New chromatographic workflows for charge variant profiling, intact mAb analysis and DAR determination

Ken Cook, Robert Van Ling
Workflows to Simplify Biopharmaceutical Analysis

• Thermo Fisher Scientific provides innovative solutions

• Solutions that consist of innovative products

• Such as our biopharmaceutical protein characterisation workflows
5 Fundamental Workflows

**Peptide mapping**
- Confirm sequence
- Analytical reproducibility
- Peak capacity

**Aggregate analysis**
- Check monomer vs Aggregates
- High salt condition, biocompatible, low dispersion, reproducibility

**Charge variant analysis**
- Check charge variation within antibody sample
- Biocompatible selectivity

**Intact protein analysis**
- Check purity of the antibody
- Biocompatible, gradient and thermostating capabilities

**Glycan analysis**
- Check glycan presence and structure
- Gradient capabilities
5 Fundamental Workflows

- **Peptide mapping**
  - Reversed Phase
  - Confirm sequence
  - Analytical reproducibility
  - Peak capacity

- **Aggregate analysis**
  - SEC
  - HIC
  - Check monomer vs Aggregates
  - High salt condition, biocompatible, low dispersion, reproducibility

- **Charge variant analysis**
  - IEX
  - Check charge variation within antibody sample
  - Biocompatible selectivity

- **Intact protein analysis**
  - RP
  - HIC
  - Check purity of the antibody
  - Biocompatible, gradient and thermostating capabilities

- **Glycan analysis**
  - HILIC
  - Mixed Mode
  - Check glycan presence and structure
  - Gradient capabilities
5 Fundamental Workflows

- **Peptide mapping**
  - Reversed Phase
  - Confirm sequence
  - UV and UV-MS
  - Analytical reproducibility
  - Peak capacity
  - Confirm sequence
  - Check monomer vs Aggregates
  - High salt condition, biocompatible, low dispersion, reproducibility

- **Aggregate analysis**
  - SEC
  - HIC
  - UV (UV-MS)

- **Charge variant analysis**
  - IEX
  - Check charge variation within antibody sample
  - UV
  - Biocompatible selectivity

- **Intact protein analysis**
  - RP
  - HIC
  - UV and UV-MS
  - Check purity of the antibody
  - Biocompatible, gradient and thermostating capabilities

- **Glycan analysis**
  - HILIC
  - Mixed Mode
  - FLD, FLD-MS, CAD
  - Gradient capabilities
Protein and MAb Separation by LC

- Protein properties:
  - Size
  - Charge
  - Hydrophobicity
  - Affinity or Recognition
Protein and MAb Separation by LC

- **Size difference?**
  - NO → Charge difference?
    - NO → Hydrophobicity difference?
      - NO → **Size Exclusion Chromatography (SEC)**
      - YES → **Reverse Phase Chromatography (RPC)**
    - YES → **Ion Exchange Chromatography (IEC) pH-Gradient**
  - YES → **Hydrophobic Interaction Chromatography (HIC)**

- **Protein properties:**
  - Size
  - Charge
  - Hydrophobicity
  - Affinity or Recognition

- **Thermo Scientific™ MAbPac™ SEC-1**
- **Thermo Scientific™ MAbPac™ SCX-10**
- **Thermo Scientific™ ProPac™ WCX-10**
- **Thermo Scientific™ MAbPac™ HIC-10**
- **Thermo Scientific™ MAbPac™ HIC-20**
- **Thermo Scientific™ MAbPac™ HIC-Butyl**
LC Systems for Bio-Therapeutic Protein Analysis

Thermo Scientific™ Vanquish™ UHPLC & Thermo Scientific™ Vanquish™ Flex UHPLC systems

Thermo Scientific™ UltiMate™ 3000 BioRS system

Distribution of applications

- Highly specific Workflows: 20%
- Standard (biopharma) applications: 80%
LC Systems for Bio-Therapeutic Protein Analysis

Thermo Scientific™ Vanquish™ UHPLC & Thermo Scientific™ Vanquish™ Flex UHPLC systems

High Resolution, Cooled Fractionation

Thermo Scientific™ UltiMate™ 3000 BioRS system

pH and Conductivity

Distribution of applications

20%
80%
Highly specific Workflows
Standard (biopharma) applications
1. **Buffers**
   - Thermo Scientific™ CX-1 pH Gradient buffers, 10X concentrated

2. **Chemistries**
   - MAbPac SCX-10 columns

3. **Separations & Detection**
   - Vanquish Flex UHPLC or UltiMate 3000 BioRS UHPLC system
   - Automated desalting on polymeric Thermo Scientific™ MSPac™ DS-10 de-salter cartridge

4. **Characterization**
   - Thermo Scientific™ Exactive™ Plus or Thermo Scientific™ Q Exactive™ Plus Mass Spectrometer
   - Thermo Scientific™ BioPharma Finder™ Software
   - Full scan method, intact mass deconvolution
Next-generation CEX Column – MAbPac SCX-10

ProPac WCX-10 column, 4 × 250 mm
Next-generation CEX Column – MAbPac SCX-10

MAbPac SCX-10 column, 4 × 250 mm

60 min. total analysis time

ProPac WCX-10 column, 4 × 250 mm
Next-generation CEX Column – MAbPac SCX-10

MAbPac SCX-10 column, 4 × 250 mm

60 min. total analysis time

ProPac WCX-10 column, 4 × 250 mm

MAbPac SCX-10 column, 4 × 150 mm

15 min. total analysis time
Next-generation CEX Column – MAbPac SCX-10

MAbPac SCX-10 column, 4 × 250 mm

Lysine Variants

Acidic Variants

Basic Variants

60 min. total analysis time

ProPac WCX-10 column, 4 × 250 mm

Lysine Variants

Acidic Variants

Basic Variants

MAbPac SCX-10 column, 4 × 150 mm

15 min. total analysis time

Improve resolution or sample throughput through column chemistry
Charge Variant Analysis by CEX

Salt gradient elution
- Based on ionic strength
- Competition / displacement for interaction with functional groups of IEX matrix
- Multiple interactions with IEX matrix

\[
\begin{align*}
\text{SO}_3^- + \text{Na}^+ &\rightleftharpoons \text{Na}_2\text{SO}_3 \\
\text{SO}_3^- + \text{Na}^+ &\rightleftharpoons \text{Na}_2\text{SO}_3 \\
\text{SO}_3^- \text{Na}^+ &\rightleftharpoons \text{Na}_2\text{SO}_3
\end{align*}
\]

pH gradient elution
- Based on pI of protein
- Loss of retention with progressive pH gradient, depending on pI
- "Single" binding event, trapping at pH < pI (for CEX)
Comparison of pH gradient buffer systems

In house buffer system 1

Thermo Scientific CX-1
pH 5.6 and 10.2

Phosphate based
Thermo Scientific CX-1 pH Gradient Buffers

• Dilute buffers 10-fold with DI water
• A linear pH gradient (pH 5.6 - 10.2) is generated by running a linear pump gradient from 100% Buffer A to 100% Buffer B
• Generic, fast & high-resolution!

<table>
<thead>
<tr>
<th></th>
<th>Buffer A</th>
<th>Buffer B</th>
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</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.6</td>
<td>10.2</td>
</tr>
<tr>
<td>Form</td>
<td>Liquid</td>
<td>Liquid</td>
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<tr>
<td>Concentrate</td>
<td>10X</td>
<td>10X</td>
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<tr>
<td>Shipping condition</td>
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<td>Room Temp</td>
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<tr>
<td>Storage condition</td>
<td>4 ~ 8 °C</td>
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Thermo Scientific CX-1 pH Gradient Buffers

- Dilute buffers 10-fold with DI water
- A linear pH gradient (pH 5.6 - 10.2) is generated by running a linear pump gradient from 100% Buffer A to 100% Buffer B
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mAb Standards Using Linear pH Gradient

MAbPac SCX 4 x 250mm

pH trace

Retention Time (min)
Fast, Generic and Linear pH Gradient – Vanquish UHPLC

pH 5.6 to 10.2 in 10 minutes, MAbPac SCX-10, 2 x 50 mm
Infliximab – Vanquish System Ultra-fast Gradients

Resolution and number of charge variants maintained in sub-minute gradients

3 steps method development
1. 10 minutes 0→100% B in 10 minutes
2. 20→40% B in 5 minutes
3. 18→27% B in 0.8 minutes
1. **Preparation**
   - Protein A, Enzymatic reduction, IEC pH-gradient, HIC, SEC,

2. **Chemistries**
   - Multi-dimension analysis
   - IEC pH-gradient, HIC, SEC

3. **Separations & Detection**
   - Vanquish Flex UHPLC or UltiMate 3000 BioRS UHPLC system
   - Automated desalting on MSPac DS-10 de-salter cartridge

4. **Characterization**
   - Exactive Plus or Q Exactive Plus Mass Spectrometer
   - BioPharma Finder Software
   - Full scan method, intact mass deconvolution
In-depth HRAM Charge Variant Characterization

1$^{\text{st}}$ dimension: IEX pH gradient + fraction collection

2$^{\text{nd}}$ dimension: Polymer RP-LC/MS
1: mAb injection (peak area = 37.97)

2: blank (Peak area = 0.233)

Column: MAbPac RP, 4 µm
Format: 3 × 50 mm
Mobile phase A: H₂O/TFA (99.9 : 0.1 v/v)
Mobile phase B: MeCN/ H₂O/TFA (90: 9.9 :0.1 v/v/v)
Gradient:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
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<tbody>
<tr>
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<td>1.0</td>
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<td>11.0</td>
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<td>100</td>
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<td>14.0</td>
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<td>0</td>
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<tr>
<td>15.0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Temperature: 80 ºC
Flow rate: 0.5 mL/min
Inj. volume: 5 µL
Detection: UV (280 nm)
Sample: mAb (5 mg/mL)
Carryover: Polymeric MAbPac RP

Column: MAbPac RP, 4 µm
Format: 3 × 50 mm
Mobile phase A: H₂O/TFA (99.9 : 0.1 v/v)
Mobile phase B: MeCN/ H₂O/TFA (90: 9.9 :0.1 v/v/v)
Gradient:

<table>
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</tr>
</tbody>
</table>

Temperature: 80 ºC
Flow rate: 0.5 mL/min
Inj. volume: 5 µL
Detection: UV (280 nm)
Sample: mAb (5 mg/mL)

Carryover <0.62%

1: mAb injection (peak area = 37.97)
2: blank (Peak area = 0.233)
Hydrophobic Interaction Chromatography

Column: MAbPac HIC-10, 4.6 × 100 mm
Competitor A (Ether), 7.5 × 75 mm
Competitor B (Butyl), 4.6 × 100 mm

Mobile phase A: 2.0 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0

Gradient:

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
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<td>40</td>
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<tr>
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<tr>
<td>1.0</td>
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<tr>
<td>20.0</td>
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<td>100</td>
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</tbody>
</table>

Temperature: 30 ºC
Flow rate: 1.0 mL/min
Inj. volume: 2 µL (4 mg/mL)
Competitor A (Ether): 4 µL
Detection: UV (280 nm)
Sample: mAb
Global Analysis of Native mAbs

Column: MAbPac HIC-10, 5 µm
Format: 4.6 × 100 mm
Mobile phase A: 2.0 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

<table>
<thead>
<tr>
<th>Time (min)</th>
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<tr>
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</table>

Temperature: 30 ºC
Flow rate: 1.0 mL/min
Inj. volume: 2 µL (4 mg/mL)
Detection: UV (280 nm)
Sample: mAb1, mAb2, mAb3, mAb4, mAb5

Absorbance (mAU) vs. Retention Time (min)
Separation of Oxidized mAb on MAbPac HIC-20

Column: MAbPac HIC-20, 5 µm
Format: 4.6 × 250 mm
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
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<td>30.0</td>
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<tr>
<td>35.0</td>
<td>0</td>
<td>100</td>
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</table>

Temperature: 30 ºC
Flow rate: 0.5 mL/min
Inj. volume: Untreated mAb: 20 µL (1.25 mg/mL)
Oxidized mAb: 20 µL (1.25 mg/mL)
Detection: UV (280 nm)
Sample: Untreated mAb

Retention Time (min) vs. Absorbance (mAU)
Separation of Oxidized mAb on MAbPac HIC-20

Column: MAbPac HIC-20, 5 µm
Format: 4.6 × 250 mm
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

<table>
<thead>
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<td>100</td>
</tr>
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</table>

Temperature: 30 ºC
Flow rate: 0.5 mL/min
Inj. volume: Untreated mAb: 20 µL (1.25 mg/mL)
Oxidized mAb: 20 µL (1.25 mg/mL)
Detection: UV (280 nm)
Sample: Untreated mAb
H₂O₂ oxidized mAb
Separation of Cys-linked ADC

Column: MAbPac HIC-Butyl, 5 µm
Format: 4.6 × 100 mm
Mobile phase A: 1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)
Mobile phase B: 50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)
Gradient:

<table>
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<tr>
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<td>100</td>
</tr>
<tr>
<td>20.0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
Temperature: 25 ºC
Flow rate: 1.0 mL/min
Inj. volume: 5 µL (5 mg/mL)
Detection: UV (280 nm)
Sample: Cys-conjugated ADC mimic
**mAb and mAb Fragments Analysis – Reversed Phase**

- **Column:** MAbPac RP, 4 µm
- **Format:** 3 × 50 mm
- **Mobile phase A:** H₂O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
- **Mobile phase B:** MeCN/ H₂O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
- **Gradient:**
  - Time (min) %A %B
  - 0.0  80  20
  - 1.0  80  20
  - 11.0  55  45
  - 12.0  55  45
  - 14.0  80  20
  - 15.0  80  20
- **Temperature:** 80 ºC
- **Flow rate:** 0.5 mL/min
- **Inj. volume:** 5 µL
- **Detection:** UV (280 nm)
- **Sample:**
  - (a) trastuzumab (5 mg/mL)
  - (b) trastuzumab + DTT (4 mg/mL)
  - (c) trastuzumab + Papain (2 mg/mL)
  - (d) trastuzumab + IdeS (2 mg/mL)
LC/MS Analysis of Reduced mAb

Column: MAbPac RP, 4 µm
Format: 3 × 50 mm
Mobile phase A: H₂O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile phase B: MeCN / H₂O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
Gradient:

<table>
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<th>Time (min)</th>
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<tbody>
<tr>
<td>0.0</td>
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</table>

Temperature: 80 ºC
Flow rate: 0.5 mL/min
Inj. volume: 1 µL
UV Detection: 280 nm
MS Detection: positive-ion mode
Mass Spec: Q Exactive Plus
Sample: reduced trastuzumab (4 mg/mL)
Heterogeneity of SiteClick™ N-glycan Labeling of Antibody

DAR 1

DAR 2

DAR 3

DAR 4
RP Separation of Unmodified mAbs and ADCs

Column: MAbPac RP, 4 µm
Format: 2.1 × 50 mm
Mobile phase A: H₂O/TFA (99.9 : 0.1 v/v)
Mobile phase B: MeCN/ H₂O/TFA (90: 9.9 :0.1 v/v/v)

Retention Time (min)

MS raw data

DAR 0
DAR 1
DAR 2
DAR 3
DAR 4

Retention Time (min)
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Thermo Fisher Scientific, UK
  Ken Cook

Thermo Fisher Scientific, Germany
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