CE-SDS reduced *Atypical* Peaks – Investigation and Root Cause Analysis

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Formycon AG – who are we?

- Based in Munich/Martinsried with ~250 employees.

- Developing biosimilar drugs for treatments for ophthalmology, immunology, and other chronic diseases, covering all stages from development to regulatory approval.

- Aim to increase patient access to essential biologic drugs by developing biosimilars with the same quality, efficacy, and safety as the reference product on the market.

My role: Head of Separation Sciences Lab

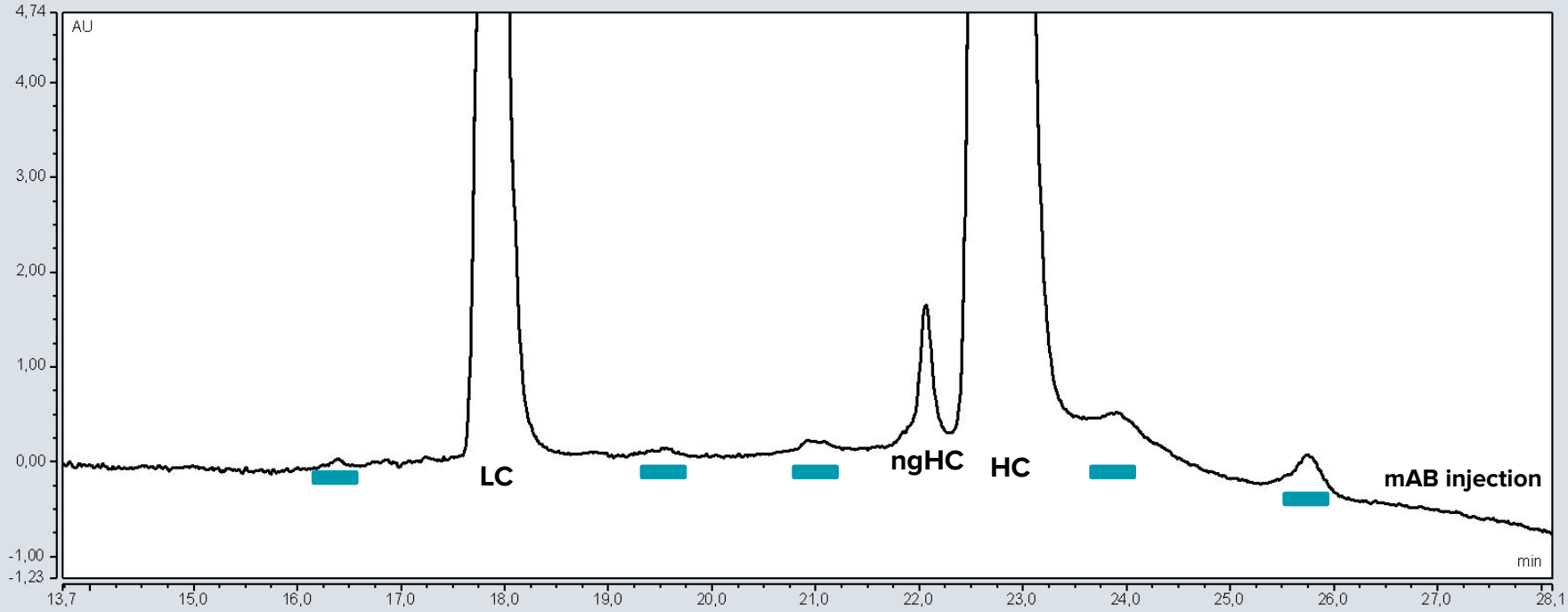
- CE and HPLC analytics: Method development, qualification/validation, transfers.
- Support Formulation development, comparability studies, forced degradation studies etc.

Atypical peaks during CE-SDS reduced analysis

Generic CE-SDS reduced analysis of an IgG1 according to Sciex manual

- 5 % **β**-ME
- 75 % SDS MW sample buffer, pH 9 (Sciex)
- Protein (IgG1) 2 mg/mL

mAb size variant peak



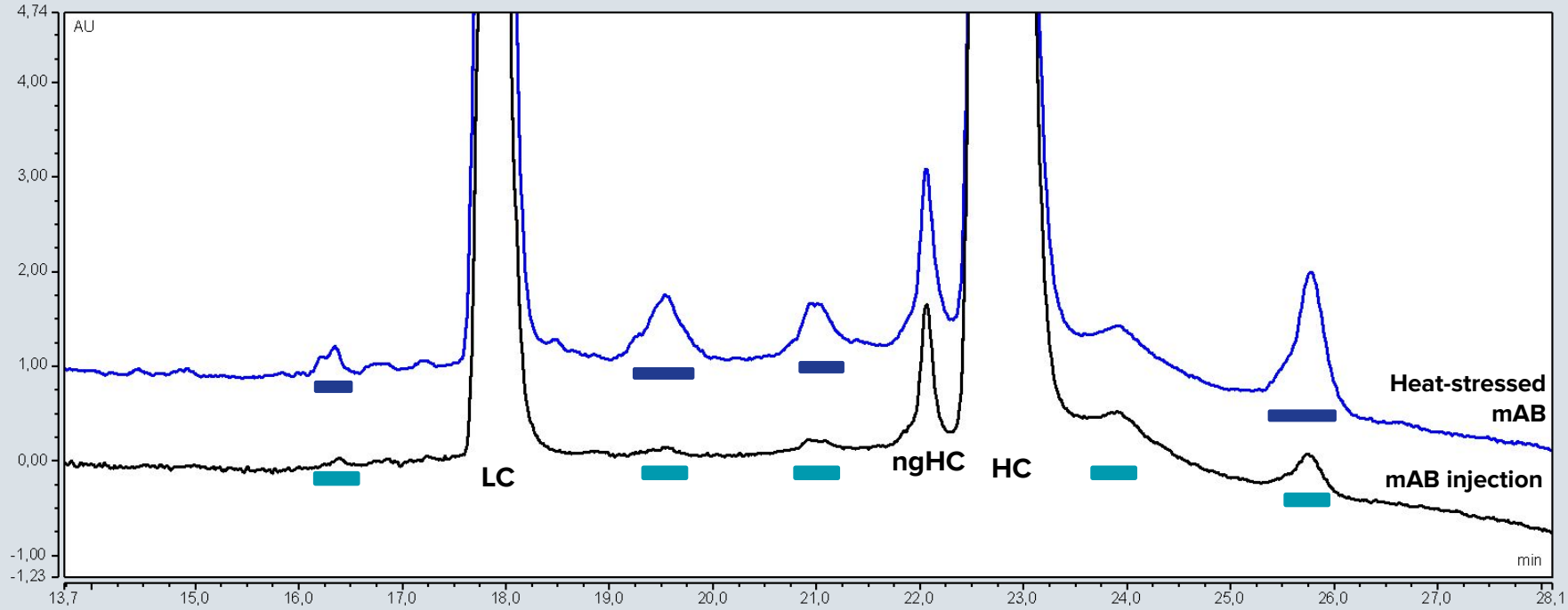
mAb peak pattern CE-SDS reduced

- Qualified test method, well established and suitable for intended use.
- Robust method performance.

Atypical peaks during CE-SDS reduced analysis

Analysis of an IgG1: Generic CE-SDS red analysis according to Sciex manual

- Peak of temperature-induced degradation
- mAb size variant peak



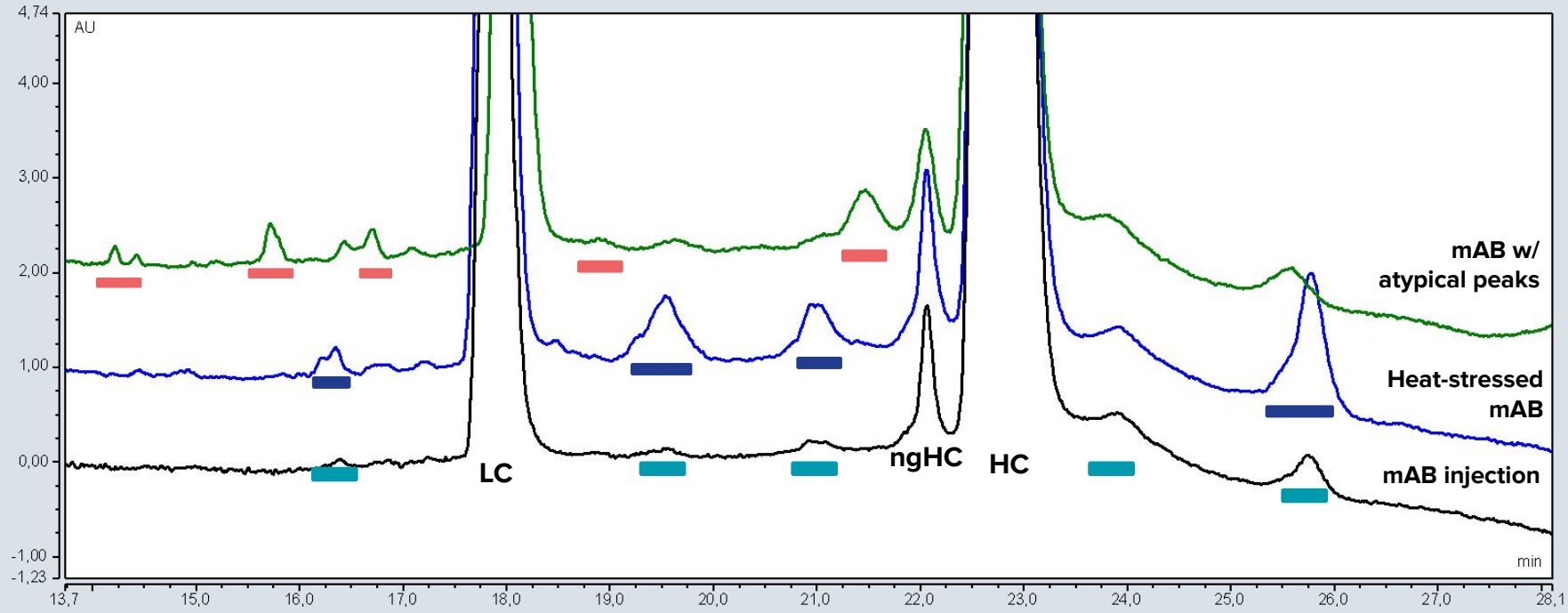
mAb peak pattern CE-SDS reduced

Temperature-induced degradation leads to distinct increase of specific LMW and HMW peaks.

Atypical peaks during CE-SDS reduced analysis

Analysis of an IgG1: Generic CE-SDS red analysis according to Sciex manual

- Atypical peak
- Peak of temperature-induced degradation
- mAb size variant peak



mAb peak pattern CE-SDS reduced

- Occasional and random appearance of atypical peaks observed in CE-SDS red analyses of mAB samples.
- Atypical peak pattern differs to ‚common' mAb degradation peak pattern (heat, pH, light)

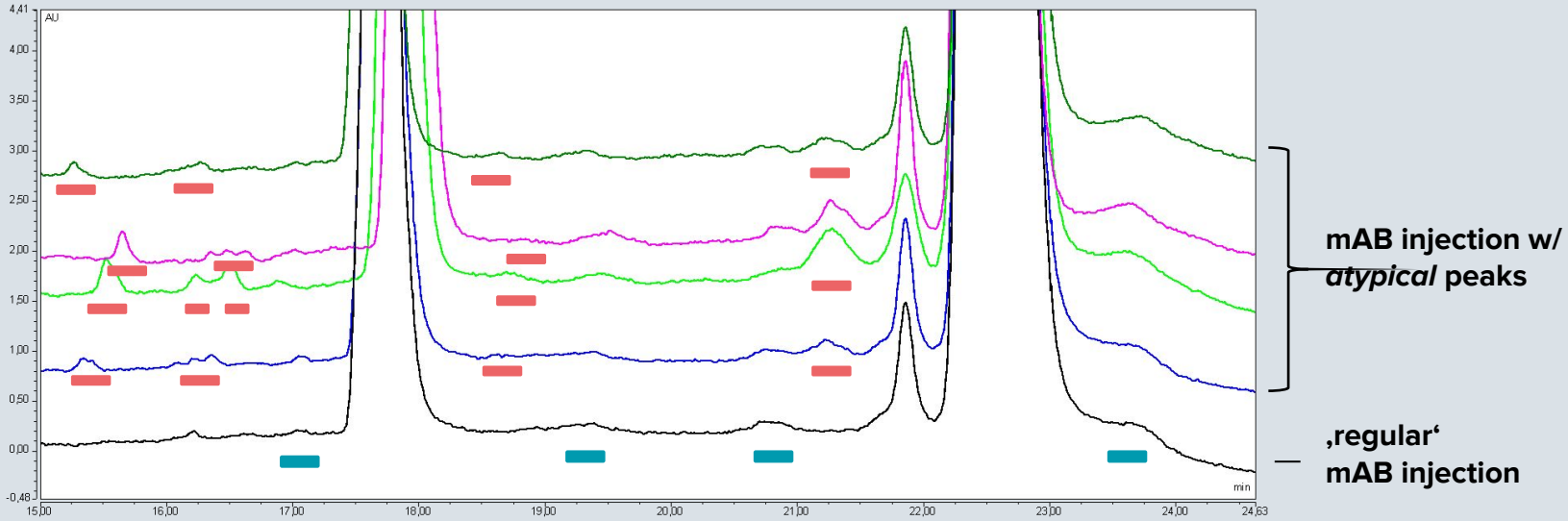
Atypical peaks during CE-SDS reduced analysis

- *Atypical* peaks appear independent from the sample type
- Similar peak profile of *atypical* peak pattern but different intensities of *atypical* peaks between affected samples (~0.2% - 3% TCA)
- Re-injection reproduces *atypical* peaks
- Re-preparation of same sample aliquot eliminates *atypical* peaks

- **Atypical peaks are linked to individual sample preparation**

Atypical peak

mAb size variant peak



Atypical peaks during CE-SDS reduced analysis

Identify the cause for atypical peaks

Initial laboratory investigation:

- No obvious errors identified during experimental execution or measurement. There were no noticeable issues with the reagents, materials, or samples.

Trending analysis

- Atypical peaks could not be linked to personal, reagents/chemicals, consumables, or analytical equipment

- Random appearance of atypical peaks which is linked to individual sample preparations

Starting point for hypothesis testing:

- Focus on disturbing factors during sample preparation that may cause atypical peaks

Identify the cause for *atypical* peaks

Hypothesis testing

Preparational steps and lab-related contaminants were tested to reproduce *atypical* peaks

- Extensive contact with reaction tubes
- Extensive contact with pipette tips
- Extensive vortexing
- Include permanent marker in sample preparation

Atypical peaks could not be reproduced consistently

- **Gloves**
- **Human-related contaminants: Skin, Coughing**
- **Human-related contaminants: Hair**
- **Dust**
- **Lab coat fibers**

Upcoming slides

Identify the cause for *atypical* peaks

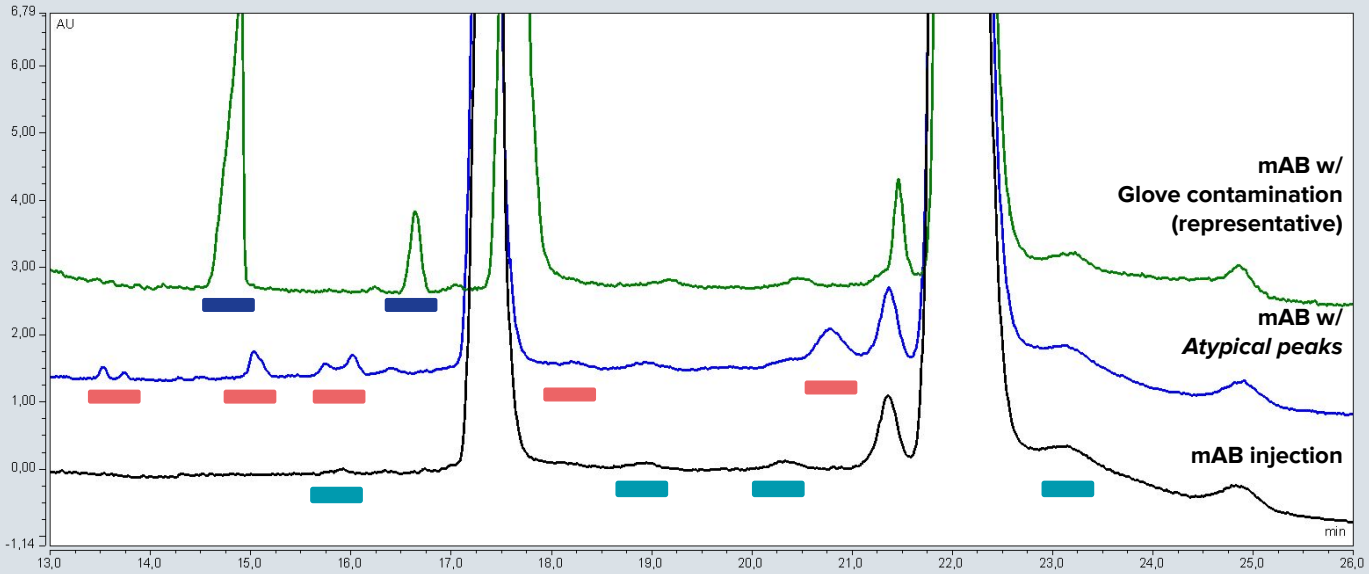
1. Test of Glove-related contaminants

Glove material was introduced as a potential contaminant during CE-SDS preparation:

- Glove abrasion
- A piece of glove added 15 min at RT
- A piece of glove added for denaturation

Five different types of gloves were tested.

- Additional ‚glove' peaks were detected when glove material was added to sample preparation.
- **The *atypical* peak pattern was not reproduced by adding glove material as contaminant**



Peak from glove contamination

Atypical peak

mAb size variant peak

Identify the cause for *atypical* peaks

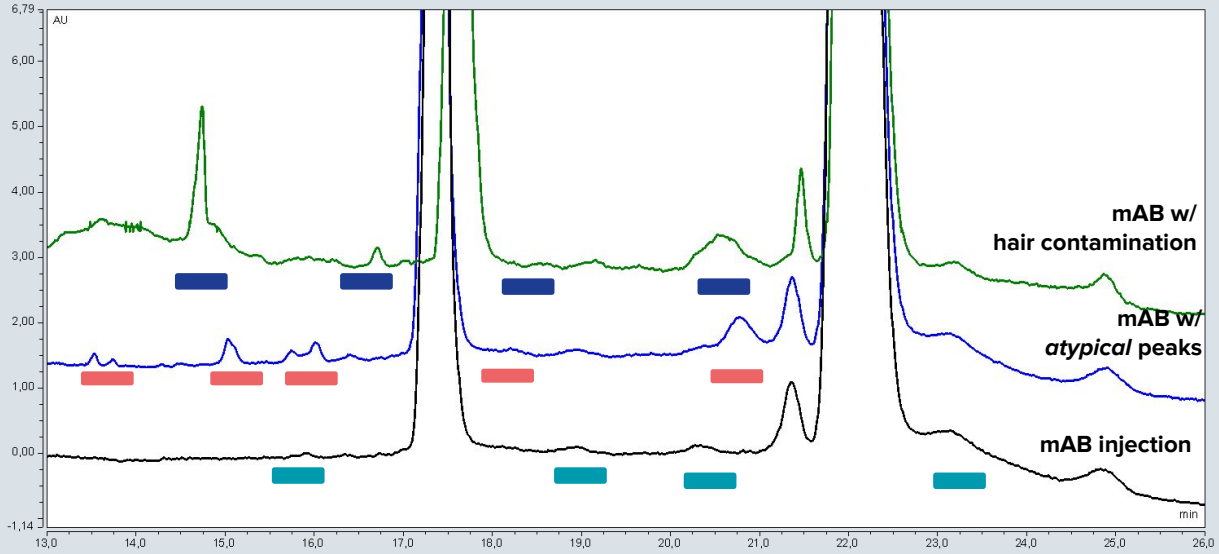
2. Test of human-related contaminants

Potentially human-related contaminations were introduced in CE-SDS red sample preparation:

- Skin
- Hair
- Coughing into vials

- No *atypical-like* peaks observed for skin and ‚coughing' samples
- *Atypical-like peaks* appeared with hair contamination

- **The peak pattern in the "hair sample" is comparable to the *atypical* peak pattern.**



Peak from hair contamination

Atypical peak

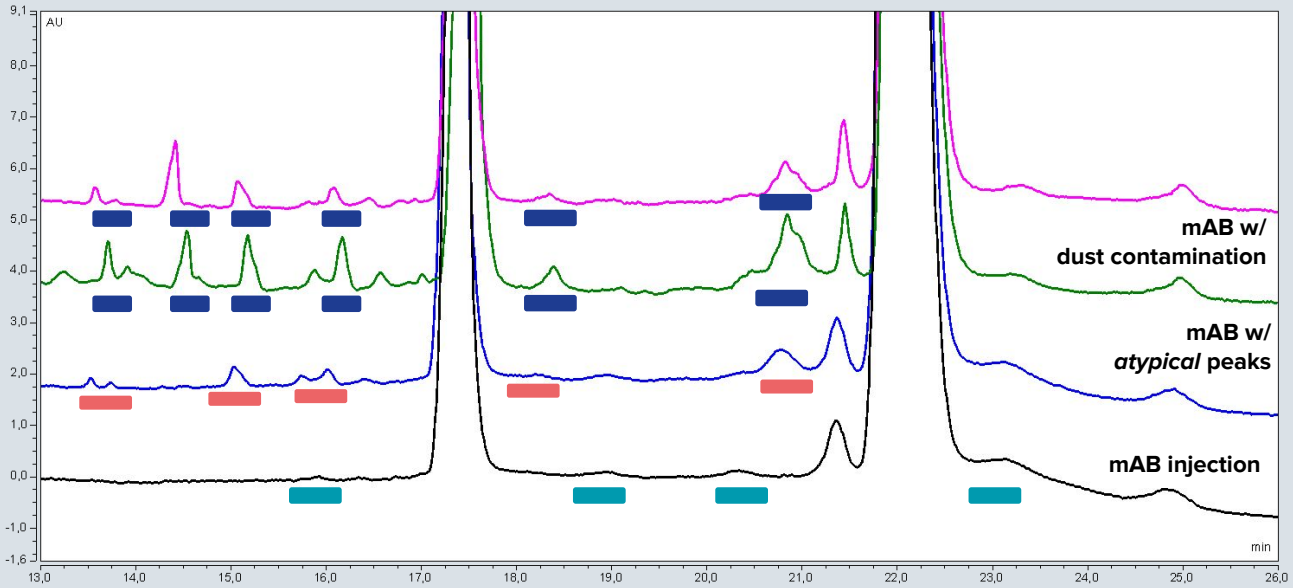
mAb size variant peak

Identify the cause for *atypical* peaks

3. Test of Dust as potential contaminant

Dust was collected from different locations and was added to CE-SDS preparation prior to denaturation.

- All dust coaining samples contained *atypical-like* peaks.
- **Peak pattern of dust-containing samples is comparable to *atypical* peak pattern**



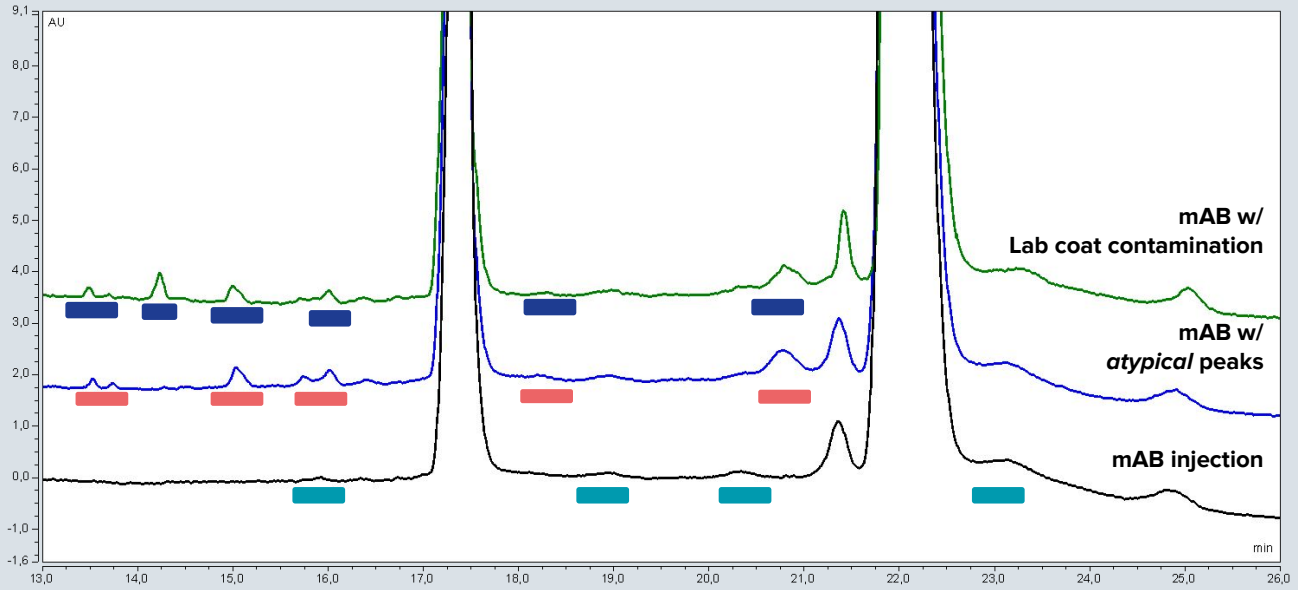
Identify the cause for *atypical* peaks

4. Test of Lab coat Fibers as potential contaminant

Different procedures to introduce lab coat-related contamination:

- Lab coat was dragged along open tube.
- Fibers and dust were collected from lab coat.
- One fiber was cut from the lab coat and was incorporated added to the sample preparation.

- <u>All</u> tested samples contained *atypical-like* peaks
- **Peak pattern of lab coat fiber containing samples are comparable to atypical peak pattern**



Identify the cause for *atypical* peaks

Conclusions

- Extensive contact with reaction tubes
- Extensive contact with pipette tips
- Extensive vortexing
- Include permanent marker in sample preparation
- Gloves
- Human-related contaminants: Skin, Coughing

No *atypical* peaks were reproduced

- **Human-related contaminants: Hair**
- **Dust**
- **Lab coat fibers**

***Atypical-like* peaks were reproduced**

Identify the cause for *atypical* peaks

Conclusions

- Extensive contact with reaction tubes
- Extensive contact with pipette tips
- Extensive vortexing
- Include permanent marker in sample preparation
- Gloves
- Human-related contaminants: Skin, Coughing

No *atypical* peaks were reproduced

- **Human-related contaminants: Hair**
- **Dust**
- **Lab coat fibers**

Atypical-like **peaks were reproduced**

- **Minor amounts of dust are most likely to cause *atypical* peaks**

Identify the cause for *atypical* peaks

In-depth analysis for the origin of atypical peaks

For upcoming tests, dust is used as *atypical* peak inducer

Composition of dust:

Human Skin Cells, Textile Fibers, Insect Parts, Inorganic Particles, Microorganisms, wear and tear from equipment

What is the cause for atypical peaks

- Dust contamination is directly responsible for the atypical peaks
- The atypical peaks result indirectly from the contamination, as it induces mAb fragmentation

Atypical peaks –

Direct contamination or protein fragmentation?

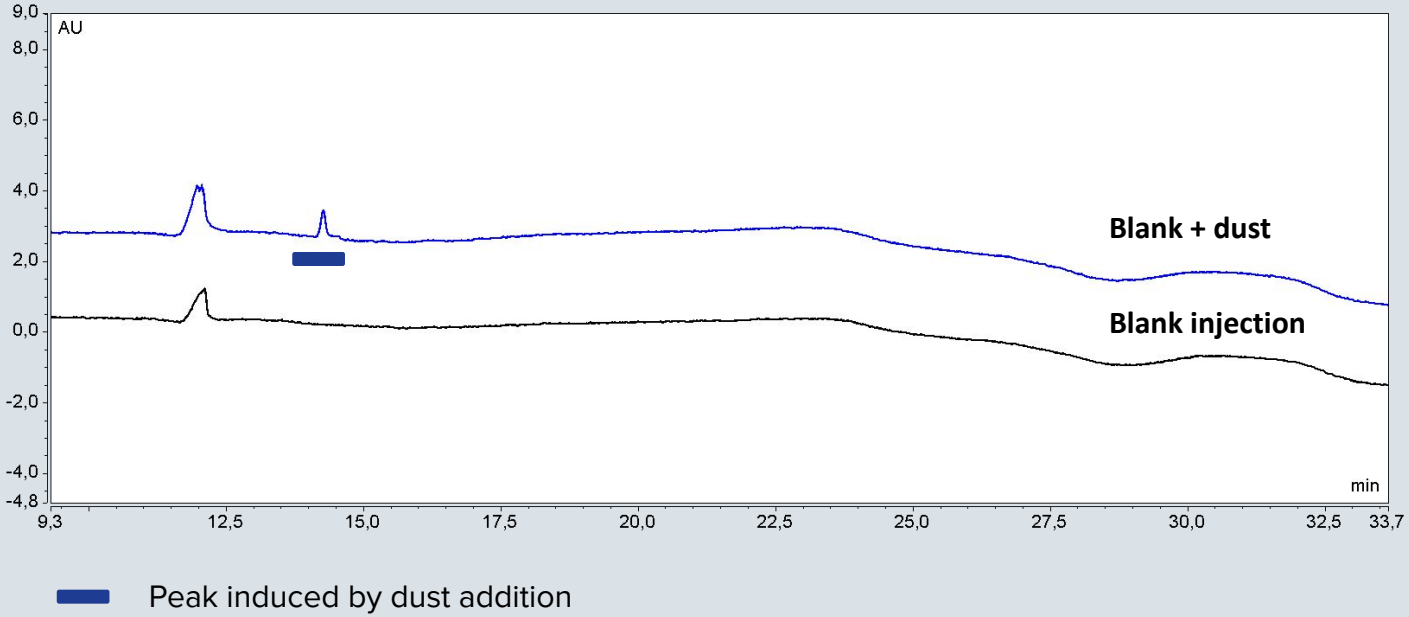
Test of blank injection

Aim:
Evaluate if the *atypical* peaks are observed in the absence of mAb

Test:
Dust was added to blank injection

- No *atypical* peaks were observed in dust-containing blank.
- One additional peak at ~14 min observed

- ***Atypical* peaks likely no direct contamination from dust**



Atypical peaks –

Direct contamination or protein fragmentation?

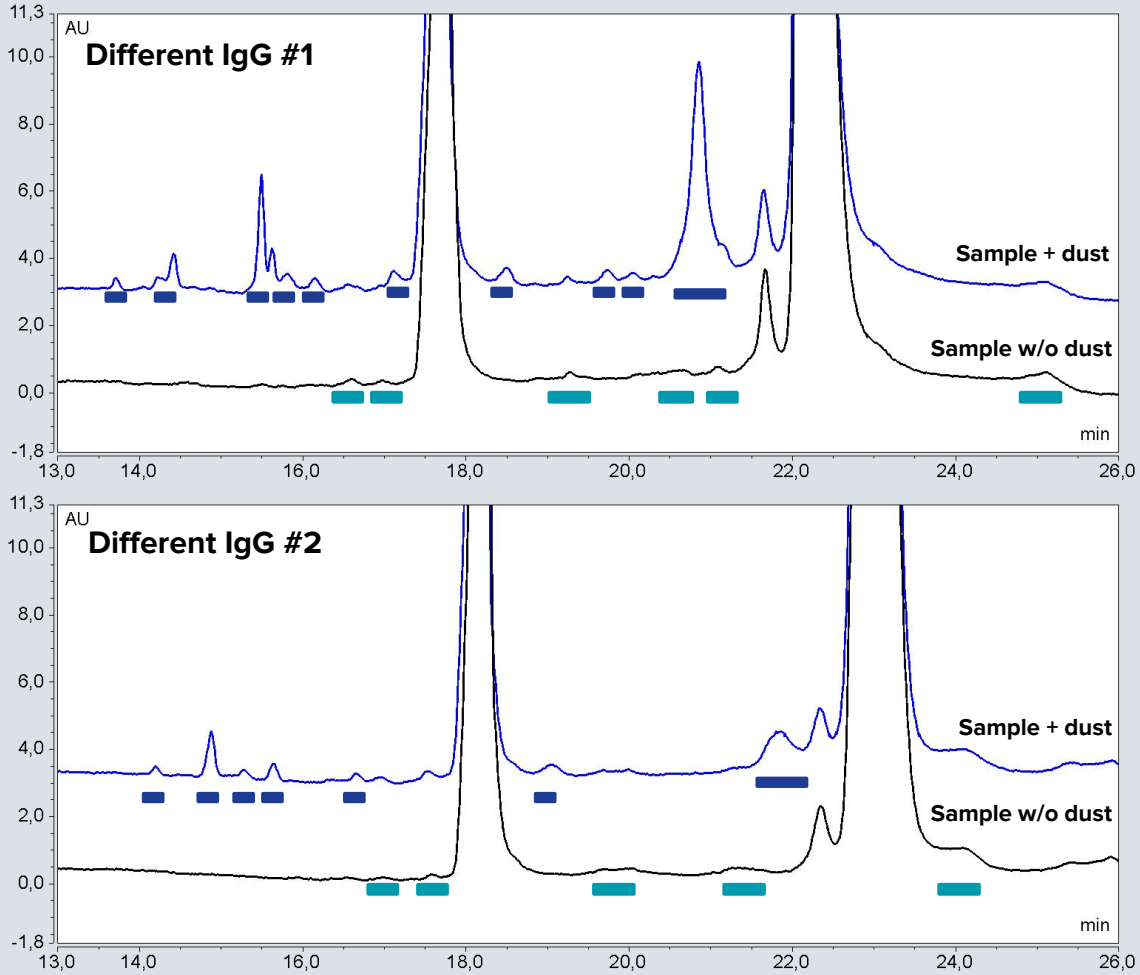
Test of other IgG molecules

Aim:
Evaluate if *atypical* peaks appear during CE-SDS red analysis of other IgG molecules

Test
Dust was incorporated to CE-SDS red sample preparation of other IgGs

- *Atypical* peaks also are also observed with other IgGs. The intensity and pattern of peaks vary among different IgGs.

- ***Atypical* peaks likely related to fragmentation of the IgG molecule.**



Atypical peaks –

Direct contamination or protein fragmentation?

Test of different molecule classes

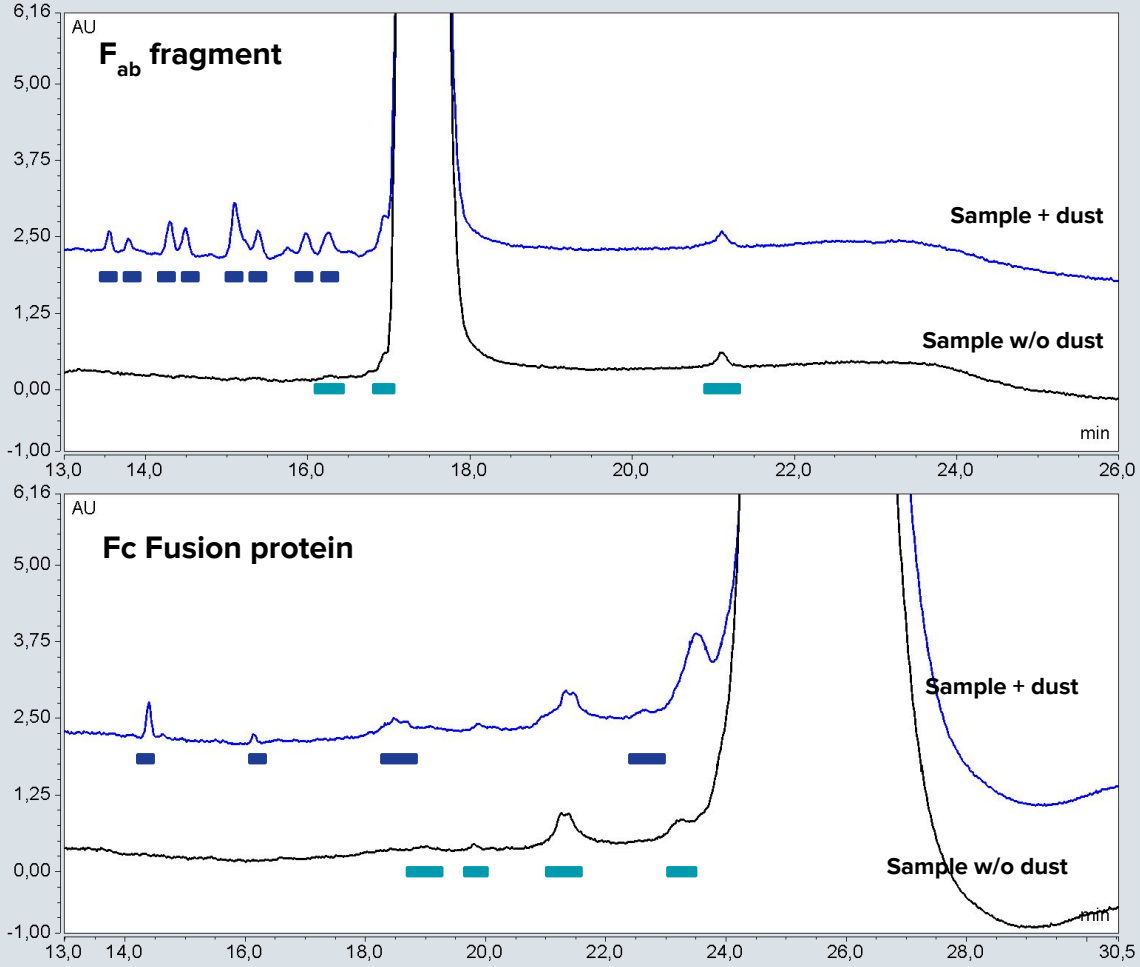
Aim:
Evaluate if *atypical* peaks appear during CE-SDS red analysis with different molecule classes

Test:
Dust was added to sample preparation of other therapeutical molecules:

1. F_{ab} fragment
2. Fusion protein

- *Atypical* peaks appear with other molecule classes in the presence of dust. The atypical peak pattern differs from that of IgGs.

- ***Atypical* peaks likely related to fragmentation of protein**



Atypical peaks –

Direct contamination or protein fragmentation?

Which component of dust is causing protein fragmentation?

Chemicals
Parameters promoting chemical fragmentation: pH, heat, metals, radicals

Elevated heat and prolonged denaturation time did not induce more pronounced *atypical* peaks

https://www.flaticon.com/free-icon/chemicals_1486187

Proteases
Proteases may cleave denatured proteins.

Do Proteases retain activity in CE-SDS sample buffer?

Acta Crystallogr D Biol Crystallogr, 1997, 53, 311-315

Atypical peaks –

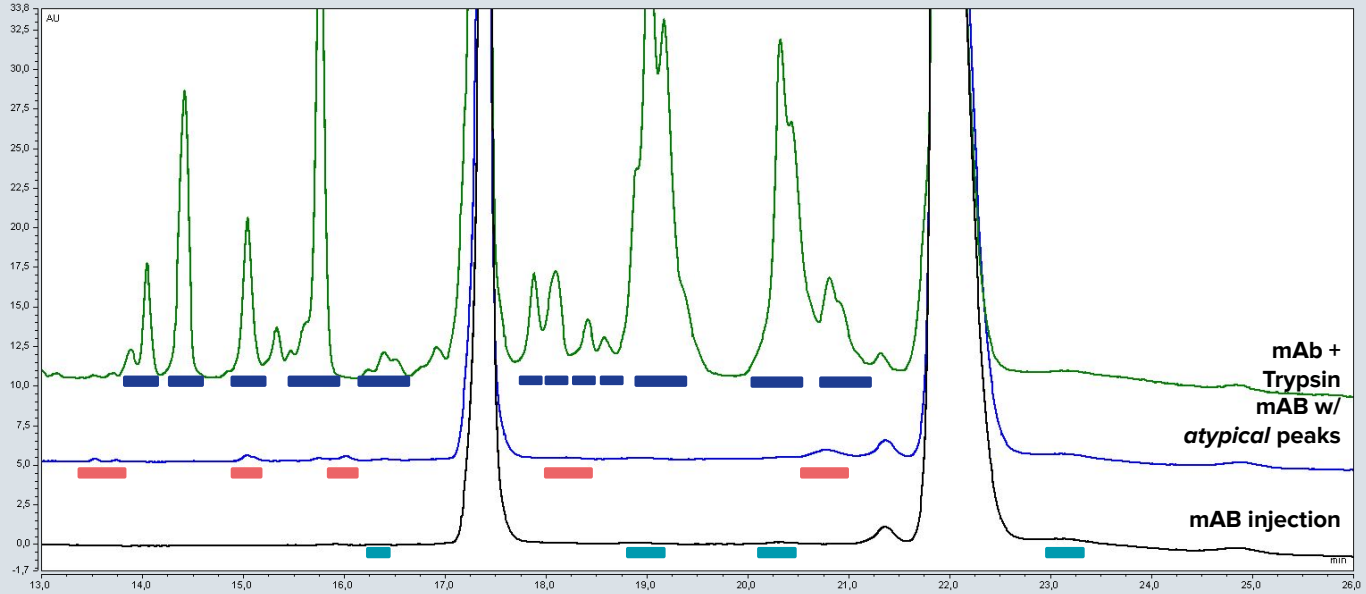
Direct contamination or protein fragmentation?

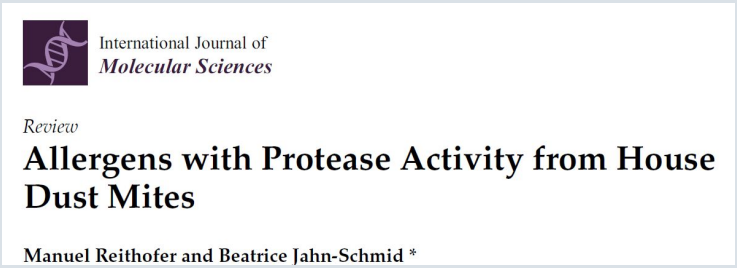
Test of protease activity in CE-SDS preparation

Aim:
Evaluate if the proteases (e.g. Trypsin) retain activity in CE-SDS red sample preparation

Test:
Minor amount of Trypsin was added to a CE-SDS red sample preparation (before denaturation).

- Major amounts of additional LMWs are detected in the CE-SDS red preparation containing Trypsin. Differences in the peak pattern are observed.




International Journal of *Molecular Sciences*

Review

Allergens with Protease Activity from House Dust Mites

Manuel Reithofer and Beatrice Jahn-Schmid *

Peak induced by trypsin addition

Atypical peak

mAb size variant peak

Atypical peaks –

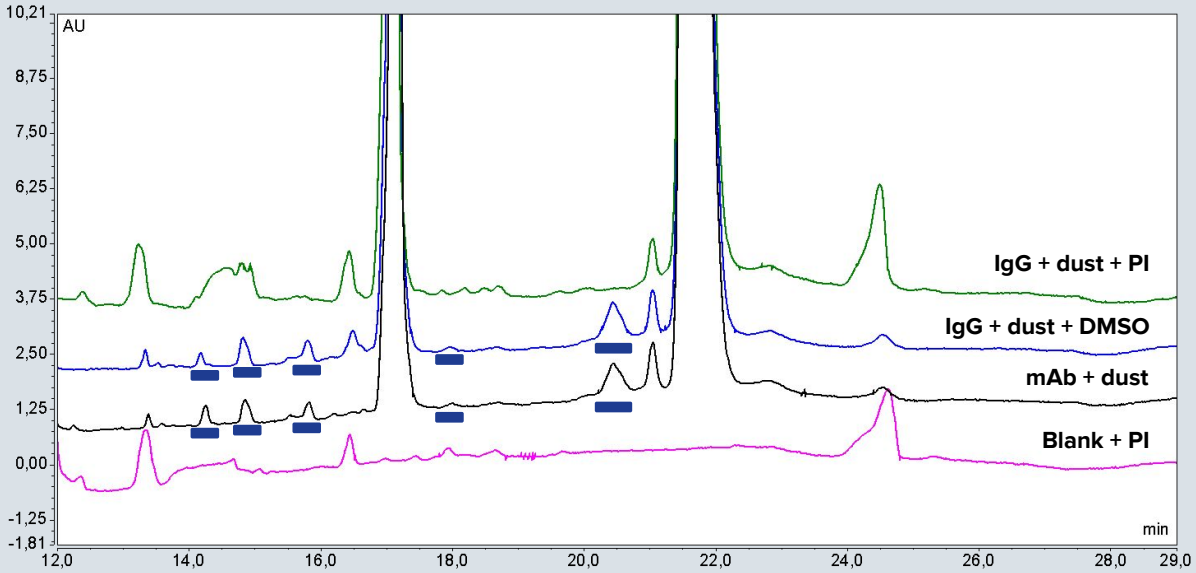
Direct contamination or protein fragmentation?

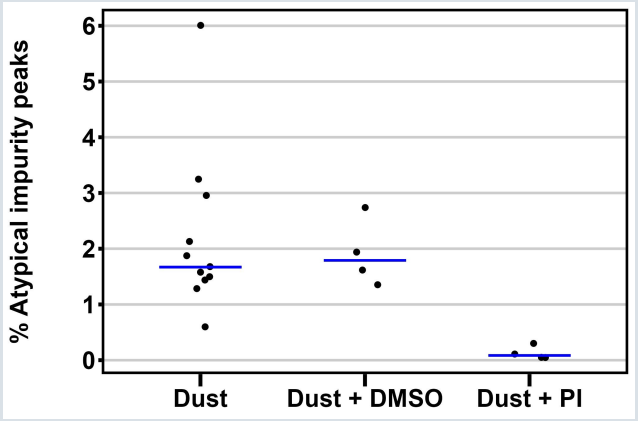
Can Protease Inhibitor counteract formation of atypical peaks?

Aim:
Test, if protease inhibitor (PI) affects formation of *atypical* peaks

Test:
1) Protease inhibitor (PI) is added to sample preparation
2) DMSO serves as neg ctrl

- Decrease of *atypical* peaks observed by PI addition.
- **Fragmentation is likely linked to proteolytic cleavage of mAb**





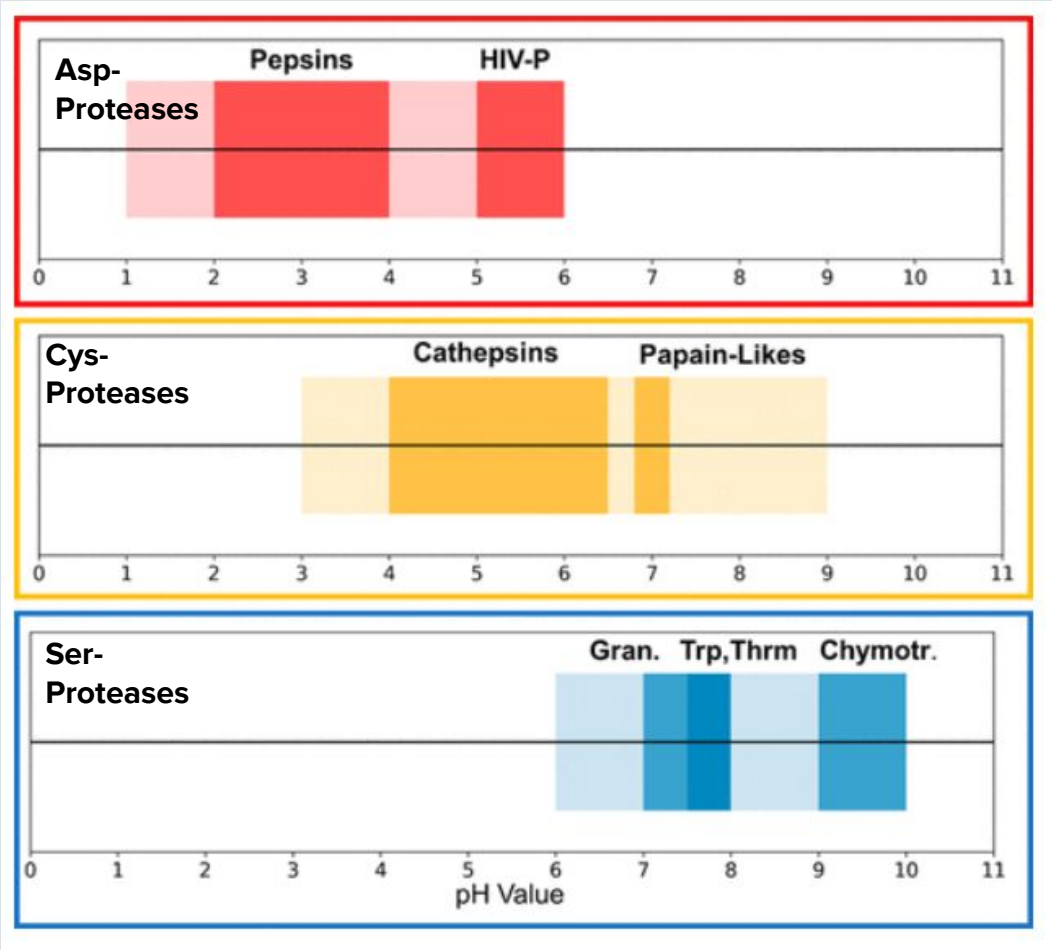
PI – Protease inhibitor cocktail

Peak induced by dust addition

Measures to prevent formation of *atypical* peaks

Change pH of sample buffer

Enzymatic function and activity of proteases is controlled by the pH value.



J. Chem. Inf. Model. 2020, 60, 3030–3042

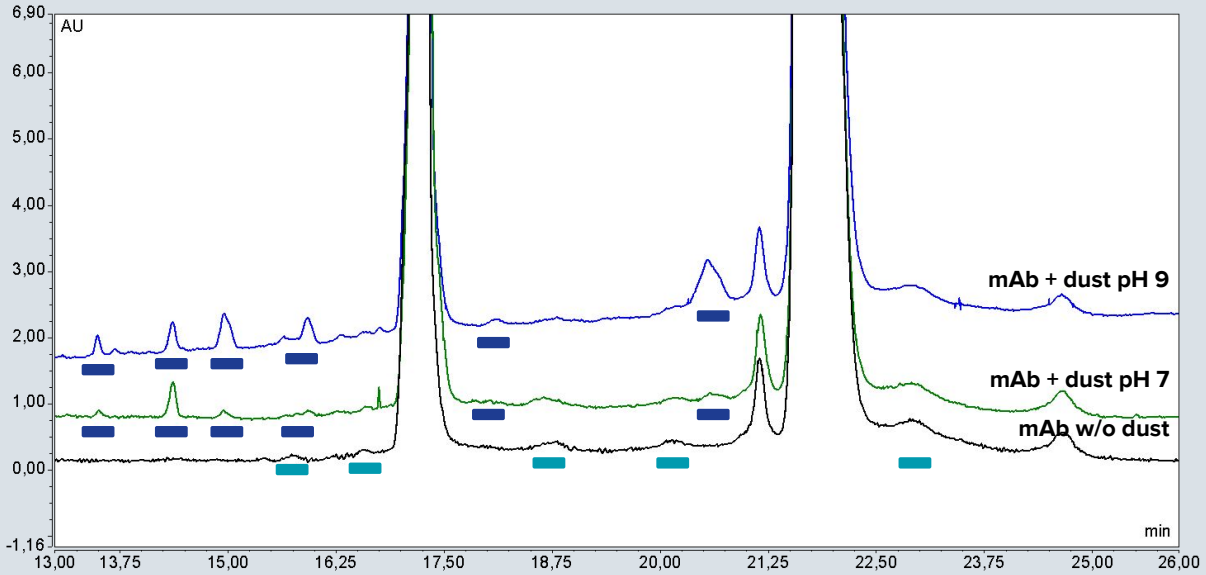
Measures to prevent formation of *atypical* peaks

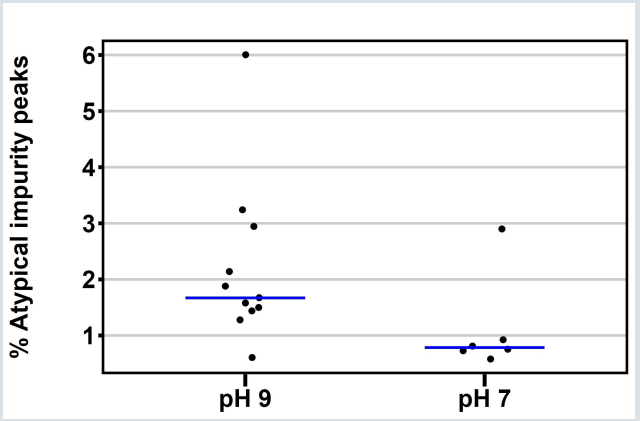
Change pH of sample buffer

Enzymatic function and activity of proteases is controlled by the pH value.

Test:
Evaluate SDS-MW sample buffer pH 9 vs SDS-MW sample buffer pH 7

- Decreasing the pH of sample buffer to pH 7.0 reduces the amount of *atypical* peaks.
- **Decreasing the pH of sample buffer to pH 7.0 leads to less pronounced *atypical* peaks**





Peak induced by dust addition

mAb size variant peak

Atypical peaks – final conclusions

1. Identify the cause for *atypical* peaks

- Dust or related components

2. *Atypical* peaks - Direct contamination or protein fragmentation?

- Data suggest that components from dust cause protein fragmentation during sample preparation.
- Fragmentation is most likely caused by proteases

3. Measures to prevent formation of *atypical* peaks

- Modifying the pH of the CE-SDS sample buffer to pH 7 avoids the pH optimum of proteases and could improve robustness against *atypical* peaks.
- Dedicated consumables (pipette filter tips and reaction tubes) should be utilized
- To avoid *atypical* peaks, routine sample preparation of CE-SDS red should be conducted under particle-reduced conditions (laminar air flow).
- Additionally, an overlay of atypical peak traces is included and described in the SOP to ensure that these peaks do not trigger OOE results

Acknowledgments

Many thanks to all involved **Formycon colleagues, especially Stefanie Baader**

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If you’re in the Munich area, stop by and visit us.

References

Slide 10:
https://www.hallohygiene.de/100x-soft-nitrilhandschuhe-medium-puderfrei-blau-30031/?gad_source=1&gclid=EAIaIQobChMIk4nYk4CkiAMVRo9QBh3mgTFrEAQYBCABEgJw2vD_BwE

Slide 11 https://wellingtondermatology.nz/services/hair-and-nail-problems/

Slide 12 https://www.collinsdictionary.com/de/worterbuch/englisch/dust

Slide 13: https://www.kokott.com/Themen-Branchen/Medizin/Oberteile/Kittel/

Slide 20: https://www.flaticon.com/free-icon/chemicals_1486187 and Acta Crystallogr D Biol Crystallogr, 1997, 53, 311-315

Slide 25 J. Chem. Inf. Model. 2020, 60, 3030−3042

Appendix - Atypical peaks

Direct contamination or protein fragmentation?

Test of CE-SDS under non-reducing conditions

Aim:
Evaluate if the *atypical* peaks are also occuring during CE-SDS NR analysis

Test:
Dust was added to CE-SDS NR sample preparation, pH 7.0

- Some additional peaks were detected migrating before the LC fragment. Peak pattern shows similarities to CE-SDS reduced *atypical* peak pattern

- ***Dust-induced atypical* also occur during CE-SDS NR analysis**