

# Kapalinová chromatografie a hmotnostní spektrometrie pro robustní analýzu biomolekul: Agilent 1290 Infinity II Bio a 6545 XT QTOF

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# Nástroje Agilent pro analýzu biologických léčiv



Příprava vzorku

Separace

Detekce

Vyhodnocení dat a reporty



BIO

Agilent 1290 Infinity II **Bio LC 1300 bar**  
Agilent 1260 Infinity II **BioInert LC 600 bar**



Agilent **AdvanceBio LC Columns**

**PLRP-S 1000 Å**

AdvanceBio RP mAb

Zorbax SB 300 RRHD

Poroshell 300

AdvanceBio Peptide Mapping

AdvanceBio Peptide Plus

AdvanceBio Oligonucleotides

Kolony pro intaktní proteiny,  
mapování peptidů, analýzu  
oligonukleotidů

# Bioinertní a Biokompatibilní

Nutnost/potřeba BIO instrumentace v laboratoři

**BIO**

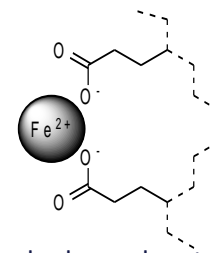
Materiály v dráze toku vzorku neobsahují žádné železo, pouze velmi ušlechtilé materiály

**BIO  
INERT**

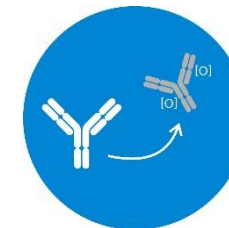
Materiály v dráze toku vzorku neobsahují žádné kovy

## Proč je to nutné?

- **Chování některých biomolekul:**
  - Chelatace – interakce biomolekuly se železem
  - Oxidace, Sorpce
- **Bio aplikace vyžadují náročné chemické požadavky**
  - Vysoká koncentrace solí
  - Vysoké nebo nízké pH → *“určitě nechceme rezavý standardní HPLC”*
- **Foto-degradace** (vysoká světelná intenzita)
- Agilent řešení: “Max Light Cartridge **LSS** DAD průtočná cela” s neutrálním šedým filtrem !



Positively charged metal ions can attract negatively charged biomolecules through **chelation**



Iron can also cause **oxidation** of some proteins



# Co je to MP35N?

- MP35N je slitina (Ni-Co-Cr-Mo → 35%, 35%, 20%, 10%)
- Odbornou veřejností akceptovaný bio-kompatibilní materiál pro UHPLC

## Corrosion Resistance

MP35N alloy possesses excellent resistance to sulfidation, high temperature oxidation, hydrogen embrittlement, saline solutions and most mineral acids.

Nitric Acid	Good	Sulfuric Acid	Good
Phosphoric Acid	Good	Acetic Acid	Excellent
Sodium Hydroxide	Good	Salt Spray (NaCl)	Excellent
Sea Water	Excellent	Sour Oil/Gas	Excellent
Humidity	Excellent		

## Type Analysis

Carbon (Maximum)	0.03 %	Manganese (Maximum)	0.15 %
Phosphorus (Maximum)	0.015 %	Sulfur (Maximum)	0.010 %
Silicon (Maximum)	0.15 %	Chromium	19.00 to 21.00 %
Nickel	33.00 to 37.00 %	Molybdenum	9.00 to 10.50 %
Cobalt	Balance	Titanium (Maximum)	1.00 %
Boron	0.010 %	Iron (Maximum)	1.00 %

## Galvanic Series of Metals and Alloys Ladder

Graphite Noble

Platinum

Gold

Titanium

MP35N®

MP159®

**AEREX™ 350** ←

Ni-Base Superalloys

Silver

A-286

Stainless Steel - Passive

Copper

Nickel-Copper Alloys

Bronzes

Brass

Nickel

Lead

Tin

Stainless Steel - Active

Carbon & Alloy Steel

Cadmium

Aluminum

Zinc

Magnesium Active

# Agilent 1290 Infinity II Bio LC



## Čerpadla

- binární a kvartérní gradient do **1300 bar**

## Autosampler

- multisampler s možností multi-wash (oplach jehly až 3 rozpouštědly, min. carryover)

## Termostat kolon

- nové termo-ekvilibrační příslušenství
- nové **bio** ventily (2pos/10port)

## Detektory

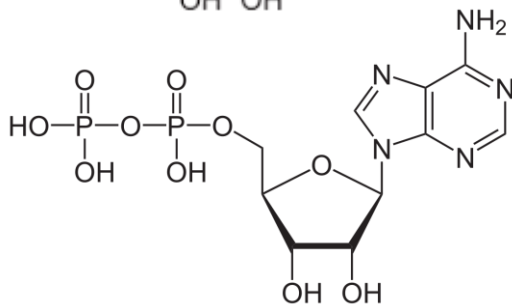
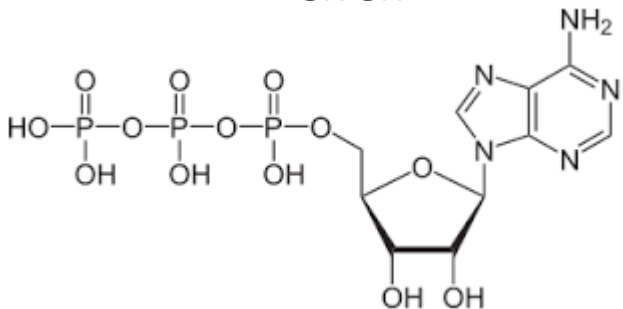
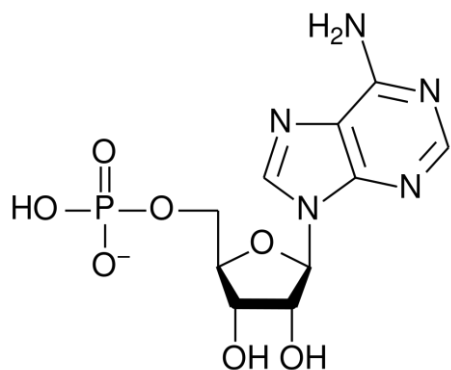
- nové 2 **bio** průtočné cely pro VWD detektor
- nová Hi-Sensitivity průtočná cela s **clonou/filtrem** pro DAD detektor



HPST



# Analýza oligonukleotidů na standardním HPLC systému



## Fosforylované sloučeniny (AMP, ADP, ATP)

- chromatografická výzva pro standardní HPLC systémy (SST průtočné cesty)
- nežádoucí interakce fosfátových skupin s povrchem průtočných cest
- chvostování píků a špatná reprodukovatelnost

## Pasivace systému

- inaktivace polárních povrchových skupin a redukce množství kovů elujícího ze standardního HPLC systému
- časově a finančně náročná operace

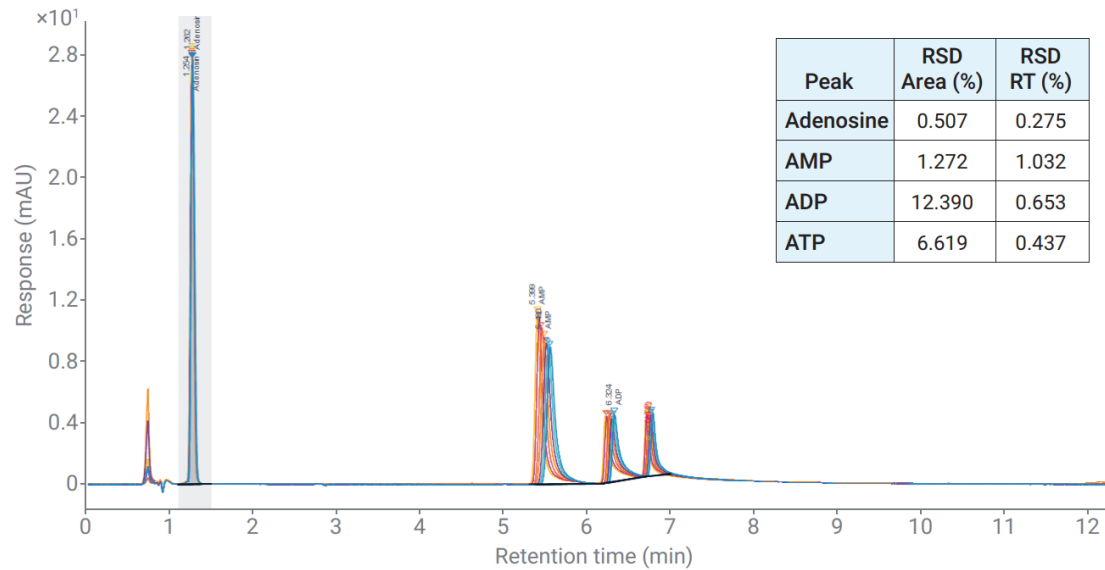
## Málo adsorpční systémy Agilent

- **BIO** INERTNÍ nebo **BIO** KOMPATIBILNÍ HPLC

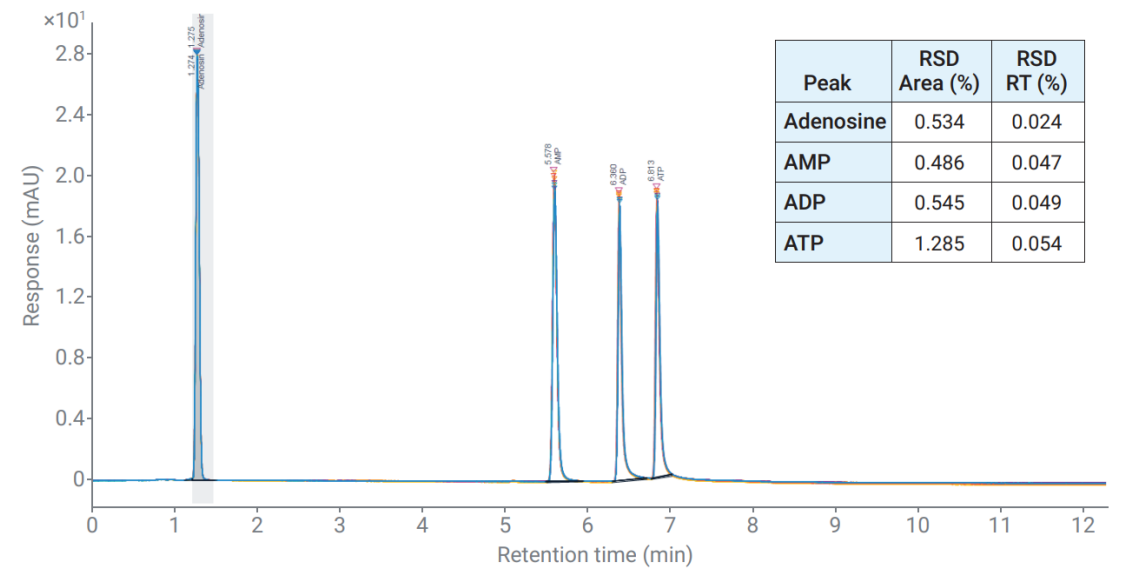


# Standardní HPLC versus BIO HPLC

## Agilent 1290 Infinity II

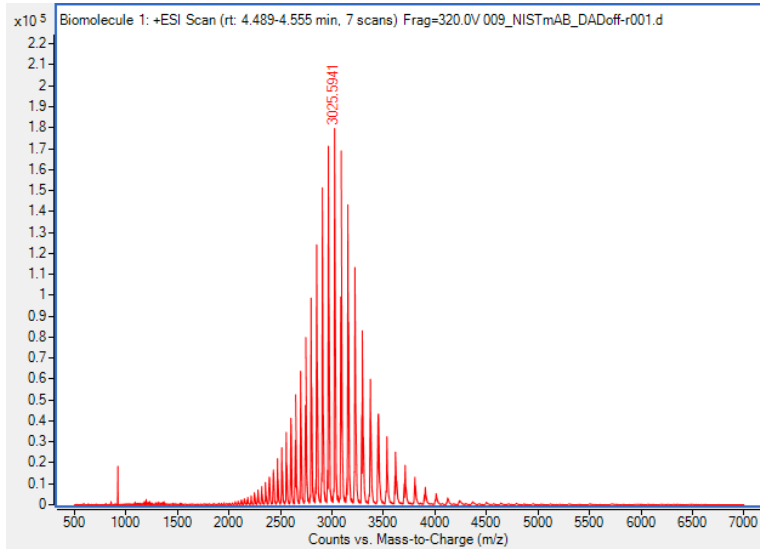


## Agilent 1290 Infinity II BIO LC

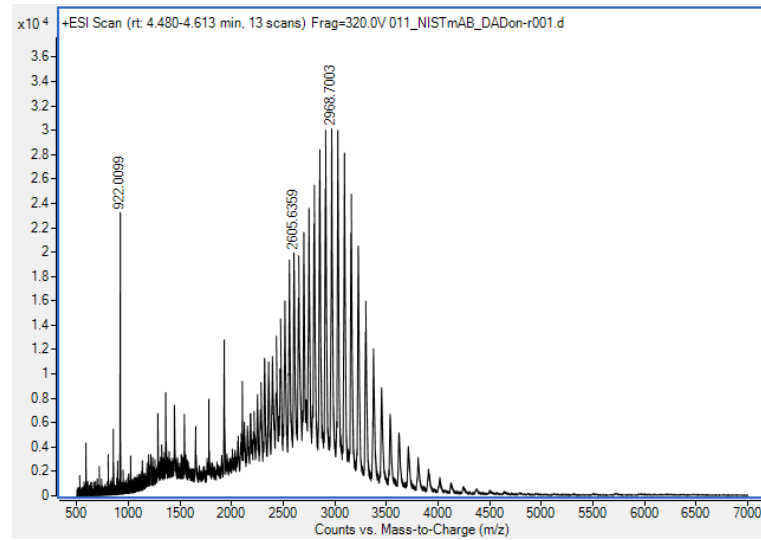




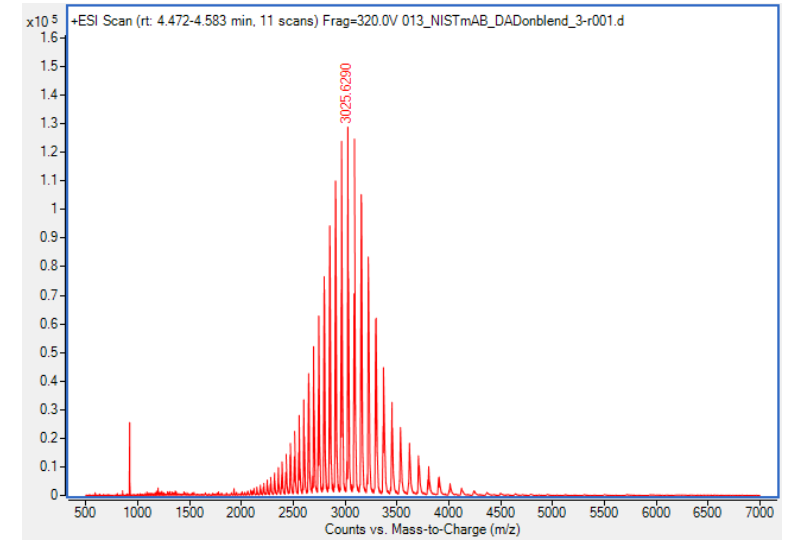
# Fotodegradace



DAD lampa off



DAD lampa on



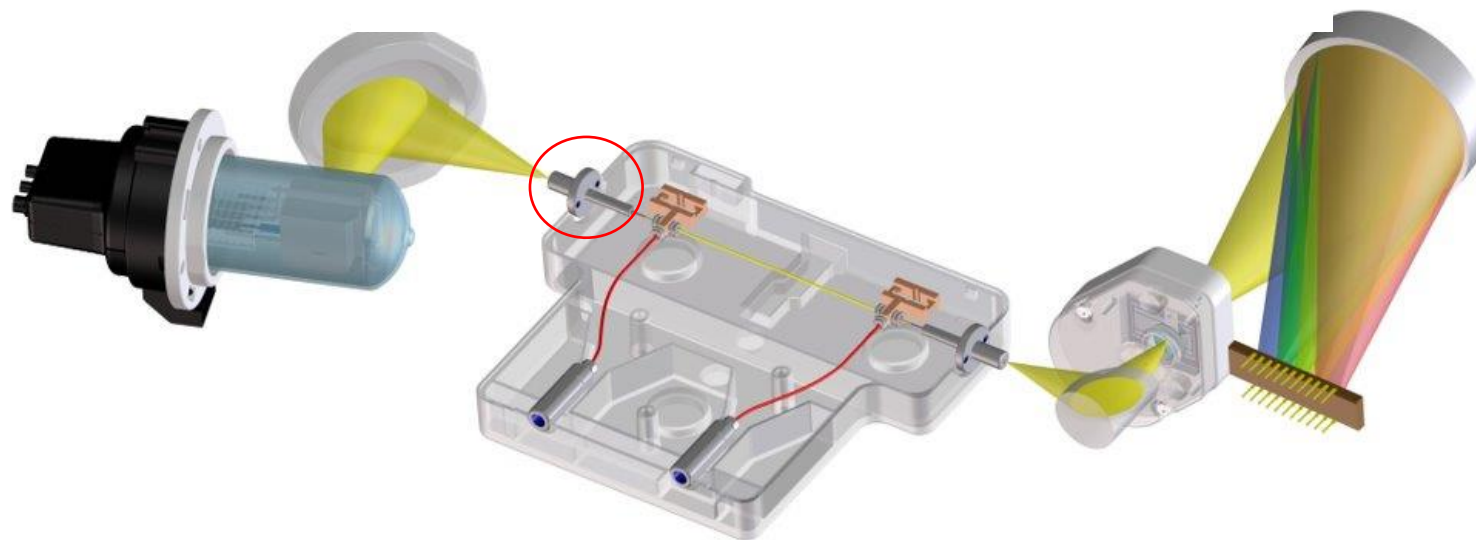
DAD lampa s clonou/filtrem



HPST



# Fotodegradace



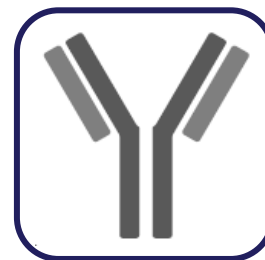
Příprava vzorku

Separace

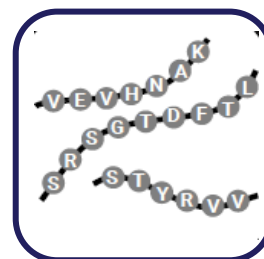
Detekce

Vyhodnocení dat a reporty

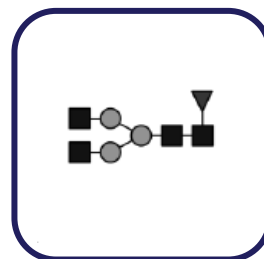
## 6545XT AdvanceBio LC/Q-TOF



**Intaktní proteiny:** vynikající citlivost a kvalita spekter



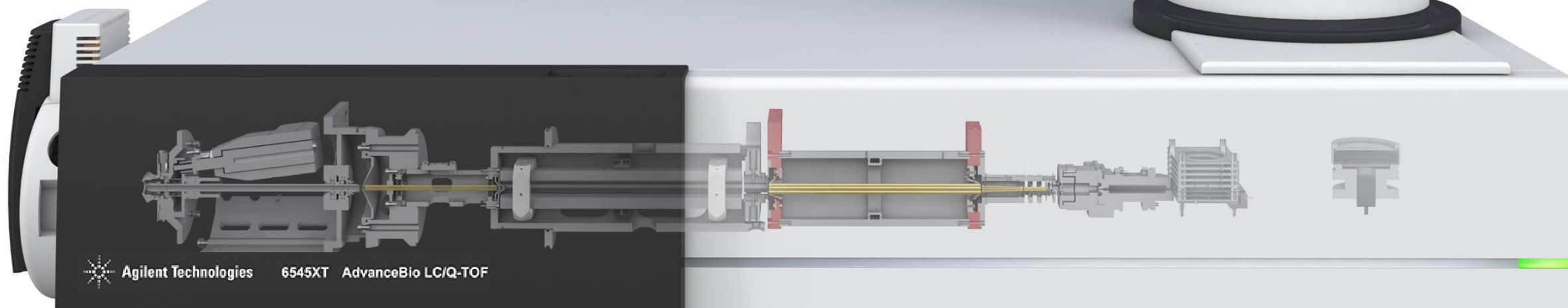
**Mapování peptidů:** rutinní konfirmace nebo hlubší charakterizace pomocí iterativního MS/MS



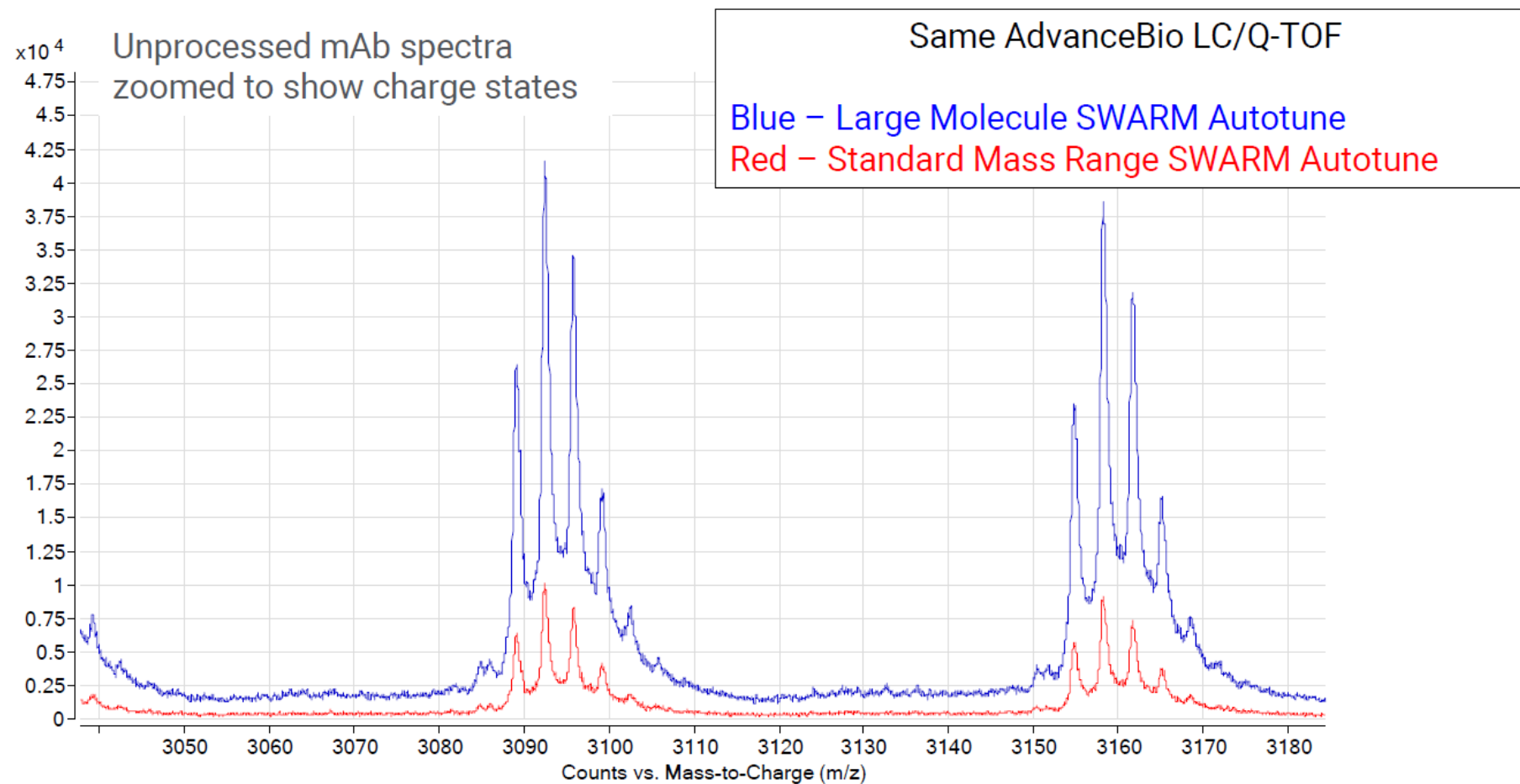
**Profilování glykanů:** plný workflow v Bioconfirm SW

# 6545XT dedikovaný LC/Q-TOF pro biomolekuly

- **Ultra nízké vakuum**  $10E^{-8}$  Torr pro minimální kolize a maximální transmisi velkých molekul
- Vynikající kvalita měření pro intaktní (velké) proteiny, i malé peptidy nebo glykany
- **SWARM autotune**: více módů optimalizace pro velké molekuly NEBO peptidy
- Hmotnostní rozsah **do m/z 30k**
- MS rozlišovací schopnost **50 000** díky lepší iontové optice
- Čistění vstupní optiky **bez zavzdušnění**

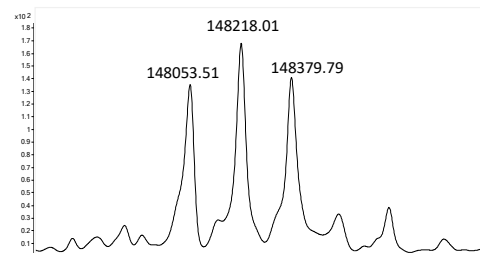
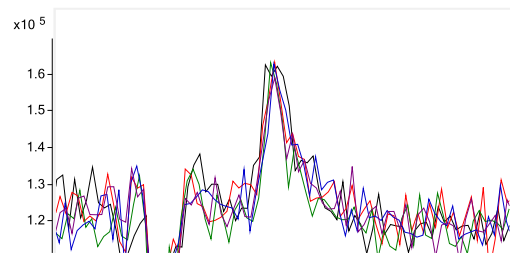


# Ladící algoritmus SWARM optimalizovaný pro velké molekuly

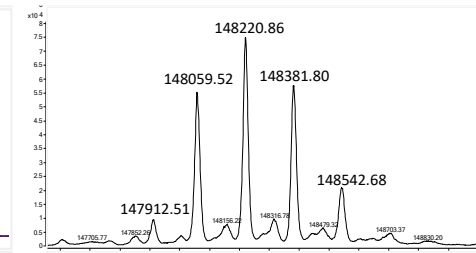
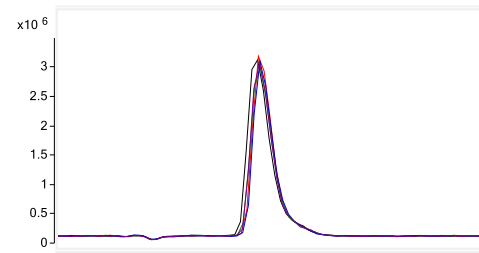


# Citlivost: Trastuzumab

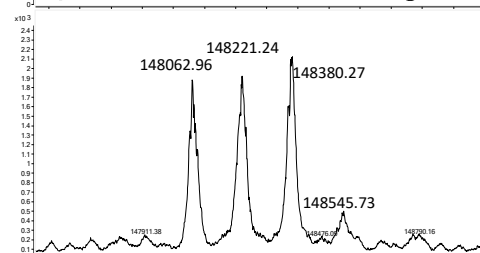
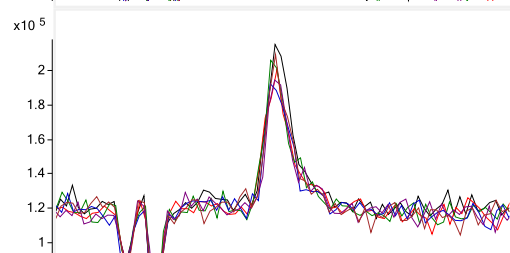
0.0316 ng



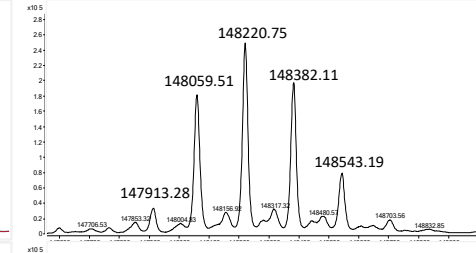
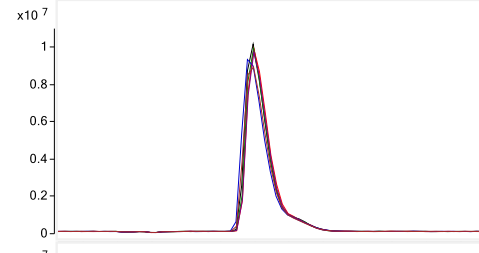
3.16 ng



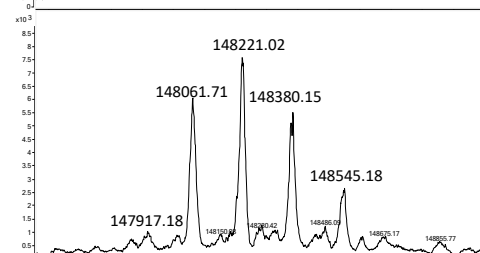
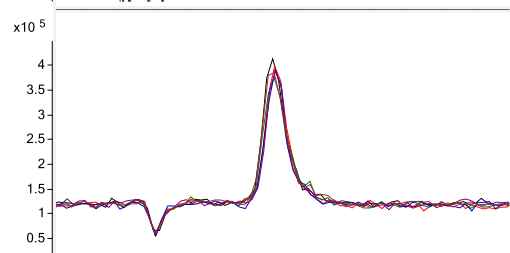
0.1 ng



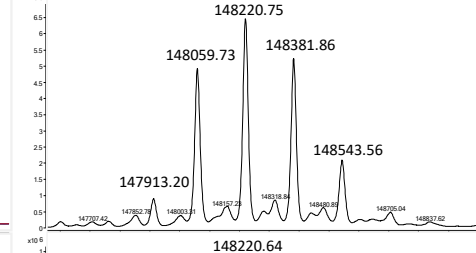
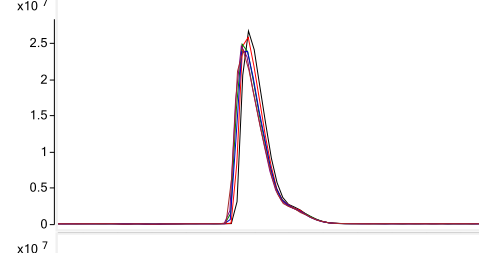
10 ng



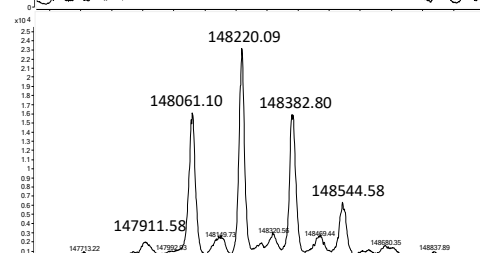
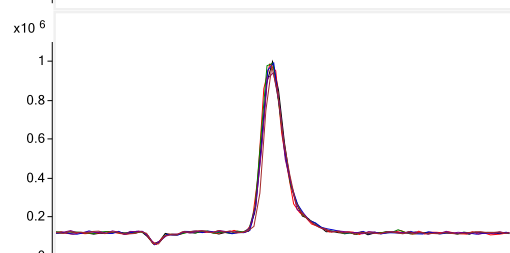
0.316 ng



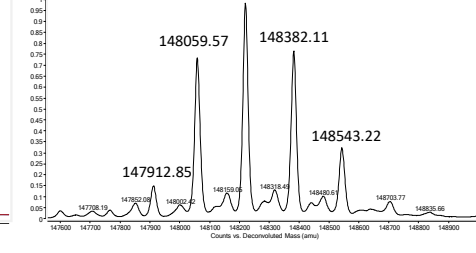
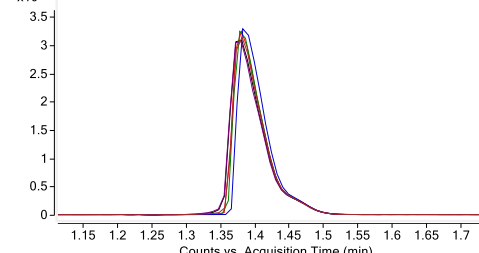
31.6 ng



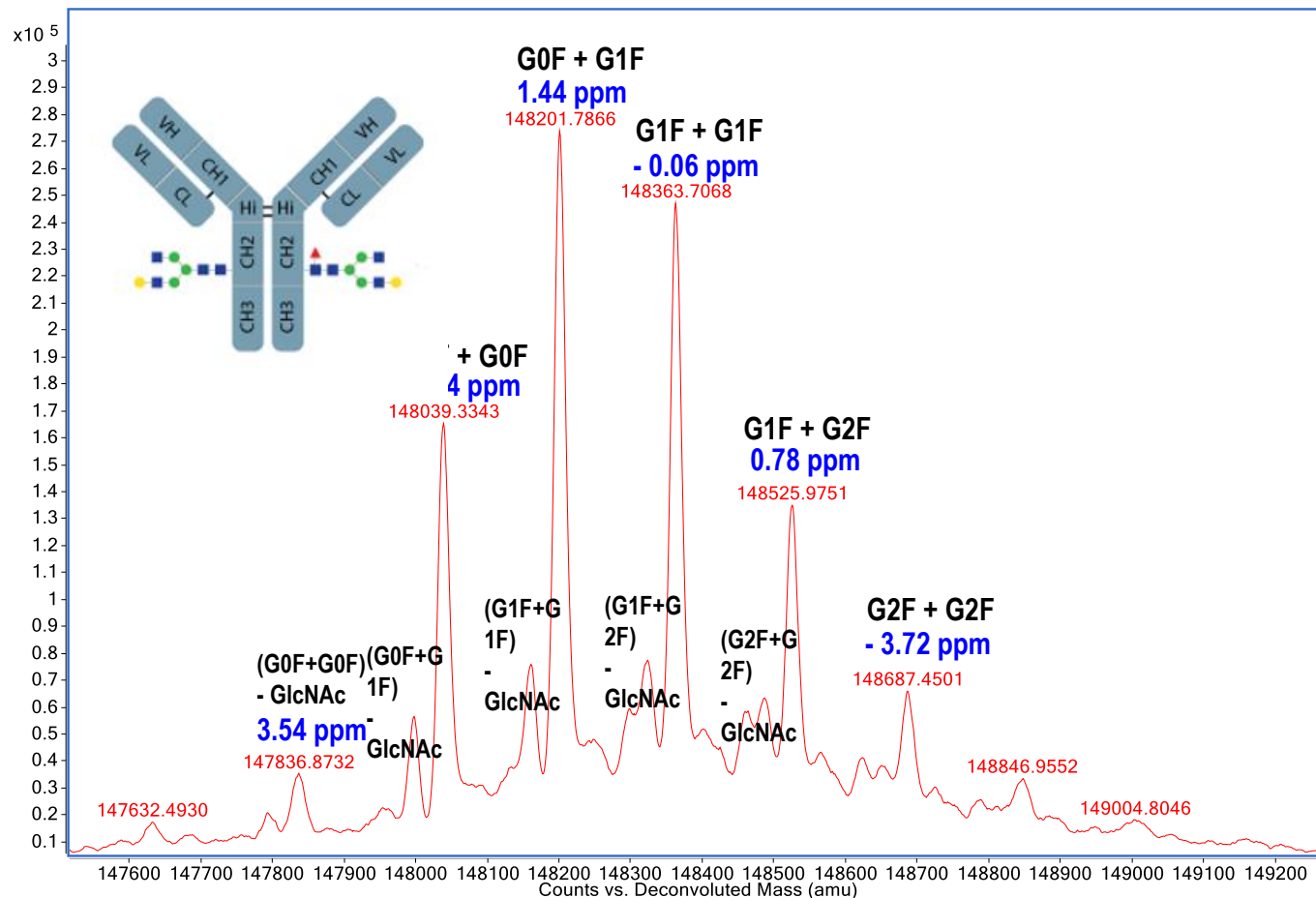
1.0 ng



50 ng



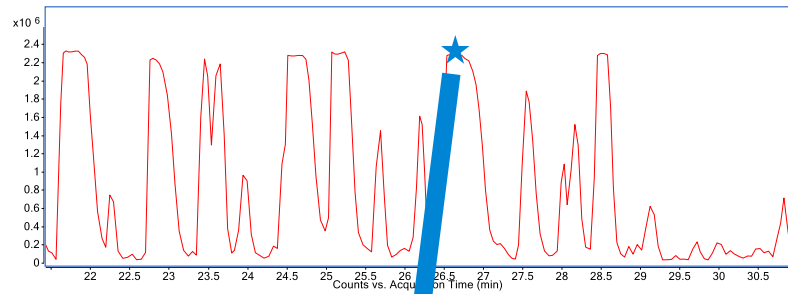
# Přesnost hmoty a detekce izoformem po dekonvoluci



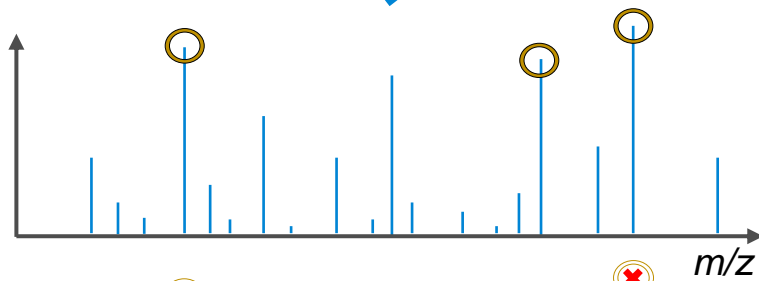
*Analýza intaktní mAb NIST (0.5  $\mu$ g nástřík),  
Nábojová dekonvoluce Maximum Entropy*

Vysoká kvalita spekter umožňuje  
vynikající přesnost hmoty glykoformem a  
detekci minoritních izoformem

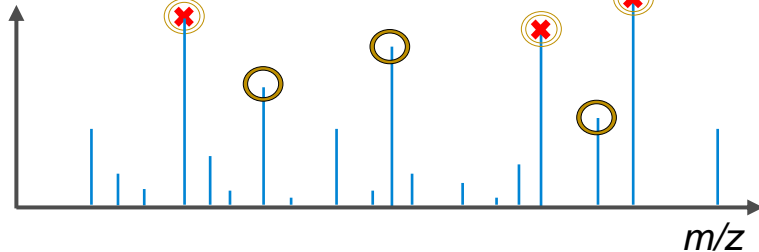
# Iterativní MS/MS – když potřebujete jít hlouběji do vzorku



LC/MS  
Analýza 1



LC/MS  
Analýza 2



Další analýzy ...

- Prekurzory vybrané pro MS/MS
- ⊗ Již dříve fragmentované prekurzory

Technical Overview



## Highly Confident Peptide Mapping of Protein Digests Using Agilent LC/Q-TOFs

### Authors

Stephen Madden,  
Crystal Cody, and  
Jungkap Park  
Agilent Technologies, Inc.  
Santa Clara, California, USA

### Introduction

The biopharmaceutical industry is adding an increasing number of protein therapeutics to its pipelines. With a five-year compound annual growth rate (CAGR) of 7.3 %, it is predicted to attain sales of \$248.7 billion worldwide by 2020<sup>1</sup>. The fastest growing segment with a five-year CAGR of 16.6 % is monoclonal antibodies (mAbs), which have been approved for a range of conditions such as oncology and autoimmune diseases. During the development of protein therapeutics, monitoring is required for a broad set of Critical Quality Attributes (CQAs), among which is confirming the protein sequence and post-translational modifications (PTMs).

Typically, a bottoms-up approach is taken in which the protein is digested with an enzyme to create peptides in the range 5 to 60 amino acids. The peptide mixture is separated by liquid chromatography then detected in a mass spectrometer (MS), which generates mass spectra containing information about them. Given the richness of MS data, there are challenges in finding spectra that have peptide information, then extracting the sequence and PTMs accurately and efficiently. A manual approach by inspection requires significant time and expertise.

The Agilent MassHunter BioConfirm B.09.00 has made a step forward in this peptide mapping workflow for finding peptides and visualizing the results. A software algorithm uses MS/MS data to narrow the list of possible peptides, saving time versus manual inspection. The results are displayed in the Sequence Coverage Map, which allows the user to review multiple samples (for example, from different digest enzymes) at the same time. A new mode in Agilent MassHunter Acquisition software on the Agilent 6545XT AdvanceBio LC/Q-TOF called Iterative MS/MS focuses on low-concentration PTMs, and enables them to be viewed in the Sequence Coverage Map.

Technical Overview P/N 5991-8552EN





Příprava vzorku

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# MassHunter BioConfirm – Vše v jednom pro vyhodnocení dat

Vstup

Sekvence proteinu



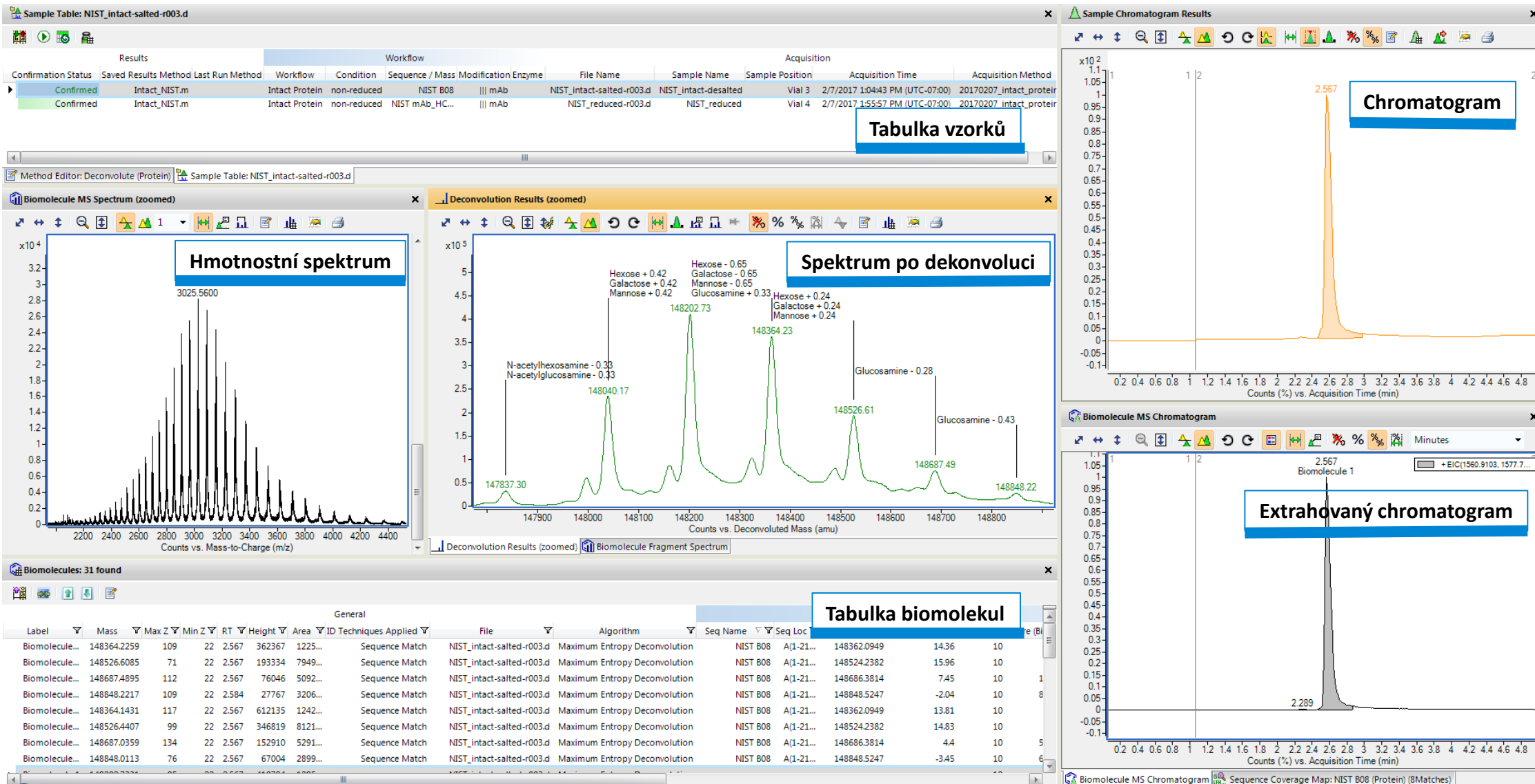
Experimentální podmínky



Výstup

- Potvrzení hmoty
- Glykoformy
- Pokrytí peptidové sekvence
- Profil glykanů

# Zobrazení pro intaktní proteiny



# Zobrazení pro mapování peptidů

Agilent MassHunter BioConfirm Software B.09.00 - BioConfirmProteinDigest-NISTmAb 2.m

File View Find and Identify Method Sequence Configuration Help

Sample Table: NIST mAb Digest\_250 ng-uL\_01.d

Results Workflow

Confirmation Status	File Name	Saved Results Method	Last Run Method	Workflow	Condition	Sequence / Mass	Modification	Enzyme
Partially confirmed	NIST mAb Digest_250 ng-uL_01.d	BioConfirmProteinDigest-NISTmAb 2.m	Protein Digest	reduced	NISTmAb	mAb     Protein Digest (Reduced + Alkylated)	Trypsin,Lys	

**Potvrzení proteinu (červená/oranžová/zelená)**

Sample Chromatogram Results

**Chromatogram**

Biomolecule MS Spectrum

**MS Spektrum**

Biomolecule Fragment Spectrum

**Anotované MS/MS spektrum**

Sequence Coverage Map: NISTmAb (Protein Digest) (64.50%)

**Mapa pokrytí sekvence**

Biomolecules: 501 found

**Nalezené peptidy**

Mining Algorithm	Z Count	ID Techniques Applied	MS/MS Count	Start	End	Seq Name	Seq Loc	Tgt Seq Mass			
Peptide Feature Extraction	1	Sequence Match	2	3.981	4.026						
Peptide Feature Extraction	1	Sequence Match	2	4.076	4.141	NISTmAb	B(60-66)/D(60-66)	857.4396	-0.68	-0.59	HYNPSLK
Peptide Feature Extraction	1	Sequence Match	2	4.102	4.156						
Peptide Feature Extraction	2	Sequence Match	4	4.102	4.201	NISTmAb	B(69-77)/D(69-77)	991.555	-0.37	-0.37	LTISKDTSK
Peptide Feature Extraction	1	Sequence Match	3	4.127	4.226	NISTmAb	B(71-77)/D(71-77)	777.4232	-0.46	-0.36	ISKDTSK
Peptide Feature Extraction	1	Sequence Match	2	4.127	4.201	NISTmAb	B(70-77)/D(70-77)	878.4709	-0.79	-0.69	TISKDTSK
Peptide Feature Extraction	1	Sequence Match	2	4.18	4.226	NISTmAb	B(72-77)/D(72-77)	664.3392	-0.27	-0.18	SKDTSK
Peptide Feature Extraction	3	Sequence Match	5	4.18	4.226	NISTmAb	B(67-77)/D(67-77)	1262.683	0.23	0.29	DRLTISKDTSK
Peptide Feature Extraction	3	Sequence Match	6	4.18	4.442						

Biomolecules: 501 found Biomolecule Identification Results: Biomolecule 181: B(69-77)/D(69-77) NISTmAb



## Příprava vzorku

Agilent AssayMAP Bravo

## Separace

**BioInertní (600 bar) a  
BioCompatibilní (1300 bar)**  
Modulární HPLC

## Detekce

**6545XT  
AdvanceBio LC/Q-TOF**

## Analýza dat

MassHunter **BioConfirm**