

A Peptide Mapping Method for Accurate Analysis of Non-Enzymatic Protein Modifications

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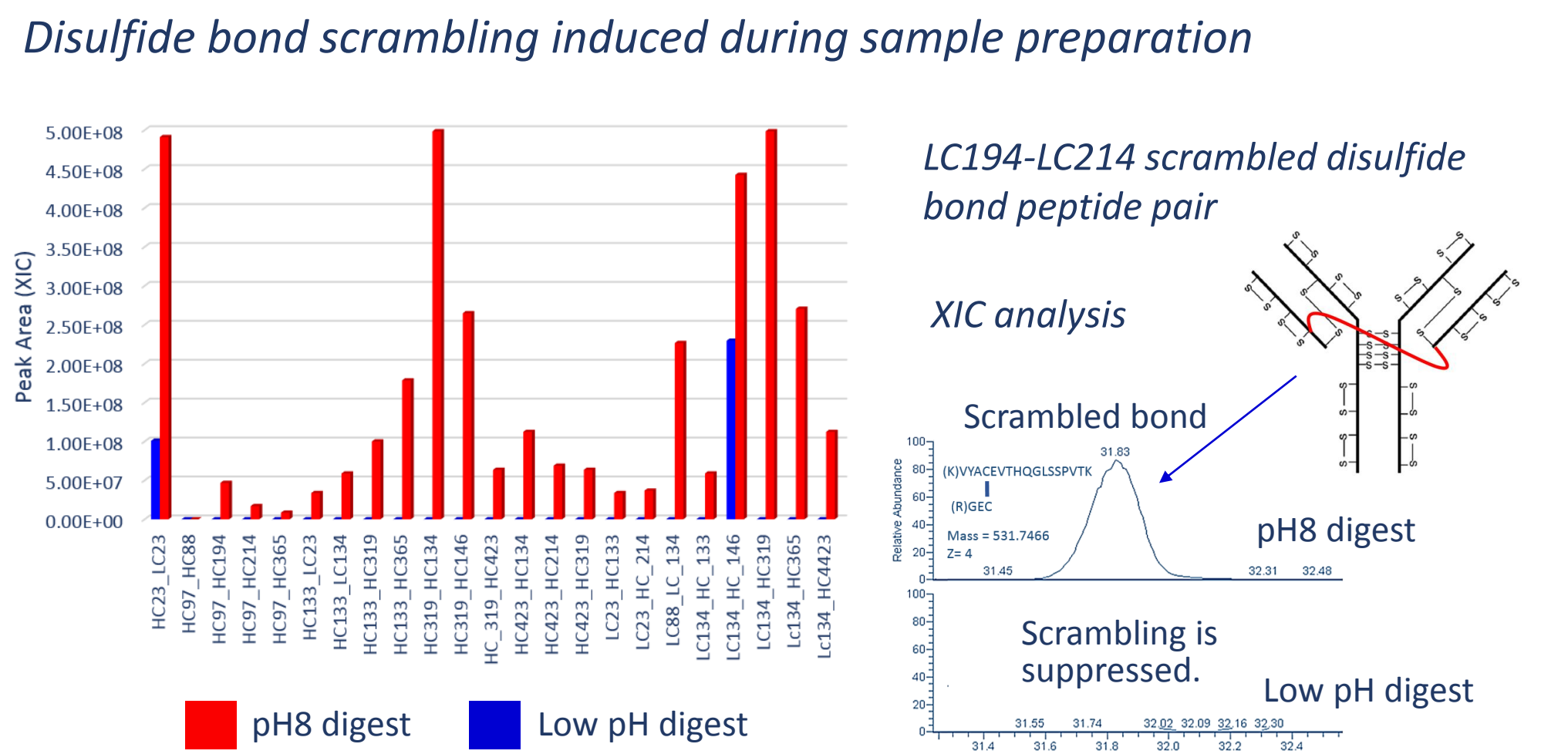
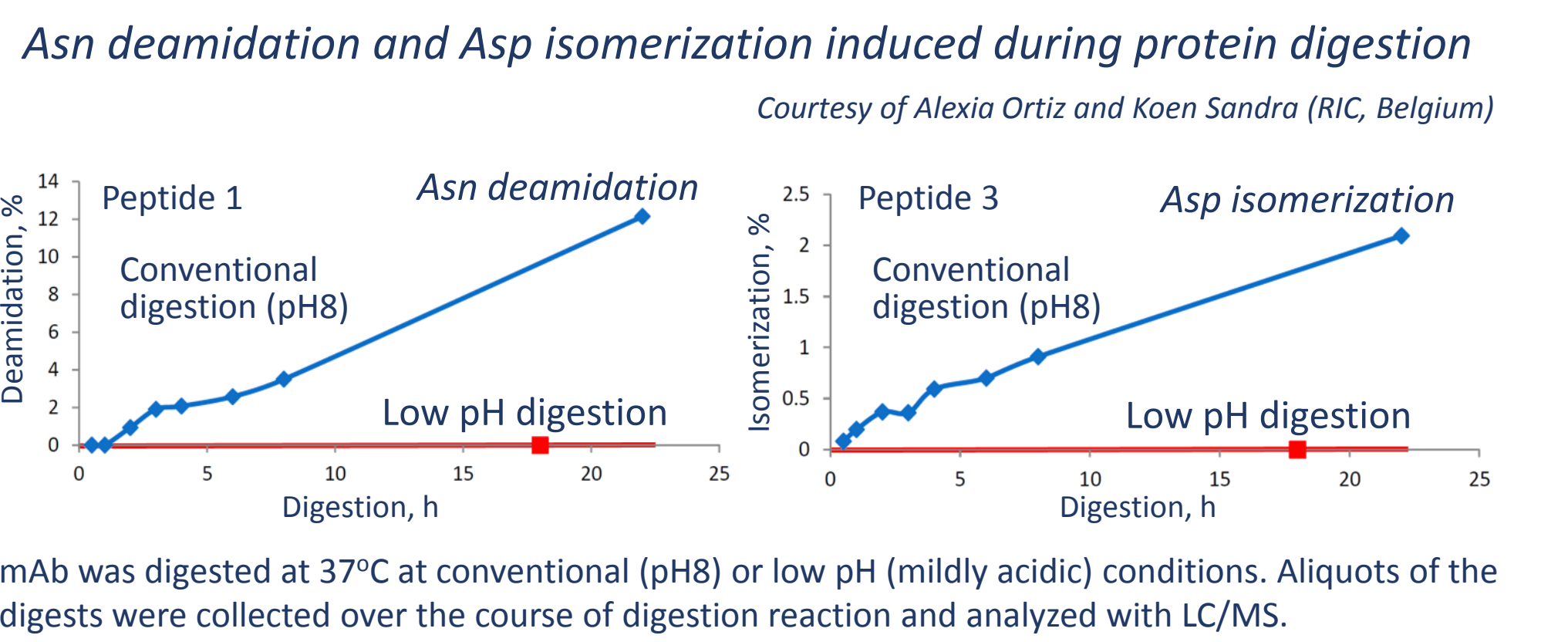
1. Introduction

Non-enzymatic chemical modifications such as deamidation, disulfide bond scrambling and oxidation can affect the stability and efficacy of biotherapeutic proteins. Peptide mapping, the method of choice for monitoring these modifications, has a significant drawback which is that peptide mapping sample preparation induces the same modifications.

We developed a sample preparation method which prevented the above side effects. In our method, all sample preparation steps including reduction, alkylation and digestion are performed at low pH (mildly acidic conditions). Efficient reduction and alkylation at low pH utilized both specialized reagents and procedural modifications. The proteolytic step represented the major bottleneck since trypsin, the most commonly used protease in peptide mapping, is inhibited at acidic pH. We overcame this problem by supplementing trypsin with a low pH resistant Lys-C protease. We also selected a reagent with oxygen scavenging activity, which was successfully applied to suppress protein oxidation during sample preparation.

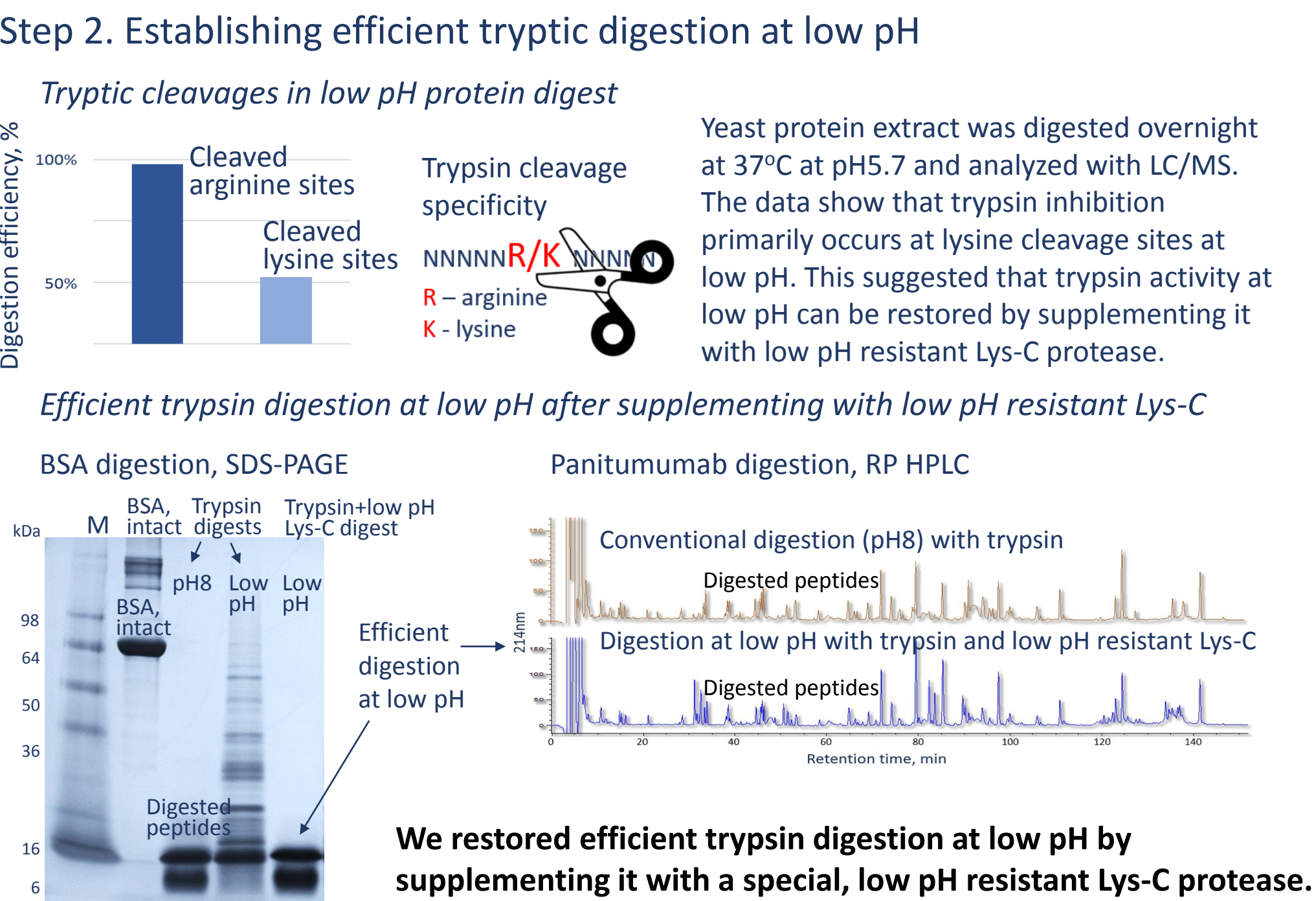
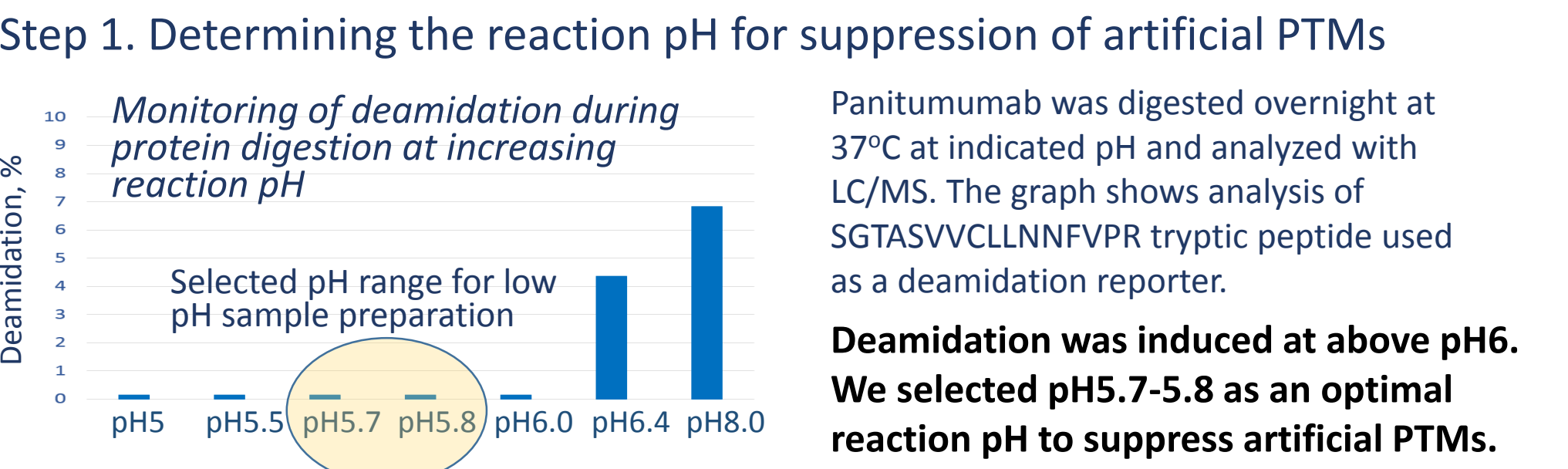
The method was optimized for use with common sample preparation procedures based on protein denaturation followed by dilution or size exclusion clean-up. Necessary optimizations were introduced to ensure efficient digestion of proteolytically-resistant protein domains, minimal baseline noise in UV HPLC and LC/MS and high analytical reproducibility.

2. Artificial non-enzymatic PTMs induced during sample preparation and low pH solution



Alkaline conditions favored by conventional sample preparation induce non-enzymatic PTMs. Suppression of these PTMs requires use of acidic conditions (low pH).

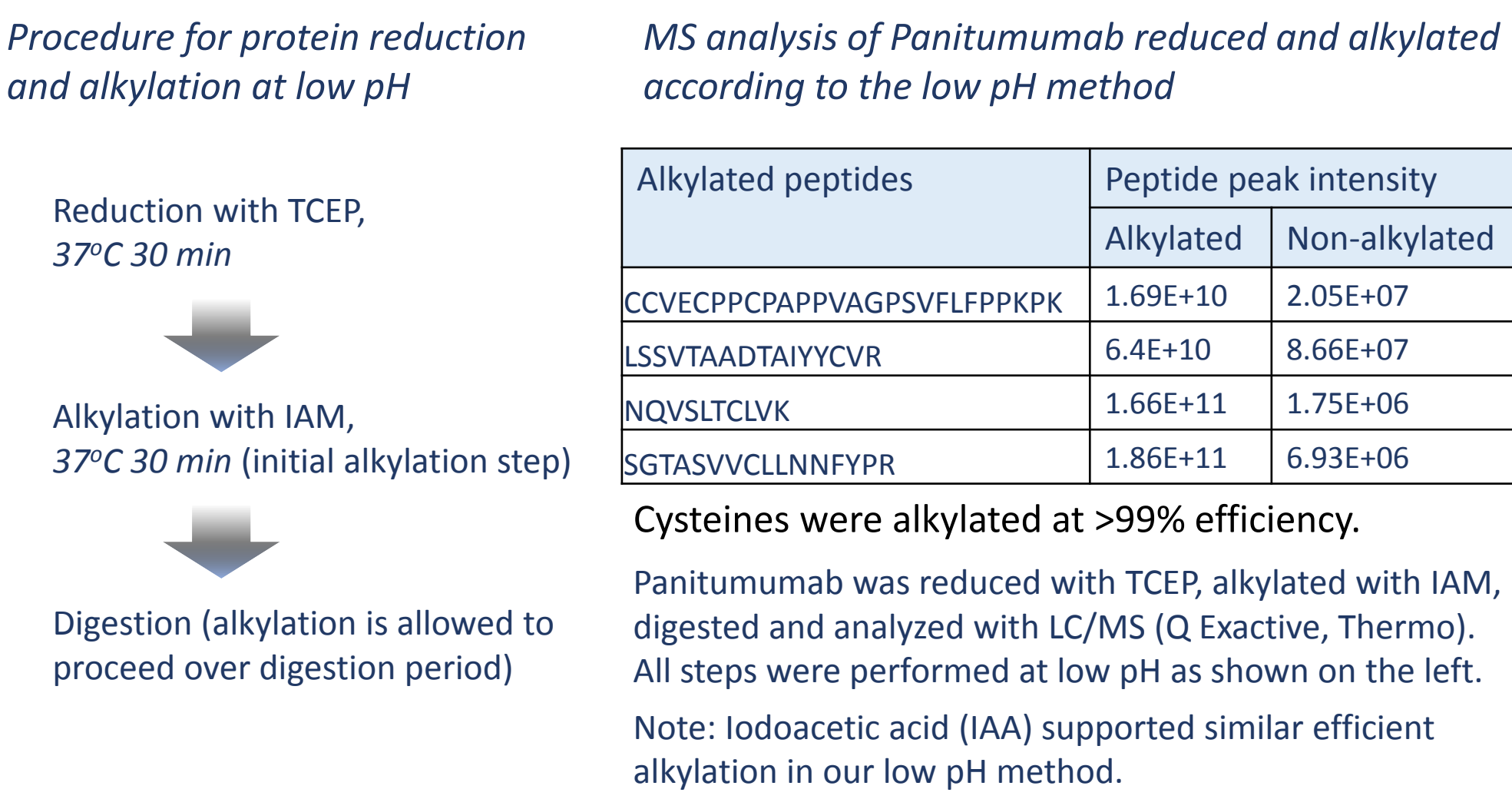
3. Establishing efficient trypsin digestion at low pH



4. Establishing efficient reduction and alkylation at low pH

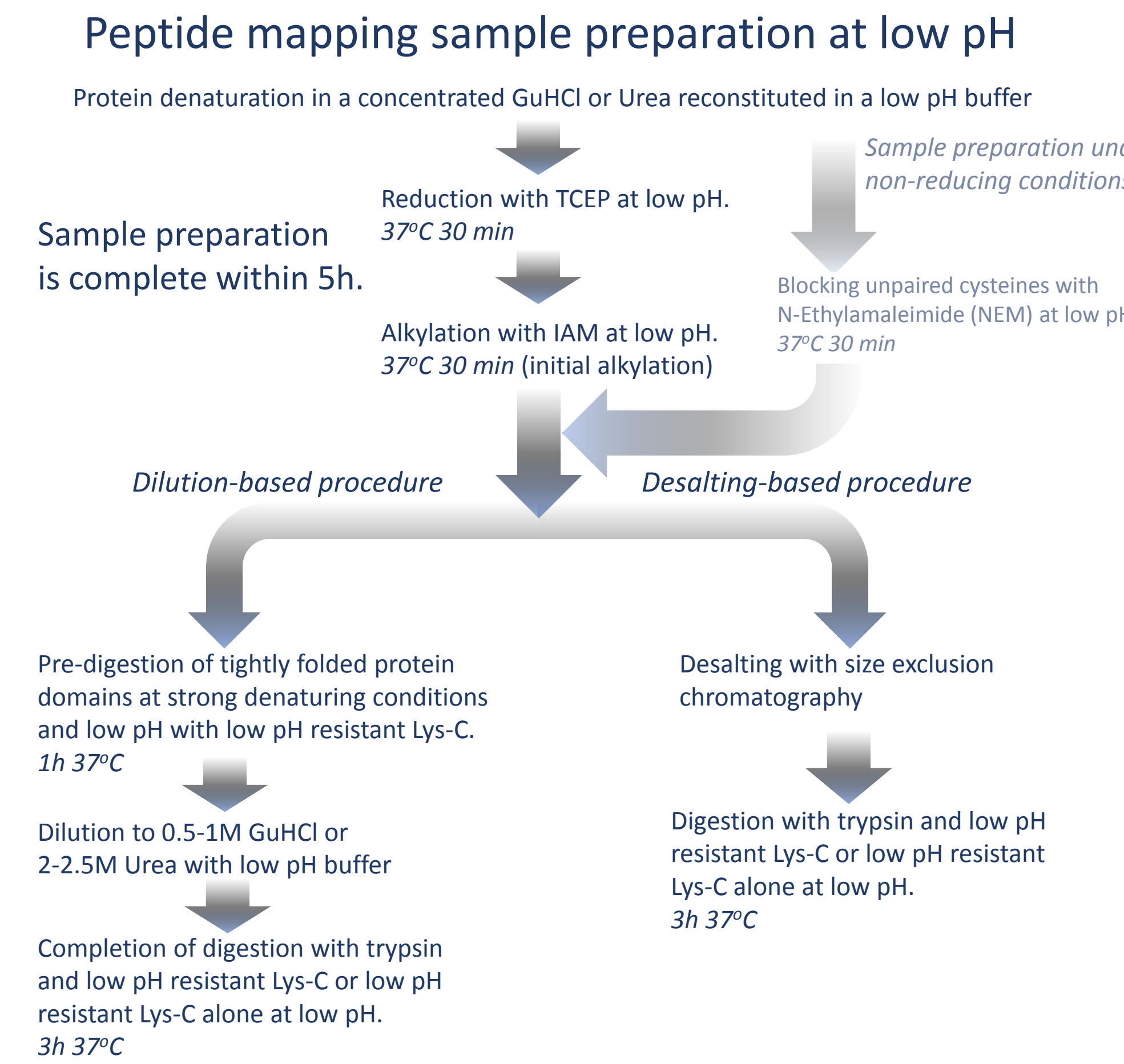
Conventional reagents used to reduce and alkylate disulfide bonds favor alkaline conditions. To achieve efficient reduction and alkylation at low pH we took the following steps:

- Tris(2-carboxyethyl)phosphine) (TCEP), a reducing reagent retaining high activity over a broad pH range including mildly acidic conditions, was selected for the method.
- Iodoacetamide (IAM) was selected as an alkylating agent. To compensate for its decreased activity at acidic pH, we allowed alkylation to proceed over digestion period.

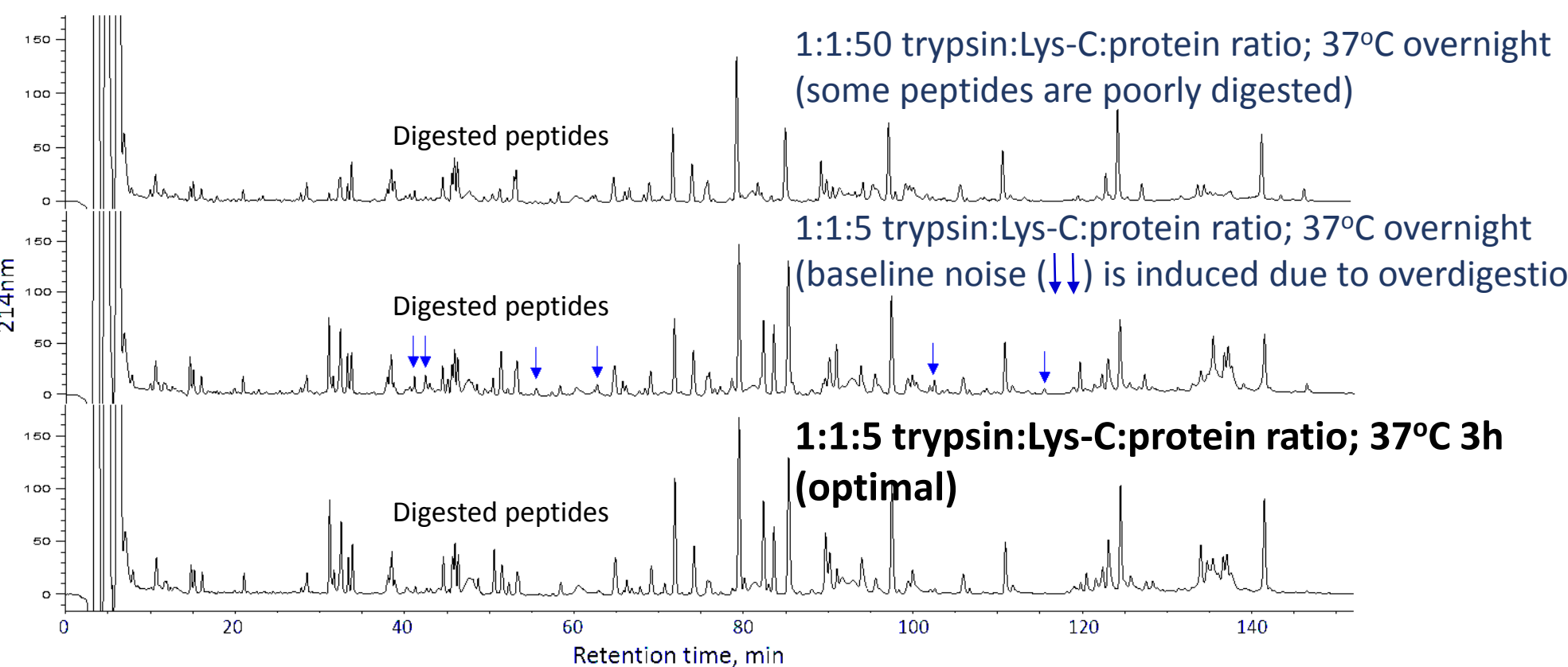


Efficient reduction and alkylation was achieved at low pH requiring minimal procedural modifications.

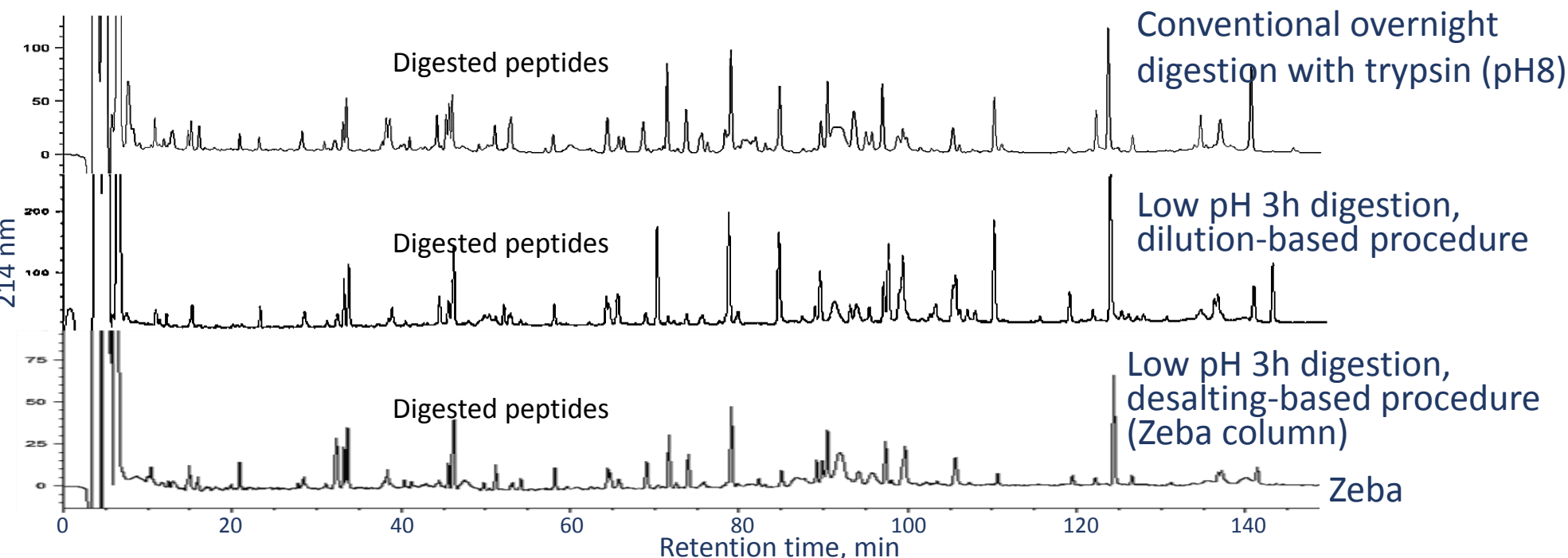
5. Adaptation of the method for common sample preparation procedures



Optimization of protein digestion at low pH (dilution-based procedure with GuHCl as a denaturing agent), RP HPLC analysis of Panitumumab digests



Side-by-side comparison of alternative digestion procedures, RP HPLC analysis of Panitumumab digests



Panitumumab was denatured, reduced, alkylated, digested as indicated and analyzed with RP HPLC (Agilent 1200 HPLC).

The low pH digestion procedures supported efficient proteolysis at low pH. The dilution-based procedure provided most efficient and reproducible (data not shown) digestion.

LC/MS analysis of IgG digestion at low pH

	Conventional digestion (pH8)	Low pH digestion
Total spectra	1197	1289
Seq. coverage	97%	98%

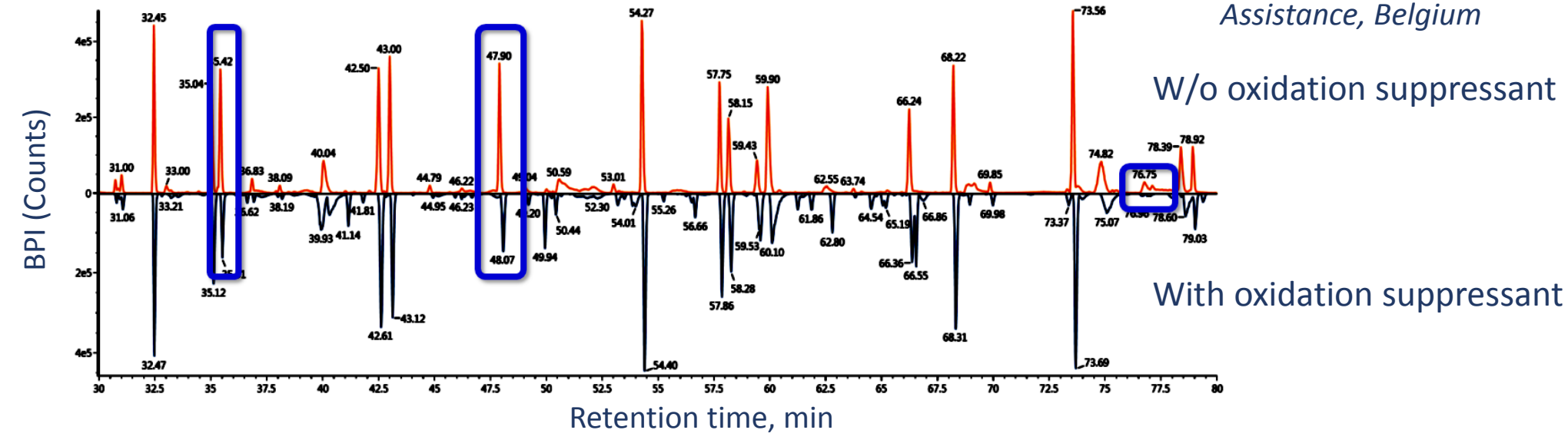
A model IgG was denatured, reduced, alkylated and digested at pH8 or low pH and analyzed with LC/MS.

No deterioration in key performance parameters was observed in low pH digest.

6. Suppression of protein oxidation with an oxygen scavenger reagent

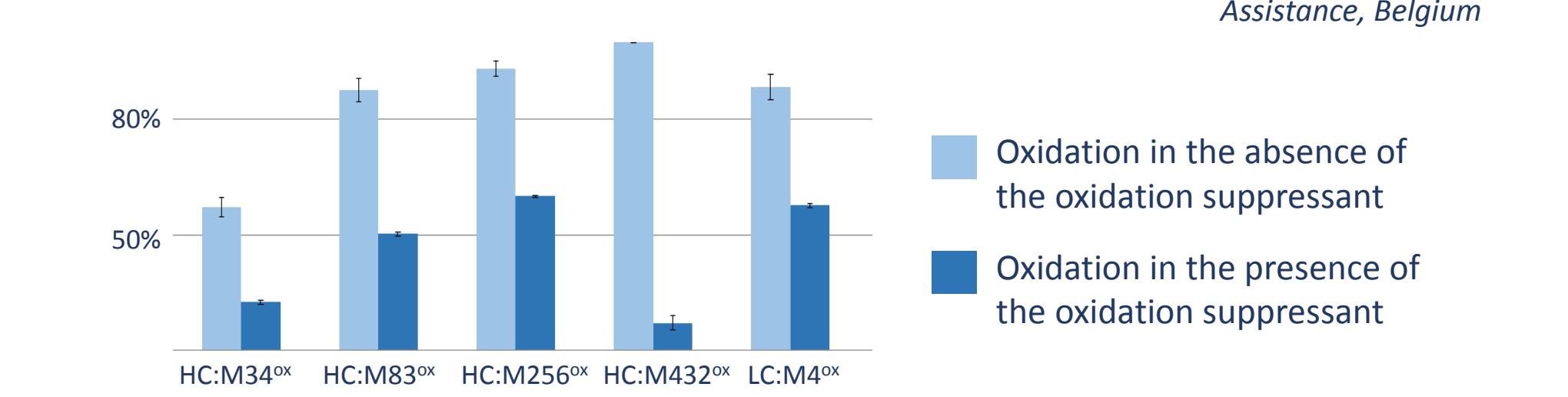
Various excipients and impurities can cause protein oxidation during sample preparation. To protect proteins from oxidation we selected a reagent with high oxygen scavenging activity ('oxidation suppressant').

Suppression of H₂O₂-induced methionine oxidation with the oxidation suppressant during IgG digestion (BPC)



A model IgG (Adalimumab) was denatured, reduced, alkylated and digested according to the low pH sample preparation procedure in the presence of an oxidation agent (hydrogen peroxide) with or without the oxidation suppressant. The digests were analyzed with LC/MS. The data indicate suppression of oxidation.

Suppression of H₂O₂-induced methionine oxidation during IgG digestion (oxidized methionine residues, %)

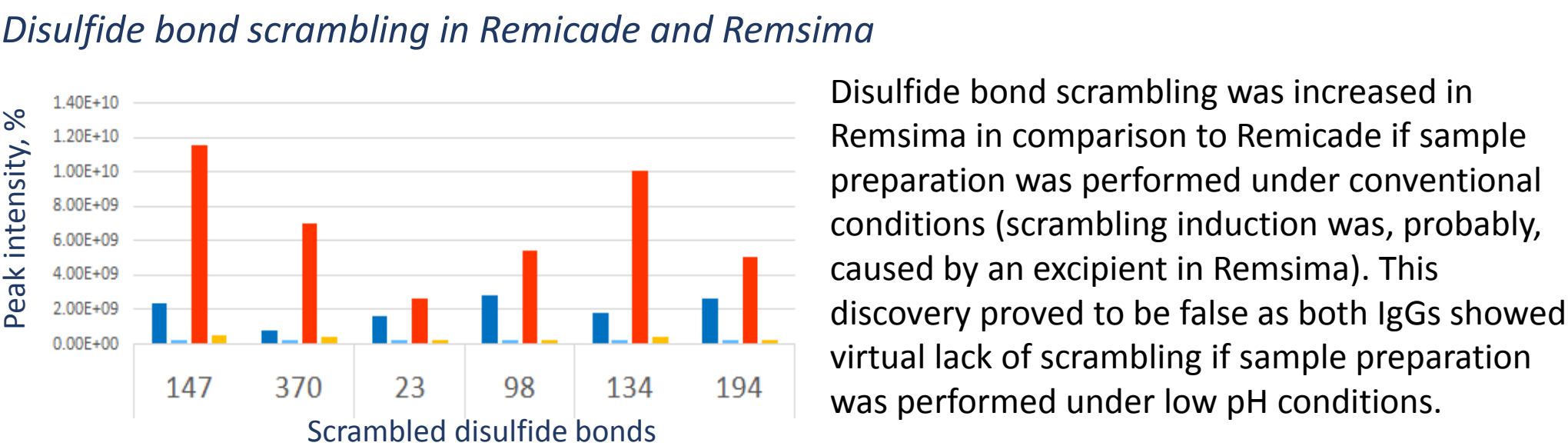
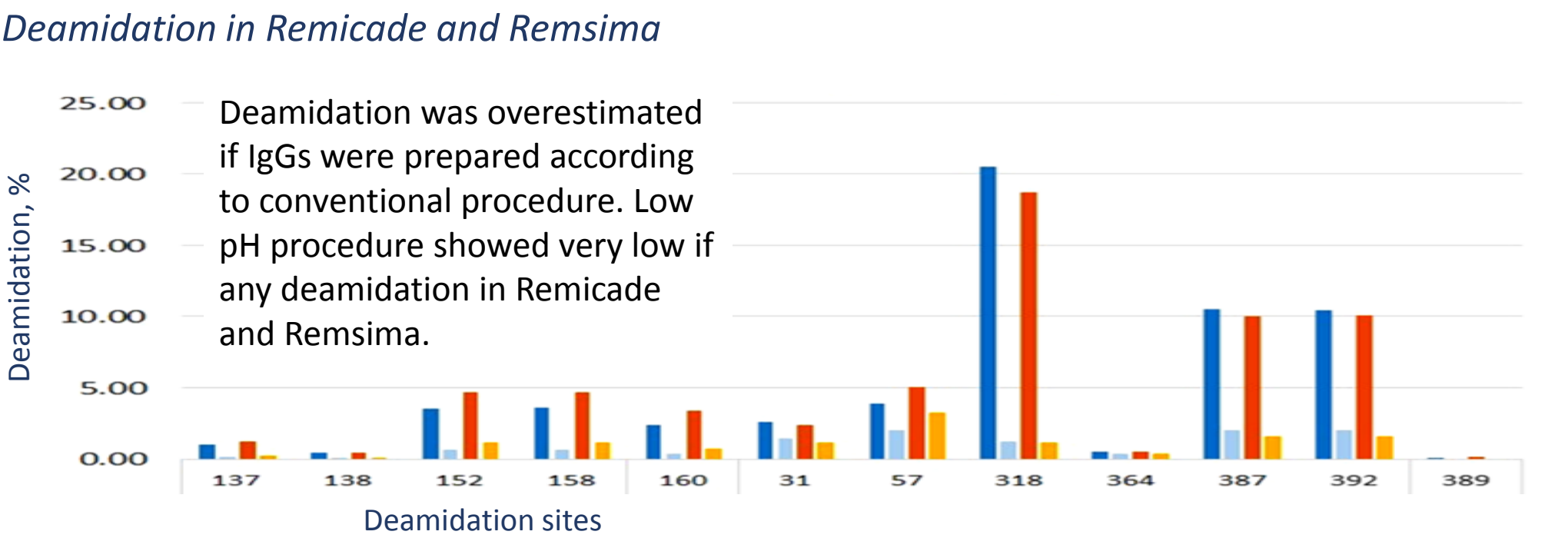


The oxidation suppressant suppressed protein oxidation during sample preparation in a model experiment.

7. Case study: BioSimilar vs Originator comparison

The low pH sample preparation method was applied to investigate structural similarity between BioSimilar (Remsima) and Originator (Remicade).

Collaborative study by Anna Schwendeman et al. (U.Michigan), MS BioWorks, LLC; Protein Metrics Inc. and Promega Corporation.



IgG was digested according to conventional (pH8) or low pH procedure and analyzed with LC/MS (Q Exactive, Thermo).

Low pH sample preparation provided the means for accurate comparison of BioSimilar and Originator.

8. Conclusions

- We have developed a new peptide mapping method in which all sample preparation steps are performed at low pH (mildly acidic conditions).
- We achieved robust tryptic protein digestion at low pH by supplementing trypsin with with a specialized, low pH resistant Lys-C.
- The method was optimized for efficient reduction, alkylation and digestion efficiency at low pH, as well as minimal baseline noise and high reproducibility. It was successfully incorporated into common sample preparation procedures based on use of denaturing agents followed by dilution or size exclusion clean-up prior to digestion.
- Sample preparation is complete within 5h in our method.
- We have also selected a reagent with high oxygen scavenging activity for optional use as a protein oxidation suppressant during sample preparation.
- The method offers an efficient means for preventing artificial non-enzymatic PTMs commonly induced during peptide mapping sample preparation.