@merckgroup.com

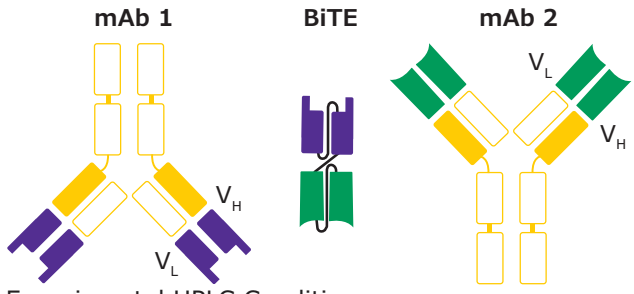
Introduction

More potent formats of monoclonal antibodies (mAbs), such as bispecific antibodies (bsAbs), are on the rise in the area of biotherapeutics. bsAbs recognize two different epitopes. This dual specificity increases the potency of these molecules compared to mAbs and expands the range of possible applications. bsAbs can be used to redirect T cells to tumor cells, block two different signaling pathways simultaneously, dually target different disease mediators, and deliver payloads to targeted sites. At this time, more than 50 bsAb products are currently undergoing clinical evaluation.[1]

Characterization of bsAbs is essential to ensuring product safety and efficacy. Size Exclusion Chromatography (SEC) coupled with Mass Spectrometry (MS) is increasingly being used to identify the accurate molecular mass of biomolecules, including bsAbs. SEC-MS, however, requires the use of mobile phases that do not contain high concentrations of non-volatile salts and the use of columns that do not exhibit column bleed, both of which will interfere with the MS signal response.

In this application, a Bispecific T cell Engager (BiTE®) consisting of two single-chain variable fragments (scFvs) recombinantly linked by a nonimmunogenic five-amino-acid chain (**Figure 1**) was analyzed by SEC-MS using a TSKgel® UP-SW3000, 2 µm column.

Figure 1. Formation of Bispecific T Cell Engager (BiTE)



Experimental HPLC Conditions

Column:	TSKgel® UP-SW3000, 30 cm x 4.6 mm, 2 µm
HPLC Instrument:	Nexera® XR UHPLC system
MS Instrument:	Q Exactive™ Plus*
Mobile phase:	20 mmol/L ammonium acetate, 10 mmol/L ammonium bicarbonate; pH 7.2
Gradient:	Isocratic
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	30 ºC
Injection vol.:	5.0 µL
Samples:	BiTE, 0.3 mg/mL (Creative Biolabs) parent mAb shown, 0.5 mg/mL (Creative Biolabs)
Ionization mode:	Electrospray ionization, positive mode
MS mode:	Scanning, m/z 800-6000

*SEC-MS analysis was performed by the Wistar Proteomics and Metabolomics Facility (*Philadelphia, PA*)

Results and Discussion

The ~55 kDa BiTE and ~150 kDa parent mAbs were injected separately onto a TSKgel UP-SW3000 column coupled to a Q Exactive Plus mass spectrometer for molar mass determination. **Figure 2** shows the (a) total ion chromatogram, (b) mass spectrum, and (c) deconvoluted mass spectrum of the BiTE. A main peak can be seen at m/z 54,143; adjacent peaks at m/z 54,181, 54,219 and 54,086 correspond to different salt adducts.

Figure 3 shows the (a) total ion chromatogram, (b) mass spectrum, and (c) deconvoluted mass spectrum of one of the parent mAbs. A main peak can be seen at m/z 149,264; adjacent peaks at m/z 149,426, and 149,592 correspond to different glycoforms. Similar results (not shown) were obtained for the other parent mAb.

These results demonstrate accurate molar mass determination for the BiTE and both parent mAbs utilizing a 20 mmol/L ammonium acetate, 10 mmol/L ammonium bicarbonate (pH 7.2) mobile phase with SEC-MS compatibility.

Prior to analysis, a blank injection was run in order to assess column bleed. **Figure 4a** shows the total ion chromatogram of a blank injection that was run on a new TSKgel UP-SW3000 column. MS data indicates that there is no column bleed from the TSKgel UP-SW3000 column prior to sample injection. Additionally, a blank injection was run between each of the sample injections in order to monitor sample carryover. **Figure 4b** shows the total ion chromatogram of a blank injection run between the BiTE and parent mAb. No evidence of carryover can be seen in the run after sample injection. The lack of column bleed and carryover indicate that the TSKgel UP-SW3000 column is suitable for use with MS.

Figure 2. SEC-MS Analysis of the BiTE. Accurate Molar Mass of the BiTE Was Calculated to be 54.1 kDa via SEC-MS

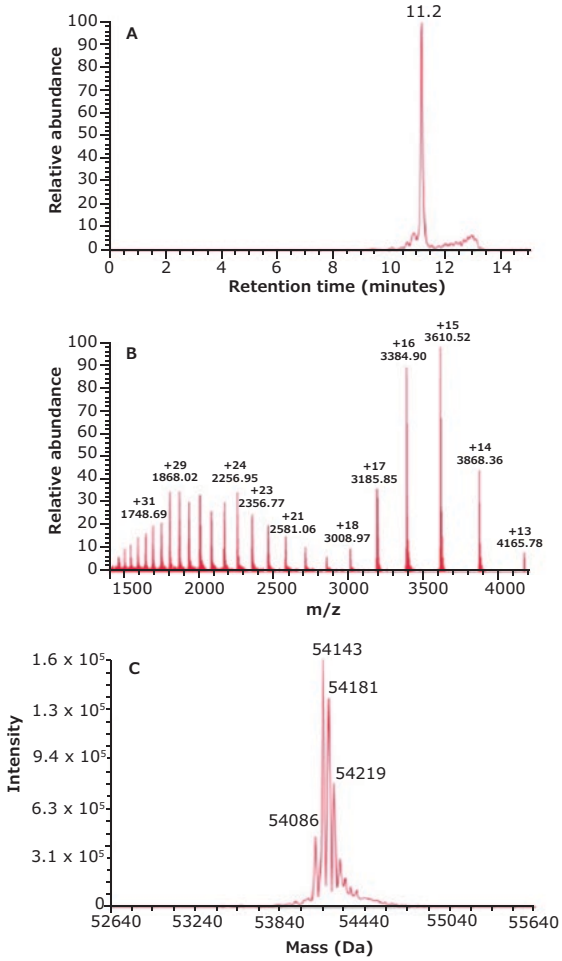
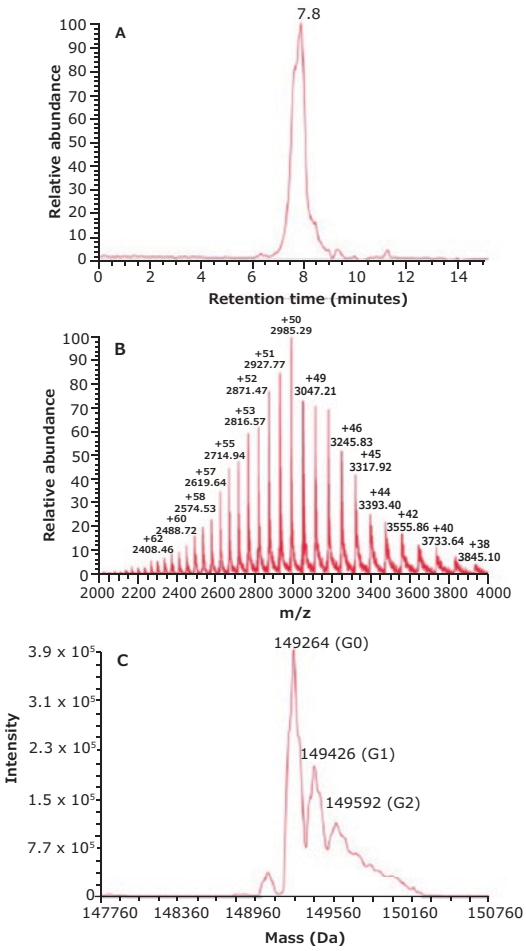


Figure 3. SEC-MS Analysis of Parent mAb. Accurate Molar Mass of the Parent mAb Was Calculated to be 149.3 kDa via SEC-MS



Conclusion

The TSKgel UP-SW3000, 2 µm SEC column can be used as a platform method for bispecific antibody accurate mass determination using SEC-MS. A MS compatible mobile phase under non-denaturing conditions was successfully used with the TSKgel UP-SW3000 column. No signs of column bleed or sample carryover, which may interfere with MS signal response, were noted with the TSKgel UP-SW3000 column.

Reference

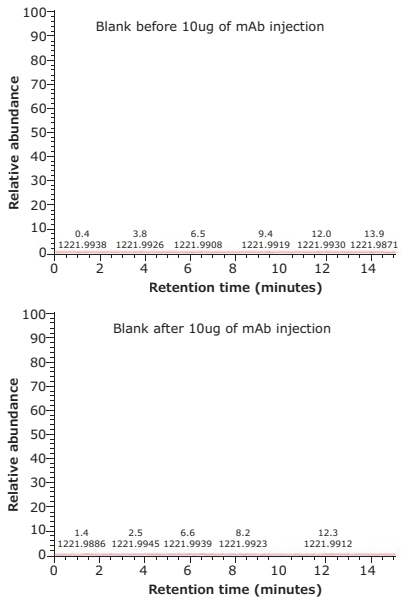
1. Trivedi, A.; Stienen, S.; Zhu, M.; Li, H.; Yuraszeck, T.; Gibbs, J.; Heath, T.; Loberg, R.; Kasichayanula, S. *Clin. Transl. Sci.*, 10, 147-162 (2017).

Featured Products

Description	Cat.No.
TSKgel® UP SW3000, 2 µm, 4.6 mm ID × 30 cm	**80023448**
Related products	
Ammonium acetate for LC-MS LiChropur®	**5.33004**
Ammonium hydrogen carbonate for LC-MS LiChropur®	**5.33005**
Water for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**

To view our broad and diverse portfolio for your large & small molecule bioanalytical workflows please visit **SigmaAldrich.com/tskgel**

Figure 4. Column Bleed and Carryover Analysis. No Column Bleed or Carryover Was Observed via MS Total Ion Chromatogram



LARGE MOLECULES

Threading the Molecular Needle: The Importance of Particle Pore Diameter for Biomacromolecule Separations

Stephanie Schuster, Application and Quality Manager, Advanced Materials Technology, Inc., Wilmington, DE
Cory E. Muraco, Senior Scientist, Liquid Separations R&D, MilliporeSigma, Analytix@merckgroup.com

Introduction

Recently, the evolution of middle to large biomacromolecules such as peptides or proteins is apparent in the market of pharmaceutical drugs in addition to traditional active ingredients based on small molecules. The use of peptides for clinical purposes has its origins in the 1920's using insulin and penicillin. In the 1960's, oxytocin and vasopressin were added to the clinician's arsenal. Leuprorelin and octreotide rounded out the first 60 years of peptide-based drugs by being introduced to the clinic in the 1980's.[1]

In the 1980's, the average length of therapeutic peptides was nine amino acids long with molecular weights of less than 5 kDa. But in the 1990's, longer peptides containing 15 – 20 amino acids became common, and, by the turn of the century, proteins with 40 to 50 amino acid residues with molecular weights approaching or exceeding 50 kDa were beginning to emerge en masse from biopharmaceutical companies. Now, in 2019, even larger proteins have taken the market by storm in the form of monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), and bispecific antibodies (bsAbs), to name just a few.

A mAb is composed of two light chains (LC) that are tethered to two heavy chains (HC) through disulfide bonds. In addition, since the LC and HC are composed of amino acids with reactive side chains, IgG's can be post-translationally modified through phosphorylation, methylation, oxidation, and nitrosylation, among other modifications. These modifications may change the binding affinity of the mAb so that it binds either the wrong antigen, does not bind any antigen, or associates with the wrong cell surface receptor. In addition, mAbs can also aggregate which can lead to allergic responses in patients. Biopharmaceutical companies need to develop rigorous methods to assess lot-to-lot reproducibility of their candidate biologic drug, and the above-mentioned modifications are known as Critical Quality Attributes (CQAs) that both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) monitor. Due to these stringent requirements from regulatory bodies, much research has been pursued in the past 20 years to develop accurate, robust, and high-throughput methods to assess biopharmaceutical purity and structure.

High performance liquid chromatography (HPLC) has been used extensively in the past several decades to characterize peptides and proteins. Since the advent of ultrahigh performance liquid chromatography-mass spectrometry (UHPLC-MS) in the mid-2000's, there has been much research in both developing new methods to characterize large biomolecules and in developing new column technology to better resolve all the different molecular entities that are present in a heterogeneous mAb therapeutic. Two main types of particle morphology are prevalent in the industry today: fully porous particles (FPPs) and superficially porous particles (SPPs, also called Fused-Core® or core shell particles). To take advantage of the low dispersion of UHPLC instrumentation, columns with sub-2 µm FPPs with pore sizes of 300 Å have been used for the analysis of larger hydrodynamic radii biomacromolecules. These columns have been the industry standard since the mid 2000's. However, these columns suffer limitations when analyzing larger or more complex proteins like mAbs and antibody-drug conjugates (ADCs). The relatively small pore size, in addition to a totally porous architecture, and overall higher surface area, restricts the free diffusion of large molecules through the particle and may cause irreversible adsorption of the protein to the stationary phase. This architecture concomitantly results in an increase in the mass transfer term and longitudinal diffusion term of the Van Deemter equation, leading to peak tailing, loss of resolution, and low recovery.

Since 2017, a new line of 1000 Å columns have been introduced that have been optimized for mAb and ADC characterization. These columns are packed with 2.7 µm SPPs that are composed of a 0.5 µm shell thickness and a 1.7 µm solid silica core. The 1000 Å pore particle permits the analysis of mAbs, ADCs, and other, much larger, biomacromolecules. Advantages over columns packed with FPPs are numerous: the SPP shows a significant advantage in mass transfer, leading to less band spreading; columns packed with SPPs are more uniformly packed than columns composed of FPPs, leading to a lower eddy dispersion (A term in the Van Deemter equation); and larger particle sized SPPs have efficiencies similar to or better than sub-2 µm FPPs, leading to the ability of the analyst to run at higher flow rates with less risk of on-column frictional

heating due to elevated column backpressure. Finally, the B-term (longitudinal diffusion) of the Van Deemter equation is also minimized with SPPs. This is due to the presence of less dead volume in the column. A column packed with FPPs will occupy only 33 % of the column volume whereas a column packed with SPPs will occupy approximately 41 % of the column volume.[2]

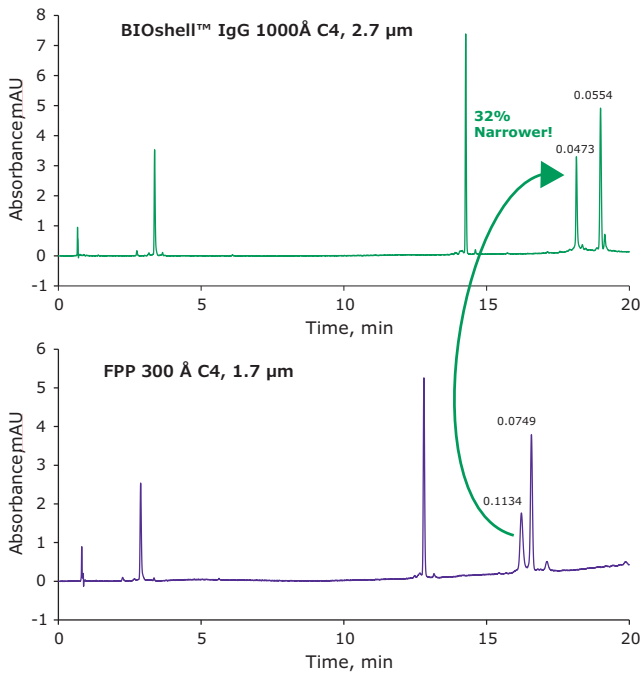
This article will further detail the reasons on why wide-pore diameters are necessary for separating biomacromolecules. Applications involving biomolecules will clearly demonstrate that pore diameters of 450 Å or less can lead to poor results and inadequate resolution of biomolecule variants.

Experimental and Results

All the BIOshell™ IgG 1000 Å SPP columns were obtained from MilliporeSigma*. The experiments were conducted using Shimadzu Nexera X2 UHPLC instruments with PDA detection. Proteins and mAbs were obtained from MilliporeSigma. NISTmAb was purchased from NIST (Gaithersburg, MD). Trifluoroacetic acid was from Pierce Chemicals (Rockford, IL). Acetonitrile was from MilliporeSigma (Gibbstown, NJ). Difluoroacetic acid was purchased from SynQuest Laboratories (Alachua, FL).

Figure 1. Comparison of 1000 Å SPP and 300 Å FPP

Column:	As indicated; 15 cm x 2.1 mm I.D.
Mobile Phase:	[A] Water (0.1 % DFA); [B] Acetonitrile (0.1 % DFA)
Gradient:	23 % B to 50 % B in 24 min
Flow Rate:	0.5 mL/min
Column Temp.:	60 °C
Detector:	UV, 280 nm
Injection:	1.5 µL
Sample:	Protein mixture, varied concentration, water



*The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the U.S. and Canada.

Larger pores enable improved access to the stationary phase. **Figure 1** shows the comparison of a separation of four proteins using a BIOshell IgG 1000 Å C4 column to a FPP 300 Å C4 column. Increased retention is observed on the BIOshell IgG C4 column indicating better access to the pores/stationary phase. The other advantage of the BIOshell IgG C4 column is that it provides narrower peak widths for all the proteins in the mix. This is especially noticeable for the largest protein in the mix – enolase. The peak width is 32 % narrower on the BIOshell IgG C4 column compared to the FPP 300 Å column.

Another advantage of BIOshell IgG 1000 Å C4 columns is how well they can resolve isoforms of IgG2 mAbs. These mAbs differ in the arrangement of the disulfide bridges that connect the heavy and light chains in the hinge region. In **Figure 2**, six different isoforms of panitumumab (trade name Vectibix) are resolved on the BIOshell IgG 1000 Å C4 column whereas the FPP 300 Å C4 column is only able to resolve approximately two isomers. In addition the back pressure is only 120 bar compared to 205 bar on the FPP 300 Å C4 column. Similar resolution advantages are found when the BIOshell IgG 1000 Å C18 column is compared to a 3 µm FPP 300 Å C18 column as shown in **Figure 3**. In this example, resolution of minor components at the base of the main NIST mAb peak are revealed when the

Figure 2. Improved Resolution of IgG2 Variants

Column:	As indicated; 15 cm x 2.1 mm I.D.
Mobile Phase:	[A] 88:10:2 Water:Acetonitrile:n-Propanol (0.1 % DFA); [B] 10:20:70 Water:Acetonitrile:n-Propanol (0.1 % DFA)
Gradient:	14 % B to 24 % B in 20 min
Flow Rate:	0.2 mL/min
Column Temp.:	80 °C
Detector:	UV, 280 nm
Injection:	2 µL
Sample:	Vectibix, 2 mg/mL, water (0.1 % DFA)

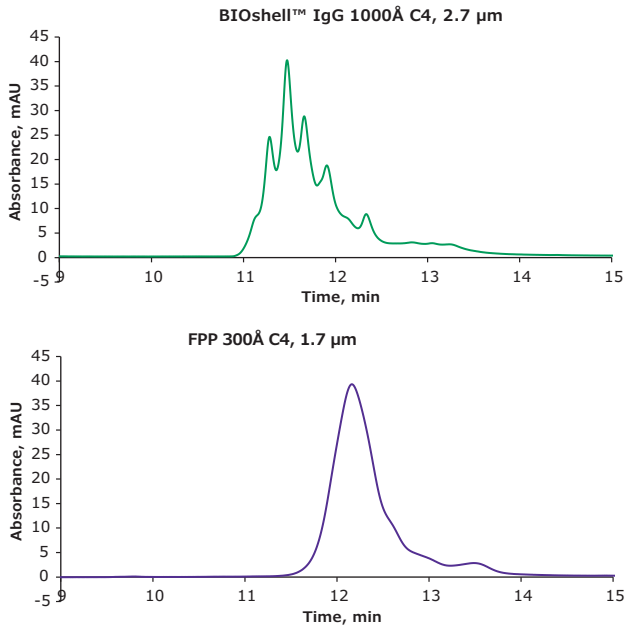
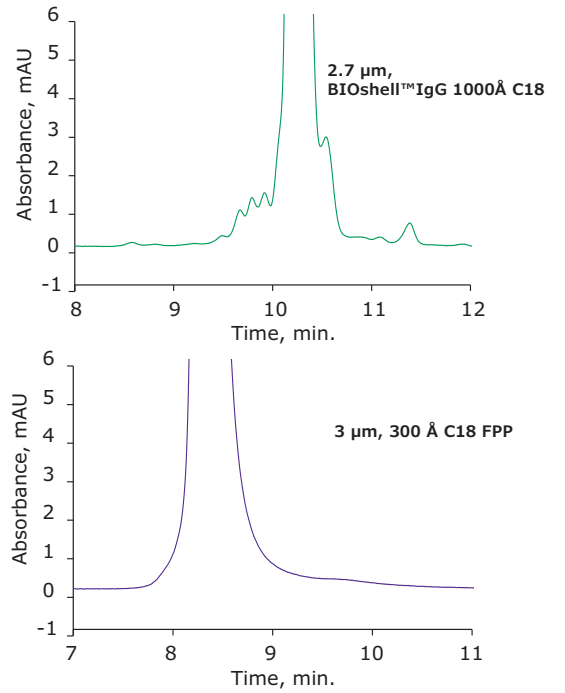


Figure 3. mAb Separation: 1000 Å SPP vs. 300 Å FPP

Column:	As indicated; 15 cm x 2.1 mm I.D.
Mobile Phase:	[A] Water (0.1% TFA); [B] Acetonitrile (0.1 % TFA)
Gradient:	36% B to 44 % B in 16 min
Flow Rate:	0.4 mL/min
Column Temp.:	60 °C
Detector:	UV, 280 nm
Injection:	2 µL
Sample:	NISTmAb, 2 mg/mL, water (0.1 % TFA)



BIOshell IgG 1000 Å C18 column is used. Conversely, none of these minor peaks are visible in the separation when the separation is run on the 3 µm FPP 300 Å column C18 column.

Increased temperature is often used as a means of increasing sample recovery when conducting protein analysis. What has been observed with BIOshell IgG 1000 Å Diphenyl phase is that recovery is equally sufficient at 40 °C as it is at 80 °C. In **Figure 4**, a separation of trastuzumab under 40 °C conditions is compared to a separation on a SPP 450 Å Polyphenyl column. Not only is the peak area greater with the novel BIOshell IgG 1000 Å Diphenyl column, but the resolution and retention are greater, too.

Sample mass on column has always been an experimental variable that chromatographers have had to consider. This variable is further magnified due to the slow diffusion kinetics of large molecules. Column overload can be monitored by observing an increase in peak width as an increasing amount of sample is injected onto the column. **Figure 5** shows the advantages of using a wider pore stationary phase material in terms of band broadening. Much lower peak widths are observed with the BIOshell IgG 1000 Å column than a 300 Å FPP column.

Figure 4. mAb Separation: 1000 Å SPP vs. 450 Å SPP

Column:	As indicated; 15 cm x 2.1 mm I.D., 2.7 µm
Mobile Phase:	[A] Water (0.1% TFA); [B] Acetonitrile (0.1 % TFA)
Gradient:	30 % B to 45 % B in 15 min
Flow Rate:	0.4 mL/min
Column Temp.:	40 °C
Detector:	UV, 280 nm
Injection:	2 µL
Sample:	Trastuzumab, 2 mg/mL, water (0.1 % TFA)

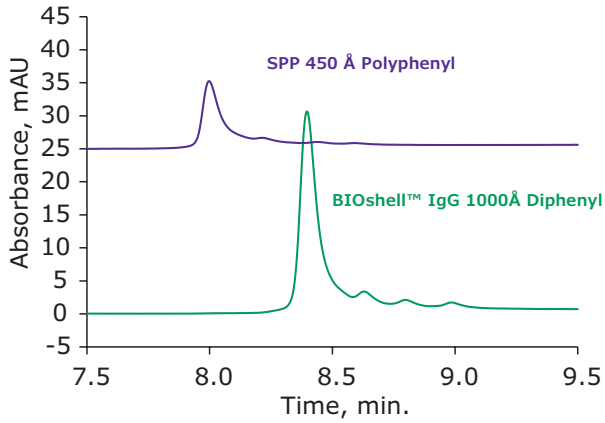
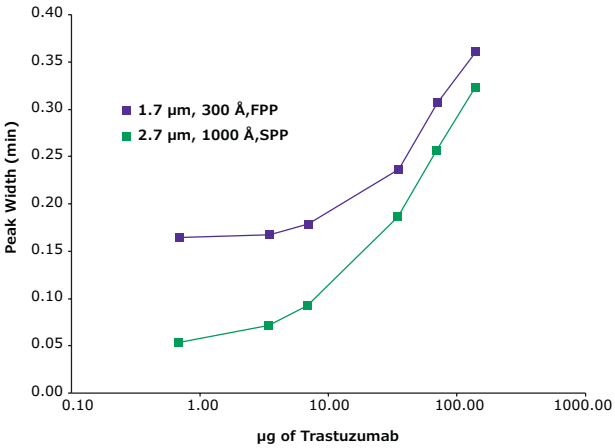


Figure 5. Dependence of Sample Mass on Peak Width

Column:	As indicated; 15 cm x 2.1 mm I.D., C4 Phase
Mobile Phase:	[A] Water (0.1% DFA); [B] Acetonitrile (0.1 % DFA)
Gradient:	27 % B to 37 % B in 10 min
Flow Rate:	0.5 mL/min
Column Temp.:	80 °C
Detector:	UV, 280 nm
Injection:	0.1, 0.5, 1, 5, 10, and 20 µL
Sample:	mAb (trastuzumab), 7 mg/mL, water



Conclusion

Biomacromolecules are complex species requiring cutting-edge chromatographic materials and methods for full characterization. One of the principle parameters of the stationary phase that must be considered when attempting to analyze biomolecules is the pore diameter of the particle. If the pore diameter is too small, steric exclusion will occur resulting in the molecule not being able to access all the available surface area within the pore of the particle. This leads to low chromatographic performance of the method. Combining the high efficiencies garnered from the SPP core-shell architecture with a 1000 Å pore diameter, biomolecules can be completely characterized at a top-down level without fear of steric hindrance or exclusion.

References

1. Ikegami, T.; "Hydrophilic Interaction Chromatography for the Analysis of Biopharmaceutical Drugs and Therapeutic Peptides: A Review Based on the Separation Characteristics of the Hydrophilic Interaction Chromatography Phases." J. Sep. Sci. 2019, 42, 130.
2. Muraco, C. E.; "Improved Biomacromolecule Separations Using Superficially Porous Particles with a 1000 Å Pore Diameter." Chromatography Today, 2018, 4, 27.

Featured Products

Description	Cat. No.
BIOshell™ IgG 1000 Å C4, 15 cm x 2.1 mm I.D., 2.7 µm	**63289-U**
BIOshell™ IgG 1000 Å C18, 15 cm x 2.1 mm I.D., 2.7 µm	**582703-U**
BIOshell™ IgG 1000 Å Diphenyl, 15 cm x 2.1 mm I.D., 2.7 µm	**577421-U**
NISTmAb, Humanized IgG1k Monoclonal Antibody	**NIST8671**

Related Products

Description	Cat.No.
Solvent & Reagents	
Water for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**
Acetonitrile hypergrade for LC-MS LiChrosolv®	**1.00029**
Trifluoroacetic acid for protein sequence analysis	**1.08178**
Guard Columns	
BIOshell™ IgG, C4 2.7 µm, 5 x 2.1 mm Guard Cartridge, pk3	**63291-U**
BIOshell™ IgG, C18 2.7 µm, 5 x 2.1 mm Guard Cartridge, pk3	**581349-U**
BIOshell™ IgG, Diphenyl 2.7 µm, 5 x 2.1 mm Guard Cartridge, pk3	**577431-U**
Ascentis® Express Guard Cartridge Holder, Pk.1	**53500-U**
Acetonitrile with 0.1% (v:v) trifluoroacetic acid for liquid chromatography LiChrosolv®	**4.80448**
Water with 0.1% (v:v) trifluoroacetic acid hypergrade for LC-MS LiChrosolv®	**4.80112**

To read more about our BIOshell line of U/HPLC columns, please visit us at **SigmaAldrich.com/bioshell**

LARGE MOLECULES

Fast Analysis of Monoclonal Antibodies Using Silica Monoliths Designed for Bioanalysis

Tom Kupfer, Egidijus Machtejevas, Gisela Jung, Peter Knoell
Benjamin Peters, Lab Head Instrumental Analytics R&D, Analytix@merckgroup.com

Introduction

In the last two decades, the pharmaceutical market has changed dramatically from small molecules to protein-based drugs and antibodies, which have a higher potential for targeting extraneous substances.[1] This is supported by the fact that six of the top ten best-selling pharmaceuticals are based on monoclonal antibodies (mAbs). In comparison to chemical entities, the analysis of mAbs is complex and requires several separation techniques, e.g., affinity chromatography, reversed phase chromatography, size exclusion chromatography and ion-exchange chromatography. In particular, the strong growth in development of biosimilars due to expiring patents of those blockbusters, led to increased requirements concerning column performance in HPLC.[2]

The analysis of biomolecules using conventional HPLC columns is usually associated with limited accessibility of surface for larger molecules, slow diffusion leading to extremely broad peaks with severe tailing, and possible conformational changes during elution. One of the reasons for those observations is the mesopore size ranging from 8-15 nm (80-150 Å). The separation quality could be improved by using column materials with wider mesopores of ca. 30 nm (300 Å) or larger.[3-5]

During product development, process monitoring, and quality control testing of mAbs, fast and precise analytical separations are necessary. The most important technique for determination of mAb titer is affinity chromatography using protein A. Protein A, a cell wall protein from *Staphylococcus aureus*, was the first isolated protein with a high affinity to the Fc region of immunoglobulin G (IgG).[6] Its high selectivity and high resistance to temperature, pH and tryptic cleavage make protein A a powerful tool in antibody purification.[7] Today, the use of protein A as a ligand on chromatographic media is one the most commonly employed methods for antibody capture during the purification process. In comparison to process protein A media, only a few vendors provide analytical protein A columns for process monitoring and quality control. Most of these columns are packed with particles limited in column backpressure, chromatographic performance, and application for matrix rich samples, such as harvested cell culture fluids. As a consequence, sample preparation is more complex and the limited column backpressure results in long analysis time due to lower flow rates.

Silica monoliths, which consist of one continuous piece of silica, are an alternative to fully porous or superficially porous particles, and are preferred for the application and fast separation of matrix-rich ("dirty") samples due to their bimodal pore structure consisting of large through pores (macropores) and mesopores.[8, 9] They are prepared according to a sol-gel process leading to a bimodal pore structure where both macropore and mesopore formation can be controlled individually.[9, 10] The design of larger macropores offers good flow characteristics resulting in much lower column backpressure as compared to columns packed with particles, and maintaining column performance even at higher flow rates.[11-13] Due to the hydrodynamic volume of mAbs (mostly 5-6 nm), the alteration of mesopore development up to a minimum of 30 nm (300 Å) is necessary to allow them to enter the pores for improved chromatographic separation and to prevent size exclusion effects. Recently, the development of silica monoliths containing larger mesopores, and their application for the analysis of biomolecules, has been reported,[14] leading to the now commercially available Chromolith® WP 300 column family.

Another sector in chromatography gaining more and more interest is the coupling of desired ligands for specific interactions with certain analytes, e.g., antibodies, aptamers or enzymes.[15-20] The majority of described protein immobilizations were performed on polymeric particles or monoliths and silica particles. In the last decade, silica-based monoliths were also used for the immobilization of several ligands to enable their use in the applications of affinity chromatography, for chiral separations, or as on-column bioreactors.[21-26] The development of wide pore silica monoliths enhanced the column properties for the immobilization and analysis of larger molecules. In this study, recombinant

staphylococcal protein A (rSPA) was immobilized onto a silica monolith containing larger mesopores suitable for the separation of mAbs. The analysis performed on the monolithic column was characterized by different parameters including detector response linearity, reproducibility and long-term stability.

Experimental

Chemicals

See the reagents & chemicals listing at the end of this article. In addition, native recombinant staphylococcal protein A (rSPA) ligand was purchased from Repligen (Waltham, MA, USA), pure cetuximab stock solution was a research sample from Merck KGaA (Darmstadt, Germany), and gammanorm IgG was obtained from Octapharma (Heidelberg, Germany).

Epoxy-modified wide pore silica monolith (25 x 4.6 mm) columns were prepared as research samples at Merck KGaA, Darmstadt, Germany (now available as Chromolith® WP 300 Epoxy).

Apparatus

All modification and cleaning steps necessary for the immobilization of rSPA ligand on epoxy-modified columns were performed using a Hitachi L-6200 HPLC Pump. The following chromatographic studies were performed on an Ultimate 3000 HPLC system consisting of LPG-3400RS HPLC pump, WPS-3000TRS autosampler, TCC-3000RS column oven compartment, and VWD-3000 UV detector from Dionex (Germering, Germany).

Preparation of rSPA silica monoliths

For this work a research prototype of Chromolith® WP 300 Epoxy was used, prepared following published processes.[27, 28, 22]

The immobilization of rSPA on wide pore epoxy-modified monoliths was done according to a dynamic process circulating ligand solution through the column. The rSPA ligand solution was dissolved in 6 mL immobilization buffer (50 mM sodium phosphate + 1.9 M ammonium sulfate pH 8.0) resulting in a rSPA concentration of 2 mg/mL. Before immobilization, columns were equilibrated with 50 mL immobilization buffer. The protein solution was circulated at a flow rate of 0.2 mL/min for 4 hours. Finally, the rSPA silica monolith was washed with 50 mL of 100 mM sodium phosphate buffer pH 7.4 and remaining epoxy functions were hydrolyzed with 150 mM phosphoric acid pH 1.5.

Results and Discussion

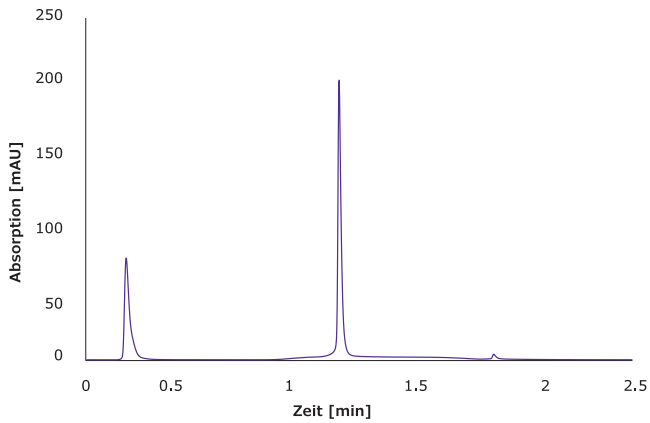
General column evaluation

Before the immobilization process, the silica monoliths were characterized by mercury intrusion porosimetry and nitrogen adsorption/desorption (BET). They consisted of macropores of 1.83 µm and mesopores of 28.6 nm (286 Å), resulting in a total surface area of 120 m^2/g silica. The final column possessed a dynamic binding capacity of 2.75 (±0.11) mg for monoclonal IgG.

The rSPA modified silica monoliths were used for separation of monoclonal antibodies from their related impurities. The separation of cetuximab from BSA impurity was performed in just over one minute and a typical chromatogram is shown in **Figure 1**. The unbound BSA eluted first followed by cetuximab, which was released due to the pH shift to pH 2.5. The rSPA silica monoliths provided sharp peaks with peak widths <0.040 minutes in a short run time which was supported by the hydrophilic column surface reducing undesirable backbone interactions with the analytes.

Figure 1. Separation of cetuximab (1 mg/mL) and BSA (1 mg/mL) by immobilized rSPA silica monolith.

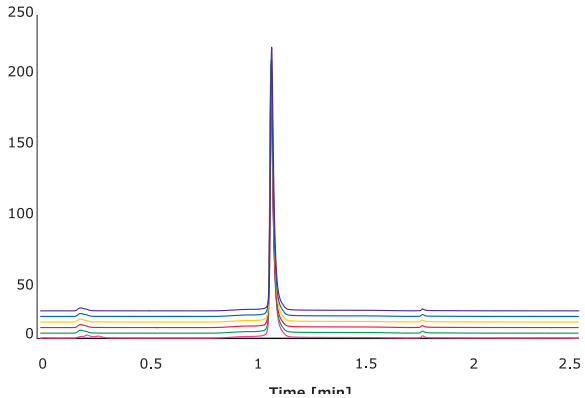
Column:	Immobilized rSPA silica monolith 300 Å, 25 x 4.6mm		
Mobile Phase:	[A] 100 mM sodium phosphate pH 7.4 [B] 100 mM sodium phosphate pH 2.5		
Gradient:	**Time (min)**	**% A**	**% B**
	0.25	100	0
	0.25-0.26	0	100
	1.00	0	100
	1.00-1.01	100	0
	1.01-2.50	100	0
Flow Rate:	2.0 mL/min		
Column Temp.:	25°C		
Detector:	UV, 280 nm		
Injection:	10 µL		



Reproducibility

The cetuximab sample was injected 50 times and all chromatographic parameters were evaluated, especially with respect to their relative standard deviation (RSD). An overlay of all 50 chromatograms is shown in **Figure 2** indicating a robust and reproducible column performance. The RSD of the retention time of eluted cetuximab was <0.1% with the peak width being equal for every run. The peak area RSD was 0.4%, supporting the previous data and assumptions of a robust column modification and reproducible column performance.

Figure 2. Analysis of 50 injections of cetuximab (1 mg/mL) on immobilized rSPA silica monolith. Only the first and every 10th chromatogram are shown. For chromatographic conditions, see **Figure 1**.



Furthermore, different batches were synthesized under exactly the same conditions and six columns of each batch were immobilized with rSPA ligand, respectively. Summaries of chromatographic data of each batch are shown in **Table 1**. The data revealed only slight differences between columns from the same batch as well as minor differences between different batches. Those results demonstrate that the immobilization process via epoxide functions is very robust and reproducible.

Table 1. Chromatographic data of separated IgG by immobilized rSPA silica monolith from different batches. For chromatographic conditions, see **Figure 1**.

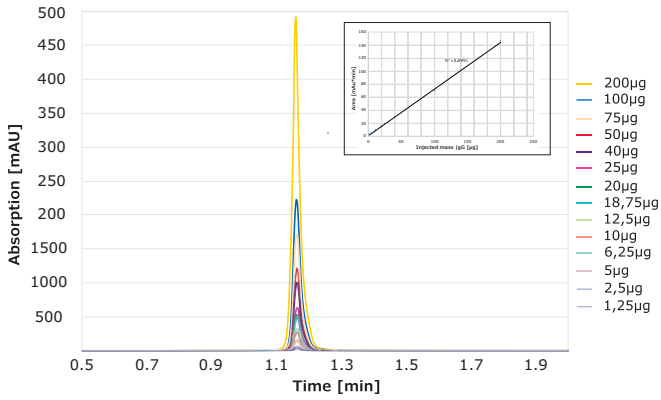
Batch No.	Retention time [min]	Peak Symmetry (USP)	Peak Width (10 %) [min]	Column back pressure [bar]
1 (n=6)	1.15 ±0.01	1.57 ±0.15	0.037 ±0.003	10 ±0
2 (n=6)	1.16 ±0.00	1.53 ±0.12	0.035 ±0.003	10 ±0
3 (n=6)	1.15 ±0.00	1.52 ±0.06	0.035 ±0.003	10 ±0
Total (n=18)	**1.15 ±0.00**	**1.54 ±0.11**	**0.036 ±0.003**	**10 ±0**

Column linear range

For titer determination, the linear range of the analytical column is a key property for its application. In antibody production, the bioreactors used contain antibody concentrations between 0.5-7 g/L, which is the minimum requirement for an analytical protein A column. The rSPA modified monolith was evaluated by analyzing different concentrations of pure cetuximab ranging from 0.0125 to 10 mg/mL. The fifteen calibration standards used covered a broad analytical range from 1.25-200 µg of injected cetuximab onto the immobilized rSPA silica monolith. The retention time of eluted cetuximab concentrations varied by only 0.1% and was confirmed by an overlay of all calibration chromatograms as shown in **Figure 3**. The correlation coefficient was higher than 0.999, supporting the hypothesis of a high column capacity.

Figure 3. Calibration curve of Cetuximab on rSPA silica monolith ranging from 1.25 µg to 200 µg.

Chromatographic conditions, same as in **Figure 1**, except injection volume: 20 µL.



Flow rate

Since monolithic silica columns exhibit a low column backpressure due to their high flow through pores, the separation capability of immobilized rSPA silica monoliths was evaluated at higher flow rates. The comparison of column performance was done with pure cetuximab spiked with BSA. It was expected that retention time and peak width of eluted cetuximab would decrease with increasing flow rate.

In **Table 2**, key data is shown for a flow rate comparison. It was evident that cetuximab column binding was not affected by flow rate and the relative peak area of eluted cetuximab was constant. Additionally, column backpressure increased linearly (R2 = 0.998) enabling high flow rates and even shorter run times with constant column performance and without any loss in binding efficiency. The high-speed separation of monoclonal antibodies at high flow rates is enabled by the high mass transfer properties of

Table 2. Chromatographic data of separated IgG (1 mg/mL) and BSA (1 mg/mL) by immobilized rSPA silica monolith at different flow rates. For chromatographic conditions, see **Figure 1**; same gradient was used as shown in **Figure 1** and was adjusted based on flow rate used; flow rate: 1.0-5.0 mL/min

Flow rate [mL/min]	Retention time [min]	Peak Width (10%) [min]	BSA area	IgG area	Column back pressure [bar]
1.0	2.31	0.058	39%	61%	3
2.0	1.16	0.032	39%	61%	6
3.0	0.77	0.024	39%	61%	10
4.0	0.58	0.021	39%	61%	13
5.0	0.47	0.020	39%	61%	17

the macropores. The flow rate data demonstrated the significant time savings and high separation efficiencies obtained by using silica monoliths.

Stability

The examination of column long-term stability was performed with IgG as a control. The stability of the monolithic structure, column modification and protein A linkage were extensively investigated by more than 5,000 runs, including 10,000 pH shifts which corresponded to more than 53,000 CV applied to the column.

The results of IgG control samples are shown in **Figure 4**. Immobilized rSPA silica monolith provided constant analysis of IgG during the complete stability test - remaining retention time, peak area, and peak width were nearly unchanged. The RSD of IgG retention time was smaller than 0.5% whereas for the peak area of eluted IgG, the RSD was below 1.1%, indicating no loss of binding capacity.

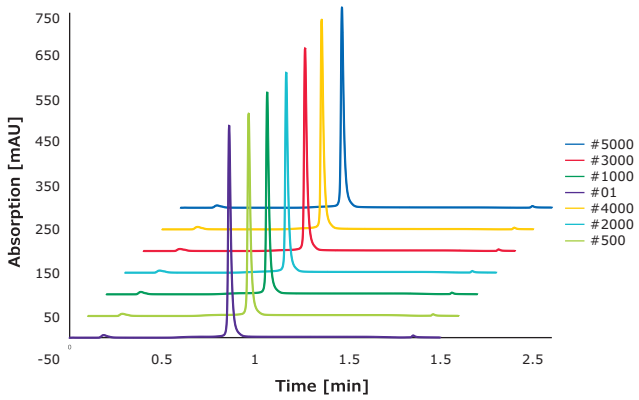
Conclusions

Recombinant protein A was covalently attached to the surface of silica monoliths with larger mesopores suited for biomolecules. The immobilized rSPA column was successfully utilized in the separation of monoclonal antibodies by affinity chromatography. The applied method for immobilization yielded a high dynamic binding capacity, leading to a broad range of applicable antibody concentrations. Furthermore, the stability test revealed a strong linkage between silica monolith and rSPA ligand due to the constant analysis of monoclonal antibodies. In conclusion, silica monoliths with bimodal pore structure immobilized with rSPA ligand are ideally suited for the chromatographic separation of monoclonal antibodies.

Figure 4. Stability test of immobilized rSPA silica monolith against 10,000 pH shifts using gammanorm IgG (1 mg/mL) as control for column performance. Chromatographic conditions, same as **Figure 1** except for gradient.

Gradient Conditions

Mobile Phase:	[A] 100 mM sodium phosphate pH 7.4 [B] 100 mM sodium phosphate pH 2.5		
Gradient:	**Time (min)**	**% A**	**% B**
	0.05	100	0
	0.05-0.06	0	100
	1.10	0	100
	1.10-1.15	100	0
	1.15-2.00	100	0



References

1. G. Walsh, Biopharmaceuticals: An overview, in: G. Walsh, B. Murphy (Eds.), Biopharmaceuticals: An Industrial Perspective, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 1-34 (1999).
2. Bell, D. LC GC N Am 34 (4) (2016) 242-252.
3. Fekete, S.; Veuthey, J.L.; Guillarme, D. J. Pharm. Biomed. Anal. 69 (2012) 9-27.
4. Guillarme, D.; Ruta, J.; Rudaz, S.; Veuthey,J.L. Anal. Bioanal. Chem. 397 (3) (2010) 1069-1089.
5. Vuignier, K.; Fekete, S.; Carrupt, P.A.; Veuthey, J.L.; Guillarme, D. J. Sep. Sci. 36 (2013) 2231-2243.
6. Forsgren, A.; Sjöquist, J. J. Immunol 97 (1966) 822-827.
7. Gorbatiuk, O.B.; Bahmachuk, A.O.; Dubey, L.V.; Usenko, M.O.; Irodov, D.M.; Okunev, O.V.; Kostenko, O.M.; Rachkov, A.E.; Kordium, V.A. Biopolym Cell 31(2) (2015) 115-122.
8. Unger, K.K.; N. Tanaka, E. Machtejevas. Monolithic Silicas in Separation Science. Wiley-VCH Verlag GMBH, Weinheim, Germany, 2011.
9. Cabrera, K. LC GC N Am 30 (4) (2012) 30-35.
10. Altmaier, S.; Cabrera, K. J. Sep. Sci, 31 (2008) 2551-2559.
11. Cabrera, K.;Lubda, D.;Eggenweiler, H.M.; Minakuchi, H.; Nakanishi, K. J High Resolut Chromatogr 23 (1) (2000) 93-99.
12. Nakanishi, K.; Ishizuka, N.; Minakuchi, H.;Hirao, K.;Tanaka, N. Colloids Surf A Physicochem Eng Asp 187-188 (2001) 273-279
13. Tanaka, N.; Nagayama, H.; Kobayashi, H.; Ikegami, T.; Hosoya, K.; Ishizuka, N.; Minakuchi, H.; Nakanishi, K.;Cabrera, K.; Lubda, D. J High Resolut Chromatogr 23 (1),(2000) 111-116.
14. Cabrera, K.; Kupfer, T.; Jung, G.; Knoell, P.; Peters, B. The Column 10 (22) (2014) 40-43.
15. Spross, J.; Sinz, A. J. Sep. Sci. 34 (2011) 1958-1973.
16. Pfaunmiller, E.; Paulemond,M.; Dupper, C.; Hage, D. Anal. Bioanal. Chem 405 (2013) 2133-2145.
17. Moser, A.; Hage, D. Bioanalysis 2 (4) (2010) 769-790.

18. Michaud, M.; Jourdan, E.; Villet, A.; Ravel, A.; Grosset, C.; Peyrin, E.J. Am. Chem. Soc. 125 (28) (2003) 8672-8679.
19. Han, B.; Zhao, C.; Yin, J.; Wang, H. J. Chrom B 903 (2012) 112-117.
20. Girelli, A.; Mattei, E. J. Chrom. B 819 (1) (2005) 3-16.
21. Lubda, D.; Cabrera, K.; Nakanishi, K.; Lindner, W. Anal Bioanal Chem 377 (2003) 892-901.
22. (22) Calleri, E.; Massolini, G.; Lubda, D.;Temporini, C.; Loiodice, F.;Caccialanza, G. J Chrom A 1031 (2004) 93-100.
23. Calleri, E.; Temporini, C.; Perani, E.; Stella, C.; Rudaz, S.; Lubda, D.;Mellerio, G.; Veuthey, J.L.; Caccialanza, G.; Massolini,G. J Chrom A 1045 (2004) 99-109
24. Temporini, C.; Calleri, E.; Campèse, D.; Cabrera, K.; Félix, G.; Massolini, G. J. Sep. Sci. 30 (2007) 3069-3076.
25. Mallik, R.; Hage, D.S. J Pharm Biomed Anal 46 (2008) 820-830.
26. Yoo, M.J.; Hage, D.S. J. Sep. Sci 32 (2009) 2776-2785.
27. Minakuchi, H.; Nakanishi, K.; Soga, N.; Ishizuka, N.; Tanaka,N. Anal. Chem. 68 (1996) 3498-3501.
28. Minakuchi, H.; Nakanishi, K.; Soga, N.; Ishizuka, N.; Tanaka, N. J. Chromatogr. 797 (1998) 121-131.

For more information on the Chromolith® columns, please request our brochure and/or visit us at **SigmaAldrich.com/Chromolith**

Featured Products

Description	Cat. No.
Chromolith® WP 300 Epoxy 25-4.6 (25 x 4.6 mm)	**1.52252**
Chromolith® WP 300 Protein A 25-4.6 (25 x 4.6 mm)	**1.52258**
Reagents & Chemicals	
di-Sodium hydrogen phosphate dihydrate for analysis EMSURE® (≥99.5 %)	**1.06580**
Ammonium sulfate for biochemistry (≥99.5 %)	**1.01211**
ortho-Phosphoric acid 85 % for analysis EMSURE® ACS,ISO,Reag. Ph Eur	**1.00573**
Sodium hydroxide solution 50 % for analysis EMSURE®	**1.58793**
Water from Milli-Q® system	**SigmaAldrich.com/labwater**

Related Products

Description	Cat. No.
Bovine Serum Albumin (BSA) heat shock fraction, pH 7, ≥98 %	**A9647**

An overview of our antibody standards for mass spectrometry can be found at **SigmaAldrich.com/silumab**

Correlation of FcR Affinity Chromatography with Glycan Pattern and ADCC Activity of a Therapeutic Antibody

Cara Tomasek, Leader, Product Management, Tosoh Biosciences, North America
Cory E. Muraco, Product Manager Liquid Chromatography Technologies, Analytix@merckgroup.com

Introduction

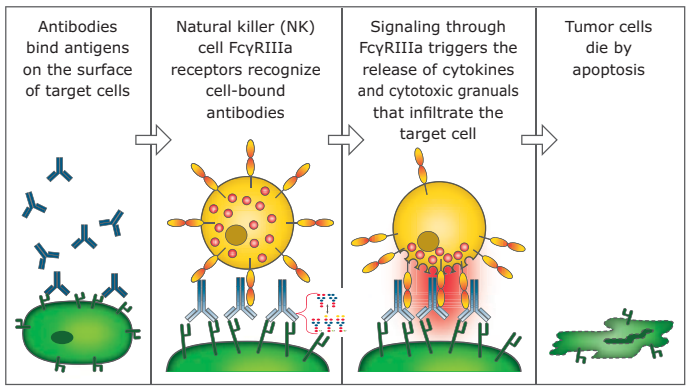
Antibody-dependent cell-mediated cytotoxicity (ADCC) is an important mechanism of action (MOA) of monoclonal antibodies used in cancer treatment. Selecting suitable cell lines and optimizing culture conditions towards the expression of antibody candidates with desired ADCC activity is an essential part of the R&D process. A fast and straightforward approach to easily access ADCC activity would facilitate screening of many clones or monitoring the effect of upstream process variations. Other stages of R&D and production could benefit from fast ADCC assessment as well: comparing biosimilar and originator, detecting lot-to-lot variations, monitoring product stability, to name but a few.

Fc Receptor and ADCC Activity

ADCC starts with the binding of the Fab region of an antibody to a target cell, *e.g.,* a cancer cell. Binding of the Fc domain of that antibody to Fcγ receptors on the outer membrane of natural killer (NK) cells triggers degranulation into a lytic synapse and finally the apoptosis of the cancer cell (**Figure 1**). The glycan microheterogeneity of the Fc domain, on the galactose and core-fucose levels, influences the binding of the Fc domain to Fcγ receptors.[1]

Current ADCC activity tests are either cell-based bioassays or surface plasmon resonance (SPR) measurements using immobilized Fcγ receptors. A new approach combines the specificity of the FcγIIIa receptor (FcγRIIIa) with the easy handling of an HPLC method.

For Fc receptor affinity chromatography, a recombinant FcγIIIa receptor ligand is immobilized on a stationary phase. Glycoforms of an antibody sample can be partly separated based on the strength of their binding to the FcR ligand. Resulting peaks can be assigned to low, medium, and high ADCC activity (**Figure 2**).



Original image by Satchmo2000, distributed under a CC-BY 3.0 license.

Figure 1. Antibody-dependent cell-mediated cytotoxicity

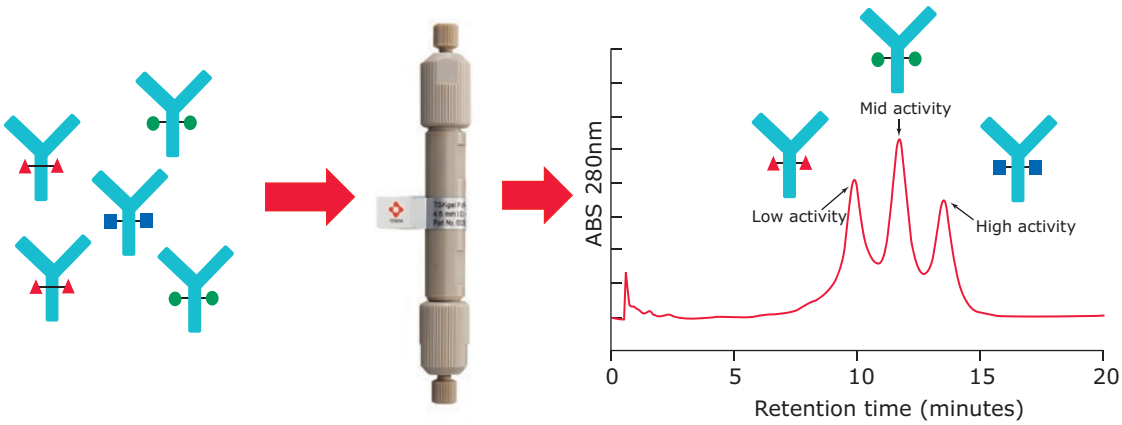


Figure 2. FcγRIIIa affinity chromatography

Taking the well-known therapeutic antibody rituximab as an example, this application note demonstrates that the pattern of FcγRIIIa affinity chromatography shows a good correlation with the results obtained by an ADCC reporter assay. Fractions collected from HPLC peaks with different receptor affinity also show different glycosylation patterns at the Fc domain.

Rituximab (**Figure 3**) is a recombinant chimeric human/mouse monoclonal IgG_1 antibody approved in 1997 and used to treat certain autoimmune diseases and types of cancer. Besides other effects of rituximab, its Fc portion mediates antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[2] N-glycans bound to the Fc domain of rituximab contain mainly G0F and G1F structures.

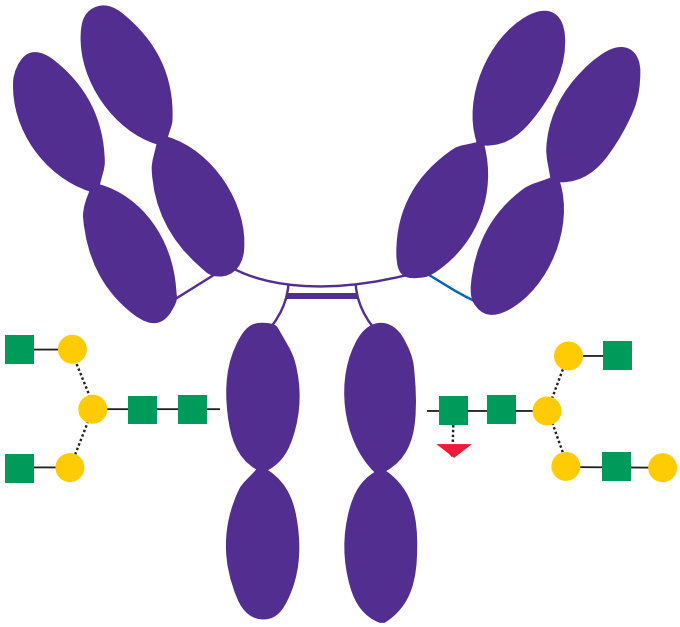
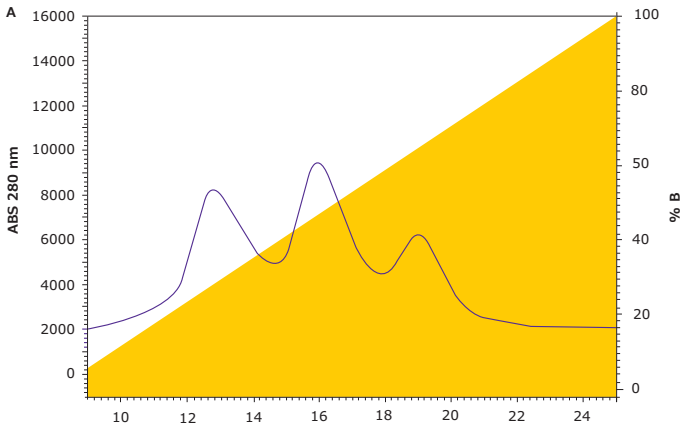


Figure 3. Schematic Diagram of Rituximab

FcR Affinity Chromatography of Rituximab

In FcγRIIIa affinity chromatography, purified antibody or cell culture supernatant is injected under conditions that promote binding of monoclonal antibodies (mAbs) to the FcγRIIIa ligand. Elution of bound mAb variants is performed by lowering the pH of the mobile phase in order to disrupt the target/ligand interactions. The higher the affinity of a mAb variant to the receptor, the higher the retention time of the respective peak.

FcR affinity chromatography analysis of rituximab on the new TSKgel® FcR-IIIA-NPR column results in three peaks representing variants with low, medium, and high FcγRIIIa affinity (**Figure 4**). Subsequent characterization of the three peaks was carried out using a semi-preparative prototype FcR-IIIA column to collect fractions. For each peak, ADCC activity was analyzed with a standard ADCC reporter bioassay kit (Promega). Glycan analysis was also carried out on each fraction using HILIC-UHPLC.



Chromatographic Conditions[3]	
column:	TSKgel® FcR-IIIA-NPR, 7.5 cm x 4.6 mm I.D., 5 µm, PEEK (**823513**)
mobile phase:	[A] 50 mM Citrate, pH 6.5 ; [B] 50mM Citrate, pH 4.5
flow rate:	1 mL/min
column temp.:	25 °C
detector:	UV, 260 nm
injection:	30 µL
sample:	Rituximab, 1 µg/µL, Rituximab kindly provided by Rentschler Biopharma

Figure 4. FcγR Affinity Analysis of Rituximab on TSKgel®I FCR-IIIA-NPR

ADCC Bioassay of Rituximab and FcR Affinity Fractions

The Fc effector reporter bioassay uses the FcγR and Nuclear Factor of Activated T-cells (NFAT)-mediated activation of luciferase activity in effector cells to determine ADCC efficacy and potency of antibodies. **Figure 5** shows the ADCC reporter bioassay response to rituximab and to the three fractions collected from FcR affinity chromatography (low, medium, high FcγR affinity). Rituximab is shown as the grey points; fraction 1, representing low FcγR affinity, is shown in blue; fraction 2, representing medium FcγR affinity, is shown in red; and fraction 3, representing high FcγR affinity, is shown in green.[3]

Table 1 shows the half maximal effective concentration (EC50) values obtained by the reporter bioassay test. The lower the EC50 value, the higher the ADCC potency. As expected, peak 3 (high FcRγ affinity, green) shows the highest ADCC potency and efficacy in the bioassay. Peak 2 shows the intermediate and peak 1 shows the lowest ADCC efficacy and potency. ADCC efficacy and potency of the original rituximab lies between the low and medium affinity fractions.

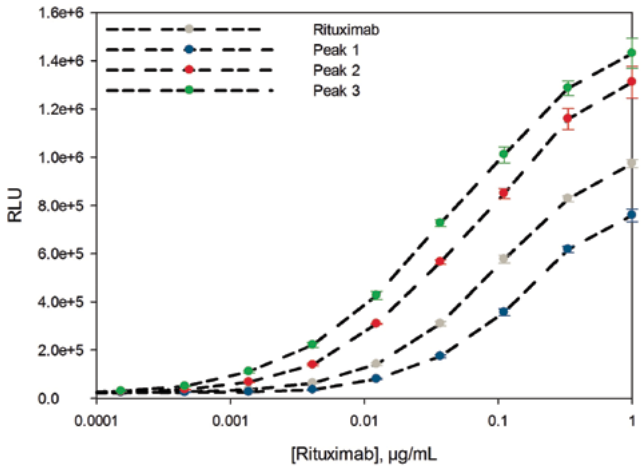


Figure 5. ADCC Reporter Bioassay Response to Rituximab

Table 1. The Dose-Response Curve of ADCC Data was fitted with a 4-Parameter Model using a Sigma Plot

EC50 values obtained by the reporter bioassay test	
Antibody	EC50 (µg/mL)
Rituximab	0.098
Peak 1	0.153
Peak 2	0.072
Peak 3	0.049

Glycan Analysis of FcR Affinity Fractions

For each peak in **Figure 4**, cleaved and 2-aminobenzamide-labeled (2AB-labeled) N-glycans were characterized by HILIC-UHPLC. **Figure 6** shows the glycan pattern of the FcR affinity fractions compared to a glycan library. The antibody glycoforms collected in peak 3 (highest affinity) show mainly galactose containing N-glycans (G1F and G2F). Peak 2 glycoforms contain more G0F glycans than peak 3 and glycoforms collected in peak 1 (lowest affinity) show predominantly fucosylated glycans without galactose units (G0F).

Conclusions

The ADCC activity bioassay results show that high retention on the TSKgel® FcR-IIIA-NPR column corresponds to a high ADCC activity. The HILIC-UHPLC glycosylation pattern analysis of the FcR affinity fractions also matches the common understanding that terminal galactose units of Fc-glycans typically enhance affinity to FcγRIIIa and ADCC activity while core fucose units decrease ADCC activity of antibodies. These

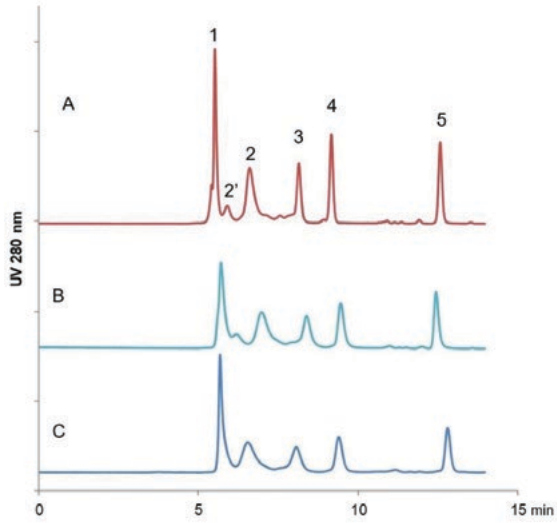


Figure 6. HILIC Analysis of Oligosaccharides of the three FcR Affinity Fractions (Peak 1 blue, Peak 2 red, Peak 3 green) compared with a 2-AB Labeled Biantennary Glycan Library (grey).[4]

results confirm that FcγRIIIa affinity chromatography allows fast assessment of biologic activity and glycoform pattern of antibodies.

References

1. D. Reusch and M. L. Tejada, Glycobiology 2015, 25 (12): 1325-1324; doi:10.1093/glycob/cwv065
2. G.J. Weiner; Semin Hematol. 2010; 47 (2): 115-123; doi:10.1053/j. seminhematol.2010.01.011
3. Master Thesis Leila Ghaleh, TU Darmstadt
4. M. Kioshi et. al. Nature Scientific Reports | (2018) 8:3955 | DOI:10.1038/s41598-018-22199-8

Featured Products

Description	Cat. No.
TSKgel® FcR-IIIA-NPR HPLC Column, 7.5 cm x 4.6 mm I.D., 5 µm, PEEK	**823513**
Sodium citrate tribasic dihydrate, > 98%	**C7254**
PNGase Fast, recombinant, expressed in E. coli	**EMS0001**
GlycoProfile™ 2-AB Labeling Kit	**PP0520**
SILu™MAb Rituximab Stable-Isotope Labeled Monoclonal Antibody	**MSQC13**
SILu™Lite SigmaMAb Rituximab Monoclonal Antibody	**MSQC17**

Rituximab was kindly provided by Rentschler Biopharma.

To see our selection of TSKgel® columns, visit us at **SigmaAldrich.com/tsk**

LARGE MOLECULES

Released *N*-Glycan Analysis of a Therapeutic Antibody Using BIOshell™ Glycan Column

Maricar Dube, Analytical Sciences Liaison
Cory Muraco, HPLC Product Manager
Judy Boland, Senior R&D Scientist
Amber Henry, R&D Scientist, Analytix@merckgroup.com

Introduction

Therapeutic monoclonal antibodies (mAbs) have seen an explosive growth since the first mAb was approved by the US FDA over thirty years ago. In fact, over the past five years, therapeutic antibodies have become the best-selling drugs,[1] and they continue to grow in terms of new approvals and targets.[2]

Monoclonal antibodies are target specific, which means that they have high-efficacy and few side effects. However, compared to chemically synthesized small molecule therapies, mAbs are considerably more complex owing to their size and the nature of their development and production. These mAbs are expressed using recombinant technologies in mammalian cell lines or other expression systems, giving rise to heterogeneity mainly through post-translational modifications (PTMs).[3] These PTMs need to be characterized as they affect the efficacy, stability, half-life, and safety of mAbs.

Glycosylation is one of the most common and important PTMs for mAbs. Glycosylation involves the attachment of glycans at specific sites on a protein, most commonly at asparagine (Asn) (*N*-linked) or serine/threonine (Ser/Thr) (*O*-linked) amino acid residues.[4] There are four levels of analytical approaches to *N*-glycan analysis: intact glycoproteins, glycopeptides, released glycans, and monosaccharide analyses.[5] This article focuses on the analysis of released *N*-glycans by high-pressure liquid chromatography (HPLC).

The steps for a released *N*-glycan analysis are outlined in **Figure 1**. The *N*-linked glycans are released by an amidase such as peptide-N-glycosidase F (PNGase F). The released glycans are then labeled with a fluorescent tag, like aminobenzamide (2-AB) or procainamide (4-amino-N-[2-(diethylamino)ethyl] benzamide). Prior to the HPLC analysis, a clean-up step is needed to remove excess tags and salts. Hydrophilic interaction liquid chromatography (HILIC) is a proven technique for the separation and quantitation of glycans over other HPLC methods (*e.g.* reverse phase, anion exchange).[4]

In this article, a BIOshell™ Glycan HPLC column is used to analyze Cetuximab (Erbitux®) *N*-glycans labeled with procainamide. BIOshell™ Glycan HPLC columns are specifically engineered to deliver a fast, high-resolution, and reproducible glycan identification using HILIC.

Experimental

Glycan Release and Labeling - PNGase Fast Kit was used for glycan release with FASP (filter aided sample prep). The released glycans were labeled using procainamide with reductive amination.

Sample Cleanup - The labeled samples were diluted with 99% acetonitrile and loaded to the conditioned Discovery Glycan SPE cartridges. They were allowed to flow through the cartridges slowly, using gravity (slight pressure or vacuum would also be suitable), making sure that the sample was entirely in the resin bed. The cartridges were washed five times with 99% acetonitrile using vacuum. After the washing step, 20% acetonitrile was added to each cartridge, and allowed to elute slowly, using gravity (applying slight pressure or vacuum would also be suitable). After all the eluent had passed into the resin bed, vacuum was used to evacuate all liquid from the SPE into the collection tube. The eluted labeled glycans were then dried by vacuum centrifugation. **Table 1** shows the SPE conditions.

Figure 1. Workflow for Released *N*-Glycan Analysis by HPLC.

Table 1. SPE Conditions

sample matrix:	Labeled sample diluted with 1 mL 99% acetonitrile/1% water
SPE tube:	Discovery® Glycan SPE Tube
conditioning:	1 mL 99% acetonitrile/1% water
sample loading:	diluted labeled sample slowly sink into bed
washing:	5 x 1mL 99% acetonitrile/1% water
eluent:	400 µL 20% acetonitrile/80% water, slowly sink into bed
eluate post-treatment:	drying by vacuum centrifugation

HPLC Analysis - The dried and labeled glycans were solubilized by dissolving in 50 µL of 75% ACN / 25% 75 mM ammonium formate pH 4.4, vortexing for 2 mins, followed by centrifugation at 16,000 x g for 2 mins. **Table 2** shows the chromatographic conditions.

Table 2. HPLC Condition for the Analysis of Procainamide Labeled Cetuximab N-Glycans

column:	BIOshell™ Glycan; 15 cm x 2.1 mm I.D., 2.7 µm
mobile phase:	[A]: 75 mM ammonium formate pH 4.4 (50 mM ammonium hydroxide, adjusted to pH 4.4 with formic acid) [B]: Acetonitrile
gradient:	75% B to 59% B in 75 min
flow rate:	0.37 mL/min
column temp.:	58 °C
detector:	Fluorescence, 308 nm excitation,359 nm emission
injection:	10 µL
sample:	dried procainamide labeled Cetuximab reconstituted in 50 µL 25% 75 mM ammonium formate pH 4.4 and 75% acetonitrile

Results

In this study, cetuximab was used as a model therapeutic mAb to analyze released N-glycans. This mAb is a chimeric mouse-human IgG1 monoclonal antibody, against the epidermal growth factor receptor (EGFR). Cetuximab is used to treat head, neck, as well as colorectal cancers. The antibody is N-glycosylated both in the fragment crystallizable (Fc) and fragment antigen binding (Fab) regions. There are numerous studies and reports showing the attachment of N-glycans to mAbs and the subsequent effect of the attachment on various biological and physicochemical processes, leading to safety and quality issues.[6,7] Some of the processes affected by the glycosylation include enhancement of the structural integrity of the mAb, serum half-life, antibody-dependent cellular toxicity (ADCC), anti-inflammatory activities, immunity, and antigen recognition. This clearly indicates towards the important need for understanding glycosylation patterns.

BIOshell™ HPLC columns are based on Fused-Core® particles (also called core-shell or superficially porous particles (SPPs)), characterized by a thin, porous shell of high-purity silica surrounding a solid, silica core. This design allows for a shorter diffusion path compared to traditional fully porous particles, as illustrated in

Figure 2. The short diffusion path accelerates mass transfer of solutes ("C" term in the van Deemter equation), concomitantly resulting in high column efficiency.

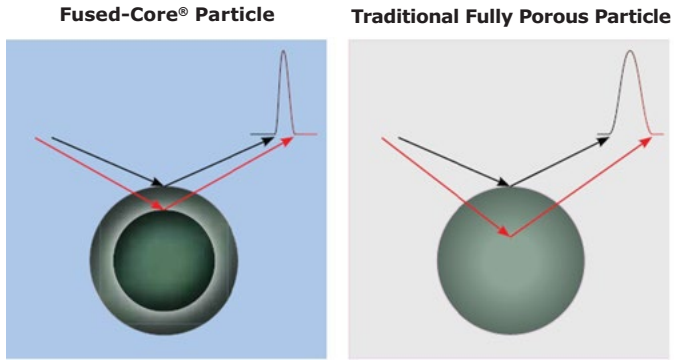


Figure 2. Fused-Core® Particles have Shorter Diffusion Paths Compared to Traditional Fully Porous Particles.

The stationary phase in the BIOshell™ Glycan column is a highly polar ligand that has five hydroxyl (-OH) groups tethered to the silica via a novel and proprietary chemical linkage. This unique column chemistry is suitable for analysis of oligosaccharides, particularly for protein-linked glycans using the typical mobile phases for HILIC of oligosaccharides.

A fluorescence chromatogram of procainamide-labeled cetuximab glycans is shown in **Figure 3**. The BIOshell™ Glycan column was able to elucidate the complex glycosylation of this mAb. The corresponding glycans were identified by MS analysis (mass spectrometry data not shown).

Figure 3. Fluorescence Chromatogram of Procainamide-Labeled Cetuximab Glycans on BIOshell™ Glycan column. LC-MS was Used to Characterize each Peak (Chromatogram not shown). A rapid Release Glycan Protocol was used.

Successful analysis of N-linked glycans by HPLC requires an efficient and reproducible glycan release step. The traditional protocol involves multiple wash steps and an overnight digestion of the native or denatured mAb. The protocol described in the experimental section of this article is a fast protocol

that uses a proprietary detergent-based buffer for rapid deglycosylation of N-linked glycans using PNGase F. In this fast protocol, complete release of N-glycans is achieved in a 15-minute incubation, compared to the traditional overnight digestion.

In another experiment, the BIOshell™ Glycan column was used to compare released glycans from cetuximab using three glycan release protocols:

- Traditional overnight protocol, denatured using guanidine hydrochloride
- Fast protocol, non-reduced (rapid deglycosylation)
- Fast protocol, reduced (rapid deglycosylation under reducing conditions using 2-mercaptoethanol)

The results are shown in **Figure 4**.

With this particular analyte (Cetuximab), all three protocols were found to be equally efficient for some glycan species, such as G0F-N, Man5, G0F, G1(F1,6), Man5G0F Hybrid, and G1F (1,3). But there were some glycans that were not efficiently released with the fast

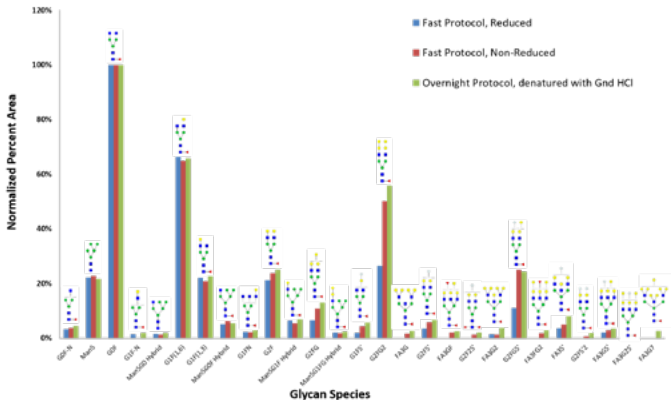


Figure 4. Comparison of Cetuximab Glycan Distribution Using three Glycan Release Protocols: ■ Fast (reduced), ■ Fast (non-reduced), and ■Traditional overnight protocol. A BIOshell™ Glycan Column was Used in all three Samples.

protocol when a reducing step using 2-mercaptoethanol was included, for example G1F5', G2FG2, FA3G, FA3GF, G2F2S', and G2FGS'. Indeed, the use of 2-mercaptoethanol in the denaturing step is not required for most proteins while using the fast protocol. But, there are proteins, like RNAse B, that seem to require it. It is recommended that as part of optimizing a method for released glycan analysis, the fast protocol must be tested with and without 2-mercaptoethanol to check for best results.

It is also worth noting that some proteins are not amenable to fast deglycosylation techniques. When working with mAbs without established protocols for glycan analysis, it is best to compare the results of the traditional overnight digest to the fast/rapid digestion protocol.[8]

Conclusion

Characterizing and monitoring the glycosylation pattern of a therapeutic mAb is required by regulatory authorities to ensure efficacy and safety of the drug. While analysis and identification of glycans can be challenging because of their structural complexity, this article has shown that a BIOshell™ Glycan HPLC column was able to elucidate the complex glycosylation of cetuximab after an appropriate glycan release and labeling protocol. Another key consideration in glycan analysis is the deglycosylation protocol. While there is a fast method that significantly saves time, it is recommended to compare the results with the traditional overnight digestion and choose the one that gives more efficient deglycosylation.

Featured Products

Description	Cat. No.
Glycan Release	
PNGase Fast Kit	**EMS0001-1KT**
30 kDa MWCO Centrifugal Filtration Units, 0.5 mL	**MRCF0R030**
Labeling	
Procainamide HCl	**SML2088**
Cleanup	
Discovery® Glycan SPE Tube 50mg / 1mL, Pk.108	**55465-U**
Visiprep™ SPE Vacuum Manifold DL (Disposable Liner), 12-port	**57044**
Acetonitrile, HPLC gradient grade or hypergrade for LC-MS LiChrosolv®	**34851 or 1.00029**
HPLC	
BIOshell™Glycan, 15 cm x 2.1 mm I.D., 2.7 µm	**50994-U**
Acetonitrile hypergrade for LC-MS LiChrosolv®	**1.00029**
Ammonium formate eluent additive for LC-MS, LiChropur™, ≥99.0%	**70221**
Formic acid 98% - 100% for LC-MS LiChropur™	**5.33002**

References

1. Lu, R.-M.; Hwang, Y.-C.; Liu, I-J.; Lee, C.-C.; Tsai, H.-Z.; Li, H.-J.; Wu, H.-C. Development of therapeutic antibodies for the treatment of diseases. J BiomED Sci 2020, 27, 1-30.
2. Wan, H. An overall comparison of small molecules and large biologics in ADME testing. ADMET & DMPK 2016, 4, 1-22.
3. Yang, X.; Bartlett, M. G. Glycan analysis for protein therapeutics. J Chromatography B 2019, 1120, 29-40.
4. Zhang, L.; Luo, S.; Zhang, B. Glycan analysis of therapeutic glycoproteins. MABS 2016, 8, 205-215.
5. Jones, A. N-Glycan Analysis of Biotherapeutic Proteins. BioPharm International 2017, 30, 20-25.
6. Giddens, J. P.; Lomino, J. V.; DiLillo, D. J.; Ravetch, J. V.; Wang, L.-X. Site-selective chemoenzymatic glycoengineering of Fab and Fc glycans of a therapeutic antibody. PNAS 2018, 115, 12023-12027.
7. Lie, S.; et. al. Comprehensive N-Glycan Profiling of Cetuximab Biosimilar Candidate by NP-HPLC and MALDI-MS. PLoS One 2017, 12, 1-14.
8. PNGase Fast Kit Technical Bulletin https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/2/ems0001bul.pdf

To see our complete offer for LC-MS, visit us at **SigmaAldrich.com/lcms**

Difluoroacetic Acid as an Effective Mobile Phase Modifier for the LC-UV/MS Analysis of Proteins

Maricar Dube, Analytical Sciences Liaison; Cory Muraco, Global Franchise Manager, Liquid Chromatography Technology; Daniel Weibel, Global Product Manager Solvents; Analytix@merckgroup.com

Abstract

Trifluoroacetic acid (TFA) is typically the mobile phase modifier used for protein analysis by reversed-phase high performance liquid chromatography (RP HPLC), especially when combined with UV detection. TFA improves retention, peak shape, and due to the latter the sensitivity. However, TFA also causes ion suppression in mass spectrometry (MS), thereby significantly reducing the sensitivity. Formic acid (FA) is generally the modifier of choice with MS detection as it provides efficient ionization. However, the use of formic acid results in less efficient chromatographic separation. Difluoroacetic acid (DFA) is a suitable alternative to TFA and FA in the LC-UV/MS analysis of proteins, allowing adequate separation efficiency when compared to FA, and better MS compatibility when compared to TFA.

Introduction

The growth of protein-based therapies and the field of proteomics have propelled technical advancements in the analysis of proteins. Characterization of these large and complex molecules utilize a range of techniques - chromatography being one of them. The analysis of intact proteins and protein digests by reversed-phase high performance liquid chromatography (RP-HPLC) provides researchers with valuable information not only about a protein's identity, but also the post-translational modifications that affect its properties.

During the analysis of proteins by RP-HPLC, trifluoroacetic acid (TFA) is added to the mobile phase to obtain sharp and symmetrical peaks with adequate retention,[1] particularly when used in conjunction with UV detection. The added TFA.

(1) lowers the pH of the mobile phase to well below the pK_a of side-chain carboxyls, thereby facilitating maximum retention of acidic moieties, and

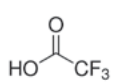
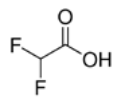
(2) acts as an effective ion-pairing agent[2] for the basic moieties of the protein. In the absence of ion pairing reagents, the residual silanols in the HPLC column interact with the protein analytes, causing band broadening, which in turn leads to reduced efficiency and sensitivity.

HPLC coupled to a mass spectrometer (specifically electrospray ionization (ESI)) has become a widely used technique in protein analysis. The mass spectrometer offers mass selectivity and higher sensitivity compared to UV, giving researchers more valuable information about analyzed protein(s). However, TFA is not compatible with MS; it suppresses signal intensity because of ion-pair formation, high conductivity and surface tension.[1] TFA also leaves background in the mass spectrometer that is difficult to remove.[3] This signal or ion suppression in turn compromises the sensitivity of the technique.

Because of TFA's incompatibility with LC-MS, investigators often use formic acid (FA) when conducting protein separations with MS detection.[4] Unfortunately, separations with FA as a modifier often result in poor peak shape and less efficient separations.[3] This result is because FA is a poorer ion-pairing agent and also a weaker acid compared to TFA.

This article explores the use of difluoroacetic acid (DFA) as an attractive acid modifier alternative in the LC-UV/MS analysis of proteins. DFA is a strong ion-pairing agent and provides the desired low pH (it has a lower pK_a compared to FA).[5] **Table 1** shows the structures and pK_a's of TFA, FA and DFA.

Table 1. Structure and pK_a of additives used in HPLC-MS

Additive	Trifluoroacetic acid	Formic acid	Difluoroacetic acid
Abbreviation	TFA	FA	DFA
Structure			
pKa	0.43	3.75	1.34

Experimental Conditions

The HPLC analyses were carried out using a Shimadzu Nexera LC system equipped with UV and MS detectors. The flow was split between detectors to minimize band broadening from the sample plug having to pass through multiple detectors in line. The conditions are listed in **Table 2**.

Table 2. HPLC conditions for the separation of proteins

Column:	BIOshell™ A400 Protein C4; 10 cm x 2.1 mm I.D., 3.4 µm (**67463-U**)
Column temp.:	50 °C
Mobile phase:	[A]: water with various acid modifiers (Table 3)
	[B]: acetonitrile (with various acid modifiers)
Flow rate:	0.35 mL/min
Gradient:	15 – 55% acetonitrile in 30 min
Injection:	10 µL
UV detection:	280 nm
MS parameter:	Single quad (ESI+); Acquisition range: 400 – 2000 m/z; Cone voltage: 3.8 kV
Cone voltage:	3.8 kV
Sample:	25 pmol each of the proteins in Table 4

Table 3. Acids used as modifier/additive

	Experiment 1	Experiment 2
Acid	**%**	**mM**
Trifluoroacetic acid (TFA)	0.1	10
Formic acid (FA)	0.25	10
Difluoroacetic acid (DFA)	0.1	10

Table 4. Proteins analyzed

Protein	Molecular Weight (kDa)
Ribonuclease (R)	13.7
Ubiquitin (U)	10.7
Lysozyme (L)	14.4
apo-Myoglobin (M)	17
Enolase (E)	46

Results and Discussion

The combination of high performance liquid chromatography with mass spectrometry (LC-MS) has become a critical tool in protein analysis. Traditionally, TFA is the mobile phase additive used as it provides adequate retention of proteins, and also gives sharp and symmetrical peaks with UV detection (**Figure 1A**). However, it is not recommended for MS detection as it suppresses the analyte signal (**Figure 1B**).

To get better MS signals, investigators use FA because it gives better ionization efficiency of the analytes (**Figure 2B**). Unfortunately, FA as a mobile phase modifier often results in poor peak shape and inefficient separations (**Figure 2A**).

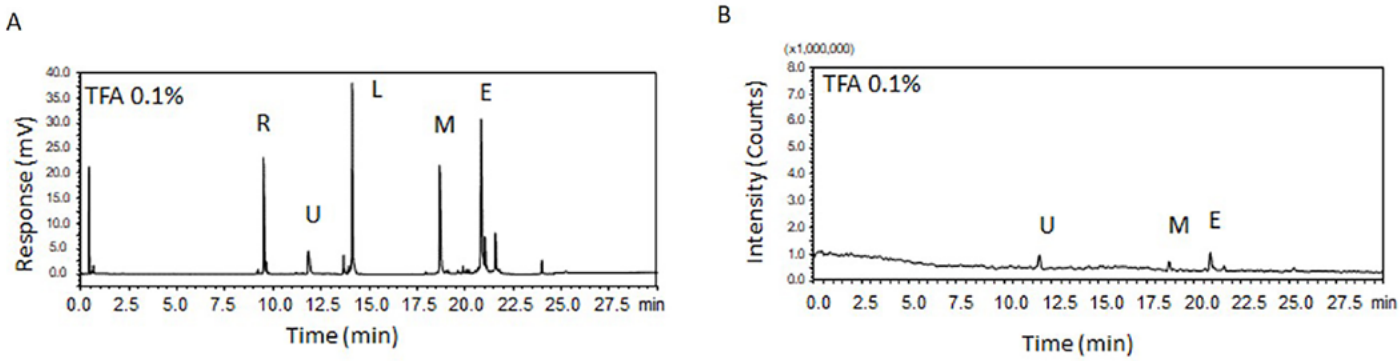


Figure 1. (A) UV and **(B)** MS chromatograms of five proteins when using TFA as modifier. R – Ribonuclease, U – Ubiquitin, L – Lysozyme, M – apo-Myoglobin, E – Enolase

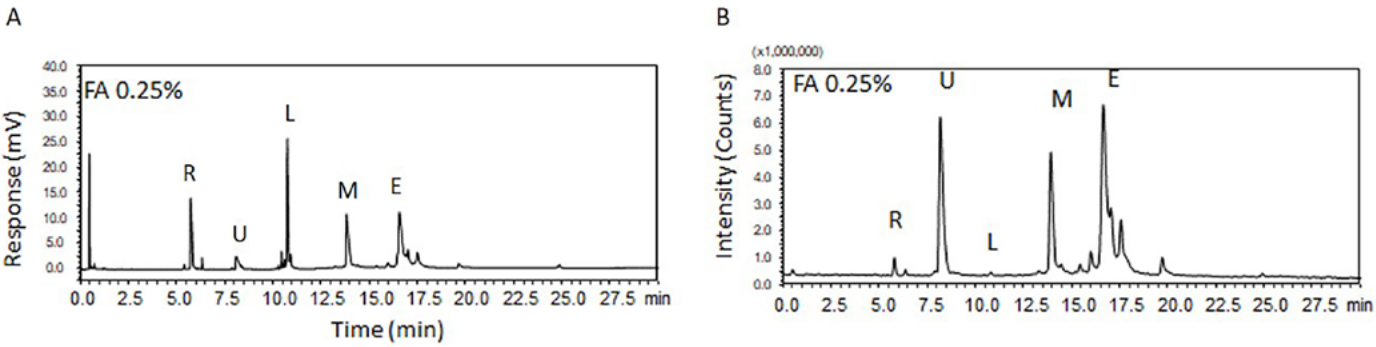


Figure 2. (A) UV and **(B)** MS chromatograms of five proteins with FA as modifier. R – Ribonuclease, U – Ubiquitin, L – Lysozyme, M – apo-Myoglobin, E – Enolase

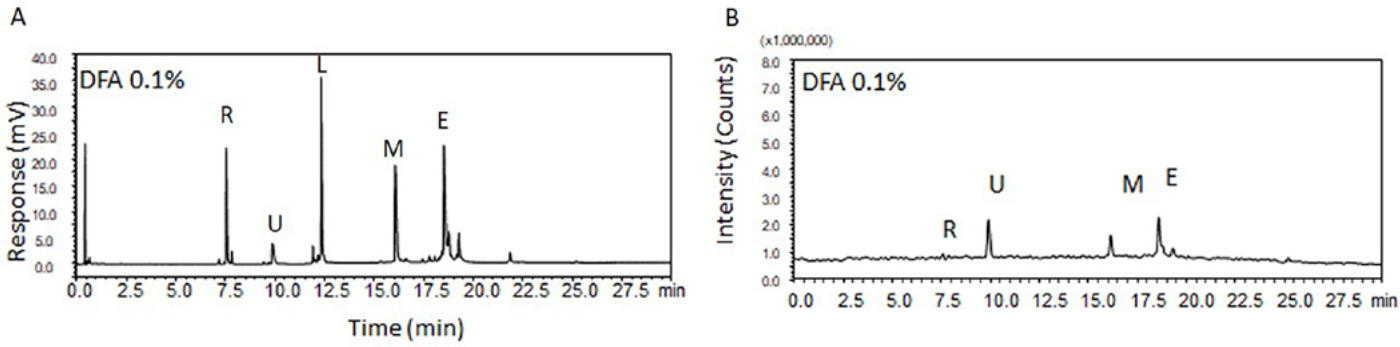


Figure 3. (A) UV and (B) MS chromatograms of five proteins with DFA as modifier. R – Ribonuclease, U – Ubiquitin, L – Lysozyme, M – apo-Myoglobin, E – Enolase

A combination of UV and MS detectors provides investigators with much richer information than just a UV detector, although using both the detectors in line will contribute to band broadening in the MS and must be considered. Using TFA to benefit UV detection does not provide any useful MS data. Using FA to benefit MS detection sacrifices separation efficiency. Therefore, there is a need to find a suitable, MS-friendly solvent additive for the HPLC analysis of proteins that will provide efficient separation without sacrificing on MS signal because of ion suppression. DFA is a viable candidate. This reagent is a stronger acid than FA and does not suppress ionization as much as TFA. **Figure 3** shows the UV and MS chromatograms of five proteins with DFA as the mobile phase modifier.

Comparing the UV chromatograms in **Figures 1A, 2A,** and **3A** illustrates the ion pairing strength of TFA, FA and DFA. TFA provides the strongest ion pairing strength, affording the strongest retention of the proteins, followed by DFA. FA is the poorest ion pairing reagent of the three.

To further explore how the different modifiers influence separation efficiency, a separate experiment was carried out where the concentrations of TFA, FA and DFA were kept the same at 10 mM. Peak widths at half height ($W_{1/2}$) were measured for each of the analyte peaks, as peak width at half height can be used as a measure of efficiency during gradient LC assays. Smaller values correspond to narrower peak width, indicating better efficiency. The results are shown in **Figure 4**.

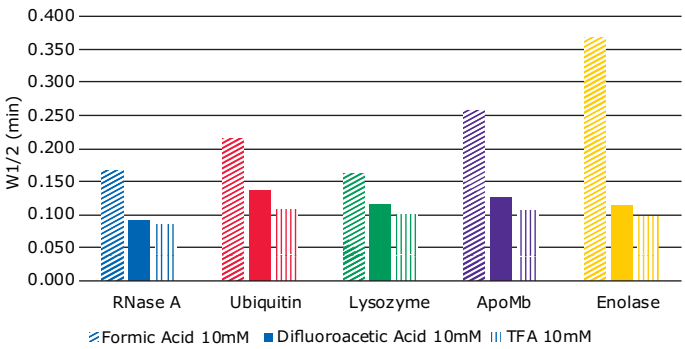


Figure 4. Peak width at half height (W1/2) of five proteins, using three different mobile phase modifiers – formic acid (FA), difluoroacetic acid (DFA), and trifluoroacetic acid (TFA)

TFA gave the narrowest peak widths, indicating better efficiency, followed closely by DFA. FA as the modifier gave the least efficient separation.

The influence of the three modifiers on the MS response can be seen by comparing **Figures 1B**, **2B**, and **3B**. Clearly, the addition of FA as modifier resulted in strong MS signals, but at the cost of separation efficiency. DFA gave better signals than TFA. This observation is also illustrated in **Figure 5A** where signals from each analyte for each of the modifiers are plotted against the total ion current (TIC). **Figure 5B** is an exploded view of just DFA and TFA MS signals, for better comparison.

As seen in **Figure 5B**, as well as **Figure 1B**, MS signals from TFA are strongly suppressed. In fact, the proteins RNase A and Lysozyme were not detected in MS. The use of DFA as modifier gave detectable MS signals as shown in **Figure 3B**.

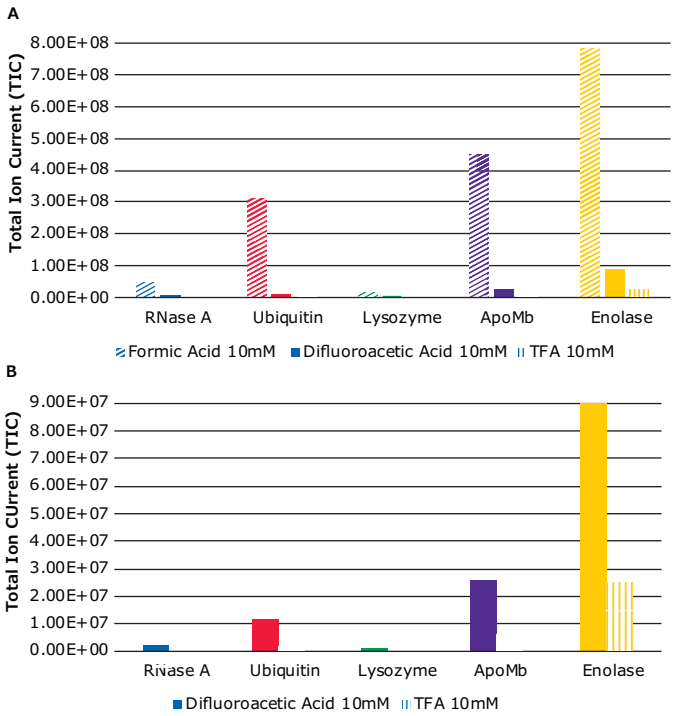


Figure 5. (A) MS signals when using three different modifiers – FA, DFA, and TFA. (B) Exploded view of MS signals when using DFA and TFA as modifiers.

Conclusion

In the HPLC analysis of proteins where combining UV and MS detectors is sometimes necessary, DFA is an attractive alternative to TFA and FA as mobile phase modifier. This reagent provides better separation efficiency than FA, and it does not cause strong ion suppression like TFA. However, DFA causes reduced MS ion yield compared to FA and slightly broader peaks than obtained with TFA. Therefore, users need to do an assessment regarding a possible and acceptable compromise for their application.

References

1. Bobály B, Beck A, Fekete J, Guillarme D, Fekete S. Systematic evaluation of mobile phase additives for the LC-MS characterization of therapeutic proteins. Talanta. 2015, 136:60-7. doi: 10.1016/j.talanta.2014.12.006
2. Corradini D, Kalghatgi K, Horváth C. Effect of mobile phase additives on peptide retention in reversed-phase chromatography with pellicular and totally porous sorbents. J Chromatogr A. 1996, 728(1-2):225-33. doi: 10.1016/0021-9673(95)01019-x
3. Wagner, B.M..; et. al. Tools to Improve Protein Separations, LCGC, 2015, 33, 856-865
4. Bobály B, D'Atri V, Lauber M, Beck A, Guillarme D, Fekete S. Characterizing various monoclonal antibodies with milder reversed phase chromatography conditions. J Chromatogr B Analyt Technol Biomed Life Sci. 2018, 1096:1-10. doi: 10.1016/j.jchromb.2018.07.039
5. Yamamoto E, Ishihama Y, Asakawa N. Application of partially fluorinated carboxylic acids as ion-pairing reagents in LC/ESI-MS. Talanta. 2014, 127:219-24. doi: 10.1016/j.talanta.2014.03.060.

Featured Products

Product	Cat. No.
BIOshell™ A400 Protein C4, 10 cm x 2.1 mm I.D., 3.4 µm	**66825-U**
Acetonitrile, hypergrade for LC-MS LiChrosolv®	**1.00029**
Water, for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**
Difluoroacetic acid (DFA), for LC-MS LiChropur™, ≥97.5% (GC)	**00922**
Trifluoroacetic acid (TFA), eluent additive for LC-MS, LiChropur™, ≥99.0% (GC)	**80457**
Formic acid, for LC-MS LiChropur™	**5.33002**

Learn more about our HPLC column portfolio at **SigmaAldrich.com/hplc**

See a comprensive overview on LC-MS workflow tools at **SigmaAldrich.com/lcms**

An optimized Protocol for Peptide Mapping of Therapeutic Monoclonal Antibodies with Minimum Deamidation and Oxidation Artifacts

Pegah Jalili, Principal Scientist, Analytical R&D
Kevin Ray, PhD, Senior Manager, Analytical R&D
Jeffrey Turner, PhD, Head of Protein Assay Development
Maricar Dube, PhD, Analytical Sciences Liaison, Analytix@merckgroup.com

Abstract

Post-translation modifications (PTM), such as oxidation and deamidation, can have serious consequences on therapeutic monoclonal antibodies. Peptide mapping is a widely used method for the identification of site-specific PTMs, but typical protein digestion workflows often end up introducing significant amounts of artifacts. Hence, to obtain an accurate assessment of the modifications, it is critical to reduce the artifacts that occur during sample preparation steps. This study used NISTmAb as a model monoclonal antibody to demonstrate an optimized peptide mapping protocol resulting in minimal artificial asparagine deamidation and methionine oxidation. The protocol utilizes shorter incubation times and an improved digestion buffer, allowing for complete sample preparation in less than six hours.

Introduction

The development, production, and storage of therapeutic mAbs must be monitored for post-translational modifications (PTMs), to assure consistent quality and safety. PTMs such as deamidation and oxidation are known to influence the efficacy, safety, and stability of therapeutic monoclonal antibodies (mAb).[1,2] Deamidation of asparagine (ASN or D) and the oxidation of methionine (Met or M) are major chemical degradation pathways for protein therapeutics and have been studied extensively.[3,4,5] Asparagine residues can form a succinimide intermediate that subsequently hydrolyzes into isoaspartic or aspartic acid (**Figure 1A**).[6,7] Whereas, hydroxyl radicals can oxidize methionine residues to form methionine sulfoxide (**Figure 1B**).[8,9]

LC-MS based peptide mapping is the method of choice for measuring the relative abundance of PTMs. The sample preparation prior to the LC-MS analysis involves three steps of denaturation/reduction, alkylation, and digestion. The digestion of different mAbs produces different peptide fragments having a wide range of sizes — from single amino acids to longer polypeptides. Since these peptides vary widely in their hydrophobicity, reversed-phase (C18) is the preferred mode of chromatography for peptide mapping.

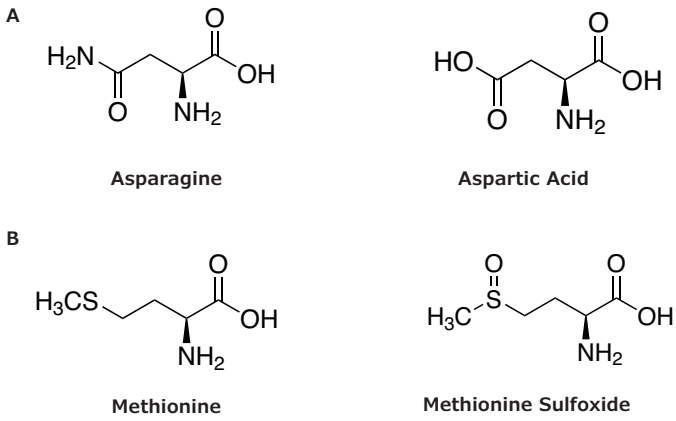


Figure 1. (A) Asparagine residues can undergo deamidation to form aspartic acid, and (B) methionine can undergo oxidation to form methionine sulfoxide.

The conventional trypsin digestion of monoclonal antibodies is lengthy, involving an overnight digestion step. The conditions and reagents used in this step are known to induce artifactual deamidation and oxidation of the mAb sample, leading to inaccurate measurement of PTMs.[10] The first part of this paper compares methionine and asparagine deamidation between conventional trypsin digestion and an optimized digestion protocol that takes less than six hours to complete. The second part compares the optimized protocol with the protocol published by NIST. All LC-MS analyses were carried out using C18 columns with superficially porous particles (BIOshell™ A160 Peptide C18).

Experimental

Sample: NIST Monoclonal Antibody Reference Material 8671 (NISTmAb)

Digestion: **Figure 2** outlines the digestion protocols.

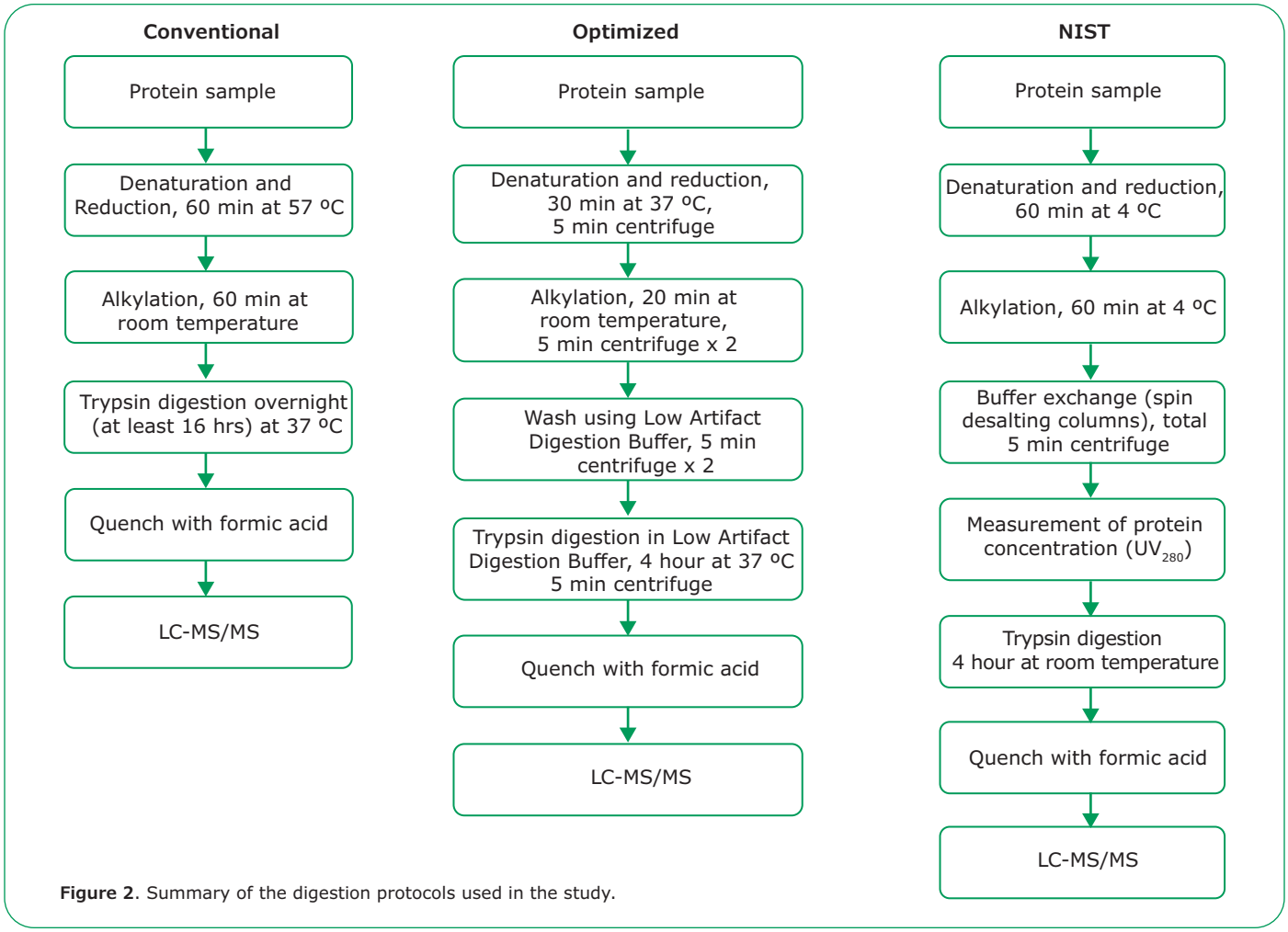


Figure 2. Summary of the digestion protocols used in the study.

The conventional protocol uses sodium deoxycholate (60 mg) in methanol (1 mL) as the denaturation solution. 20 mM TCEP (tris(2-carboxyethyl)phosphine) and the denaturation solution were mixed in 1:1 (v/v), to which 20 µL of dried down sample was added and incubated at 57 ºC for one hour. The sample was brought back to room temperature and centrifuged at 14,000 x g per gram for 30 seconds. This was followed by the alkylation step using 5 µL of 200 mM iodoacetamide (in 50 mM ammonium bicarbonate) and subsequent incubation for one hour in the dark at room temperature. Digestion was carried out by adding enough trypsin solution (trypsin in 50 mM ammonium bicarbonate) to have an enzyme:protein ratio of 1:20 and incubating at 37 ºC overnight (at least 16 hours) on a thermo-shaker. 2 µL neat formic acid was used to quench the digestion.

Detailed procedure for the optimized protocol is described in the technical bulletin for Low Artifact Digestion Buffer.[11] A NIST[12] paper describes the protocol provided by NIST. The reagents used in each protocol are shown in **Table 1**.

Table 1. Reagents used in the protocols of the study.

Reagent	Conventional	Optimized	NIST
Denaturing solution/buffer	Sodium deoxycholate	Urea	Guanidine HCl
Reduction	TCEP	TCEP	DTT
Alkylation	Iodoacetamide	Iodoacetamide	Iodoacetamide
Digestion buffer	Ammonium bicarbonate	Low Artifact Digestion Buffer	Urea
Trypsin	SOLu-Trypsin	SOLu-Trypsin	Recombinant, proteomics grade, expressed in Pichia pastoris

LC-MS Conditions HPLC:

HPLC Conditions	
Instrument:	Waters Acquity UPLC
Column:	BIOshell™ A160 Peptide C18, 15 cm x 1.0 mm, 2.7 µm particles (**67099-U**), two columns in series
Mobile phase	[A] 0.1% formic acid in water [B] 0.1% formic acid in acetonitrile
Gradient:	**Time (min) / %B** 0 / 1 120 / 35 121 / 97 136 / 97 137 / 1 162 / 1
Flow rate:	80 µL/min
Column temp.:	room temperature
Injection:	10 µL (3-4 µg MAb digest)

Mass Spectrometry:

MS Conditions	
Instrument:	Thermo QE Plus
Polarity:	Positive
Spray voltage:	4.0 kV
Capillary temp:	320 ºC
Sheath gas:	10
Aux gas:	5
S-Lens:	50 V
m/z range:	300-4000

Data analysis

The raw MS files were subjected to BioPharma Finder™ 3.0 (Thermo Fisher Scientific) for peptide mapping. The peptide identifications were performed by searching the processed data against the NISTmAb sequence-based accurate mass of a full mass scan and assignments of product ions in MS/MS spectra. The data was filtered to report only the peptides with a mass tolerance of ±10 ppm. The % deamidation and oxidation were calculated by BioPharma Finder software using the mapping tab. Also, the result was manually checked by creating the extracted ion chromatograms (XICs) for unmodified and modified peptide within 10 ppm mass error. Equations 1 and 2 were used to calculate the % modification (oxidation, deamidation) and % missed cleavage (% MC), respectively.

Equation 1:

$$\% \text{ Modification} = \frac{\text{area under the peak of XIC of modified peptide}}{\text{area under the peak of XIC of modified peptide + area under the peak of XIC of un-modified peptide}} \times 100$$

Equation 2:

$$\% \text{ MC} = \frac{\text{area under the peak of XIC of MC peptide}}{\text{area under the peak of XIC of standard peptide + area under the peak of XIC of MC peptide}} \times 100$$

Results and Discussions

Peptide mapping using LC-MS has become a routine analysis in the development and manufacture of therapeutic mAbs. Traditional sample preparation procedures used prior to LC-MS are often cumbersome. These procedures generally involve chemical denaturation, reduction and alkylation, buffer exchange, and overnight protease digestion of the protein sample at elevated pH and temperature. Asparagine deamidation and methionine oxidation take place during these various steps, the extent of which depends on the conditions such as reagents used, ionic strength, temperature, pH, incubation time, digestion buffer, and presence of trace metals (in the case of methionine oxidation).[7] A simpler, shorter method with minimal artifacts is certainly desired to obtain accurate endogenous levels of deamidation and oxidation.

Figure 3A is the base peak chromatogram of tryptic digested NISTmAb, showing examples of typical tryptic peptides used to measure the levels of Met (M) oxidation and Asn (D) deamination in this work. **Figure 3B** is the extracted ion chromatogram and MS spectrum of the peptide DTLMISR (t_R = 51 min) and the peptide with an oxidized methionine residue (position HC:M255), t_R = 44 min. The oxidation of methionine rendered the molecule less hydrophobic, thus less retentive on the BIOshell™ A160 Peptide C18 column. **Figure 3C** is the XIC and MS spectrum of the peptide GFYPSDIAVEWESNGQPENNYK (t_R 88.90 min) and the deamidated peptide (position HC:N387). The deamidated forms (isoASP and ASP) elute before and after the unmodified peak at ~87.91 and 91.92 min, respectively.

Optimized versus Conventional Protocol

The optimized and conventional protocols use the same reduction (TCEP) and alkylation (iodoacetamide) reagents, but they differ in the denaturing solution used, incubation times, and temperature (see **Figure 2** and **Table 1**). The conventional protocol has much longer incubation times and uses higher temperature for the denaturation/reduction step. The digestion step happened overnight with the conventional protocol at a higher pH of 8.5

With the optimized protocol, digestion took only four hours. The digestion buffer used was specifically developed to minimize deamidation and oxidation during the digestion step without sacrificing the digestion efficiency. The buffer was formulated at an optimal pH and contained a proprietary antioxidant.

In both protocols, the protease used was SOLu-Trypsin, a proprietary formulation of recombinant Trypsin (porcine sequence expressed in *Pichia pastori*) and stable in solution when refrigerated.

The deamidation levels between the protocols were extremely different at the two sites (**Figure 4A**). The biggest difference was observed at site HC:N387, where deamidation was 41.1% for the conventional

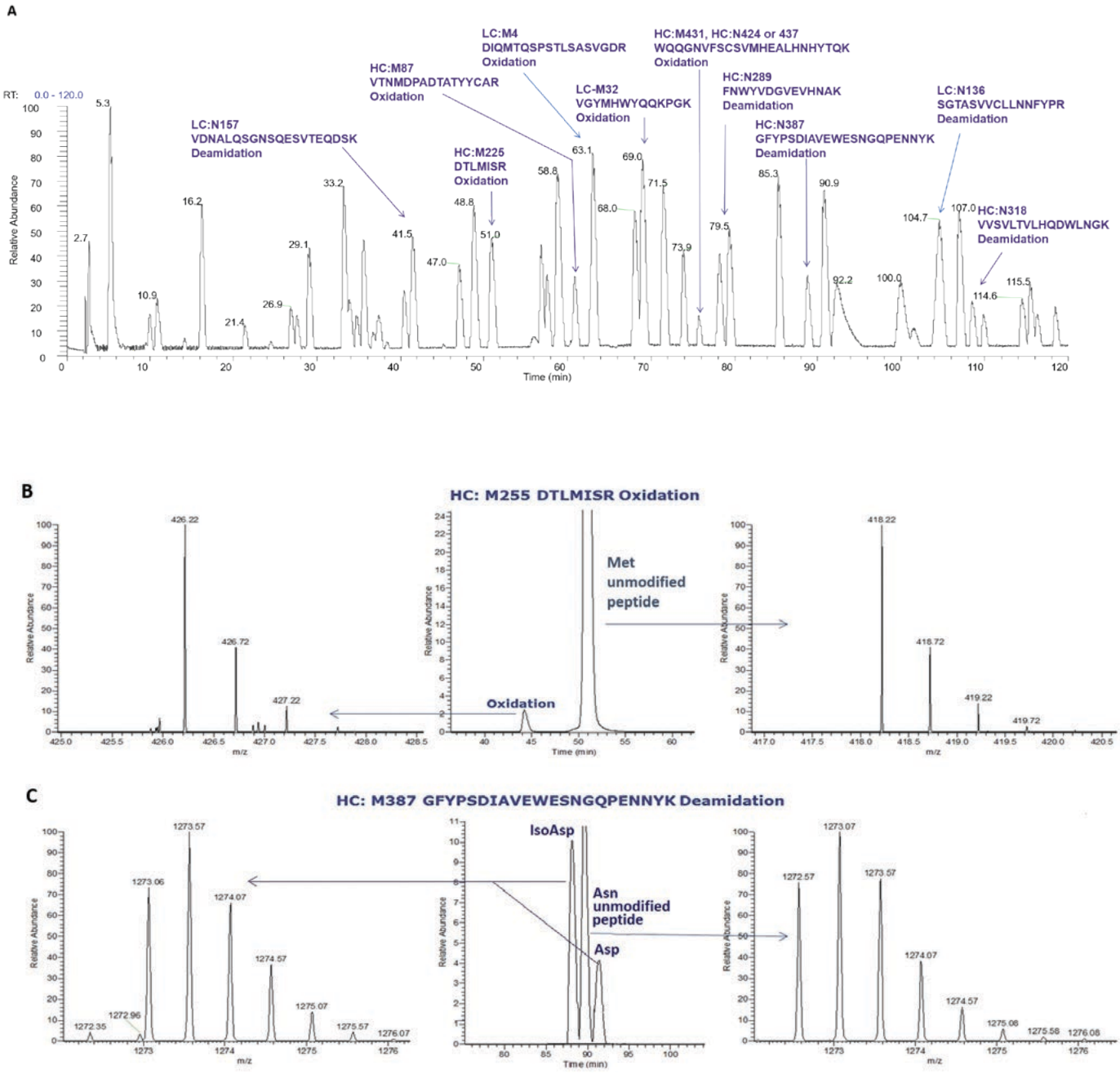


Figure 3. Analysis of tryptic digested NISTmAb (A) Base peak chromatogram of tryptic digested NISTmAb. Labeled peptides are examples of typical tryptic peptides used for determining Met oxidation and Asn deamination levels. (B) Extracted ion chromatogram and spectra of unmodified DTLMISR peptide and the peptide with oxidized Met. (C) Extracted ion chromatogram and spectra of unmodified GFYPSDIAVEWESNGQPENNYK peptide and the peptide with deamidated Asn.

protocol and 0.6% for the optimized one. At site HC:N318, a 21.2% deamidation was observed for the conventional protocol and none was observed for the optimized protocol. These results are not surprising. It is well known that the incubation times of protein samples in the denaturing/reduction and alkylation steps, and to a larger extent, the length of digestion, are directly proportional to the levels of artificial modification.[13] It has been reported that deamidation artifacts are reduced at lower temperatures;[14] in the conventional protocol, the denaturation/reduction step was carried out at an elevated temperature (57 ºC).

The difference in % oxidation was not as high as observed for deamidation. At site LC:M32, the conventional protocol had 2.9% higher oxidation than the optimized protocol, and it was 4.2% higher at site HC:M255 (**Figure 4B**).

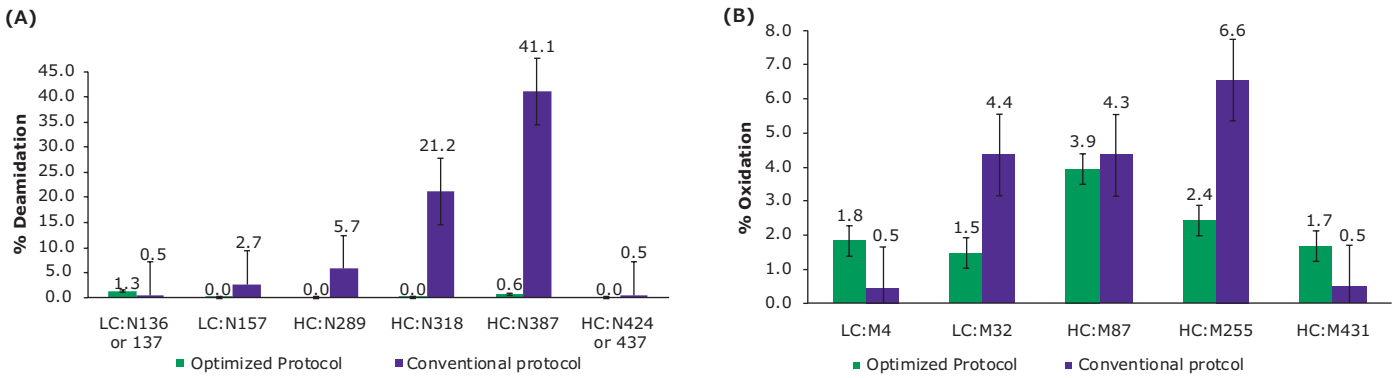


Figure 4. Levels of (A) Asn deamidation and (B) Met oxidation of NISTmAb tryptic peptides using the conventional and optimized protocols.

Optimized Protocol versus NIST Protocol

In 2018, NIST published a paper wherein they described the development of a tryptic digestion protocol used for peptide mapping. Their study focused on parameters such as buffer concentration, digestion time and temperature, and the source and type of trypsin used.[12] (See **Figure 2** for the outline of the protocol and **Table 1** for the reagents used.)

The denaturation/reduction and alkylation steps were carried out at a very conservative temperature (4 ºC) with incubation times much longer than the one for the optimized protocol. In addition, the protocol required a buffer exchange step (into the urea containing digestion buffer) before the tryptic digestion. Overall, the NIST protocol requires more time for reagent and sample preparation compared to the optimized protocol.

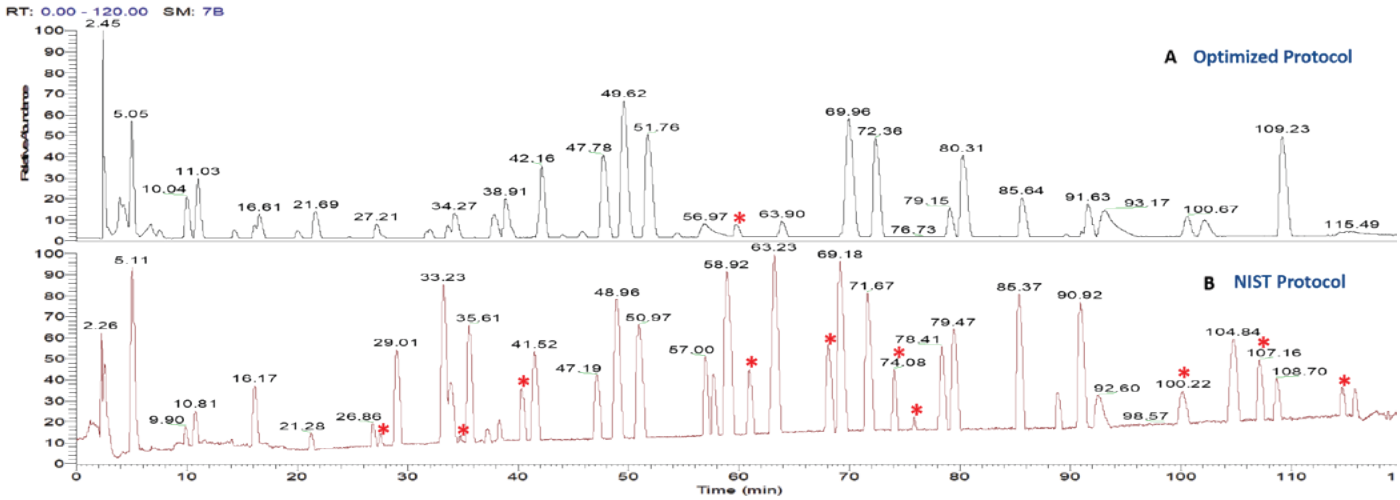


Figure 5. Comparison of base peak chromatograms of tryptic digested NISTmAb using the (A) optimized and (B) NIST protocols. Missed cleavage peptides are labeled with a red asterisk (*).

Figure 5 compares the base peak chromatograms of the digested NISTmAbs using the optimized protocol and the NIST protocol. The profile for the optimized digestion protocol is less complex. The chromatogram from the NIST protocol exhibited many extra peaks which were identified as missed cleavage peptides. The average percent missed cleavage was 16% for the optimized protocol and 35% for the NIST protocol (**Figure 6**).

In terms of Met oxidation and Asn deamidation, the performance of the two protocols is similar. The level of Met oxidation for both the methods is <5% and for deamidation <1.7% (**Figure 7**).

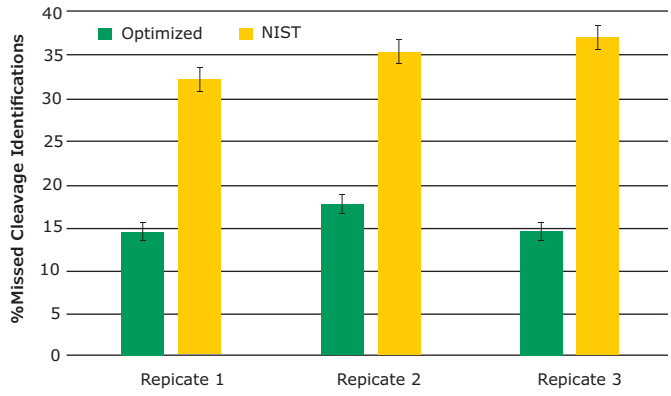


Figure 6. Average percent missed cleavage for the optimized and NIST protocols.

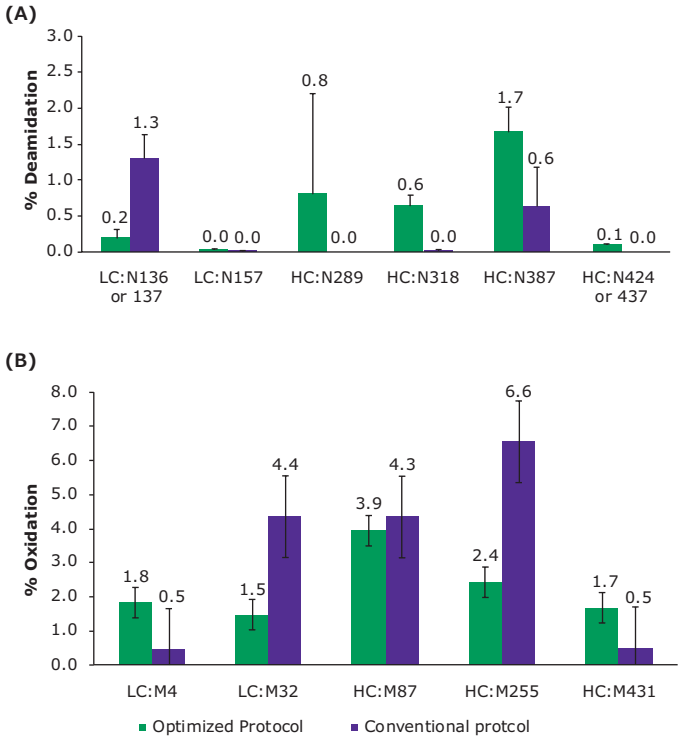


Figure 7. Levels of (A) Asn deamidation and (B) Met oxidation of NISTmAb tryptic peptides using optimized protocol and NIST protocol.

It is important to note that the HPLC conditions are also critical for the study. To enhance resolution and sequence coverage, two BIOshell™ A160 Peptide C18 15 cm x 1.0 mm columns arranged in series were used to provide for a total effective column length of 30 cm. Hydrophilic peptides with less than five amino acids such as VDK, TISK, EYK that typically elute in flow-through, could be retained on the longer column and be subsequently analyzed by mass spectrometry. In addition, the BIOshell™ columns are composed of superficially porous particles (SPPs) containing a solid, nonporous silica core with a porous silica outer layer, providing higher separation efficiency. This particle attribute results in a narrower peak width and improved resolution of the peptide analytes. Together with the optimized gradient conditions shown in the experimental section, a good separation of the unmodified and modified peptides was achieved. This result is well illustrated in **Figure 3B**, where the unmodified DTLMISR peptide and the peptide with oxidized Met were well resolved, allowing for accurate quantitation of each species.

Conclusions

The optimized protocol gave significantly lower levels of Asn deamidation compared to the conventional protocol, particularly at two sites, HC:N387 (over 40% lower) and HC:N318 (over 20% lower). The levels of oxidation (<5%) and deamidation (1.5 %) were comparable with the NIST protocol. In addition, more missed cleavage peptides were observed with the NIST protocol (35%) compared to the optimized protocol (16%). The optimized protocol also offers the advantage of allowing complete digestion in less than 6 hours, with minimal deamidation and oxidation artifacts. The use of two BIOshell™ A160 Peptide C18 (15 cm) columns in series allowed the successful separation of peptides in the tryptic digestion.

Featured Products

Description	Cat.No.
HPLC	
BIOshell™ A160 Peptide C18, 15cm x 1.0 mm, 2.7 µm	**67099-U**
Acetonitrile with 0.1% (v/v) Formic acid, hypergrade for LC-MS LiChrosolv®	**1.59002**
Water with 0.1% (v/v) Formic acid, hypergrade for LC-MS LiChrosolv®	**1.59013**
Formic acid 98% - 100%, for LC-MS LiChropur™	**5.33002**
Standards, Reagents, and Accessories	
NISTmAb, Humanized IgG1k Monoclonal Antibody	**NIST8671**
Low-Artifact Digestion Buffer	**EMS0011**
SOLu-Trypsin	**EMS0004**
Microcon-30kDa Centrifugal Filter Unit with Ultracel-30 membrane	**MRCF0R030**

REFERENCES

1. X. Xu, Y. Huang, H. Pan, R. Molden, H. Qiu, T. J. Daly, and N. Li. PLOS ONE. https://doi.org/10.1371/journal.pone.0223899
2. Y. Wang, X. Li, Y. -H. Liu, D. Richardson, H. Li, M. Shameem, and X. Yang. MAbs. 8, 1477–1486 (2016).
3. I. Schmid, I., Bonnington, L., Gerl, M. et al. Commun Biol. 1, (2018). https://doi.org/10.1038/s42003-018-0032-8.
4. A. L. Pace, R. L. Wong, Y. T. Zhang, Y.-Hs. Kao, Y. J. Wang, Journal of Pharmaceutical Sciences. 102, 1712-1723 (2013).
5. M. E. Alam, et al. Journal of Pharmaceutical Sciences, 109, 656-669 (2020.
6. S. Liu, K. R. Moulton, J. R. Auclair, and Z. S. Zhou. Amino Acids. 48, 1059–1067 (2016).
7. V. Timm, P. Gruber, M. Wasiliu, H. Lindhofer, and D. Chelius. J Chrom B 878, 777-784 (2010).
8. K. Sankar, K. H. et. al. MAbs. 10, 1281–1290 (2018).
9. X. M. Lam, J. Y. Yang, and J. L. Cleland. Journal of Pharmaceutical Sciences, 86, 1250-1255 (1997).
10. D. Wang, C. Nowak, B. Mason, A. Katiyar, and H. Liu. Journal of Pharmaceutical and Biomedical Analysis, 183, 113-131 (2020).
11. Low Artifact Digestion Buffer Technical Bulletin https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/2/ems0011bul.pdf
12. T. Mouchahoir and J. E. Schiel. Analytical and Bioanalytical Chemistry 410, 2111–2126 (2018)
13. D. Ren, et. al. Anal. Biochem. 392, 12-21 (2009).
14. S. D. Stroop. Rapid Commun. Mass Spectrom. 21, 830-836 (2007).

Find more about BIOshell™ U/HPLC columns at **SigmaAldrich.com/hplc**

LARGE MOLECULES

Three Methods for Critical Quality Attribute Determination of Monoclonal Antibodies

Geoffrey Rule, Principal Scientist; Romana Rigger, Senior Scientist; Pegah Jalili, Principal Scientist; Uma Sreenivasan, Director; Kevin Ray, Senior Manager; Stephan Altmaier, Senior Manager; Analytix@merckgroup.com

Abstract

Three step-by-step protocols for the monitoring of critical quality attributes (CQAs) of monoclonal antibodies were developed using adalimumab as an example. Monoclonal antibodies are glycoproteins used as drugs in the treatment of a variety of cancers and immune system disorders. These large biomolecules have complex structures making thorough characterization essential to ensure the safety and efficacy of drugs. Here, we provide an overview of the different workflows that can be used in these molcules intact and reduced mass analysis, primary structure determination (i.e. peptide map), and N-linked glycan analysis to enable an accurate characterization of their CQAs.

Introduction

Increasing importance of monoclonal antibody (mAb) therapeutics has fundamentally changed the pharmaceutical market in recent years. These macromolecules are used in cancer therapies and in combating autoimmune and other diseases. Biomolecules are produced using recombinant techniques in mammalian cell lines, expressing the mAbs in large bioreactors. mAbs are glycoproteins with a molecular weight of approximately 150 kDa. Antibodies are composed of two light chains and two heavy chains (LC and HC, respectively) linked to one another through covalent inter- and intra-chain disulfide bonds. The individual chains are composed of amino acids and can be post-translationally modified (PTM). The different modifications include glycosylation, phosphorylation, methylation, oxidation, and nitrosylation.

A reliable characterization of mAbs is essential to ensure the safety and efficacy of both the innovator and biosimilar drugs.[1] Therefore, the critical quality attributes (CQAs) are defined for each protein along with their acceptable ranges. For example, the extent of deamidation, pyroglutamination, oxidation, or formation of lysine variants in mAbs is quantified in relation to their respective native forms.[2] Common analytical techniques employed to verify CQAs being within the set limits include capillary zone electrophoresis, isoelectric focusing, ion exchange, size exclusion (SEC), reversed phase (RP) or hydrophilic interaction liquid chromatography (HILIC), coupled with ultraviolet (UV), fluorescence, or mass spectrometric (MS) detection.[3,4] Strategies used to aid the analysis include chemical or enzymatic fragmentation of the protein into subunits,[5] removal and analysis of glycans, and proteolytic digestion of the protein into smaller peptides followed by their sequencing. A combination of these techniques can also be applied. For the analysis of mAbs from cell culture supernatants, an affinity purification step is often used to prepare samples prior to their analysis.[6]

Recently, we prepared step-by-step workflows for three approaches commonly used in mAb characterization.[7-9] These approaches determine:

1. the mass of the intact molecule and of the heavy and light chains (intact and middle-up mass analysis),
2. the amino acid sequence (peptide mapping), and
3. the glycosylation state (glycan analysis).

Intact mass analysis relates to the measurement of the mass of an intact mAb without its dissociation into subunits. Middle-up experiments are performed after cleaving the mAbs into several large fragments, or subunits, via chemical reduction or proteolytic digestion. This can provide information on the sites of modification.

In peptide mapping, the protein is cleaved into fragments with a protease enzyme, most commonly trypsin. Prior to the enzymatic digestion, it is common to denature the protein to expose internal amino acids. As in middle-up analysis, the protein may be treated chemically to break disulfide bonds linking different portions of the protein. This reduction step is typically followed by alkylation of the exposed free thiols to prevent reformation of disulfide bonds. Minimizing the introduction of modifications to individual amino acids during the analytical stages of peptide mapping is also important. For example, under appropriate conditions, asparagine can be deamidated and methionine can be oxidized, both of which alter the mass of the peptide.

Glycan analysis is a third means of characterizing CQAs of mAb therapeutics. Glycans are polysaccharide chains that are attached to proteins (glycoprotein) or lipids (glycolipid). They exist as an array of different sugar units attached through different linkages and sometimes existing in complex di-, tri-, and tetra-

antennary structures. Glycans are typically described as "N-linked," when attached at the side chain nitrogen of asparagine, or "O-linked", when linked to the side chain oxygen of serine or threonine. Glycan profiles can affect the stability and bioavailability of mAb therapeutics and can vary with manufacturing conditions. Characterization of glycans is essential to ensure the equivalence of newly released mAb lots to the approved drug.

Glycan analysis is achieved by releasing glycans from the protein by using chemical or enzymatic methods followed by derivatization and fluorescence or mass spectral analysis. Common derivatization agents include 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), and procainamide. The latter has properties suited to both fluorescence and MS detection. Fluorescent derivatization allows relative quantification of glycan species while mass spectral analysis provides structural confirmation.

Figure 1 illustrates how the three approaches fit into the overall characterization of a mAb, along with a technique for purification of mAbs when necessary. Next, we look at each of the three workflows in a bit more detail.

Intact Mass Analysis

Non-reducing (intact) and reducing (middle-up) SEC-MS workflows for mAb analysis provide a high-level characterization of the protein structure and subunits.[10] Intact analysis yields mass measurement of the entire mAb, without dissociation of the subunits. Use of high resolution, high mass accuracy mass spectrometers to verify molecular weight can be utilized to reveal information about stoichiometry and proteoforms that may exist. Multiple charge state spectra are deconvoluted to allow correlation of observed masses with expected or theoretical masses.

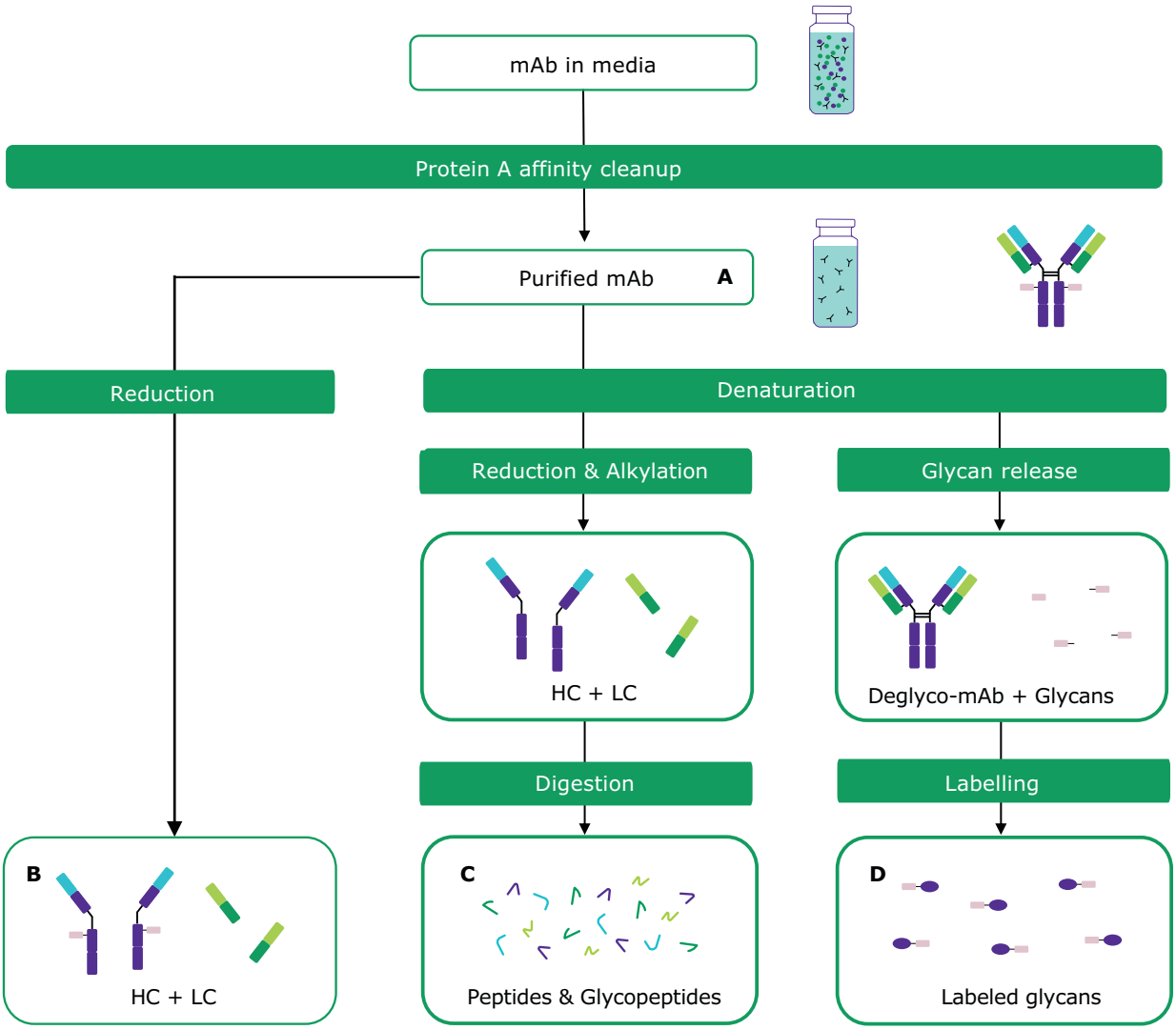


Figure 1. Overview of analytical techniques for antibody sample preparation and analysis showcased in this work. **A:** Intact mass analysis. **B:** Middle-up analysis of mAb after reduction. **C:** Peptide mapping after denaturation, reduction, alkylation, and tryptic digestion. **D:** Glycan analysis after denaturation, glycan release and labelling with procainamide.

In SEC, analytes enter, or are excluded from, the pores of the chromatographic support based on size. In a mixture of mAb, mAb aggregates, and antibody fragments, large aggregates elute first, followed by the antibody and then the fragments. In a middle-up analysis, HCs can be separated from LCs in this same fashion to identify the glycosylation sites on one or the other chains.

A photodiode array (280 nm) trace, total ion chromatogram, MS spectrum, and deconvoluted MS spectrum of adalimumab are shown in **Figures 2** and **3** (see conditions in **Table 1**). Observed masses of the non-reduced mAb are found to correlate well with the theoretical masses and observed mass error was found to be 0.010% or less. Deconvolution of the spectrum revealed several different glycoforms.

Table 1. SEC method conditions for intact mass analysis

SEC Method Conditions	
Column:	Tosoh TSKgel® SW3000XL, 30 cm x 2.0 mm I.D., 4 µm (**821485**)
Mobile phase:	Acetonitrile/water 30/70 (v/v) containing 0.1% trifluoroacetic acid (TFA) (v/v)
Isocratic elution:	0.1 mL/min
Column temp.:	Ambient
Detection:	UV 280 nm and MS
Injection:	20 µL

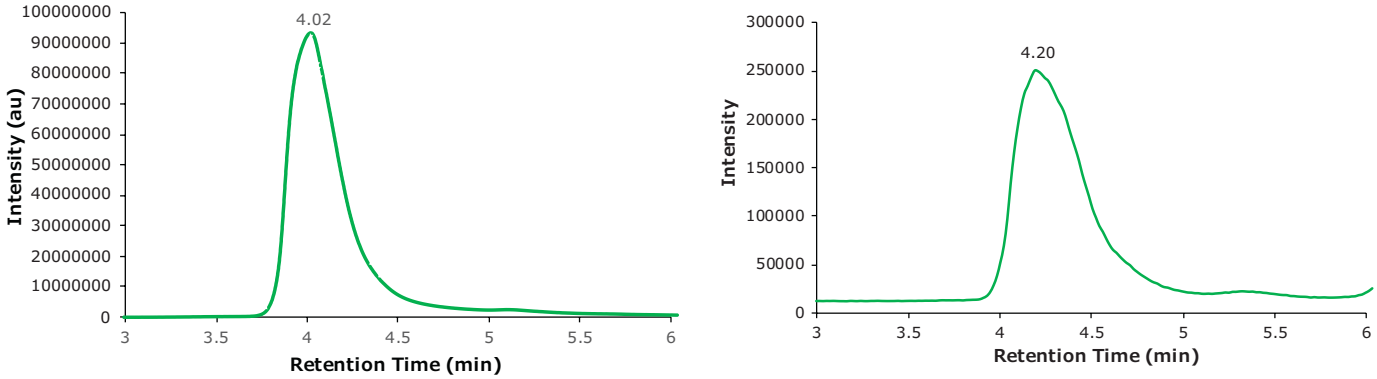


Figure 2. UV photodiode array (280 nm, left) and TIC traces (right) of intact adalimumab.

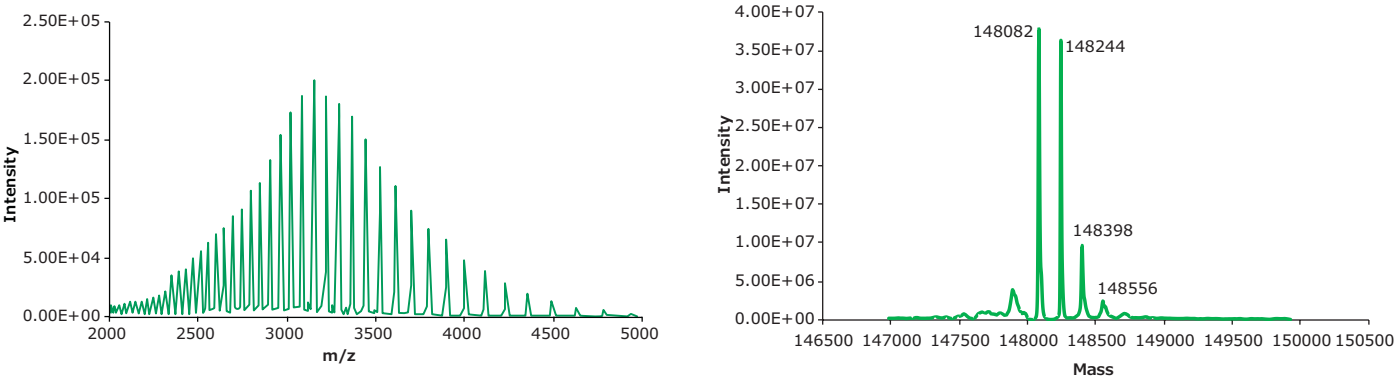


Figure 3. MS spectra for intact adalimumab. Left: summed spectrum; Right: deconvoluted spectrum showing several different glycoforms. (MS spectra generated on a QTOF instrument)

Peptide Mapping

Peptide mapping attempts to characterize the exact sequence of amino acids in a protein along with any modifications to those amino acids. In this work, sample proteins were digested with trypsin in a low-artifact digestion buffer[11] and utilizing filter-assisted sample preparation (FASP).[12] The latter makes use of a molecular weight cut-off filter, in microcentrifuge tube format, to separate peptide fragments from whole protein. The low-artifact digestion buffer minimizes amino acid modification through the digestion process to provide a more accurate depiction of the protein being produced.

The digest is injected onto an HPLC column capable of resolving as many of the peptide fragments as possible, including those bearing glycans, prior to MS detection. HPLC columns based on superficially porous particles (SPP) like Ascentis® Express and BIOshell™ columns are suitable to obtain high efficiencies and resolution. **Figure 4** shows the chromatographic profile obtained for the model mAb, adalimumab while **Table 2** displays the method conditions. Coupling two 15 cm columns provided greater separation of peptides across the run as well as slightly better retention of hydrophilic peptides.

The depth of information provided by peptide mapping has led to the development of multi-attribute methods (MAM) that are used to ensure quality and consistency of protein therapeutics with a single method.[13] With MAMs, a list of PQAs (product quality attributes) is generated from an in-depth characterization of the protein-of-interest using high-resolution, high-mass accuracy MS/MS, and peptide mapping experiments. These analyses include characterization of PTMs, particularly glycosylation. Results of the in-depth characterization are used to create a library of features expected from the therapeutic products during their manufacture. A simpler, MS only instrument

Table 2: Peptide mapping method conditions

Columns:	Two coupled Ascentis® Express Peptide ES-C18 columns, 15 cm x 1.0 mm, I.D. 2.7 µm (**53561-U**)
Mobile phase:	[A] 0.1% formic acid (FA) in water (v/v); [B] 0.1% FA in acetonitrile (v/v)
Gradient:	

Time (min)	A (%)	B (%)
0.00	99	1
120	65	35
121	3	97
136	3	97
137	99	1
162	99	1

Flow:	0.08 mL/min
Column Temp.:	Ambient
Detection:	MS/MS
Injection:	10 µL

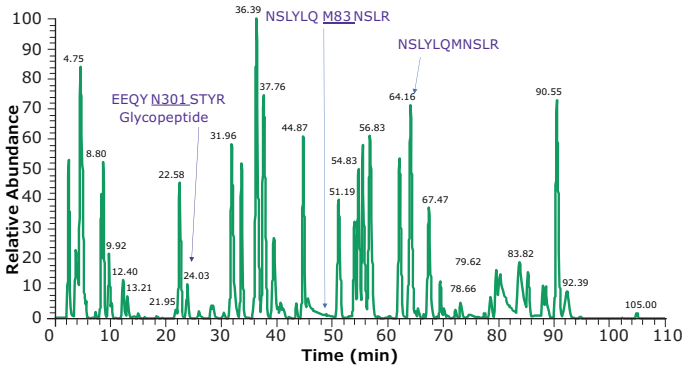


Figure 4. Base peak chromatogram of adalimumab tryptic peptides. Heavy chain glycosylated peptide (EEQYN301STYR) is observed at 24.03 min. Using a dual column set up, we observe hydrophilic peptides such as PGK, SCDK, VDK eluting at under 4 min. An example peptide and its oxidized form are NSLYLQM83NSLR and NSLYLQMNSLR eluting at 49.30 and 64.16 min, respectively.

can then be used to monitor the essential PQAs, or CQAs, during on-going monitoring of drug manufacture. The occurrence of newly identified, missing, or changed peaks during analysis triggers further investigation. The mild, rapid digestion provided by FASP and the low artifact digestion buffer, along with the excellent sequence coverage here, lends itself to MAM quality control of protein therapeutics.

N-linked Glycan Analysis

A prerequisite for accurate quantification of glycans is their complete release and isolation from the protein. Enzymatic cleavage with PNGase F is remarkably effective for achieving this. PNGase F is characterized by its high activity, broad substrate specificity, and its property of cutting most asparagine linked (N-linked) glycans. The glycan residue remains intact and can be subjected to further analysis. Here, a PNGase Fast Kit was used which, in combination with the enzyme, provided drastically reduced reaction time but with an accuracy equivalent to traditional methods.

Among glycan derivatization reagents, procainamide offers better fluorescence and electrospray ionization, and therefore better sensitivity than the more traditional 2-AB and 2-AA labeling. Here, released N-glycans were analyzed by a UHPLC-FLR-MS method that takes advantage of the quantitation provided by fluorescence detection and mass spectrometric identification. In total, 16 glycan features of adalimumab were observed. **Figure 5** illustrates the fluorescence chromatogram of adalimumab glycans. while **Table 3** provides the chromatographic conditions. Glycan peak areas were integrated, and the MS spectra were used to confirm glycan identities (**Table 4**). In total, twelve different glycans were quantified using this approach. The glycan profile, including qualitative and quantitative aspects, is comparable to the results obtained elsewhere.[14,15]

Table 3: Glycan analysis method conditions

Column:	BIOshell™ Glycan 15 cm x 2.1 mm I.D., 2.7 µm (**50994-U**)
Mobile phase:	[A] 75 mM ammonium formate, pH 4.4 in water; [B] acetonitrile
Gradient:	

Time (min)	A (%)	B (%)
0.00	75	25
75	59	41
76	75	25

Flow rate:	0.3 mL/min
Column Temp.:	58 °C
Detection:	1:1 split to both fluorescence detector, Ex: 308 nm, Em: 359 nm, and MS
Injection:	10 µL

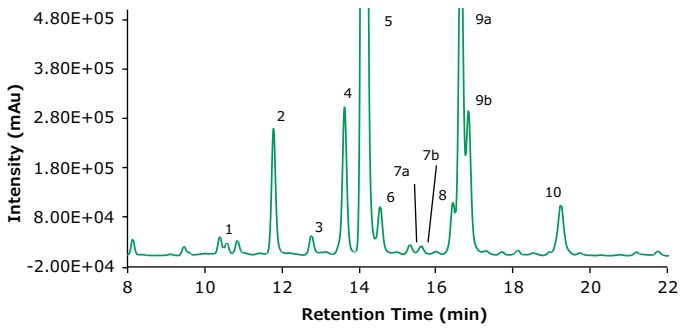


Figure 5. Fluorescence chromatogram of adalimumab after PNGase release and derivatization with procainamide. Peak annotations correspond to glycans shown in Table 4.

Table 4. N-Glycans, monosaccharide composition, and content in adalimumab

Peak #	Glycan	Monosaccaride Composition	Content (%)
1	G0-N	Man3 GlcNAc3	0.6
2	G0F-N	Fuc1 Man3 GlcNAc3	4.1
3	G0	Man3 GlcNAc4	0.9
4	Man5	Man5 GlcNAc2	5.3
5	G0F	Fuc1 Man3 GlcNAc4	65.0
6	G1F-N	Fuc1 Man3 Gal1 GlcNAc3	1.7
7a	G1(1,6)	Man3 Gal1 GlcNAc4	0.4
7b	G1(1,3)	Man3 Gal1 GlcNAc4	0.4
8	Man6	Man6 GlcNAc2	1.8
9a	G1F(1,6)	Fuc1 Man3 Gal1 GlcNAc4	12.1
9b	G1F(1,3)	Fuc1 Man3 Gal1 GlcNAc4	5.1
10	G2F	Fuc1 Man3 Gal2 GlcNAc4	2.7

Conclusion

Characterization and monitoring of therapeutic mAbs is required by regulatory authorities to ensure efficacy and safety of these drugs. Workflows for three important approaches to mAb analysis were developed and presented elsewhere as detailed step-by-step procedures.[7-9]

References

1. Berkowitz SA, Engen JR, Mazzeo JR, Jones GB. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. Nat Rev Drug Discov. 2012,11(7):527-40. doi: 10.1038/nrd3746.
2. Song YE, Dubois H, Hoffmann M, D Eri S, Fromentin Y, Wiesner J, Pfenninger A, Clavier S, Pieper A, Duhau L, Roth U. Automated mass spectrometry multi-attribute method analyses for process development and characterization of mAbs. J Chromatogr B Analyt Technol Biomed Life Sci. 2021,1166:122540. doi: 10.1016/j.jchromb.2021.122540
3. J. H. Knox, High Performance Liquid Chromatography, Edinburgh University Press, 1978.
4. D'Atri V, Fekete S, Beck A, Lauber M, Guillarme D. Hydrophilic Interaction Chromatography Hyphenated with Mass Spectrometry: A Powerful Analytical Tool for the Comparison of Originator and Biosimilar Therapeutic Monoclonal Antibodies at the Middle-up Level of Analysis. Anal Chem. 2017,89(3):2086-2092. doi: 10.1021/acs.analchem.6b04726.
5. Sjögren J, Olsson F, Beck A. Rapid and improved characterization of therapeutic antibodies and antibody related products using IdeS digestion and subunit analysis. Analyst. 2016,141(11):3114-25. doi: 10.1039/c6an00071a.
6. Beck A, Wagner-Rousset E, Ayoub D, Van Dorsselaer A, Sanglier-Cianférani S. Characterization of therapeutic antibodies and related products. Anal Chem. 2013, 85(2):715-36. doi: 10.1021/ac3032355.
7. Altmaier S, Jalili P, Sreenivasan U, Ray K, Protocol for Purification, Optional Reduction, and SEC-MS Analysis of a Monoclonal Antibody, https://www.sigmaaldrich.com/technical-documents/protocol/pharmaceutical-and-biopharmaceutical-manufacturing/biologics-biosimilars-characterization/protocol-for-analysis-of-a-monoclonal-antibody (accessed March 2022)
8. Rule G, Jalili P, Sreenivasan U, Ray K, Peptide Mapping LC-MS/MS Analysis Workflow for Adalimumab, https://www.sigmaaldrich.com/technical-documents/protocol/pharmaceutical-and-biopharmaceutical-manufacturing/biologics-biosimilars-characterization/peptide-mapping-lc-ms-ms-analysis-workflow-for-adalimumab (accessed March 2022)
9. Rigger R, Jalili P, Sreenivasan U, Ray K, Step-by-Step Protocol for Procainamide Labelled Glycan Profiling of a Monoclonal Antibody, https://www.sigmaaldrich.com/technical-documents/protocol/pharmaceutical-and-biopharmaceutical-manufacturing/biologics-biosimilars-characterization/released-n-linked-glycan-analysis-workflow (accessed March 2022)
10. Lermyte F, Tsybin YO, O'Connor PB, Loo JA. Top or Middle? Up or Down? Toward a Standard Lexicon for Protein Top-Down and Allied Mass Spectrometry Approaches. J Am Soc Mass Spectrom. 2019, 30(7):1149-1157. doi: 10.1007/s13361-019-02201-x
11. P. Jalili, K. Ray, J. Turner, M. Dube, LCGC Europe ebook, 3-12, March 2021.
12. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009, 6(5):359-62. doi: 10.1038/nmeth.1322
13. Rogers RS, Nightlinger NS, Livingston B, Campbell P, Bailey R, Balland A. Development of a quantitative mass spectrometry multi-attribute method for characterization, quality control testing and disposition of biologics. MAbs. 2015,7(5):881-90. doi: 10.1080/19420862.2015.1069454.
14. Lee N, Lee JJ, Yang H, Baek S, Kim S, Kim S, Lee T, Song D, Park G. Evaluation of similar quality attribute characteristics in SB5 and reference product of adalimumab. MAbs. 2019, 11(1):129-144. doi: 10.1080/19420862.2018.1530920
15. Tebbey PW, Varga A, Naill M, Clewell J, Venema J. Consistency of quality attributes for the glycosylated monoclonal antibody Humira® (adalimumab). MAbs. 2015, 7(5):805-11. doi: 10.1080/19420862.2015.1073429.

Featured Products

Product	Cat. No.
Columns	
Ascentis® Express Peptide ES-C18 15 cm x 1.0 mm, 2.7 µm	**53561-U**
BIOshell™ Glycan 15 cm x 2.1 mm, 2.7 µm	**50994-U**
Tosoh TSKgel® SW3000XL, 30 cm x 2.0 mm, 4 µm	**821485**
System Suitability Standards and Samples	
SILu™Lite SigmaMAb Universal Antibody Standard human	**MSQC4**
SILu™Lite SigmaMAb Adalimumab Monoclonal Antibody	**MSQC16**
Sample Preparation	
SOLu-Trypsin	**EMS0004**
PNGase Fast Kit	**EMS0001**
Low Artifact Digestion Buffer	**EMS0011**
Tris(2-carboxyethyl) phosphine BioUltra	**68957**
Dithiothreitol BioXtra	**D5545**
Procainamide hydrochloride	**PHR1252**
Protein A–Agarose Fast Flow 50%, aqueous suspension	**P3476**
Other Solvents and Reagents	
Water, LiChrosolv® for LC-MS	**1.15333**
Acetonitrile for UHPLC-MS LiChrosolv®	**1.03725**
Trifluoroacetic acid, LiChropur™ for LC-MS	**80457**
Formic acid, LiChropur™ for LC-MS	**5.33002**

See our application collection for Biopharmaceutical Characterization at our Pharma QC page **SigmaAldrich.com/pharmaqc**

For our portfolio of HPLC columns, visit **SigmaAldrich.com/hplc**

REFERENCE MATERIALS

Elemental Impurities

Certified Reference Materials for ICH Q3D, USP<232> & <2232> and Ph.Eur. 5.20

Ingrid Hayenga, Product Manager Reference Materials, Analytix@merckgroup.com

Metallic contamination in drug products, referred to as elemental impurities, may arise from several sources. They may be added intentionally in synthesis, or may be present as contaminants, (e.g., through interactions with processing equipment or by being present in components of the drug product) and are consequently detectable in the drug product. Since elemental impurities pose a risk to patient health due to toxicological effects, element impurity levels should be controlled within acceptable limits in the drug product.[1]

Evolution of ICH Q3D guidelines

In 2009 the International Conference on Harmonization (ICH) proposed the development of a new harmonized guideline to provide a global policy for limiting metal impurities in drug products and ingredients. This approach should provide clear regulatory guidance on specification limits for elemental impurities worldwide and logically should have an impact on the work of the national regulatory bodies in having transparent and comparable results.

In a step 4 version of its "Guidelines for Element Impurities" document, the ICH categorized the various elemental impurities in four different classifications which were intended to facilitate decisions during the risk assessment process:

Class 1: impurities are significantly toxic to humans and have limited or no use in the manufacture of pharmaceuticals. They can be found as impurities from commonly used materials (e.g., mined excipients). All four elements require evaluation during the risk assessment across all potential sources of elemental impurities and routes of administration.

The class 1 elements are: As, Cd, Hg, Pb.

Class 2: impurities are generally considered route-dependent human toxicants. These impurities are further divided into two sub-classes, 2A and 2B, based on their relative likelihood of occurrence in the drug product.

- Class 2A elements have relatively high probability of occurrence in the drug product and thus require risk assessment across all potential sources of elemental impurities and routes of administration (as indicated). The class 2A elements are: Co, Ni and V.
- Class 2B elements have a reduced probability of occurrence in the drug product related to their low abundance and low potential to be co-isolated with other materials. As a result, they may be excluded from the risk assessment unless they are intentionally added during the manufacture of drug substances, excipients or other components of the drug product.

Class 2B elements are: Ag, Au, Ir, Os, Pd, Pt, Rh, Ru, Se and Tl.

Class 3: includes elements which have relatively low toxicity at oral administration but may require a risk assessment if applied via inhalation or parenteral routes.

Class 3 elements are: Ba, Cr, Cu, Li, Mo, Sb and Sn.

Other elements: There are some elemental impurities for which Permitted Daily Exposures (PDEs) have not been established due to their low toxicities and/or differences in regional regulations. If they are present in a drug product, they are addressed by other guidelines and/or regional regulations.

These elements are: Al, B, Ca, Fe, K, Mg, Mn, Na, W and Zn.

Evaluation of USP and EP

Up to 2010, the USP and EP proof of heavy metal contamination in drugs was obtained via a colorimetric analytical method based on the precipitation of a metal sulfide in a sample and comparing it to a lead standard (USP <231> and Ph.Eur. 2.4.8).

Based on the Guideline for Elemental Impurities (Q3D) which was published by the International Conference on Harmonization (ICH) in 2010, the USP proposed three new General Chapters covering impurity limits, analytical procedures in pharmaceutical products and raw materials, and elemental contaminants in dietary supplements.

- Chapter USP <232>, Ph.Eur. 5.20: Elemental Impurities in Pharmaceutical Products - Limits
- Chapter USP <233>: Elemental Impurities in Pharmaceutical Products – Procedures
- Chapter USP<2232>: Elemental Contaminants in Dietary Supplements

In January 2015, the USP established January 1, 2018 as the new date of applicability for General Chapters <232>, <233> and <2232>. The implementation should align with limits and timelines set down by other pharmaceutical and medical agencies such as the ICH Q3D Step 4 Guidelines for Elemental Impurities announced on December 16, 2014.

The Pharmacopoeia Europe announced in July 2014 their strategy regarding elemental impurities and the implementation of the ICH Q3D. Nearly one year later, in April 2015, they published their policy on elemental impurities and timelines for revision of general and individual texts. In August of the same year, clarification was given for products outside the scope of ICH Q3D.

The implementation of the guideline compliances should start in June 2016 for products with new marketing authorization, either containing new active substances or already approved substances.

Marketed products, including new mutual recognition applications of already approved substances, should comply with the Guideline from December 2017.

The implementation of the General Test 5.20 and the General Method 2.4.20 replaced the EMA guideline on metal catalysts and metal reagents by the principles of the ICH. The publication was done in the Ph.Eur. Suppl. 9.3 (implementation date January 1, 2018), having no test for elemental impurities in the individual monographs except for substances of natural origin. Given the intrinsic nature of elemental impurities in these substances, they are among the major potential sources of elemental contamination in medicinal products. The Ph.Eur. Commission has also specifically recommended keeping the different tests for elements for which no PDE limits have been established, i.e., those identified as “other elements” in the ICH Q3D guideline in individual monographs.[2]

Analytical methods

Concerning new analytical methods, ICH Q3D does not include any recommendation on instrumental methods but the following analytical procedures are suggested in USP<233> dependent on the expected concentration of the elemental impurity in the product or component:

- Parts-per-million (ppm) concentrations ICP-OES or atomic absorption
- Parts-per-billion (ppb) concentrations ICP-MS

ICH Q3D limits for elemental impurities

For a total of 24 elements, toxicity limits are specified and defined as maximum PDE levels in mg/day for the four major drug delivery categories. **Table 1** lists the PDE values in µg/day, valid for drug products with an intake of ≤10 g/day.

Table 1. Permitted Daily Exposure (PDE) for Elemental Impurities

Element	Class	Oral PDE (µg/day)	Parenteral PDE (µg/day)	Inhalation PDE (µg/day)
As	1	15	15	2
Cd	1	5	2	2
Hg	1	30	3	1
Pb	1	5	5	5
Co	2A	50	5	3
V	2A	100	10	1
Ni	2A	200	20	5
Tl	2B	8	8	8
Au	2B	100	100	1
Pd	2B	100	10	1
Ir	2B	100	10	1
Os	2B	100	10	1
Rb	2B	100	10	1
Ru	2B	100	10	1
Se	2B	150	80	130
Ag	2B	150	10	7
Pt	2B	100	10	1
Li	3	550	250	25
Sb	3	1200	90	20
Ba	3	1400	700	300
Mo	3	3000	1500	10
Cu	3	3000	300	30
Sn	3	6000	600	60
Cr	3	11000	1100	3

Table 2 lists the elements to be considered in the risk assessment

For the new adapted USP <232> and Ph.Eur.Suppl. 9.3 chapters, we offer three *Trace*CERT® element mixes with element ratio corresponding to the oral concentrations of the ICH Q3D guideline, mix I covers class 1, 2A and some of 2B elements; mix II covers the remaining 2B class elements; mix III covers all class 3 elements.

A second series of three mixes covers the parenteral concentration ratios.

All products with their element respective concentrations (mg/L) are listed in **Table 3**.

Table 4 lists the features of the *Trace*CERT® Certified Reference Material (CRM) solutions.

Table 2. Elements to be Considered in the Risk Assessment

Element	Class	If Intentionally Added (all routes)	If not intentionally added		
			Oral	Parenteral	Inhalation
As	1	Yes	Yes	Yes	Yes
Cd	1	Yes	Yes	Yes	Yes
Hg	1	Yes	Yes	Yes	Yes
Pb	1	Yes	Yes	Yes	Yes
Co	2A	Yes	Yes	Yes	Yes
V	2A	Yes	Yes	Yes	Yes
Ni	2A	Yes	Yes	Yes	Yes
Tl	2B	Yes	No	No	No
Au	2B	Yes	No	No	No
Pd	2B	Yes	No	No	No
Ir	2B	Yes	No	No	No
Os	2B	Yes	No	No	No
Rb	2B	Yes	No	No	No
Ru	2B	Yes	No	No	No
Se	2B	Yes	No	No	No
Ag	2B	Yes	No	No	No
Pt	2B	Yes	No	No	No
Li	3	Yes	No	No	No
Sb	3	Yes	No	No	No
Ba	3	Yes	No	No	No
Mo	3	Yes	No	No	No
Cu	3	Yes	No	No	No
Sn	3	Yes	No	No	No
Cr	3	Yes	No	No	No

Table 4. Features of the TraceCERT® CRMs

*Trace*CERT® Solutions
Unique level of accuracy and lot-specific value
Produced according to ISO Guide 34 and analyzed in our ISO/IEC 17025 accredited lab; traceable to at least two independent references (NIST, BAM or SI unit kg)
Sophisticated packaging and comprehensive documentation including proper uncertainty calculation, expiry date and storage information
Packaged in opaque and gas-tight aluminum foil bags for extended stability. Certificates are included and list up to 70 trace impurities for the *Trace*CERT® products.
250 mL package size*

For more information and to view sample certificates, please visit
SigmaAldrich.com/ICHQ3D

References:

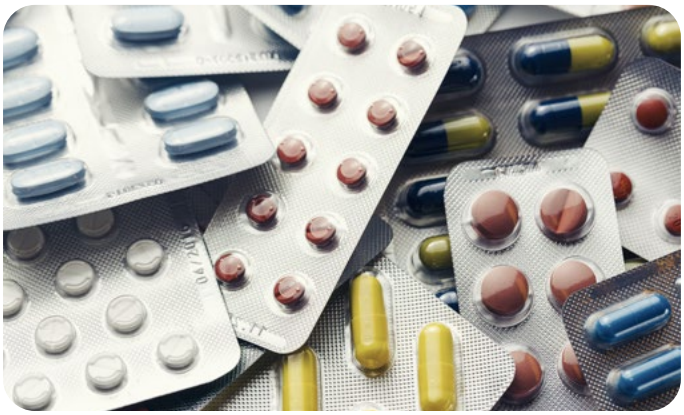
1. ICH Q3D limits from Step 4 version, December 16, 2014 Option 1
2. Thermo Fischer, the Medicine Maker, Edition 4 – August 2016100

Table 3. Suitable Multi-Element CRM Solutions According to ICH Q3D

Element	Class	*Trace*CERT® Elemental Impurities Mix according to ICH Q3D oral			*Trace*CERT® Elemental Impurities Mix according to ICH Q3D parenteral		
		Standard 1	Standard 2	Standard 3	Standard 1	Standard 2	Standard 3
		Cat. No.	Cat. No.	Cat. No.	Cat. No.	Cat. No.	Cat. No.
		19041	73108	69729	89118	89922	07368
		In 12% HNO_3	In 10% HCl	In 5% HNO_3 & HF<0.5%	In 12% HNO_3	In 10% HCl	In 5% HNO_3 & <0.5% HF
Ag	2B	150 mg/L			10 mg/L		
As	1	15 mg/L			15 mg/L		
Au	2B		100 mg/L			100 mg/L	
Ba	3			140 mg/L			70 mg/L
Cd	1	5 mg/L			2 mg/L		
Co	2A	50 mg/L			5 mg/L		
Cr	3			1100 mg/L			110 mg/L
Cu	3			300 mg/L			30 mg/L
Hg	1	30 mg/L			3 mg/L		
Ir	2B		100 mg/L			10 mg/L	
Li	3			55 mg/L			25 mg/L
Mo	3			300 mg/L			150 mg/L
Ni	2A	200 mg/L			20 mg/L		
Os	2B		100 mg/L			10 mg/L	
Pb	1	5 mg/L			5 mg/L		
Pd	2B		100 mg/L			10 mg/L	
Pt	2B		100 mg/L			10 mg/L	
Rh	2B		100 mg/L			10 mg/L	
Ru	2B		100 mg/L			10 mg/L	

Reference Materials for Extractables and Leachables Testing

Matthias Nold, Product Manager Reference Materials, Analytix@merckgroup.com



Extractables and Leachables (E&L) are chemical compounds with the potential to migrate into pharmaceutical or clinical products from packaging materials, tubing, or medical devices, resulting in patient exposure.

Manufacturers of pharmaceutical products and medical devices are obligated to perform extensive E&L studies to identify compounds that might leach into the product and, if necessary, assess the toxicity of these chemicals.

Since it is never entirely predictable which migrants may occur, it is crucial that no analytes are overlooked. Depending on the nature of the packaging material, the product, and the applied conditions, new unexpected or unknown compounds can be found. Therefore, there is no finite list of analytes to be tested for. However, there are certain monomers or additives that are very often detected in extractables and leachables studies.

Recently we added a series of sixteen new reference materials to this range, which are listed below. These new products are provided with a NIST SRM traceable value determined by quantitative NMR (qNMR) in accordance with ISO/IEC 17025. The values are given with an uncertainty which takes into account the stability and homogeneity of the material. We have also developed certified reference material (CRM) mixes for LC and GC which cover a broad range of compound classes that are most commonly found in extractables and leachables studies. Please regularly check our website for the most recent product additions.

LC Mix

Product Number	**95636**
Product Name	Extractables and Leachables Screening Standard for LC
Suffix	Certified Reference Material TraceCERT
Solvent	Acetonitrile
Concentrations	50 µg/mL per component
Package Size	1 mL

Product Name	CAS	Name	CAS
Irganox 1010 (Ir 1010)	6683-19-8	Butylhydroxytoluene (BHT)	128-37-0
Irganox 1076 (Ir1076)	2082-79-3	1,3-Di-tert-butyl-benzene (DBB)	1014-60-4
Dometrizol (Dome) / Tinuvin P/2-(2H-Benzotriazol-2-yl)-p-cresol	2440-22-4	Oleamide (Ole)	301-02-0
ε-Caprolactam (CAP)	105-60-2	Bis(2-ethylhexyl) phthalate (DEHP)	117-81-7
Dibenzylamine (DBA)	103-49-1	Stearic acid (SA)	57-11-4
Benzoic acid (BA)	65-85-0	Erucamide (Eruca)	112-84-5
2-Mercaptobenzothiazole (2-MBT)	149-30-4	Irganox 3114 (Ir3114)	27676-62-6
Bisphenol A (BPA)	80-05-7	Irgafos 168-oxide	95906-11-9
2-Ethylhexanoic acid (EHA)	149-57-5	2,4-di-tert-Butylphenol	96-76-4
Bis(4-chlorophenyl)sulfone (CPS)	80-07-9	Palmitic acid	57-10-3
2,6-Di-tert-butyl-4-hydroxymethyl-phenol (DBOHP)	88-26-6		

GC Mix

Product Number	**01829**
Product Name	Extractables and Leachables Screening Standard for GC
Suffix	TraceCERT® Certified Reference Material
Solvent	TBME
Concentrations	50 µg/mL per component
Package Size	1 mL

Product Name	CAS	Product Name	CAS
Irganox 1076 (Ir1076)	2082-79-3	Bis(2-ethylhexyl) phthalate (DEHP)	117-81-7
ε-Caprolactam (CAP)	105-60-2	Stearic acid (SA)	57-11-4
2-Mercaptobenzothiazole (2-MBT)	149-30-4	Erucamide (Eruca)	112-84-5
Bisphenol A (BPA)	80-05-7	Irgafos 168-oxide	95906-11-9
Butylhydroxytoluene (BHT)	128-37-0	2,4-di-tert-Butylphenol	96-76-4
1,3-Di-tert-butyl-benzene (DBB)	1014-60-4	2,6-di-tert-Butylphenol	128-39-2
Oleamide (Ole)	301-02-0	Palmitic acid	57-10-3

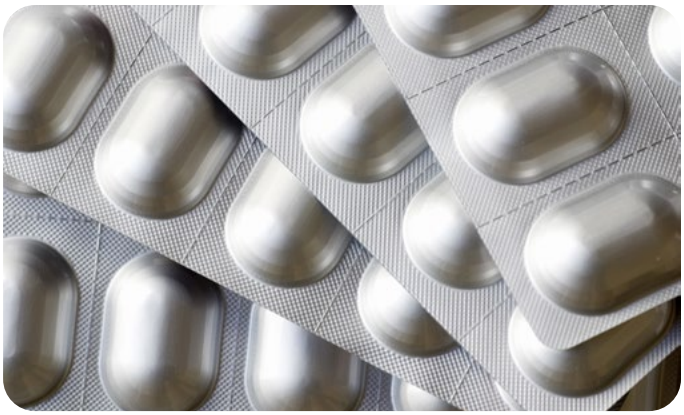
Cat.No.	Product Name	Synonym	CAS	Package Size
78274	Benzophenone		119-61-9	100 mg
90048	Bis(2,4-di-tert-butylphenyl) phosphate		69284-93-1	100 mg
CRM96153	Bis(4-chlorophenyl) sulfone	CPS	80-07-9	100 mg
CRM96142	Bis[4-(glycidyloxy)phenyl]methane, mixture of isomers	Bisphenol F diglycidylether (BPFE)	2095-03-6	100 mg
CRM01483	e-Caprolactam	CAP	105-60-2	100 mg
78270	1-Decene		872-05-9	100 mg
CRM95728	Dibenzylamine	DBA	103-49-1	100 mg
78132	Didodecyl 3,3′-thiodipropionate		123-28-4	100 mg
78478	7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione		82304-66-3	50 mg
CRM96857	3,5-di-tert-butyl-4-hydroxybenzyl alcohol	2,6-Di-tert-butyl-4-hydroxymethyl-phenol (DBOHP)	88-26-6	100 mg
CRM96659	1,3-Di-tert-butylbenzene	DBB	1014-60-4	100 mg
CRM00437	2,4-Di-tert-butylphenol		96-76-4	100 mg
CRM96852	2,6-Di-tert-butylphenol		128-39-2	100 mg
78290	Docosane		629-97-0	100 mg
CRM01374	cis-13-Docosenoamide	Erucamide (Eruca)	112-84-5	100 mg
78292	Eicosane		112-95-8	100 mg
78699	Ethyl 4-ethoxybenzoate		23676-09-7	100 mg
78263	Ethylene glycol butyl ether		111-76-2	100 mg
78293	Hexadecane		544-76-3	100 mg
CRM96697	2-(2-Hydroxy-5-methylphenyl)benzotriazol	Dometrizol (Dome) / Tinuvin P	2440-22-4	100 mg
78345	Isophorone		78-59-1	100 mg
78266	Isovaleric acid		503-74-2	100 mg
CRM96051	2-Mercaptobenzothiazole	2-MBT	149-30-4	100 mg
78289	2-Methyldodecane		1560-97-0	100 mg
78294	Octadecane		593-45-3	100 mg
78295	1-Octadecene		112-88-9	100 mg
CRM00318	Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate	Irganox 1076 (Ir1076)	2082-79-3	100 mg
78340	1-Octene		111-66-0	100 mg
CRM96709	Oleamide	Ole	301-02-0	100 mg
CRM96656	Pentaerythritol tetrakis(3,5-di-tert-butyl-4-hydroxyhydrocinnamate)	Irganox 1010 (Ir 1010)	6683-19-8	100 mg
CRM96839	Tris(2,4-di-tert-butylphenyl)phosphate	Irgafos 168-oxide	95906-11-9	100 mg
CRM96737	Tris(3,5-di-tert-butyl-4-hydroxybenzyl) isocyanurate	Irganox 3114 (Ir3114)	27676-62-6	100 mg

For a complete overview on our reference materials for pharma applications visit us at **SigmaAldrich.com/standards** and see our Pharma Industry Segment specific page

Secondary Standards for Pharmaceutical Quality Control Testing

An attractive alternative to in-house produced working standards

Michael Hurst, Global Product Manager - Reference Materials; Katherine Stenerson, Lead Expert – Product & Portfolio Management, Cannabis & Pharma QC Reference Materials, Analytix@merckgroup.com



Merck's pharmaceutical secondary standards provide pharmaceutical testing laboratories and manufacturers with a convenient and cost-effective alternative to making in-house working standards.

Using these commercially available secondary standards frees up time in the laboratory by allowing precious resources to be focused on tasks other than the time-consuming steps involved in the preparation of standards for QC testing.

Some key features of our pharmaceutical secondary standards are:

- Traceability to the United States Pharmacopeia (USP) and to the European and British Pharmacopeias (Ph. Eur., BP) - if available.
- Certified purity value according to ISO Guide 34 and ISO/IEC 17025 using the mass balance approach. This requires the use of multiple techniques to measure impurities which may be present. Purity is reported along with the expanded uncertainty value.
- Comprehensive certificate according to ISO Guide 31.

Our secondary standards are certified reference materials (CRMs) produced in an ISO/IEC 17025 and ISO Guide 17034 accredited facility. A comprehensive certificate of analysis is provided with each product that demonstrates traceability to USP primary standards and Ph. Eur. BP if available. In addition, an independent certified purity value is provided, which allows these secondary standards to be used as reference materials for quantitative applications.

The United States Food and Drug Administration (FDA), USP and Ph. Eur. all recognize secondary standards or working standards which have an established reference to the corresponding primary standard (reference to this policy available upon request).

Comprehensive Certificate

The certificate provided with our pharmaceutical secondary standards contains these key elements:

- Handling and storage instructions.
- Traceability results versus the pharmacopeial primary standard.
- A certified purity value, determined by mass balance (according to ISO/IEC 17025).
- Qualification data along with comprehensive details on the analytical methods used.

An excerpt from an actual certificate that shows an example of traceability is shown below. You can also view and download an example of a full certificate for any pharmaceutical secondary standard from our website at SigmaAldrich.com/pharmastandards, by using the lot number provided under "footnote" on the product description page. Click on the product number of interest and scroll down to the "footnote" heading. Then, enter the provided lot number in the "documentation" section further down the page.

It is easy to maintain the latest certificate for your standards

The values on the certificate provided with secondary standards are always traceable to the current pharmacopeial lots. If a valid pharmacopeial lot changes, the corresponding pharmaceutical secondary standard will be re-certified with traceability to the new lot and a new certificate will be available on-line. For this reason, the valid certificate for your pharmaceutical secondary standard should always be downloaded from our website prior to use of the material. To do this, find your standard on SigmaAldrich.com using the product number or via the Standards Explorer tool (SigmaAldrich.com/standards-explorer) and click on the product number shown below the product description. Click on the COO/COA search tool on the left-hand side and enter your lot number in the resulting search box.

An example of traceability assay data for an Ibuprofen pharmaceutical secondary standard (PHR1004)

Comparative assay demonstrates direct traceability to Pharmacopeial Standards*

Specification: 97.0–103.0% (anhydrous, USP/NF) 1
98.5–101.0% (dried substance, EP)

Determination Method: HPLC
(ref., Current Compendial Monograph: Ibuprofen)

Column: Ascentis C18, 4.6 x 250 mm, 5 µm 2

Mobile Phase: Acetonitrile/Water/Chloroacetic acid (600:400:4)

Column Temperature: 30 °C

Flow Rate: 2 mL/min

Injection: 5 µL 3

Detector Wavelength: 254 nm

Assay vs. USP Reference Standard (as is basis)

Assay Value	**vs. USP LOT** 4
99.5% 5	R024X0 Labeled Content = 0.998 mg/mg

1 Analytical specification

2 Reference to Pharmacopeial monograph (if available) used for the certification

3 Analytical conditions of the method used

4 Reference to the Pharmacopeial lot of the primary standard

5 Assay value (traceable to the given Pharmacopeial lot)

*This is an extract of the certificate of traceability assay results (versus the USP reference standard) for Ibuprofen (PHR1004)

You can also sign up for monthly updates on the certificates for your standards. By signing up, you will be notified in advance of any changes that may occur due to re-qualification, etc. This will help to ensure that you are always using the most current version of a product's certificate.

Expiration Date

During the development of this product line, we investigated what the compendial producers were using as expiration policies. We found that USP, Ph. Eur. and BP give no fixed expiration dates for their materials. USP specifically "expires" a standard 1 year after lot depletion while the Ph. Eur. does the same at 6 months. The BP designated an expiration of 3 months from dispatch, with no guidance on lot depletion. We received feedback from customers asking for a certified reference material with a fixed expiration that could be used in their laboratories as a working standard. In response, we have set a fixed expiration date for each product that is a minimum of one year from receipt, with many materials having an expiration of greater than 1 year.

A Large Portfolio of Pharmaceutical Secondary Standards

Our portfolio of pharmaceutical secondary standards is constantly growing and includes products for assay, performance and impurity testing. See what is new by clicking on the link below:

NEW Pharmaceutical Secondary Standards

To see our entire portfolio of pharmaceutical secondary standards, visit:
SigmaAldrich.com/pharmastandards

To learn more about pharmaceutical secondary standards, visit:
SigmaAldrich.com/secondarystandards

Standards for Pharma

SmartChemicals Improve Titration Data Integrity & Efficiency

Bettina Straub-Jubb, Product Manager Titration, Analytix@merckgroup.com

Titration Goes Digital - a joint project

In a joint project, METTLER TOLEDO, Switzerland, known as a manufacturer of high-end titration instruments, and Merck KGaA, Darmstadt, Germany, known as a producer of highest-quality analytical reagents and certified reference materials, have together developed this new titration technology to improve data integrity. With combined expert knowledge, we created a user-friendly and easy to use tool for transferring data wirelessly from the reagents to the titrator to open a new page of titration.

Introduction

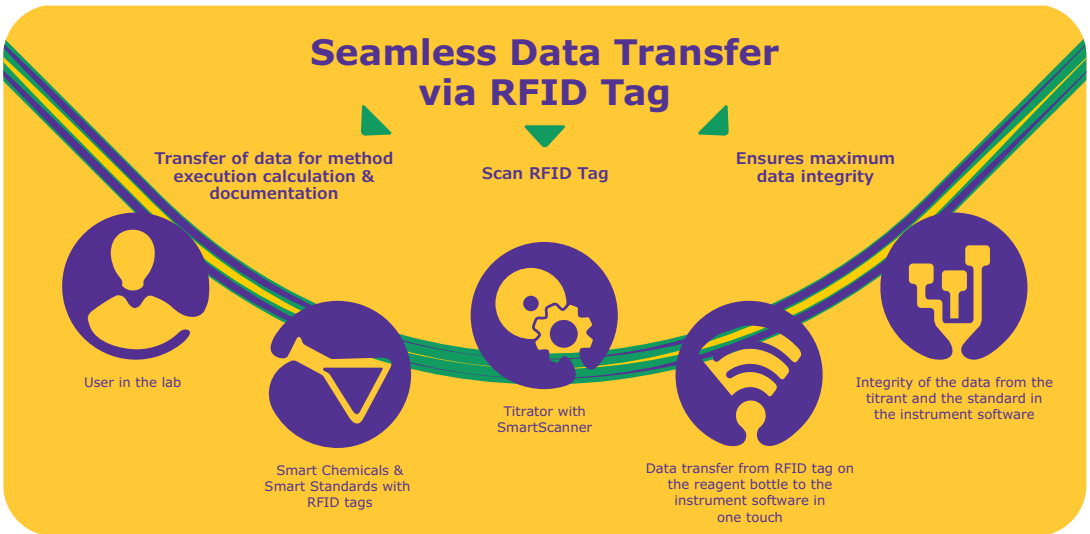
This new technology using SmartChemicals in your titration process can secure your data transfer. Additionally, it saves time by eliminating manual data transmission.

Certificates of analysis often need to be downloaded and data subsequently copied manually to the titrator software for the correct calculation of the results and for documentation. This process is time consuming and creates sources of errors - such as typing mistakes.

The use of SmartChemicals eliminates these time-consuming steps and the potential for human errors by transferring all data wirelessly and instantly to the titrator software (see below). Volumetric solutions, Karl Fischer titrants and all standards are embedded with an RFID (Radio Frequency Identification) tag bearing all relevant information from the Certificate of Analysis.

Table 1 lists the information stored on the RFID tag for SmartTitrants and SmartStandards. Just a brief touch of the titrator's SmartReader on the SmartChemical tag on the bottle (**Figure 1**) conveys all relevant data to the titrator software, saving time, reducing errors and ensuring maximum data integrity.

Secure data integrity in the titration process was never so easy



RFID tag on the SmartChemical label contains all relevant data

All data needed for execution of the method, result calculation and documentation, such as product name, catalog number, lot/batch number, concentration/ purity, date of release, shelf life/expiry date, are stored on the RFID tag on the SmartChemical label (**Figure 1**) for seamless transfer to the titrator software. For standards, additional data is transferred, including molecular weight, supplier/producer name, uncertainty and compliance according to Pharmacopeias or ISO 17034 (**Table 1**).

Once the information is transferred from the RFID to the titrator, it is stored in the titration software and displayed on the touchscreen (**Figure 2 & 3**).

Previously opened chemicals are also recognized, and the initial opening date is stored on the RFID

Figure 1. Titrator's RFID Reader and Example Label of a SmartChemical (RFID Tag Marked)

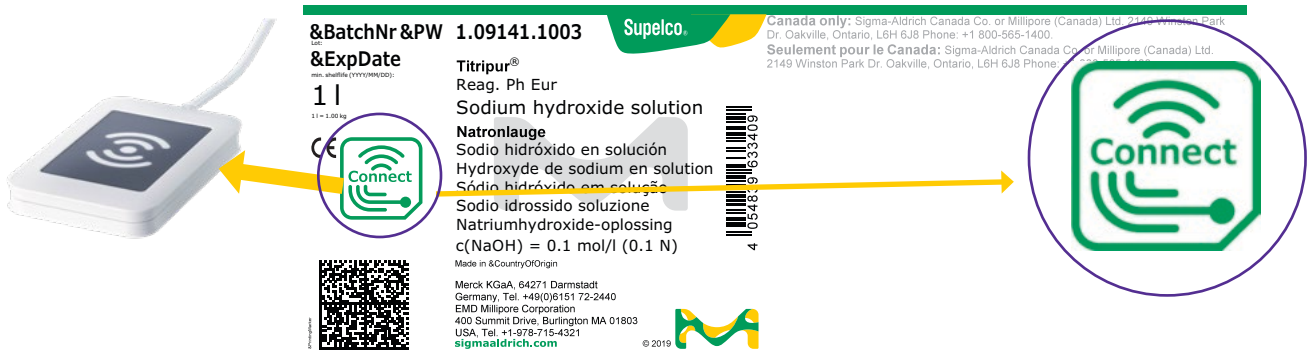


Figure 2. Information on SmartTitrant as Displayed in the Titrator Software

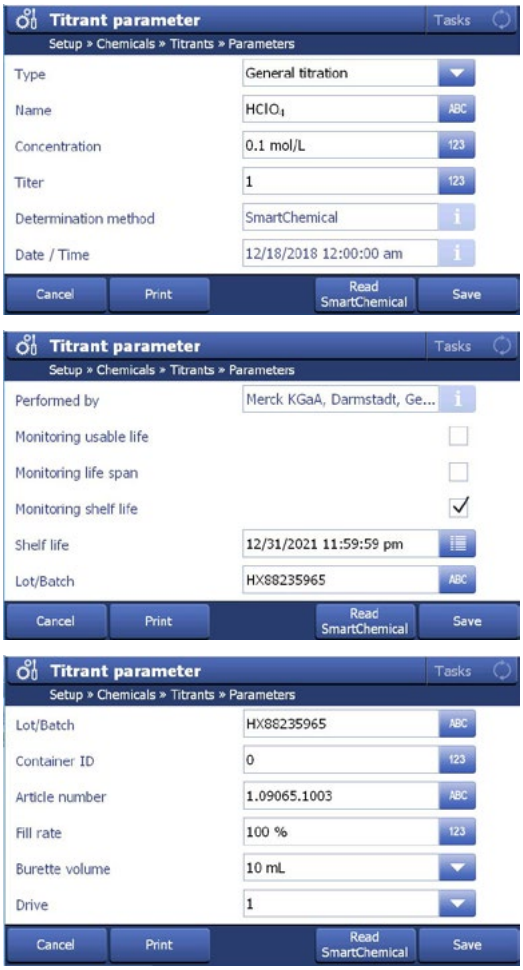
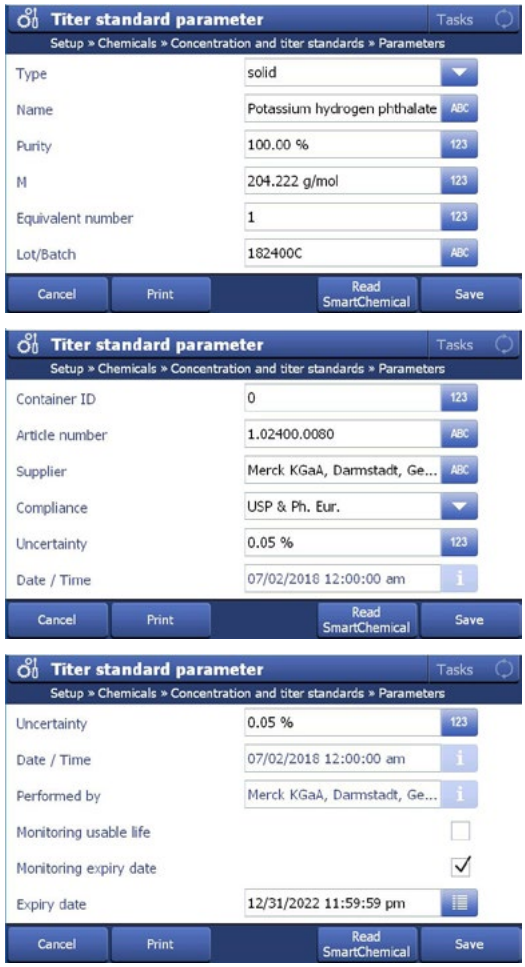


Figure 3. Information on SmartStandards as Displayed in the Titrator Software



tag and is shown on the instrument's touchscreen. Expired chemicals are automatically blocked from use preventing an incompliance of the measurement. In addition, user-specific guidelines regarding use period after opening can be set up and monitored by entering an individual life span into the software.

Table 1. RFID Data Content

SmartTitrant	SmartStandard
Name	Name
Concentration	Concentration/Purity
Article number	Article number
Lot/Batch no.	Lot/Batch no.
Shelf life	Expiry date
Initial opening date	Initial opening date
Date of release (Date / Time)	Date of release (Date / Time)
	Molecular weight
	Compliance
	Uncertainty

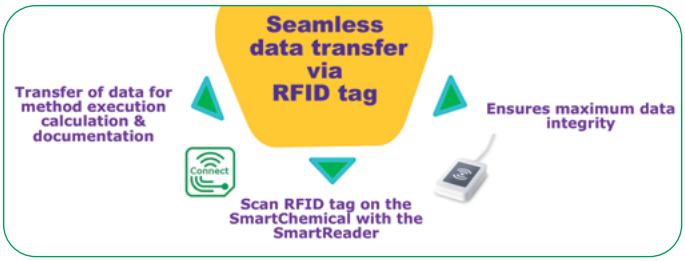
SmartChemicals are compatible with the METTLER TOLEDO Instruments:

- Excellence Titrator models T5, T7, T9
- Compact Titrator models G10S, G20S, V30S, V20S, V10S.

Conclusion

SmartChemicals with the RFID tag on the label provide

- Secure data transfer – ensures complete and correct reagent data
- Ease of use – intuitive operation and convenient data transfer in one touch
- Extended quality management – titer determination, shelf life, compliance data, initial opening date
- Improved efficiency – fast data transfer - saves time - no manual writing and no need to employ the four-eyes principle



Featured Products

Description	Cat. No.*
Titripur® Volumetric Solutions	
Perchloric acid in acetic acid 0.1 mol/L, 1 L	**1.09065.1003**
Potassium hydroxide solution in ethanol 0.5 mol/L, 1 L	**1.09114.1003**
Potassium hydroxide solution in ethanol 0.1 mol/L, 1 L	**1.09115.1003**
Hydrochloric acid solution 0.1 mol/L, 1 L	**1.09060.1003**
Sodium hydroxide solution 0.1 mol/L, 1 L	**1.09141.1003**
Sodium thiosulfate solution 0.1 mol/L, 1 L	**1.09147.1003**
Sodium thiosulfate solution 0.1 mol/L , 4 L Titripac®	**1.09147.4003**
Certipur® Volumetric Standards (CRMs)	
Potassium hydrogen phthalate, Certified Reference Material, 80 g	**1.02400.0083**
Benzoic acid, Certified Reference Material, 60 g	**1.02401.0063**
TRIS(hydroxymethyl)aminomethane, Certified Reference Material, 80 g	**1.02408.0083**
Zinc, Certified Reference Material, 100 g	**1.02409.0103**
Aquastar® Karl Fischer Titrants	
CombiTitrant 5, Karl Fischer one component reagent 5 mg H_2O/mL, 1 L	**1.88005.1003**
Titrant 5, Karl Fischer two component reagent 5 mg H_2O/mL, 1 L	**1.88010.1003**
Titrant 5, Karl Fischer two component reagent 5 mg H_2O/mL (for North America only), 1 L	**1.88010.1043**
CombiTitrant 2, Karl Fischer one component reagent 2 mgH_2O/mL, 1 L	**1.88002.1003**
CombiTitrant 2, Karl Fischer one component reagents 2 mg H_2O/mL, 1 L (for North America only)	**1.88002.1043**
Aquastar® Water Standards (CRMs)	
Water Standard 1% in ampoules, Certified Reference Material, 10x8 mL	**1.88052.0013**
Water Standard 1% in ampoules (for North America only), Certified Reference Material, 10x8 mL	**1.88052.0313**
Water Standard 0.1 % in ampoules, Certified Reference Materials, 10x8 mL	**1.88051.0013**
Water Standard 0.1 % in ampoules (for North America only), Certified Reference Material, 10x8 mL	**1.88051.0313**

*The catalog numbers for our SmartChemicals are the same as the existing products, only the last digit has been changed from a 0 to a 3 e.g. 1090651000 becomes 1090651003 for the SmartChemical with an RFID tag.

More products, packsizes and Titripac's are avialable!

For an up-to-date listing, visit us at **SigmaAldrich.com/smartchemicals**

For our complete portfolio of titration products visit us at **SigmaAldrich.com/Titration**

Making Science Run Like Clockwork

The Supelco® portfolio provides a comprehensive range of analytical products and services designed to meet the breadth of your specific needs.

From analytical grade solvents & reagents, reference materials, and chromatography columns to rapid chemical tests and a wide safety portfolio, our extensive range of analytical products delivers the accuracy and precision to make your science run like clockwork, **every time.**

Explore the comprehensive Supelco® analytical product portfolio at

SigmaAldrich.com/supelco

Supelco®
Analytical Products

To place an order or receive technical assistance

Order/Customer Service: **SigmaAldrich.com/order**
Technical Service: **SigmaAldrich.com/techservice**
Safety-related Information: **SigmaAldrich.com/safetycenter**

SigmaAldrich.com

Lit. No. MK_BR5393EN Ver. 2.0
42582
09/2022