Development of an SDS-CGE method for purity determination of acellular *Bordetella pertussis* vaccines

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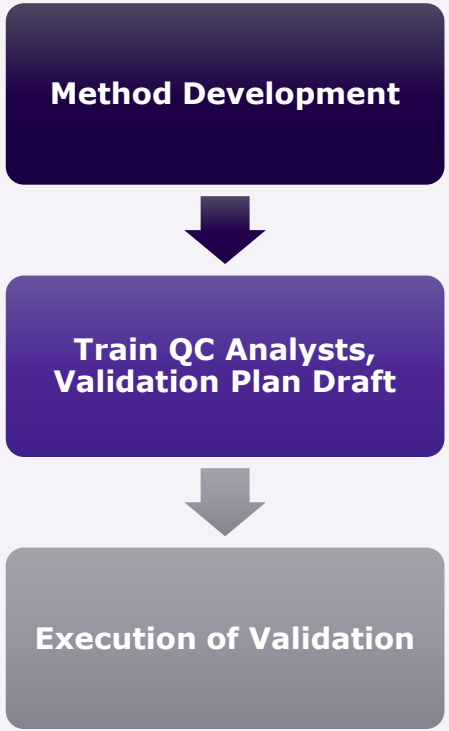
Overview



Objective:

Development of an SDS-CGE method for purity determination of acellular component Bordetella pertussis drug substances (DS)

- **Current QC release assay for DS component pertussis (cP) purity:** SDS-PAGE with densitometry

- **Six matrices (manufactured from cell-derived process):**
  - Pre-adsorbed Fimbriae 2/3 (FIM) – release
  - Pre-adsorbed Pertactin (PRN) – release
  - Pre-adsorbed Pertussis Toxin (PT) – release
  - Pre-adsorbed Filamentous Hemagglutinin (FHA) – release
  - Adsorbed Fimbriae 2/3 (FIM-ads) – stability monitoring
  - Adsorbed Pertactin (PRN-ads) – stability monitoring

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Capillary Gel Electrophoresis (CGE) for Purity Determination

- **Analytical Target:**
  - Sensitivity: 1% impurity detection
  - Resolution: baseline separation of antigen peaks
  - Unified method: suitable for all four antigens
- **Method parameters already established:** antigen precipitation with TCA, instrument parameters, sample preparation
- **Method parameters to be evaluated:**
  - Optimization of method for antigen-specific challenges – reducing agent optimization, identification of unknown peaks, resolution optimization, low peak area
  - Resolution stress and assay validity criteria
  - Method robustness
  - Validation execution

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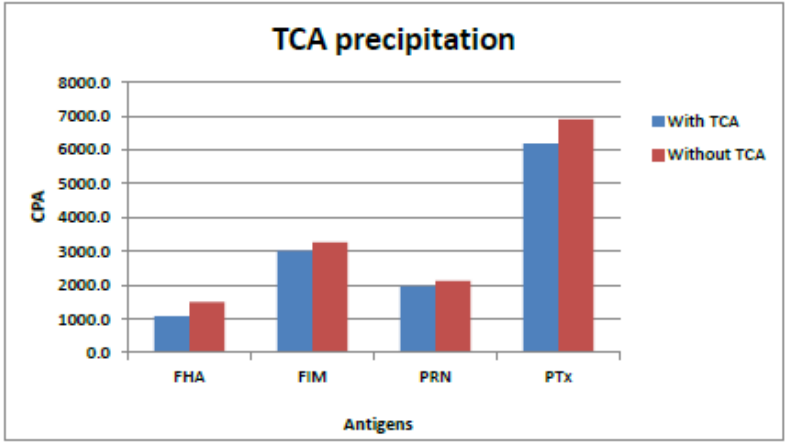
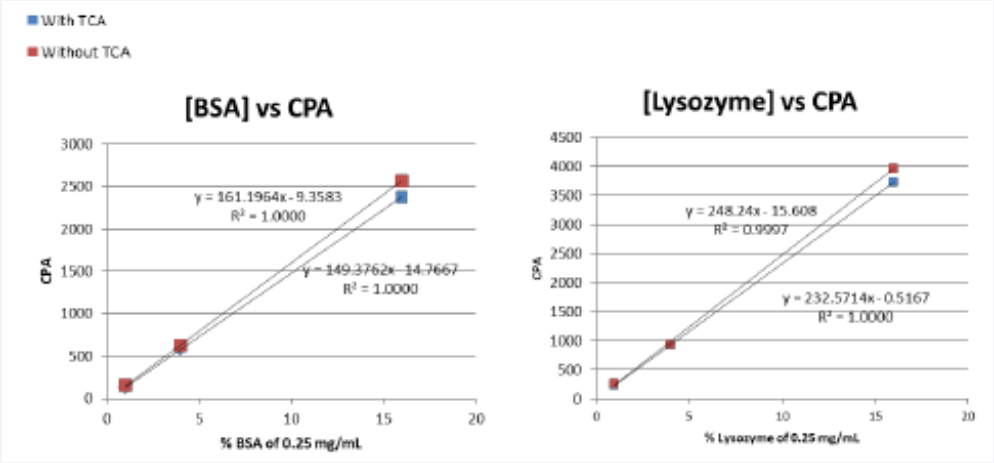
Trichloroacetic Acid (TCA) Precipitation





- **TCA precipitation:** prevent matrix effects, concentrate samples
- Corrected peak area (CPA) of antigen peaks not significantly different with TCA precipitation
- Mock impurity with antigens spiked with BSA or lysozyme: mock impurity recovery is not significantly different with and without TCA precipitation

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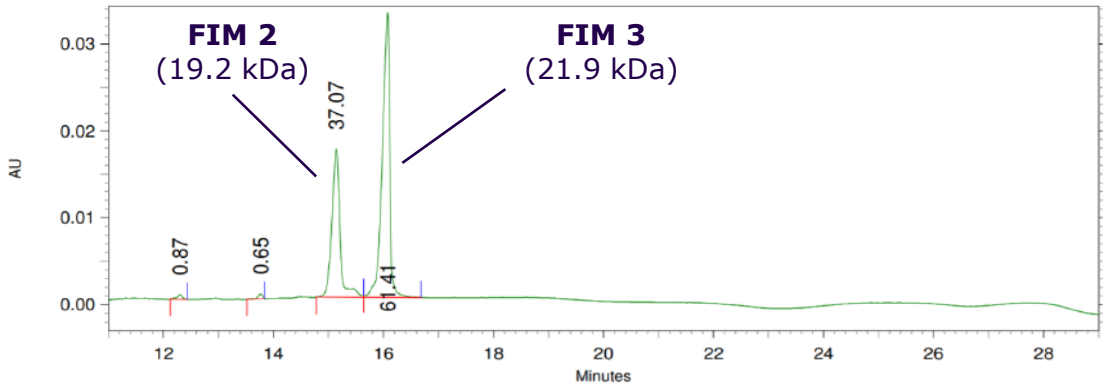
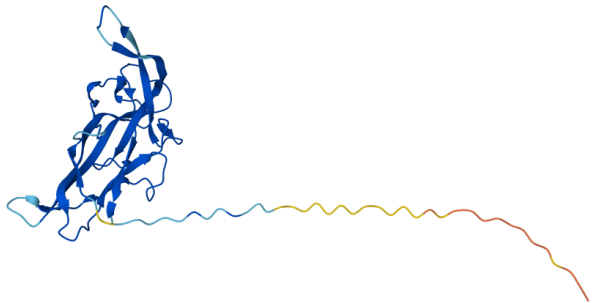
Fimbriae 2/3 (FIM)

- **Fimbriae:** hair-like, appendages extending from bacterial surface for motility, adhesion, conjugation, intercellular interactions, DNA uptake, and biofilm formation
- Two serologically distinct but similar structures that are co-purified, both α-helical – known to form oligomers by dynamic light scattering (DLS)



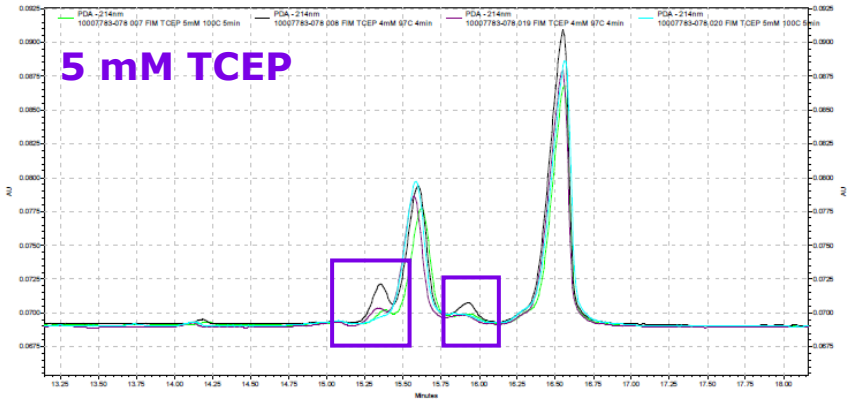
FIM 2 Structure

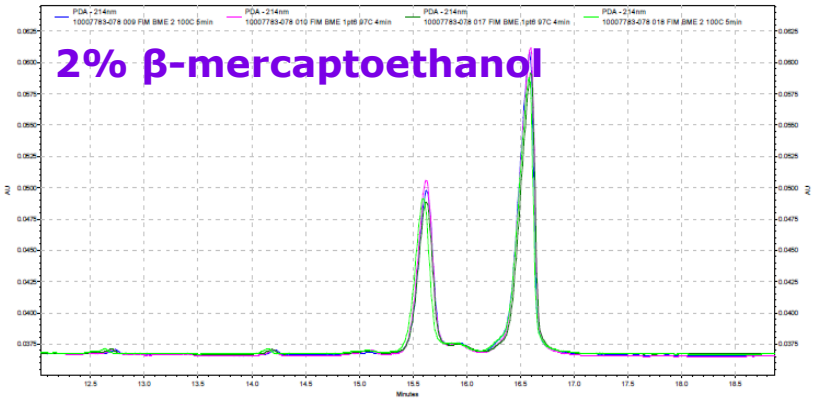


Source: AlphaFold (P05788)

- **Specific challenge:** Complete reduction of disulfide bonds

Fimbriae 2/3 (FIM) – Reducing Agent Selection

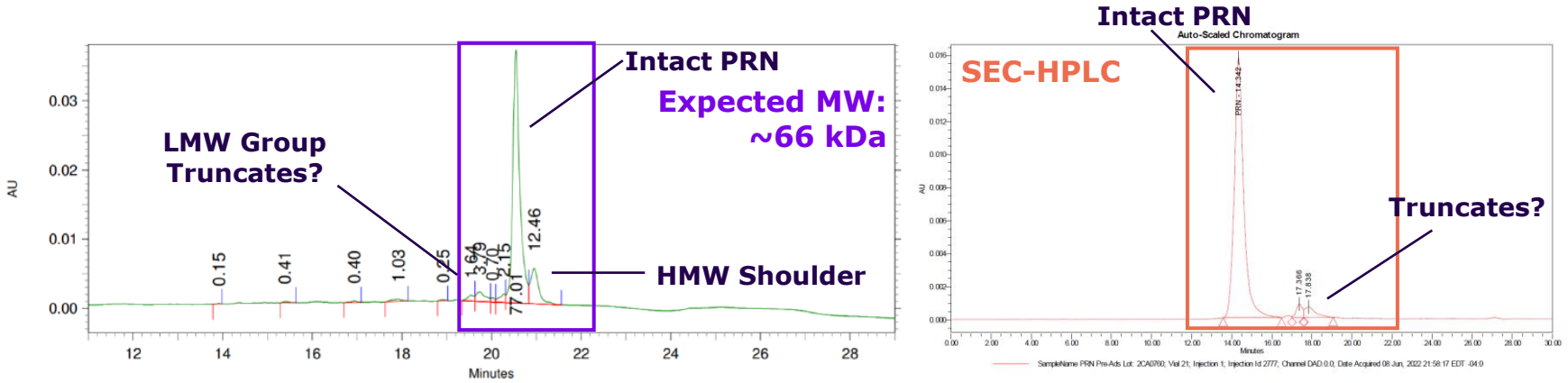




- **Tris(2-carboxyethyl)phosphine (TCEP):** aim to replace β-mercaptoethanol as reducing agent with a preferred health & safety alternative
  - Recovery loss observed with TCEP with lower total electropherogram CPA by up to 49%
  - Appearance of FIM shoulders – incomplete reduction, instability throughout sequence
- **Decision:** Use of TCEP not feasible as reducing agent for this workflow

Pertactin (PRN)

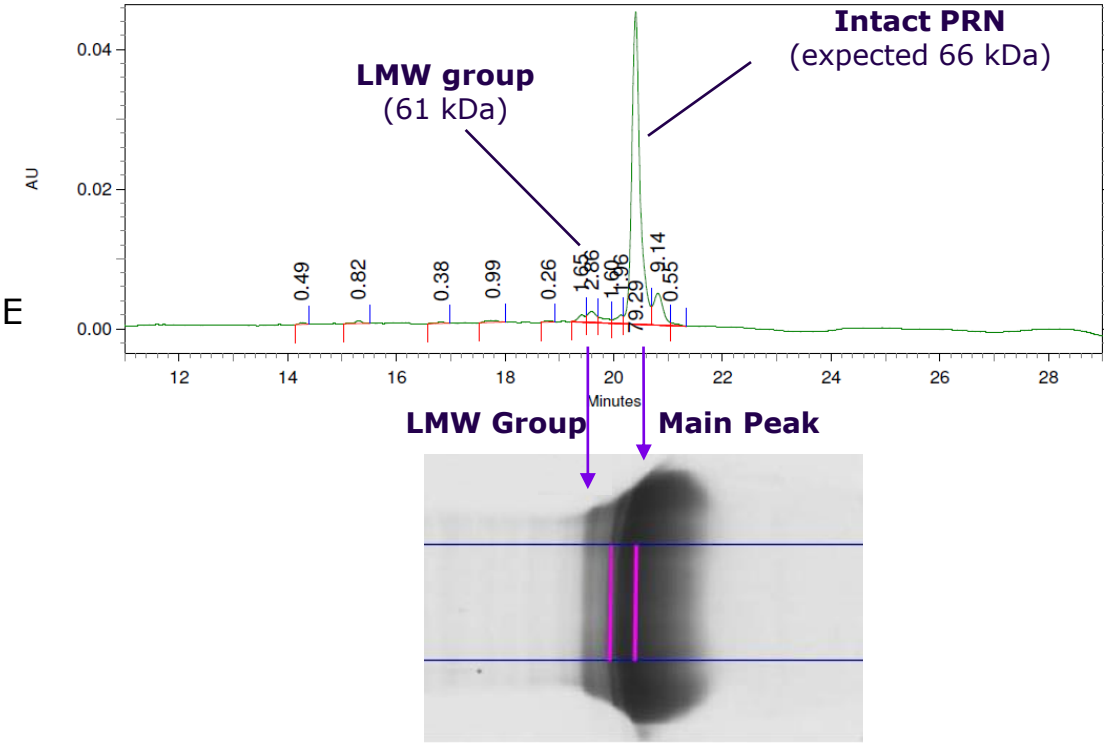
- **Pertactin:** outer membrane protein responsible for epithelial adhesion, 16-stranded parallel β-helix



- Presence of LMW group and HMW shoulder in addition to PRN main peak
- SEC-HPLC confirms presence of LMW group
- **Specific challenge:** Identification of product-related LMW and HMW peaks

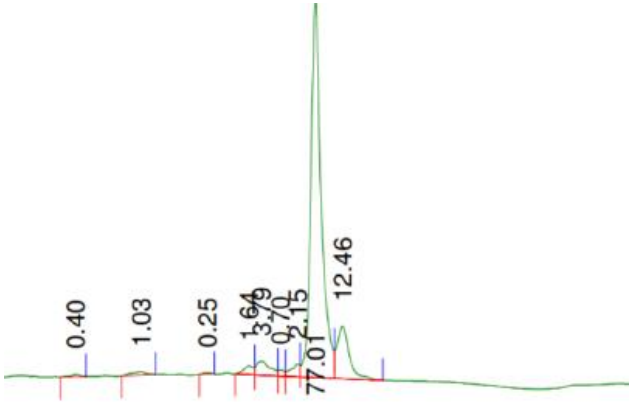
Pertactin (PRN) Peak Identification – LMW Group

- PRN LMW group present in electropherogram and MW matches with SDS-PAGE gel
- Mass spectrometry analysis of gel digest band from SDS-PAGE revealed product-related substance (truncate)
  - **Decision:** LMW group is included in purity value to be consistent with current SDS-PAGE purity release method



Pertactin (PRN) Peak Identification – HMW Group

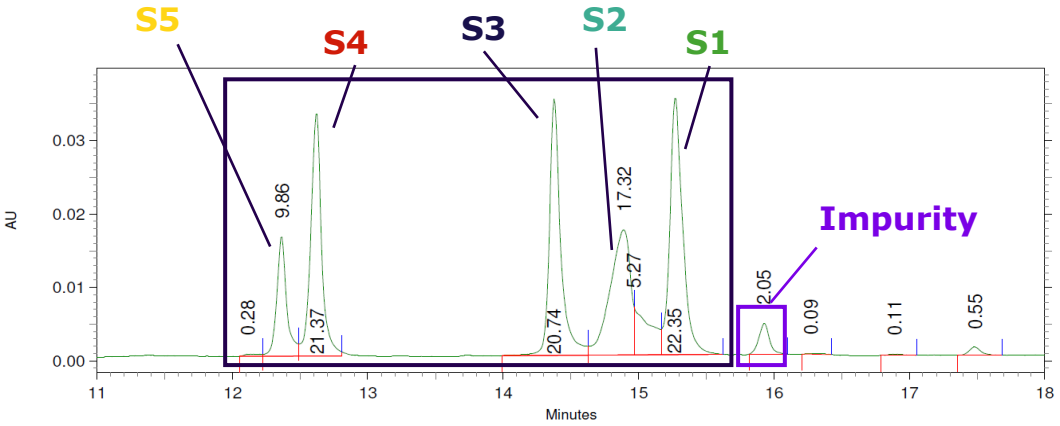
- **Ion mobility spectrometry (IMS):** this workflow was used to measure collisional cross-sectional area in gas phase to determine different conformational states of same MW
  - Major and minor conformational species corresponding to main peak and HMW respectively
- **SDS-CGE:** May adopt folded or partially denatured state even in presence of detergent, core may remain undenatured
- **Decision:** HMW shoulder to be included in purity value (consistent with current SDS-PAGE purity release method)

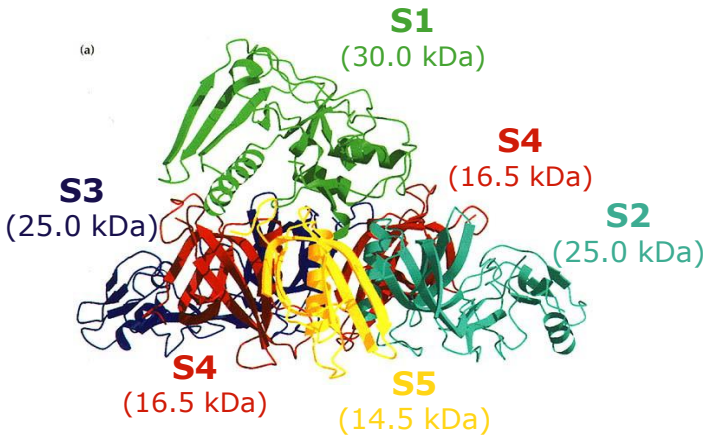


Emsley, P *et al.* (1996). *Nature.*

Pertussis Toxin (PT)

- Hexamer – five subunits (two S4 subunits per hexamer), intermolecular disulfide bonds, subunits on electropherogram assigned based on MW





Stein, PE *et al.* (1994). *Cell.*

- **Specific challenges:**
  - Identification of the impurity peaks and S2 HMW shoulder
  - Improve peak resolution

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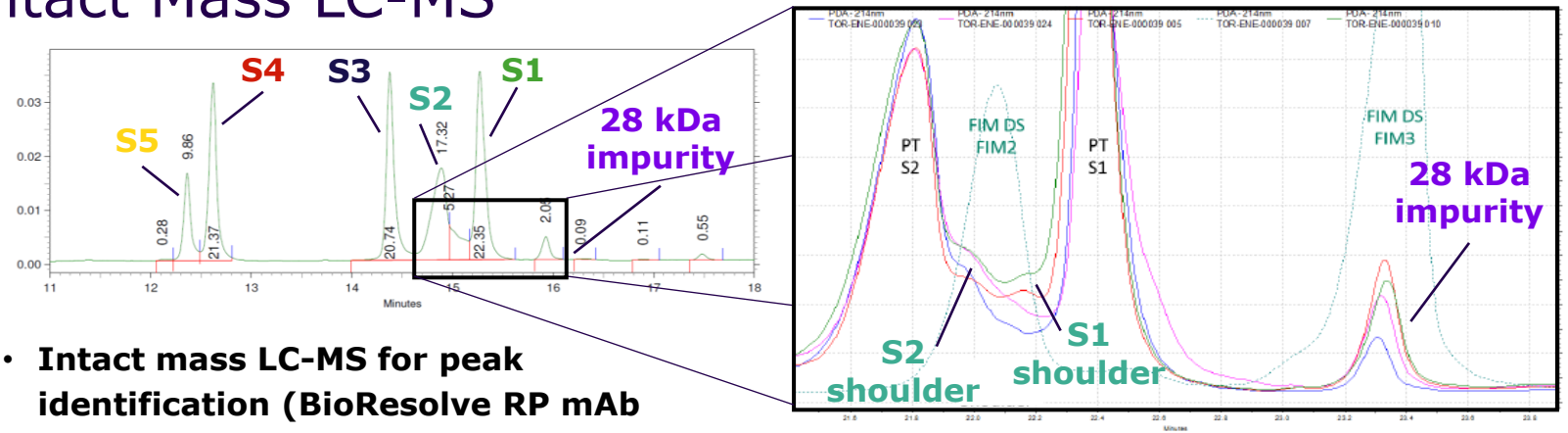
Pertussis Toxin (PT) – Impurity Peak Identification by Intact Mass LC-MS



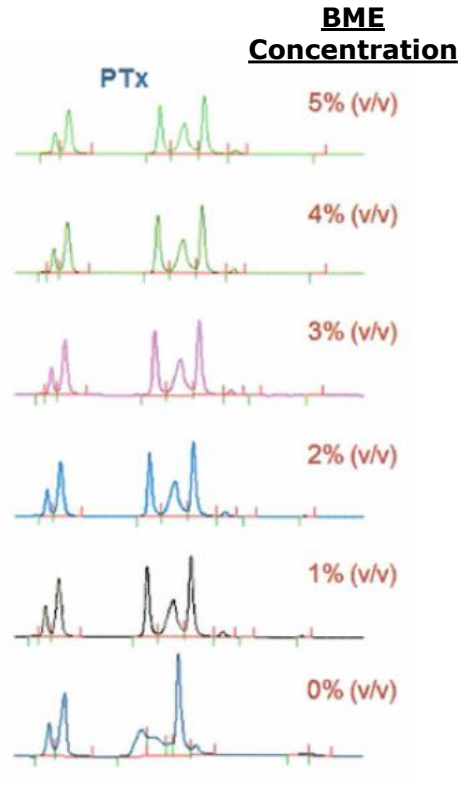
- **Intact mass LC-MS for peak identification (BioResolve RP mAb Polyphenyl Column with Q-TOF) performed on full sample**

1. Impurity peak baseline resolved from PT S1 – consistently detected at the same relative quantity as in SDS-CGE, MW matches to FIM3 (decision to treat as impurity)
2. PT S2 shoulder – identified as oxidized S1 and FIM2 – neither shoulder aligns with FIM2, LMW S1 shoulder increases in MW upon H_2O_2 incubation with oxidized S1 observed in LC-MS at ~2-7% abundance (decision to include in purity value)

Pertussis Toxin (PT) – Baseline Separation of Subunits

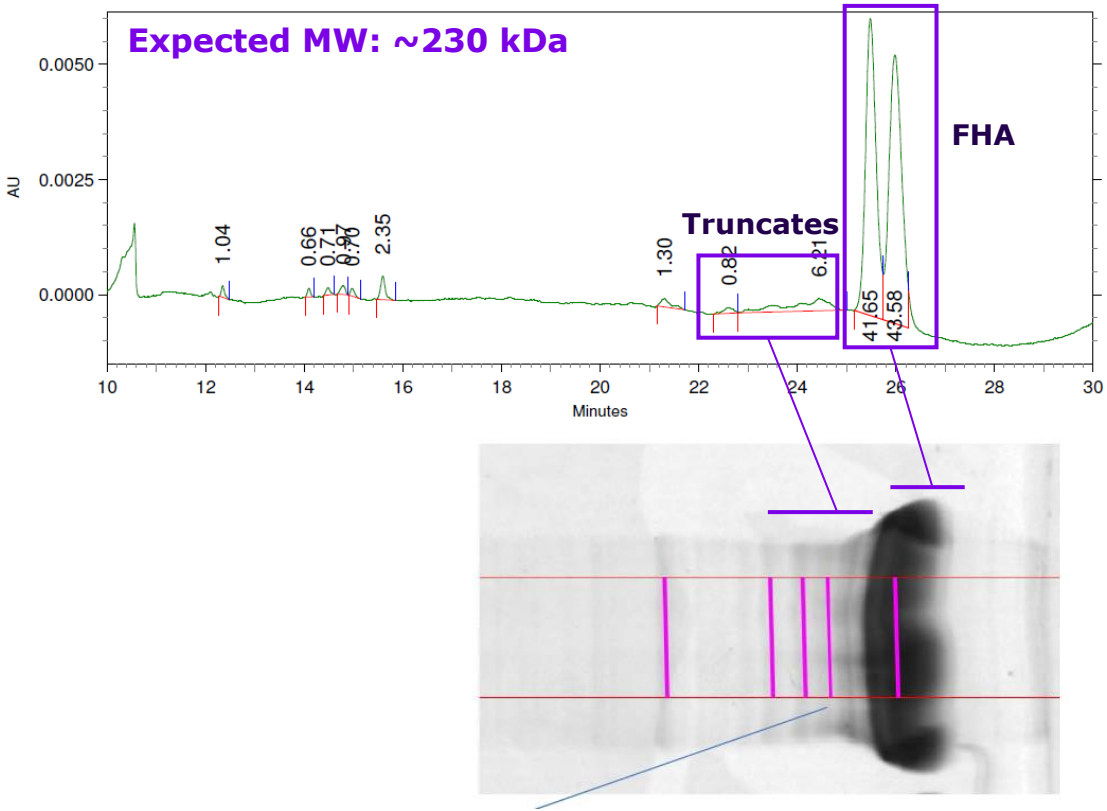
- Parameters assessed to improve resolution and potentially achieve baseline separation of peaks:

Parameter	Range Assessed	Condition Providing Optimal Resolution
Aperture	200-800 µm	200 µm
Separation Voltage	10-15 kV	15 kV
β-mercaptoethanol (BME) Concentration	0-5% v/v	2% v/v
Mode of Injection	Hydrodynamic vs. Electrokinetic Injection	Electrokinetic Injection



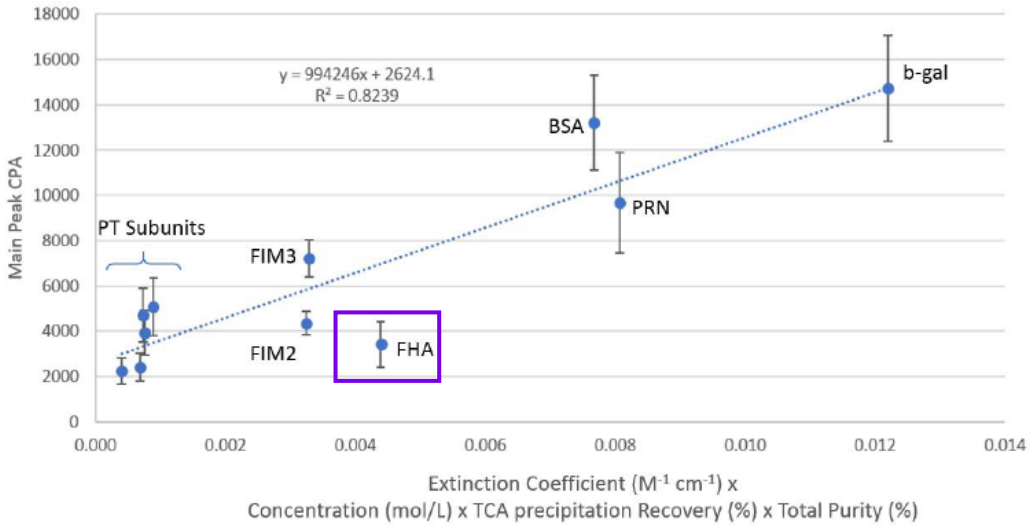
Filamentous Hemagglutinin (FHA)

- **FHA:** cell surface protein, functions as an adhesin
- **Most difficult antigen for method development**
- **Specific challenges:**
  - Low total CPA compared to other antigens (due to lower content of aromatic amino acids)
  - Peak identification
  - Joule heating (long separation time) results in baseline instability, inconsistent peak integration

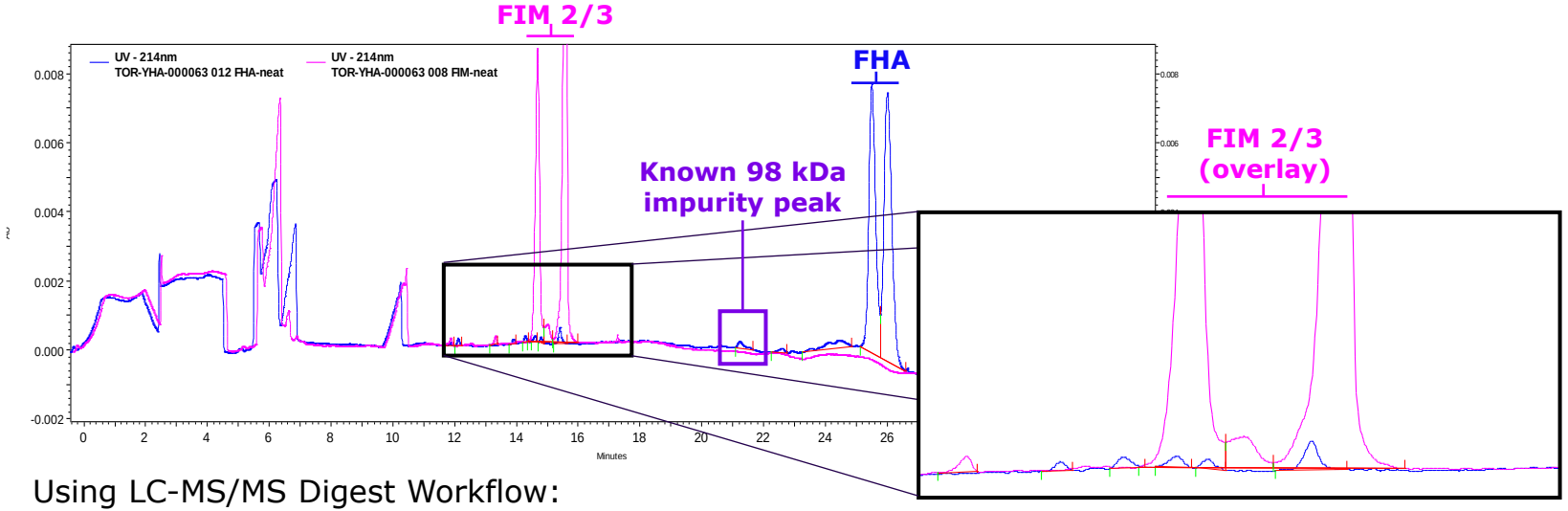


Filamentous Hemagglutinin (FHA) – Recovery

- **Aim:** Demonstrate appropriate FHA recovery despite CPA being lower
- Attempted to increase FHA signal at 214 nm (buffer exchange, detergent addition, increase SDS, increase precipitation, longer chain detergents injection mode)
- FHA loaded at target load and LOD consistently detects impurities with <0.3% variability
- **Decision**: modelling demonstrates appropriate recovery is achieved despite low apparent CPA

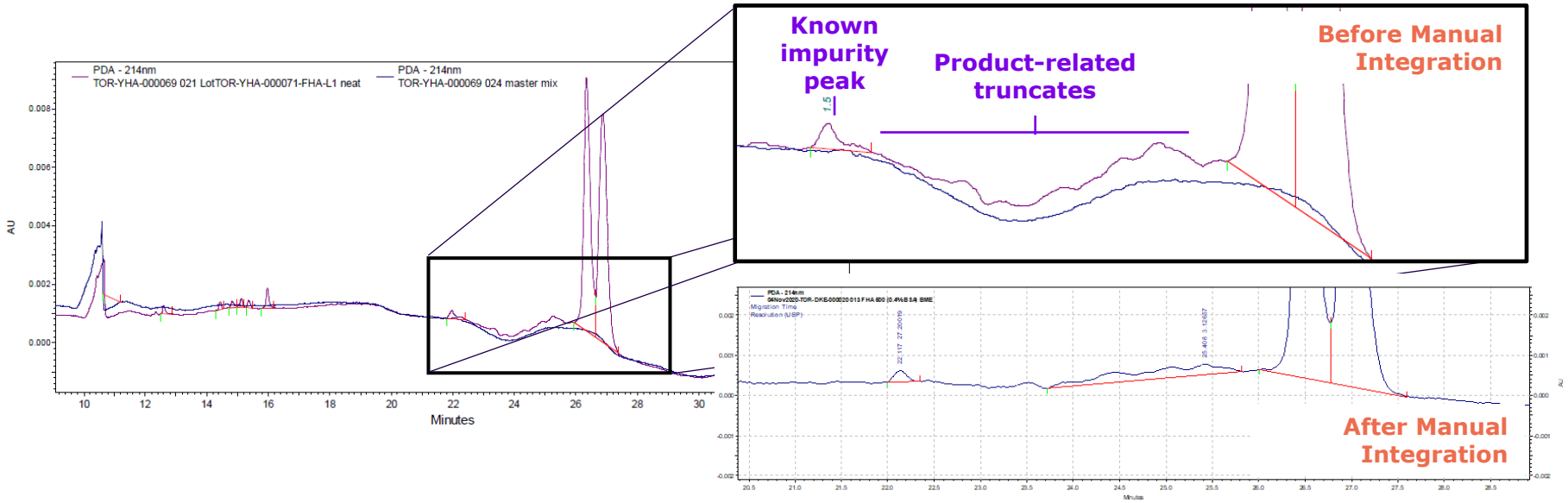


Filamentous Hemagglutinin (FHA) – Peak Identification



- Using LC-MS/MS Digest Workflow:
  1. FIM 2/3 detection? FIM 3 detected, not FIM 2
  2. Identity of 98 kDa impurity peak (also observed on SDS-PAGE)? HCP (multiple candidate proteins in MW range)
  3. Explanation for 2 FHA peaks. Differential truncation/maturation, or could be due to two Fha proteins (FhaS and FhaL)

Filamentous Hemagglutinin (FHA) – Joule Heating



- Peaks in 23-28 min region are antigen product-related truncate peaks and included in purity value
- Joule heating of SDS gel buffer – baseline instability, inconsistent peak integration of truncates
- **Decision:** Use manual integration for appropriate integration of truncate peaks

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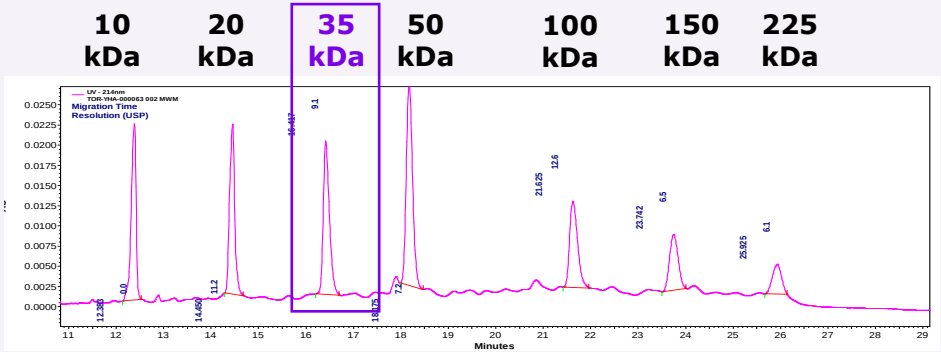
Assay Validity Criteria and Resolution Stress

Stress	Sep. Volt. (kV)	Cap. Temp. (°C)	Suitability Observations	Suitable?	35 kDa Res. Factor (USP)
1	20	40	Lower resolution, baseline instability	No	7.4
Poor Capillary	15	25	LOD not detected	No	7.7
2	18	30	PT purity 0.04% different from target	Yes	8.8
3	17	28	PT purity 0.03% different from target	Yes	8.6
Target	15	25	N/Ap	Yes	8.7-10.0
4	14	23	PT purity 0.3% different from target	Yes	9.8
5	12	17	PRN LMW peaks (included in purity) below LOD	No	11.4
6	10	15	Wider peaks, PRN and FHA not visible on egram	No	12.0



- Assay validity criteria to be set based on USP Resolution of 35 kDa peak
- 32 Independent replicates of MWM also fall within range
- **Set USP resolution to:** 7.7-11.4

Robustness Assessment of Optimized Method

Sample Preparation

- 500 µg/mL target load
- TCA precipitation
- Heat at 100°C for 5 min to denature
- 2% β-mercaptoethanol
- 1% SDS 100 mM Tris-HCl Sample Buffer

Instrument Parameters

- PDA Detector (detection at 214 nm)
- Uncoated capillary (30.2 cm)
- Electrokinetic injection (5.0 kV, 30 sec)
- Aperture 200 µm
- Separation 15.0 kV, 30 min
- 25°C capillary temperature
- 20°C sample storage

- **Robustness assessment for all antigens:**
  - BME: 1.75-2.25%
  - Heat temperature: 97-103°C
  - Heat time: 4-6 min
- **No statistically significant effect for any of the parameters assessed for robustness**

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Method Validation Design

Target Purity Decrease (%)	Level	Analyst/Day (# of Determinations)		
		Run 1 A1D1	Run 2 A1D2	Run 3 A2D3
0	1 (neat)	3	3	3
4	2	1	1	1
8	3	3	3	3
12	4	1	1	1
16	5	1	1	1
20	6 (<spec)	3	3	3
Total determinations per day		**12**	**12**	**12**

- **Validation parameters:** precision (intermediate and repeatability), linearity, accuracy, range, specificity, LOQ
- Validation executed with most complex matrix for each antigen:
  - FIM (adsorbed)
  - FHA (pre-adsorbed)
  - PRN (adsorbed)
  - PT (pre-adsorbed)
- Spiked with BSA or β-galactosidase to mock impurity
- Six levels – level 6 selected to be below specification for all antigens

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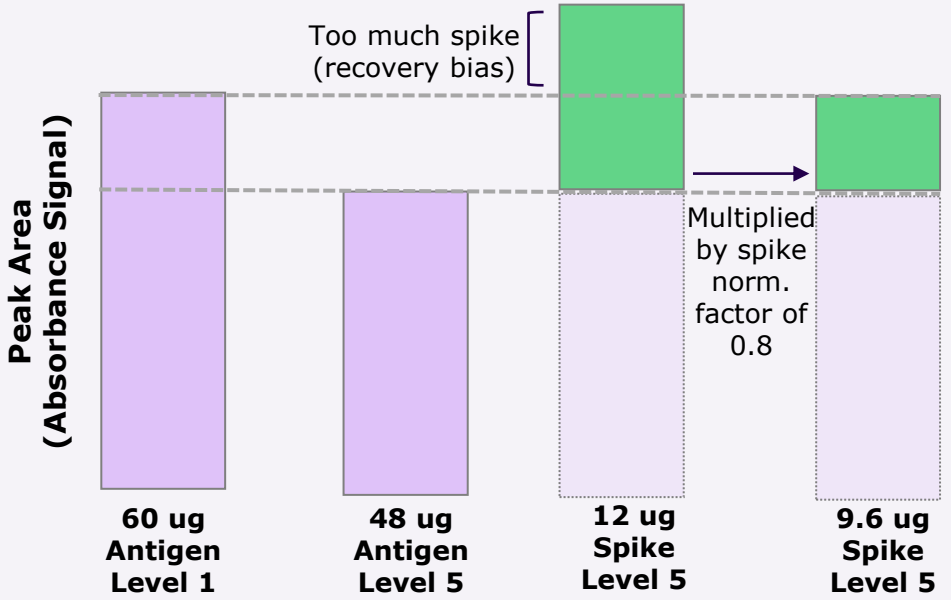
Spike Normalization Factor

- **For method validation, linearity and accuracy assessment requires different 'levels' to force reportable value lower than the neat purity**
  - Spiked levels created with mock impurity BSA or β-galactosidase
  - Extinction coefficient different between antigens – levels not simply created based on %w/w value
  - Normalization based on extinction coefficient to meet target purity decrease



Antigen	Spike	Spike Norm. Factor
FIM	BSA	0.82
PRN	β-gal	0.88
PT	BSA	2.18
FHA	β-gal	0.40

Method Validation Results Summary

- **Aim:** Demonstrate method suitability for replacement of SDS-PAGE as release test
- Accuracy and precision assessed across 3 levels
- Dose linearity: Purity vs. spike amount, assessed across 5 levels
- **LOD: detection of impurities ≥1%**
- **Conclusion:** method suitable, recommendation to replace existing SDS-PAGE method for purity assessment

	Dose Linearity (R^2)	Accuracy (%Recovery)	Intermediate Precision (%CV)
FIM-ads	1.00	99-102%	0.2-0.4%
FHA	0.99	97-100%	0.2-0.8%
PRN-ads	0.99	96-103%	0.3-0.9%
PT	1.00	100-105%	0.6-1.0%

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Conclusions

- Successfully developed and validated an SDS-CGE method for replacement of SDS-PAGE for purity determination of cP antigens – implementation in QC as a release test
- **Antigen specific optimization:**
  - FIM 2/3: TCEP reducing agent not a suitable alternative for β-mercaptoethanol
  - PRN: identification of LMW and HMW peaks
  - PT: identification of impurities, optimized sample preparation and instrument parameters to maximize resolution of subunits
  - FHA: CGE-based purity suitable despite lower observed CPA, identification of impurities, and manual integration of truncates
- **Validation:** demonstrated method is suitable for intended purpose based on method performance (linearity, accuracy, precision, specificity) and is an option to replace existing SDS-PAGE method for antigen purity assessment

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