Pharmacokinetics and Metabolism: Analysis of Low-abundant Deamidation and Oxidation Modifications in Therapeutic Species in Complex Biological Matrices by Liquid Chromatographic and Mass Spectrometry Methods

by

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List of abbreviations

AGC	Automatic gain control
Asn	Asparagine
Asp	Aspartic acid
AUC	Area under the curve
BCA	Bicinchoninic acid assay
BLAST	Basic local alignment search tool
CDR	Complementarity-determining regions
CE	Collision energy
cQA	Critical quality attribute
CV	Coefficient of variation
Cys	Cysteine
Deam	Deamidation
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EFGR	Epidermal growth factor receptor
EIC	Extracted-ion chromatogram
ELISA	Enzyme-linked immunosorbent assay
ETD	Electron-transfer dissociation
FA	Formic acid
Fab	Antigen-binding fragment
Fc	Crystallizable fragment
FcRn	Neonatal Fc receptor
FDA	Food and Drug Administration
Gln	Glutamine
Gly	Glycine
GMP	Good manufacturing practice
H_2O_2	Hydrogen peroxide
HC	Heavy chain
HCD	Higher-energy collisional dissociation
HCL	Hydrogen chloride
HER	Human epidermal growth factor receptor
HILIC	Hydrophilic interaction liquid chromatography
His	Histidine
HPLC	High-performance liquid chromatography
IAM	Iodoacetamide
IEF/c-IEF	Isoelectric focusing / capillary isoelectric focusing
IgG	Immunoglobulin G
IS	Internal standard

Iso-Asp	Iso-aspartic acid
IT	Injection time
IV	Intravenous
LC	Light chain
LC-MS	Liquid chromatography–mass spectrometry
LLOQ	Lower limit of quantification
Lys-C	Lysobacter enzymogenes protease
mAb	Monoclonal antibody
MAM	Multi attribute method
Met	Methionine
MS	Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut off
n.a.	Not applicable
NaOH	Sodium hydroxide
Ox	Oxidation
PBS	Phosphate-buffered saline
pH	Potential of hydrogen
PIMT	Protein L-iso-aspartyl methyltransferase
PK	Pharmacokinetics
PKP	Pharmacokinetic profile
PRM	Parallel reaction monitoring
Pro	Proline
PTM	Post-translational modification
QC	Quality control
QQQ	Triple quadrupole
rcf	Relative centrifugal force
RP	Reverse phase
RPLC	Reverse phase liquid chromatography
rpm	revolutions per minute
RSD	Relative standard deviation
RT	Retention time
SDS	Sodium dodecyl sulfate
s/n	Signal-to-noise ratio
Ser	Serine
SIL	Stable isotope labeled
SPE	Solid phase extraction
SRM/MRM	Selected reaction monitoring / multiple reaction monitoring
STD	Standard deviation
TCEP	tris(2-carboxyethyl) phosphine

TFA	Trifluoroacetic acid
Trp	Tryptophan
Tyr	Tyrosine
UPLC	Ultra-performance liquid chromatography
USP	Unites States pharmacopeia

Zusammenfassung

Die Charakterisierung und Quantifizierung von Modifikationen wie die Deamidierung, die in therapeutischen Proteinen nach deren Injektion in das Blut von Patienten auftreten können, sind von immenser Bedeutung, da sie pharmakokinetische Folgen nach sich ziehen können. LC-MS/MS-basierte Methoden, die mehrere Modifikationen in präklinischen/klinischen Studien gleichzeitig überwachen, gibt es kaum. Eine Analyse von therapeutischen Proteinen im Blut von Patienten ist besonders anspruchsvoll, da ähnliche körpereigene Proteine in diesen Proben vorhanden sind. Pertuzumab ist ein vollständig rekombinanter, humanisierter monoklonaler Antikörper (MAK), der für die Behandlung von HER2-positivem, metastatischem Brustkrebs genutzt wird. Wie andere MAKs kann Pertuzumab nach der Anwendung Biotransformationen unterliegen. Dennoch wurden Methoden, die in vivo den Abbau von Pertuzumab überwachen und auf LC-MS/MS-Methoden basieren, bisher nicht entwickelt. In dieser Arbeit war es das Ziel eine LC-MS/MS-Methode zu erarbeiten, mit der die gering abundanten Deamidierungs- und Oxidationsmodifikationen in Pertuzumab in Serum quantifiziert werden können. Hierfür wurden verschiedene Proben-Vorbereitungsverfahren für die Verdauung der Proben in Bezug auf Verdauungseffizienz und Deamidisierungs-/Oxidationsniveau verglichen, welche als Nebenreaktionen durch die Probenvorbereitungsbedingungen verursacht wurden. Mittels LC-MS/MS-Analysen wurden die Aminosäuren identifiziert, die besonders empfindlich gegenüber Deamidierungs- und Oxidationsmodifikationen sind und die pharmakokinetische Relevanz besitzen und die zugehörigen tryptischen Peptide für die weitere Methodenentwicklung ausgewählt. Eine Umkehrphasen-LC-Chromatographie wurde solange optimiert, bis eine Basislinientrennung zwischen den amidierten und deamidierten Spezies erreicht wurde. MS-basierte gezielte Methoden wie PRM (parallel reaction monitoring) und SRM (selected reaction monitoring) wurden angewandt und bezüglich Sensitivität und Spezifizität verglichen. Sensitivität, Robustheit und Reproduzierbarkeit in einer optimierten PRM-Methode wurden auf einer unteren Quantifizierungsgrenze von 2ug/ml für Pertuzumab in Kaninchenserum erzielt. Die entwickelte Methode wurde gemäß FDA-Richtlinien validiert und alle untersuchten Kriterien für eine bioanalytische Methodenvalidierung wurden erfüllt. Während der Methodenvalidierung wurden verschiedene Quantifizierungsstrategien untersucht, die alle auf dem relativen internen Standard (IS) basierten, um sich einer akuraten Quantifizierungsrate unter Berücksichtigung von Matrixeffekten in Serum zu nähern. Es konnte ein erhöhte Matrixeffekte in individuellen Seren im Vergleich zu Serumsammelproben beobachtet werden. Schließlich wurden Genauigkeit und Präzision bei der Quantifizierung von niedrig abundanten modifizierten Peptiden durch die IS-basierte Quantifizierungsstrategie erreicht. Die validierte Methode wurde verwendet, um Deamidierungs- und Oxidationsraten zwischen in-vivo- und in-vitro-Proben im zeitlichen Verlauf zu untersuchen und zu vergleichen. Interessanterweise wurde eine individuelle Variabilität der Deamidierungsraten in vivo im Zeitablauf festgestellt. Zudem unterschieden sich die Deamidierungsraten über die Zeit unter denselben Bedingungen zwischen in-vivo- und in-vitro-Modellen. Aus diesem Grund war es nicht möglich, ein in-vitro-Modell, das angereicherte Serumsammelproben nutzt, zur Vorhersage von in-vivo-Deamidierung bei individuellen Kaninchen zu verwenden. Dennoch konnte eine Korrelation zwischen Deamidierungsraten, die im Zeitablauf in der Fc-Region auftraten, und Deamidierungsraten, die in der CDR-Region auftraten,

in Kaninchen beobachtet werden. Dies erlaubt es, Abbauuntersuchungen, die sich auf die Fc-Region fokussieren, als Indikator für die Empfindlichkeit gegenüber Modifizierungsreaktionen in CDR-Regionen heranzuziehen. Zusammenfassend können die Ergebnisse dieser Arbeit als Basis für Studien zur MAK-Stabilität und deren Empfindlichkeit gegenüber Modifizierungsreaktionen in vivo dienen. Die vorgeschlagene IS-basierte Strategie für eine akurate Quantifizierung trotz Matrixeffekt ist auf die Untersuchung jedes beliebigen MAKs und jeder beliebigen Modifikation auf Peptidniveau übertragbar. Die optimierte Probenverdauung ist für Bottom-Up-Studien anwendbar, in denen geringe Deamidierungs- und Oxidationsnebenreaktionen wichtig sind. Der beobachtete Unterschied in Bezug auf Deamidierungsneigung für Fc- und CDR-Regionen zwischen verschiedenen MAKs ist auch von Interesse, wenn es um die Untersuchung von Protein-3D-Strukturen und um die Stabilität für Biotherapeutika und Biosimilars geht.

Abstract

Characterization and quantification of modifications such as deamidation occurring in therapeutic proteins after clinical administration are of extreme importance as they may have a pharmacokinetic impact. LC-MS/MS-based methods monitoring several modifications at a time in exploratory animal study/clinical samples are limited. Analysis of in vivo samples increases the complexity of the assay due to the presence of endogenous proteins. Pertuzumab, is a fully recombinant humanized monoclonal antibody (mAb) used in the treatment of HER2-positive metastatic breast cancer. As other mAbs, Pertuzumab may be subject to biotransformation after administration. However, methodologies for monitoring its degradation in vivo based on LC-MS/MS methods have not yet been thoroughly described nor explored. Within this work, an LC-MS/MS method capable of quantifying low abundant deamidation and oxidation modifications in Pertuzumab in serum as biological matrix was developed. For this purpose, distinct sample digestion preparations were compared in terms of digestion efficiency and induced levels of deamidation and oxidation side reactions due to sample preparation conditions. Through forced degradation studies and LC-MS/MS analysis, peptides of interest were selected based on their propensity to deamidation and oxidation modifications and their pharmacokinetic relevance. A reversed-phase LC method was optimized until a baseline separation between deamidated species was obtained. MS-based targeted methods such as PRM and SRM were applied and compared in terms of sensitivity and specificity. Sensitivity, robustness, and reproducibility in an optimized PRM method at a low limit of quantification of 2ug/ml of Pertuzumab in animal serum was demonstrated. The developed method was validated according to the FDA guidelines and all studied criteria for bioanalytical methods validation were fulfilled. During method validation, different relative internal standard (IS)-based quantification strategies were explored to approach accurate quantification rates despite a matrix effect of serum. An exacerbated matrix effect was observed within individual serum compared to pooled serum. Nevertheless, accuracy and precision in the quantification of low abundant modified peptides were obtained through a proposed IS-based quantification strategy. The validated method was applied to study and compare deamidation and oxidation rates between in vivo and in vitro samples over time. Interestingly, an individual variability of deamidation rates in vivo over time was observed. Furthermore, the rates of deamidation over time under the same conditions differed between in vivo and in vitro models. Therefore, an in vitro model using spiked pooled serum was not possible to be used for predicting in vivo deamidation of individual animals. However, a correlation between deamidation rates over time occurring in the Fc region deamidation rates occurring in the CDR region was observed within individual animals. This allows degradation studies focusing on the Fc region to serve as an indicator for degradation propensity of CDR regions. In conclusion, the results observed in this work serve as a basis to study mAb stability and individual degradation in vivo. The proposed IS-based strategy for accurate quantification despite a matrix effect is applicable to the study of any other mAb and any other modification at peptide level. The optimized sample digestion is applicable for bottom-up studies where low deamidation and oxidation side reactions are required. The observed difference in deamidation propensity for Fc and CDR regions between mAbs under the same conditions and in vivo is of interest within the study of protein 3D structure and stability for both biotherapeutics and biosimilars.

Introduction

HER2-positive breast cancer

Breast cancer is not a single disease but a comply of several tumor subtypes with different molecular causes. That variety of different origins leads to different required treatments. One possible cause of breast cancer is the overexpression of HER2. The HER family comprises for members HER 1-4, while the overexpression of HER2 counts for 20% of all breast and gastric cancer cases. The HER receptors share a similar transmembrane glycoprotein structure with four sub-domains allowing the binding of different ligands and receptor dimerization. The ligand binding results in either homodimerization or heterodimerization between different HERs, induce tyrosine kinase phosphorylation and lead to cell migration, proliferation, and cell survival[1].

HER2 overexpression in a significant percentage of invasive breast cancer cases has prognostic and predictive implications. Up to 25 to 50 copies of HER2 genes and up to 40- to 100-fold increase in the HER2 proteins results in 2 million receptors expressed at the tumor cell surface[2]. HER2 gene amplification is clinically associated with shorter disease-free and overall survival/time to relapse in breast cancer[3]. HER2 amplified breast cancers have also increased sensitivity to some cytotoxic chemotherapeutic agents, resistance to some hormonal agents, and increased propensity to brain metastasis[4].

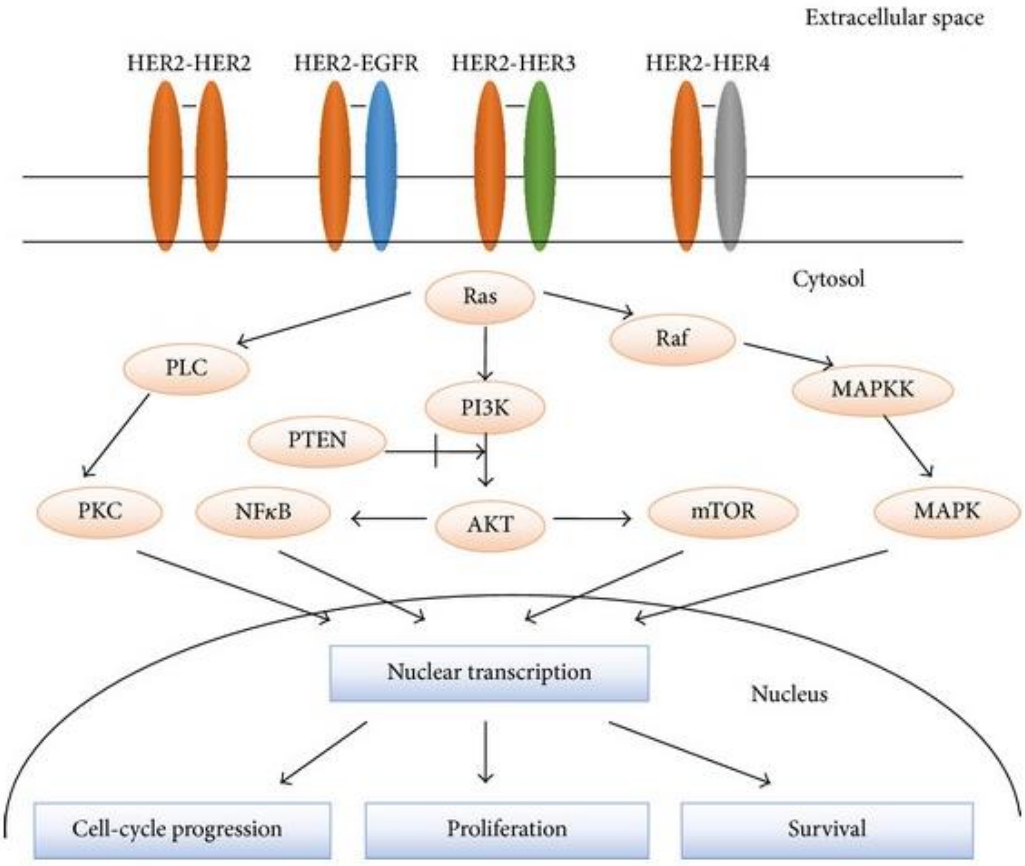


Image. Homodimerization or Heterodimerization of HER receptors lead to downstream signaling pathways promoting cell growth, proliferation, and cell survival. (Iqbal N, 2014, Mol Biol Int)

Monoclonal antibodies and biosimilars

Antibodies are proteins of the immune system produced by B-lymphocytes which recognize and bind to targets (antigens). Monoclonal antibodies (mAbs) are produced by single B-Cells. Their production using cell fusion techniques and hybridoma cell production was introduced in 1975. However, the therapeutic emergence was boosted when these mAbs were found to be able to bind biological targets like tumor antigens, molecules involved in autoimmune and infectious disease related molecules, etc[5]. Their promising therapeutic effect in the treatment of cancers, autoimmune and infectious diseases, etc. uplifted its research and production. Currently there are more than 50 therapeutic mAbs produced on the market with a value of over 120 billion USD global market share[5]. However, development costs of these therapies are very high and patient accessibility is limited. For these reasons, biosimilar versions start to emerge after the patent protection expires in originator products.

Biosimilars are biological drugs highly similar but not identical to the originator biological reference. While the synthesis of the active ingredient may not represent a significant problem in the development of small molecules, mAbs are complex macromolecules, whose production process involves highly complex and sensitive regulatory mechanisms of microorganisms, glycosylation processes, isolation, and purification, etc. Because of that, the end product is expected to be slightly different with respect to e.g. post-translational modifications even if the peptide sequence of a biosimilar is identic to the originator[6,7]. Therefore, production of biosimilars undergo an extensive scrutiny of regulation to assure their bioequivalence and validity through comparability exercises as well as their similarity to the innovator[7,8]. The presence of biosimilars as a cheaper alternative to the patient justifies its economical nature need. However, a strict regulation environment and extensive testing is needed prior to release.

Pertuzumab and Trastuzumab

Pertuzumab and Trastuzumab are fully recombinant humanized monoclonal antibodies based on human immunoglobulin IgG1. They target the HER2 dimerization (receptor pairing) for the treatment of HER2 positive breast cancer[9]. Dimerization of the human EFGR family mediates intracellular signaling events, which promote cancer proliferation survival and therapeutic resistance. Pertuzumab has been therapeutically used for its binding to HER2 causing the blockage of the binding pocket necessary for receptor dimerization and signaling. This inhibits signaling pathways of cell survival and growth such as mitogen-activated protein kinase pathway and PI3K pathway[10–12]. Pertuzumab is usually administrated among with Trastuzumab. Both molecules bind to different regions on HER2 and therefore having a synergistic activity. Trastuzumab was approved in 1998, however the lack of gene amplification or overexpression was later identified as a possible reason of Trastuzumab's inefficacy in other types of solid tumors[13–15]. One mechanism of Trastuzumab's resistance was HER3 overexpression. By Trastuzumab binding the extracellular domain of HER2, downstream signaling was blocked and it was not effective in inhibiting heterodimerization especially in HER2-HER3. This led to the interest for a novel therapeutic class of HER2 targeted therapies, such as the use of Pertuzumab. The development of Pertuzumab aimed at a different epitope than Trastuzumab. It binds to the dimerization domain II of HER2 and therefore prevents the formation of the ligand induced HER2 heterodimer. These properties are responsible for activating mitogenic and survival signal pathways. Although Pertuzumab has shown limited activity when used as monotherapy, the combination with Trastuzumab and Docetaxel represents a novel standard for the first-line treatment of HER2-positive metastatic breast cancer[9].

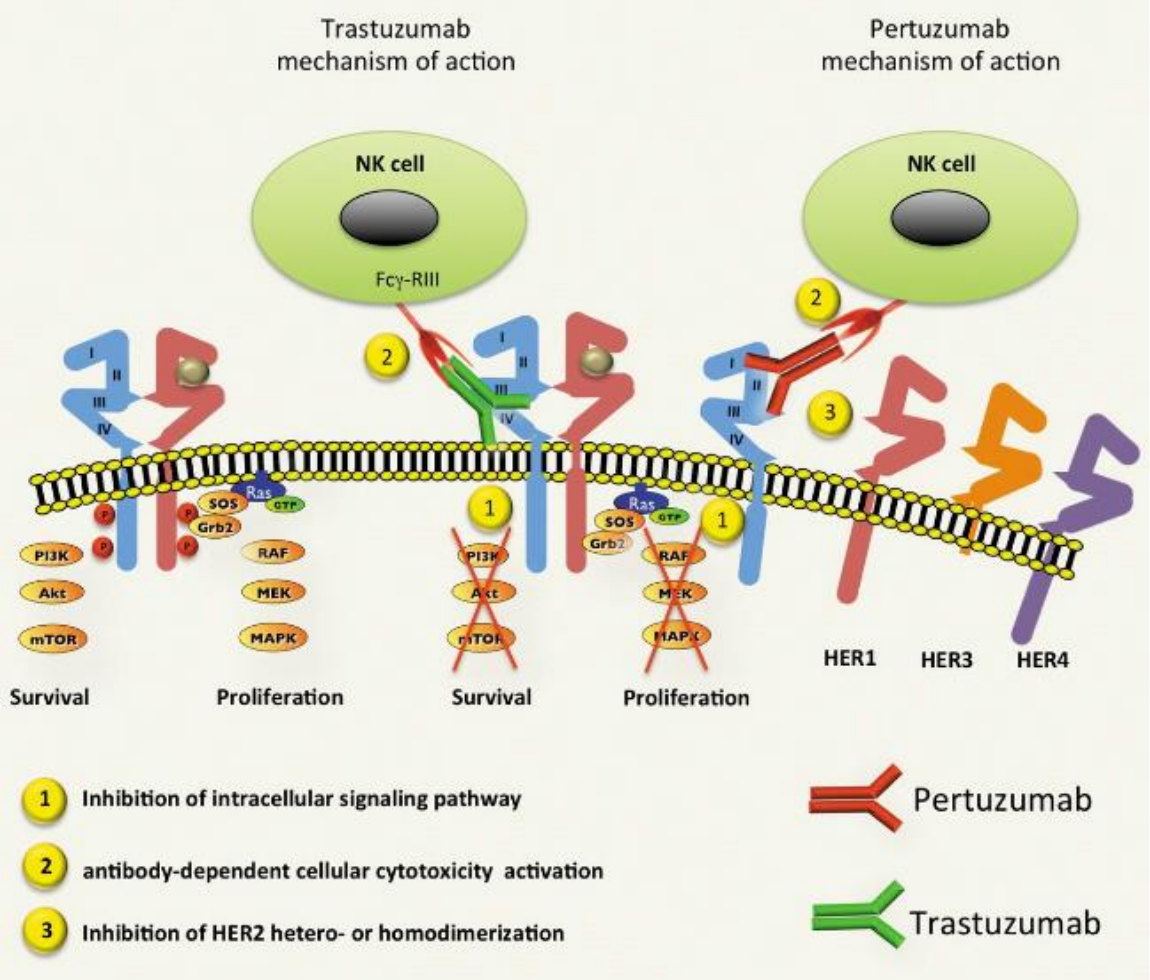


Image. Trastuzumab's and Pertuzumab's mechanism of action (Barthelemy P. 2014, Anticancer Research)

Post translational modifications and its impact on pharmacokinetics

Since the appearance of biological therapeutics (such as mAbs) as a promising therapeutic platform for the treatment of various human conditions, an increasing effort has been performed in understanding the multiple pathways that may lead these molecules to degradation during formulation, storage, and after administration to the patient. The modifications occurred during the protein's lifespan may be traduced to a risk of immunogenicity, safety, stability, and changes in the pharmacokinetics by affecting the efficacy profile, biological activity, and therapeutic effect[16–19]. Apart from biologically induced post-translational modifications, protein heterogeneity is also caused by chemically induced modifications. These have their origin in the potentially reactive side chains of most amino acids. The protein's metabolism may lead to deamidation of Asparagine and Glutamine, oxidation of amino acids as Cysteine, and Methionine, oxidative modification of Tyrosine, Tryptophan and Histidine, nitration of Tyrosine and Tryptophan and so on[20]. The proper characterization and control of these modifications is a critical aspect for quality assurance as well

as for proper therapeutic effects, safety, and comparability to originator molecules in the case of biosimilars[21].

Prior to approval and release, biological products must be subject to an extended analysis to assess the nature of the parameters considered as critical quality attributes (cQAs). cQAs are those molecular features that may affect the function, stability or immunogenicity of the mAb if not present within a determined range[22]. For example, the glycan composition is an important feature to be considered as the lack of core fucosylation enhances binding affinity and increases antibody-dependent cellular cytotoxicity. In turn, the presence of non-human N-glycan moieties increases immunogenicity and drug clearance. Presence of oxidation influences the half-life as well as the affinity. Modifications such as deamidation may reduce the activity and potency of the mAb[22–28].

Chemical modifications of therapeutic proteins can occur both in vivo and in vitro during all their lifespan[29], from biosynthesis to their complete clearance[30]. During manufacturing and storage, modifications are monitored with control and analytical testing strategies. However, once the protein is administered to the patient it can undergo additional changes that are partially understood due to the complexity of the blood environment, but still require a better understanding[16]. Conditions present in the blood such as specific pH (7.35-7.45), temperature (37° C), the presence of protein-modifying enzymes, etc. may vary between patients and trigger changes not previously assessed during development. Such changes can potentially lead to an effect on pharmacokinetic properties (such as serum half-life and clearance) as well as on the safety and efficacy profile and/or trigger of an immunogenic response[16,18].

Some modifications have been previously described as responsible for the degraded forms of therapeutic proteins such as glycosylation, N-terminal pyroE formation, C-terminal lysine removal, glycation, aggregation, deamidation, oxidation, alternative disulfide bond linkage as trisulfide bonds, thioether formation, racemization etc[16,18,31]. A better understanding of the conditions, heterogeneity, and immunogenicity caused by these is essential to improve protein efficacy, safety, and quality[30].

Oxidation

Oxidation of Methionine and Tryptophane has been well characterized in the literature. In that case, two conserved Met residues were identified in the Fc region (Met 252 and Met 428) which are highly susceptible to oxidation under certain conditions during manufacturing or storage, such as contact to metal surface and exposure to light[18,31–35]. Several studies have well characterized that oxidation on these two residues lead to a decrease in affinity towards the neonatal receptor FcRn[25,36,37]. These studies forced an oxidation at highly unnatural levels up to close to 80% of Methionine oxidation. In contrast to that, studies with a lower level of oxidation (as 7%) did not show any results in affinity decrease[37]. Furthermore, it has been also defined that only molecules with both heavy chains oxidized show a significantly faster clearance, as a single oxidized chain conserves the molecule's binding activity to the FcRn[18,38]. Therefore, oxidation does not represent a major impact on biological activity. However, it becomes of interest when comparing in vivo and in vitro spiked models. It has been proven that when studying these major degradation

pathways occurring in vivo, spiked PBS models may correlate and predict the same levels of modification seen in vivo except for oxidation. In the case of oxidation, results in correlation with in vivo samples were only able to be seen when using a serum spiked model[39]. This shows that the presence of serum is necessary to reproduce and track all in vivo product oxidation reactions.

Deamidation

Deamidation is the major degradation pathway of Asparagine and Glutamine resulting in Aspartate, Iso-aspartate, and Glutamate formation. This degradation pathway has been seen in vivo and implicated in aging and age-related diseases[40]. The process of protein deamidation is characterized by the attack of the peptide bond nitrogen of the N+1 amino acid on the carbonyl carbon of the asparagine under alkaline pH. That creates an intermediate succinimide ring which is quickly hydrolyzed either at the a or b carbonyl groups, forming then iso-Asp and Asp at a 3:1 ratio[40]. On the other hand, at presence of acidic or neutral pH, Asp residues isomerization occurs through dehydration of Asp and the deamidation of Gln through the formation of a six-ring glutanimide[41]. This has been a modification reported among the major causes of charge heterogeneity in therapeutic proteins by introducing acidic species due to the addition of a negatively charge Asp and the insertion of a methyl residue in the polypeptide backbone. The introduction of acidic species generates acidic isomers of aspartic acid and iso-aspartic acid with a 0.984 Da mass shift and the decrease of the isoelectric point[40,42]. Deamidation is highly dependent on conditions such as pH, temperature, and ionic strength[18,30] as well as on the sequence surrounding the Asn and Gln residues. Proximity of small and/or hydrophilic residues as Ser and Thr to the Asn residue can increase the deamidation rate, while an Asn followed by a Gly residue has a great susceptibility to be deamidated[30]. Gly and Ser located C-terminally to the deamidation site have been found to be most destabilizing[43,20]. Under physiological conditions, enzymatic deamidation reactions represent spontaneous protein damage signaling aged proteins for degradation[44]. For some proteins, deamidation might serve as molecular clock of cellular biological function and it is involved in the enzymatic aging process, in which non-enzymatic spontaneous deamidation may harm many proteins. Therefore, deamidation is involved in many human disorders with aged tissue[45].

While in vivo deamidation is an irreversible process, isomerization may be repaired with a conversion rate between 15% and 25% of the original L-iso-Asp to L-Asp by enzymatic methylation followed by spontaneous ester hydrolysis through the PIMT enxyme[46–48] (Figure 1). Although Gln residues can also be deamidated, their rate is much slower than the rate for Asn residues (1-500 days for Asn while 100-5000 days for Gln at neutral pH and 37°C)[43]. Deamidation of Asn may change the structure and function of a protein through the introduction of unfavorable negative charge. This changes its hydrophobicity decreases its bioactivity while altering the pharmacokinetics and antigenicity[43,49]. When deamidation occurs in the Fab region, a change in thermal stability of the molecules has been reported, while in the CDR region changes in affinity and potency have been suspected[18,31]. The occurrence of iso-aspartate formation in one of these CDRs may lead to a high loss of antigen binding capacity[50].

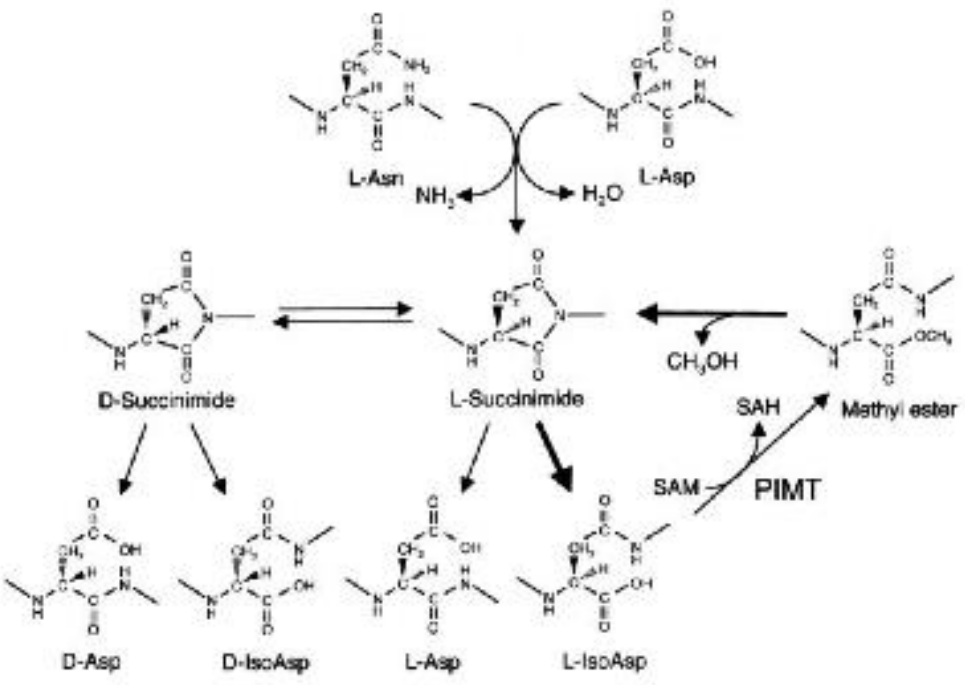


Figure 1. Deamidation of asparagine, isomerization of aspartic acid residues and repair process (Yang H and Zubarev RA 2010, Electrophoresis).

Deamidation and oxidation as PTMs of interest

Even though also other modifications have been seen also in vivo, they have not been identified to represent a major impact in biological activity, pharmacokinetic properties, or efficacy. For example N-terminal modifications such as N-terminal Pyroglutamate formation are non-enzymatic spontaneous reactions that cycle the N-terminal Gln forming pyroE. However, no effect on pharmacological properties, safety, pharmacodynamics, or pharmacokinetics have been seen[18,51,52]. C-terminal Lysine removal has no effect on structure, thermal stability, antigen binding, potency FcRn binding, or pharmacokinetics[31,53–55]. C-terminal Pro amidation has been observed to increase on a medium level with the copper addition of culture. It also increases in biological active peptides in humans, for which it is considered an unnatural modification. In the end, it has been observed to not have an effect on antigen binding and Fc effect on function[31,56,57].

On the other hand, spontaneous occurrence of deamidation in vivo may lead to the modification of the therapeutic proteins' bioactivity after administration. By this, the response alters over time with regards to the therapeutic agent and the drug efficacy[58]. Although protein deamidation occurs both in vivo and in vitro, limited literature and studies of in vivo occurrence have been available. Nevertheless, this field is of major and critical importance as seen in the anticancer agent Trastuzumab (Herceptin) and the possible decrease of its biological activity when a signature peptide presents deamidation after long exposure to the treatment on patients[20,28]. In the case of

oxidation, its monitoring represents a high interest as this is the only modification seen that enables to to correlate with in vitro with in vivo results by using serum as biological matrix.

Need for further analysis of biopharmaceuticals in vivo development

Mass Spectrometry analysis has gained use in the study of therapeutic proteins due to the specificity of this methodology. While protein quantification has been traditionally performed through ligand-binding assays, these give a single readout and it is not possible to obtain information from the structural modifications that may have induced the modification[59]. Protein biotransformation and the monitoring of in vivo drug metabolism have been somehow remained unexplored despite their impact on therapy efficiency and biopharmaceutical's pharmacokinetics. LC-MS/MS approaches provide the opportunity to study structural modifications on therapeutic proteins, while creating the chance to analyze modifications occurring during the protein's metabolism such as deamidation, oxidation, etc. Mass spectrometry relies on the unique combination of the precursor ions' molecular mass, the number of fragment ions for a specific protein in the sample, and the principle that no other protein would produce a peptide after proteolytic digestion with the same mass to charge (m/z) ratio for the precursor ion and all the same fragment ions. The combination of MS with separation and enrichment methodologies increases selectivity and reduces complexity for quantification. However, in vivo analysis present the challenge of a complex biological matrix and the potential interference of endogenous proteins present in vivo with the assay[60].

The heterogeneity of monoclonal antibodies is a critical aspect to be studied and considered during therapy. The reason for that is the possibility of these minor forms to influence pharmacokinetic properties. Trastuzumab's "Hot spots" for deamidation have been proposed through protein instability studies. Six Asn-Gly and Asn-Ser sequences can be found in Trastuzumab. Three of these labile residues (Asn55, Asn30 and Asp102) are located in the CDR regions[28]. The presence of two degradation spots in the CDR region (LC-Asn30 and HC-Asp102) prevents Trastuzumab from being able to be supplied as a liquid formulation[61].

Some studies[39] have aimed to compare in vivo with in vitro degradation rates for several modifications including oxidation, deamidation, isomerization, glycation, and N-terminal pyroglutamate formation through LC-MS peptide mapping and intact mass analysis. However, such methodologies do not study individual variability and rely on an affinity purification approach (based on an anti-idiotypic antibody to separate the mAb from other proteins), for which the study of modifications occurring in CDR regions is not possible. Further optimization is required in in vivo studies for purification and analysis. Although some in vitro studies for deamidation have been performed, few studies have assessed in vivo deamidation[59,28]. The importance of having the possibility to characterize the in vivo fate of therapeutic proteins with in vitro methods has its cause in the lack of clinical samples during early development.

As other mAbs, Pertuzumab may be subject to biotransformation and PTMs, for which peptides of interest (susceptible to changes in forced degradation studies) should be monitored for their possible impact on efficacy and safety. However, methodologies for monitoring Pertuzumab's

degradation products in vivo based on LC-MS/MS methods have not yet thoroughly been described or explored in detail.

Deamidation and oxidation artifacts caused by sample preparation

Sample preparation during multi-attribute methods can lead to the introduction of artifacts derived from the conditions used. It is important to optimize the proper conditions depending on the method and antibody to minimize these artifacts. Some approaches focused on determining artificial levels of deamidation and oxidation have been developed under this premise. For instance, levels of deamidation occurring during sample preparation steps through the usage of ^{18}O-water compared against MiliQ water in the reagent's preparation during sample preparation has been explored[62]. Through this approach, deamidation occurring during sample preparation would have a molecular weight increase of 3 Da (2 Da extra from the incorporation of ^{18}O into the newly formed carboxyl group when hydrolyzation of the succinimide intermediate) compared to the 1 Da mass increase for the deamidation occurring naturally. Therefore, this approach permits to differentiate the isotopic peak cluster of the peptides from deamidation products during analysis[62]. However, this approach generates the disadvantage of a challenging mass spectrum containing several overlapping isotopic distributions[62,63]. An analytical method based on the analysis of only b ions of peptides containing N-terminal amino acids was proposed to overcome this complicated generated mass spectrum[63].

Similarly, methods seeking to determine the level of Methionine oxidation in sample preparation through isotope labeling and LC-MS peptide mapping have been developed[64,65]. Methionine residues may be fully oxidized using hydrogen peroxide enriched with ^{18}O atoms prior to sample preparation to quench any possibility of the occurrence of oxidation artifacts during sample preparation and analysis. The level of original oxidation before the treatment may be assessed due to the 2 Da molecular difference between Methionine with ^{16}O and ^{18}O. The advantage of this method is that it may help to distinguish caused oxidation not only from sample preparation, but also from analysis (by capillary zone electrophoresis and electrospray mass spectrometry due to the electrolysis of water as well as from the increase of the potential applied to the electrospray needle)[64,65].

Other strategies explored to avoid artificial deamidation and oxidation make use of lower temperatures and lower pH values during digestion. However, these conditions might affect the activity of commonly used proteases[63]. During tryptic digestion, peptides are completely exposed to the solvent for longer periods of time in alkaline buffers. Approaches as reducing times of IgG digestion to as few as 30 minutes (by maximizing trypsin activity through complete removal of guanidine from the digestion buffer to avoid its inhibition activity on trypsin)[66] have been explored. Although diminished artificial modifications, cleaner tryptic maps due to less trypsin self-digestion, and fewer nonspecific cleavages were observed, the protein recovery free of guanidine after the desalting steps (to remove guanidine carried over to the digestion buffer) was reported as of only 70%[66].

Besides different methodologies, the influence of commonly utilized buffers optimal for maintaining trypsin activity has also been questioned on causing or reducing Asn deamidation artifacts[67]. For instance, based on the premise that organic solvents have previously showed to decrease artificial deamidation due to the decreased dielectric strength as well as reported cases of enhancing trypsin activity[68,69], the possibility of their addition was assessed within a study[67]. It was observed that a simple addition of 10% acetonitrile yielded a considerably lower level of artifacts. This simple but useful determination could be of high assistance when considering a digestion protocol.

Many other approaches have been explored in the literature, including ultrafast multi-attribute liquid chromatography-mass spectrometry methods[21,70], the use of controlled microwave radiation during digestion[71], the use of microwave assisted hydrolysis techniques and its effect on deamidation,[22] etc. However, some of these approaches require extra instrumentation under very controlled conditions and may increase the complexity of the assay.

The proper conditions of sample preparation must be tested per method depending on the question of interest. An equilibrium and compromise between efficiency of sample preparation (digestion completeness, carry over, used buffers) and the derived artificial modification from the experiment conditions must be found.

Challenges of monitoring deamidation and current analytical methods used

Mass spectrometry analysis of proteoforms is an essential aspect of protein characterization and of understanding the complexity of the regulation in the cell's physiology. Many post-translational modifications influence the activity and regulation of proteins by the introduction of a modified group or amino acids (Table 1). For instance, kinase cascades and enzyme activity are triggered and inhibited by reversible addition and removal of phosphate groups[72–74]. The current race for analysis of proteoforms and modified peptides and their impact on functionality is crucial for both discovery and protein characterization. However, analysis of PTMs is challenging due to their low stoichiometric abundance. In the case of deamidation the low mass shift introduced presents an additional challenge. Analytical methods must be sensitive and with the capability for high resolution to overcome the challenge of separation and analysis of antibody variants which differ greatly despite the small differences in mass. A big effort on method development and optimization is required to study the mentioned types of PTMs and their impact on biological functions.

Post-translational modification* (modified residues)	Change in mass (Δm, Da)	Example biological functions and comments
Phosphorylation		
(Ser, Thr, Tyr)	80	Signal transduction, regulation of enzyme activity, involved in protein–protein and protein–ligand interactions; phosphorylation and sulphation have the same Δm (80 Da), but can be distinguished by positive- or negative-ion mass-spectrometry analysis
Glycosylation		
N-linked (Asn) O-linked (Ser, Thr) GPI anchor	> 800 203, > 800‡ > 1,000	Protein stability, solubility, secretion signal, regulator of interactions, extracellular recognition and interactions; modification by a GPI anchor is coupled to protein processing
Acylation		
Palmitoylation Farnesylation Myristoylation	238 204, 206§ 210	Protein localization and activity, involved in protein–protein and protein–membrane interactions
Sulphation		
(Tyr)	80	Signalling and protein localization, involved in protein–protein interactions; phosphorylation and sulphation have the same Δm (80 Da), but can be distinguished by positive- or negative-ion mass-spectrometry analysis
Ubiquitylation		
(Lys)	> 1,000	Protein degradation signal, involved in protein–protein interactions; observed as a +114-Da mass tag (Gly–Gly) after the tryptic digestion of a modified protein
Methylation		
(Lys mono-, di- and trimethylation, Arg mono- and dimethylation)	14, 28, 42	Regulates protein activity, protein–protein and protein–nucleic-acid interactions, chromatin dynamics and gene activity (histone modification); note that trimethylation and acetylation have similar mass increments
Acetylation		
(N-terminal residue, Lys)	42	Protein stability and activity, regulates protein–protein and protein–ligand interactions; note that trimethylation and acetylation have similar mass increments
Disulphide-bond formation		
(Cys)	–2	Stabilizes protein structure and activity, involved in redox processes
Oxidation		
(Met) (Trp)	16 4, 16, 32‖	Might regulate protein activity; often a chemical artefact
Deamidation		
(Asn, Gln)	1	Associated with ageing, might regulate protein activity and interactions; often a chemical artefact
Hydroxylation		
(Pro)	16	Structural stability (collagens)

Table 1. A selection of post-translational modifications, their relevant mass values, and biological function (Jensen ON, 2006, Nat Rev Mol Cell Biol).

Monoclonal antibodies are commonly quantified by immunoaffinity-based assays like enzyme-linked immunosorbent assays (ELISA)[75,76]. Although these assays can have high sensitivity and throughput, they also have limitations due to the fact of being labor-intensive and requiring specific reagents. Consequently, the development time of such assays can be quite long. Moreover, changes occurring in the CDR regions, PTMs and, biotransformation of the mAb are not possible to be characterized just by the result of a binding or non-binding event in an ELISA analysis. This inevitably leads to a loss of information. Therefore, quantitative technologies that are complementary to these assay types are crucial.

Within the currently used analytical methods for studying deamidation, there are separation techniques including electrophoretic (IEF, cIEF, ureal gel electrophoresis) and chromatographic (reversed phase, ion exchange and hydrophilic interaction) methods. Identification of the separated species may be achieved through mass spectrometry and N-terminal sequencing methods. LC/MS-

MS approaches have recently emerged to evaluate in vivo deamidation[43]. These approaches prove the feasibility of the methodology to characterize in vivo deamidation of a mAb in a sensitive and rapid analysis.

Targeted analytical methods in LC-MS/MS analyses to study biotransformation

New mass spectrometry methods such as selected/multiple and parallel reaction monitoring (SRM/MRM and PRM) have emerged as promising tools for data-dependent analyses in complex samples. In targeted proteomics, MRM is used to monitor selected precursor-fragment transitions of targeted peptides performed on a hybrid quadrupole linear ion trap or other fast scanning tandem quadrupole instruments. PRM methods are based on targeted MS/MS analyses for which only the selected transitions are being measured and the full fragment ion spectrum of each precursor in an inclusion list is recorded continuously in contrast to SRM methods. The term PRM was created to specify the experimental setup being carried out in an orbitrap instrument. These methods offer advantages for targeted proteomics in specificity, reproducibility, sensitivity, linearity, and suitability for accurate quantitative analysis and on complex samples[77,78].

Approaches using MRM/PRM as techniques for absolute quantification of the proteins of interest in complex matrices have been started to be developed in recent years[79,80]. However, many of these methods do not focus on biotransformation products, but more on the quantification of undegraded mAbs[79]. These approaches make use of surrogate peptides for quantification in human serum. However, there are still few approaches focusing on the relative or absolute quantification of biotransformation products.

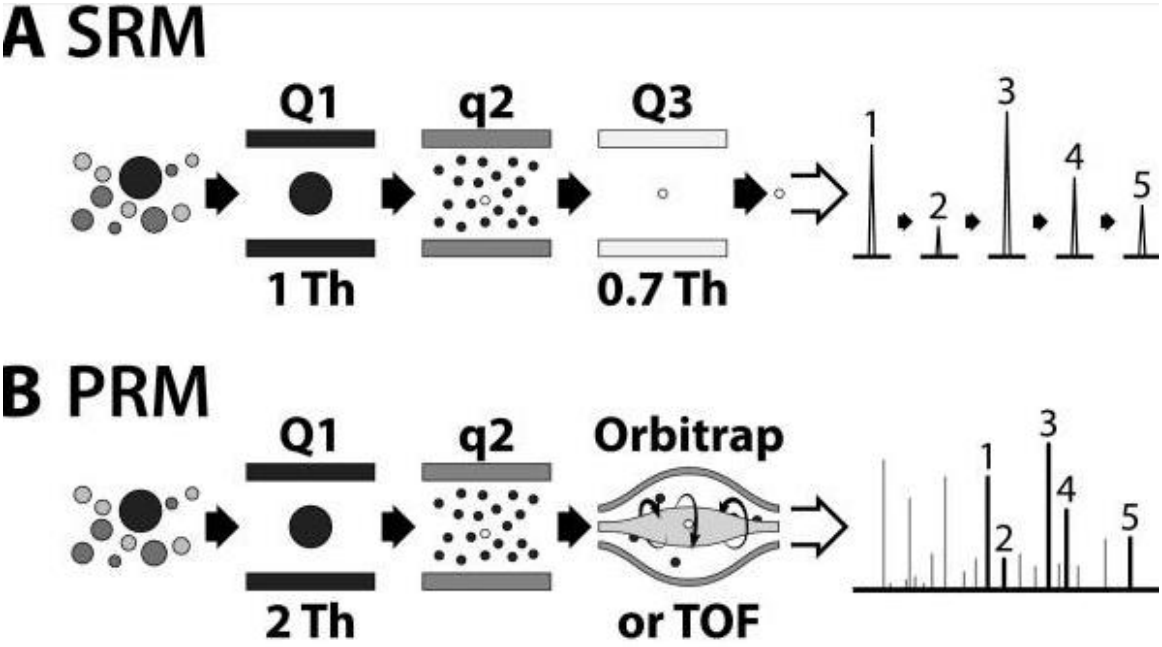


Image: Schematic representation of SRM in QQQ instruments and PRM in QQOrbitrap or QQTOF instruments (Peterson A, 2012, Mol Cell Proteomics).

Current interest in the development of multi-attribute methods

Multi-attribute methods (MAMs) have gained popularity within the biopharmaceutics industry and researchers measure multiple critical quality attributes (cQAs) simultaneously (both from subunits of a mAb or from a protein digest) due to the provided opportunity. cQAs are those molecular variants characteristics that compromise the product's quality if not within a proper range or distribution [81]. Monitoring the small mass changes present in therapeutic proteins is an essential step in the biopharma's development and characterization workflow, enabling to characterize a molecule and identify/quantify the cQAs' linked efficacy, potency, safety etc. properly and thoroughly. Many different methods have been used by the biopharmaceutical industry during early development phases of bioproducts. All of them are showing a different coverage and different challenges. While conventional methods include immune assays, SDS gel-based approaches, electrophoretic and biophysical approaches, these methods usually monitor only one characteristic. By that, achieving a comprehensive bioproduct characterization becomes highly time consuming and costly[82].

Different methods used in the industry yield several drawbacks to be considered. For instance, although reducing capillary electrophoresis is used to detect polypeptide clips in the purified product, it does not yield any information on the location of the clip's occurrence. Ion exchange chromatography misses to specify where the modification leading the readouts of acidic and basic species is in the sequence. In the case of the overall distribution of the N-glycans in an HILIC glycan assay, the percentage occupancy at the potential glycation sites is missing. In methodologies such as chromatography and electrophoresis methods it is not possible to monitor at amino acid level, but only at an intact level lacking site-specific information[83,84].

However, the use of chromatography coupled to MS combined with powerful data processing in MAMs may address these problematics while providing the possibility to identify and minimize product variants and impurities during production that could contribute to the formation of heterogeneity or variants[81]. The use of MS analysis in biologics has been estimated to have arisen from 20% to 80% in recent years[83]. The use of MAM provides the capability of simultaneously monitoring over 20 different quality attributes as glycation, oxidation, deamidation, glycosylation, fragmentation, fucosylation, etc[82]. The increasing appliance and popularity of MAMs within the industry rely on their advantages over conventional purity methods to quantify multiple molecular product attributes at once (in a controlled GMP environment and in alignment with quality-by-design) during process development and to provide a specific and quantifiable molecular characterization[81].

For protein therapeutics, MAMs represent a large-scale targeted search of the peptide mapping data through complex software and predefined EIC parameters. By obtaining a site-specific

characterization and quantification of the modification under consideration, it is possible to obtain a readout of modified percentage compared to unmodified peptides and an identification of new peaks in comparison to a reference to detect impurities in samples[81]. However, MAMs also have some limitations that include the measurement of DNA level or other non-protein molecules that can be analyzed through peptide mapping and that could be present in the formulation. Also self-association and higher order structure can be seen as limiting factors[81]. Additionally, data analysis can be complex and the equipment might require a high maintenance effort that traduces to an expensive application in quality control[83].

Some MAMs have been developed to monitor and quantify multiple PTMs of biotherapeutic molecules based on a quality-by-design approach recommended through health regulatory agencies[82,85] (a quality-by-design approach seeks to deliver methods that provide a complete analysis of the quality profile by reducing costs and replacing assays that are time consuming and provide just an indirect analysis of quality attributes). Similarly, targeted quantitation of PTMs in a mAb through MAMs has been explored[83]. The goal was to characterize drug substance, stability samples, support cell culture process development, downstream development, and protein characterization in serum incubation studies.

The impact of multi-attribute methods in the industry

The development and use of multi-attribute technologies and the demand for compatible MAM data analysis software aiming at biotherapeutics characterization has increased in recent years. The complexity and the large number of generated spectra requires the use of algorithms and software packages with the power of identifying proteins based on the primary sequence analysis and the peptides[86]. To quote only some developments on available platforms: GRAMS AI software launched by Thermo Scientific, Finder and Chromeleon by BioPharma, Enpower 3 software by Waters, MassMap by MassMap GmbH, and Genedata Expressionist platform by Genedata. [82]

The impact of MAMs within the pharmaceutical and biotechnological industry is tangible in the creation of forums such as the MAM Consortium. Efforts for translating MAM processes from the development phase to quality control activities are visible. They show the work and organization of analytic experts and agencies to bring together all advances in process and product development of MAMs. This collaboration seeks to strengthen the communication between industry parties and regulators and to facilitate the presentation of data in a common way. Members of this consortium include not only leading biopharmaceutical companies, but also equipment and software providers and regulatory entities.[87]

The trend topics and recent developments of the industry are regularly discussed in different symposia and seminars with the aim of connecting industry experts and sharing innovations in workflows and software solutions for peak detection. Companies are currently seeking to offer high-resolution mass spectrometry-based workflows. An example is the Thermo Scientific HR Multi-Attribute Method, which is looking to offer a hardware-software workflow for MS-peptide

mapping-based methods. These high-resolution mass spectrometry-based workflows have the purpose of quantifying multiple critical quality attributes simultaneously, enabling characterization and monitoring while also providing purity testing with new peak detection features. These features yield the main goal for a standardization of biotherapeutic characterization during development and manufacturing. Moreover, the replacement of other methods with lower resolution and more time needed can be seen as additional value of the mentioned workflows. The industry requires and demands efforts for workflow developments to be compliant-ready and standardized, aiming to help the industry's transition into such an approach through data acquisition during analytical processes. Companies, contract developers, and manufacturing organizations are now also evaluating the implementation of MAM methods, for which analytical equipment, software, and reagent vendors are currently focusing on offering more products tailored to their use[88].

Given the promising potential of MAMs when it comes to improving processes for biomolecules characterization, great interest is seen in embarking this innovation. Synchronization between companies and vendor-related analytics, as well as regulatory review processes are sought. The perspective of modern and future technologies relies on the measurement of quality attributes based on quality-by-design approaches with a robust and sensitive process capable of giving a product quality profile with the minimum number of assays. Therefore, the impact of incorporating MAMs into the current process development has arisen interest in the industry. As stated by Mire-Sluis from Amgen during the plenary session held at CASSS Well Characterized Biotechnology Products 2017:

"...Once jurisdictions get used to this type of methodology, import testing becomes much easier when regulatory authorities like to retest your material during development. It is a single method, rather than transferring eight or nine methods around the world to multiple jurisdictions... Once these regulatory authorities have these mass specs, it should be easier to transfer tests around the world" [87]

However, we still see a slow real penetrance in the use of such methods in the industry due to challenges that their development inherently bring. Within this work, a discussion of the learnings acquired from a MAM development for studying low abundant PTMs will be presented. These learnings can exemplify some of the restraints slowing down the implementation of MAMs approaches in the industry. The complex analysis and long optimization approaches to reliably monitor/quantify peptides of interest, as well as data validation exercises, are key points where higher efforts should be developed in the forthcoming research.

Goal

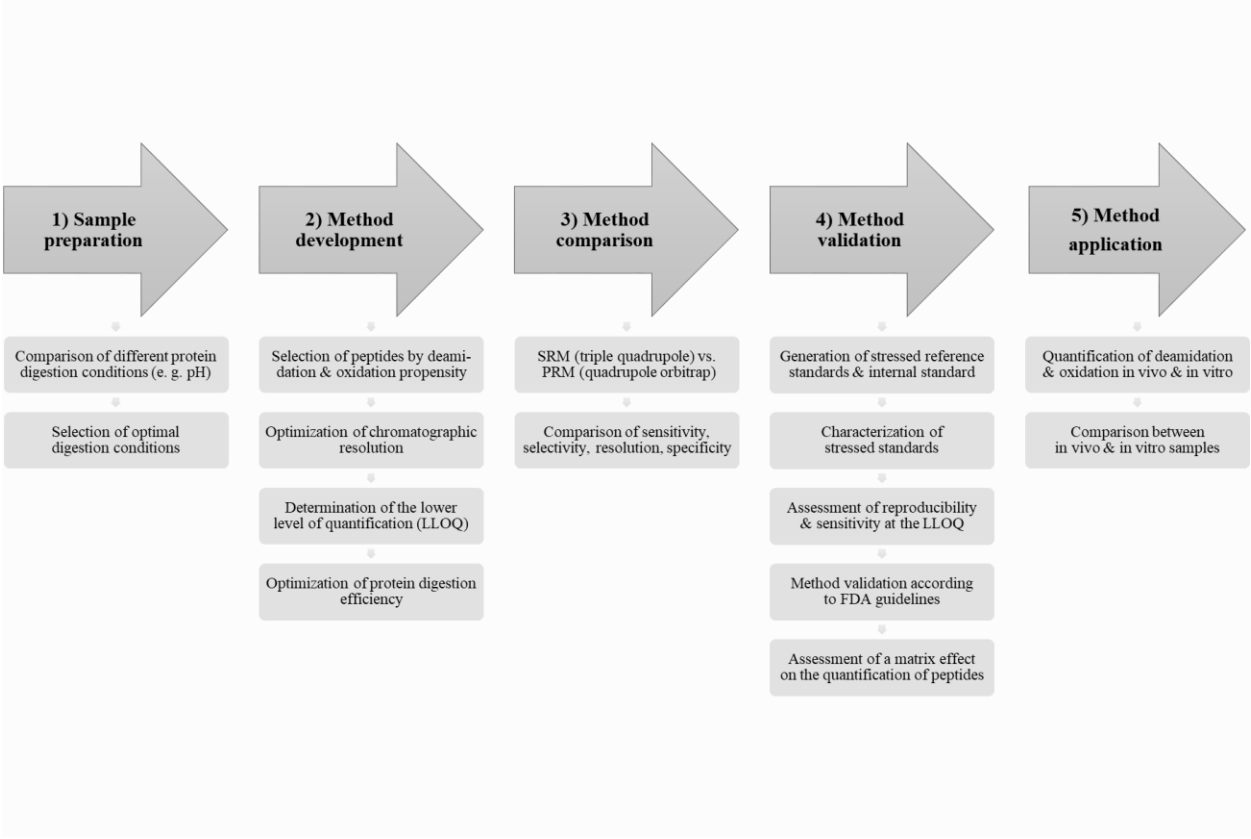
The underlying rationale of this project is that therapeutic proteins can be subjected to conversion reactions. These can lead to protein modifications and occur throughout the whole biotherapeutics' lifespan: during production, storage, or after administration to the patient. Common modifications are e. g. oxidation, deamidation, or loss of ammonia. These are usually present in very low abundancy. The presence of these modifications may have an impact on efficacy, bioactivity, safety, and pharmacokinetics within patients. In particular, the occurrence of deamidation may decrease the efficacy of therapeutic monoclonal antibodies (mAbs)[18,31,50]. Thus, for the success of mAb-based therapies in the clinic it is important to study the following questions:

1. How fast and to which extent is the protein modified after administration to the patient?
2. Are the observations of question 1 different from individual to individual?
3. Is it possible to answer questions 1 and 2 through an in vitro model?

For answering these questions, a reliable strategy for the quantification of non-modified mAbs and their modified variants is required. The aim of this study was to develop these urgently needed quantification strategies by the means of a bottom-up LC-MS/MS method and to apply them for:

1. Quantifying low-abundant modifications such as deamidation and oxidation at peptide level in biological matrices
2. Assessing if the extent of modifications over time differs between individuals
3. Comparing an in vitro model to available in vivo samples

Workflow


1) Sample preparation
Comparison of different protein digestion conditions (e. g. pH)
Selection of optimal digestion conditions
2) Method development
Selection of peptides by deamidation & oxidation propensity
Optimization of chromatographic resolution
Determination of the lower level of quantification (LLOQ)
Optimization of protein digestion efficiency
3) Method comparison
SRM (triple quadrupole) vs. PRM (quadrupole orbitrap)
Comparison of sensitivity, selectivity, resolution, specificity
4) Method validation
Generation of stressed reference standards & internal standard
Characterization of stressed standards
Assessment of reproducibility & sensitivity at the LLOQ
Method validation according to FDA guidelines
Assessment of a matrix effect on the quantification of peptides
5) Method application
Quantification of deamidation & oxidation in vivo & in vitro
Comparison between in vivo & in vitro samples

Results and Discussion

I. Comparison of sample digestion parameters in terms of efficiency and side reactions including deamidation and oxidation

Results

To study biotherapeutics through a bottom-up approach, enzymatic protein digestion is required. During the digestion process, the therapeutic proteins can be subjected to conversion reactions. Deamidation and oxidation reactions may derive from the incubation conditions needed for protein digestion and electrospray ionization in LC-MS methods (in the case of oxidation). Such an introduction of modifications during sample preparation and analysis is a main challenge in multi-attribute methods. To analyze rates of modifications occurring in vivo over time, a sample preparation with minimal induction of modifications is crucial. Proper selection and optimization of conditions for sample preparation must be performed per method and per antibody under investigation. To achieve this, different conditions for sample preparation were compared to select those conditions yielding minimal rates of deamidation and oxidation.

Deamidation (a post-translational modification (PTM) of major interest within this work) can be actively influenced by varying conditions of temperature and pH. Most of the enzymatic digestion reactions within digestion protocols are usually carried at temperatures of around 37 °C and pH values between 7 and 8, being these optimal values for proteolytic activity. Five protocols using different enzymes and different ranges of temperature and pH for the digestion of both Pertuzumab and Trastuzumab were tested and compared. This comparison was done to find the sample preparation setup which yields the minimum extra number of modifications due to proteolytic digestion. The digestion protocol introducing the lowest yield of deamidation and oxidation rates due to digestion conditions among the tested protocols was subject to further optimization.

Note: Whenever the term "deamidation/oxidation rate" is used within this study, it refers to the ratio of the area under the peak of a deamidated/oxidized species in a tryptic peptide divided by the sum of all peak areas from the modified and non-modified species of the same peptide analyzed through LC-MS/MS. This area under the peak may or may not be corrected by an internal standard (IS).

A selection of one protocol optimized for NIST mAb peptide mapping at two different pH values[89] (A and B), one protocol optimized for mAb peptide mapping analysis (E), and one protocol obtained from a vendor (Promega) in an acquired digestion kit (C and D) were compared (see Material and Methods section I, 1). Compared digestion protocols used the following parameter values:

A. *Enzyme: Trypsin, pH value: 7.0*

B. *Enzyme: Trypsin, pH value: 7.8*
C. *Enzymes: Recombinant Lys-C + Recombinant trypsin, pH value: 5.5*
D. *Enzyme: Recombinant Lys-C, pH value: 5.5*
E. *Enzyme: Lys-C, pH value: 7.0*

Other enzyme-pH-combinations (e. g. Lys-C at pH 7.8) were decided to not being explored at this stage for cost reasons. Two monoclonal antibodies (Pertuzumab and Trastuzumab) were digested in triplicates according to each of the five protocols (see Material and Methods section I, 1) and analyzed through an HPLC (high-performance liquid chromatography) coupled to a tandem mass spectrometer (quadrupole orbitrap). Acquisition was obtained through a peptide mapping method (see Material and Methods section I, 2). Sequence coverage as well as deamidation rates, oxidation rates, and ammonia loss rates were relatively quantified and evaluated. Quantification was obtained as the ratio of the peak area from the modified peptide against the total sum of the peak areas from modified and non-modified species of the peptide.

In the investigation of achieved digestion efficiency for both mAbs, sequence coverage per digestion protocol was assessed. The sequence coverage achieved per experiment for both mAbs is shown in Table 2 and was calculated permitting 3 enzymatic mis-cleavages. Total sequence coverage was obtained through assignation to a certain amino acid sequence based on the tryptic peptide precursor mass and confirmed in addition by spectra generated from the precursor's fragmentation into y and b ions.

1. **Sequence coverage:** In the proteolytic digestion of Pertuzumab, a higher sequence coverage was obtained through protocols B and C compared to A, D, and E (Table 2). Protocol C yielded the best sequence coverage. Also, reproducibility was significantly better for digestions following protocol C (RSD of 1.15% between triplicates vs. 13.18% and 8.16% for protocols A and B, respectively). For digestion of Trastuzumab, all protocols showed a sequence coverage between 80.62% and 89.50% and relative standard deviations between 0.98% and 4.29%.

Sample preparation protocol per mAb	Sequence coverage	STD	RSD
Pertuzumab			
A) Digestion with trypsin at pH value of 7.0	74.87%	0.1184	13.18%
B) Digestion with trypsin at pH value of 7.8	81.27%	0.0785	8.16%
C) Digestion with recombinant Lys-C + recombinant trypsin at pH value of 5.5	84.53%	1.14%	1.15%
D) Digestion with recombinant Lys-C at pH value of 5.5	70.24%	0.1456	17.09%
E) Digestion with Lys-C at pH value of 7.0	69.01%	0.1415	16.84%
Trastuzumab			
A) Digestion with trypsin at pH value of 7.0	81.33%	0.0413	4.29%
B) Digestion with trypsin at pH value of 7.8	89.50%	0.0098	0.98%
C) Digestion with recombinant Lys-C + recombinant trypsin at pH value of 5.5	80.62%	0.0493	5.16%
D) Digestion with recombinant Lys-C at pH value of 5.5	83.45%	0.0170	1.72%
E) Digestion with Lys-C at pH value of 7.0	82.55%	0.0353	3.62%

Table 2. Sequence coverage for digestion of Pertuzumab and Trastuzumab in tested digestion protocols. Standard deviations between triplicates are depicted in absolute (STD) and relative (RSD) values. Proteins' sequence coverage obtained as: assignation to a certain amino acid sequence based on the tryptic peptide precursor mass and confirmed in addition by the spectra generated from precursor's fragmentation into y and b ions. A) Digestion with trypsin at pH of 7.0. B) Digestion with trypsin at pH of 7.8. C) Digestion with Lys-C at pH of 7.0. D) Digestion with Lys-C at pH 5.5. E) Digestion with Lys-C and trypsin at pH 5.5.

As next step, the rates of ammonia loss, deamidation, and oxidation reactions occurring during proteolytic digestion of Pertuzumab derived from the distinct protocols were quantified. These rates were compared between all five protocols (A – E). Dimensions of this comparison were pH value and the enzyme used for digestion:

2. **pH comparison:** The two protocols performed at pH value of 5.5 yielded a lower rate of deamidation in the amino acid sequence containing the PENNY motif (subsequently referred to as PENNY peptide). This peptide contains several asparagine residues and an NG motif that increases chemical propensity to deamidation. Deamidation rates at the PENNY peptide were observed to be of only 1.05% on average in the protocols performed at pH of 5.5, whereas deamidation rates increased to 2.03% and 2.17% on average in protocols at pH of 7 and 7.8, respectively (Table 3). Although other sites of deamidation than the ones shown in Table 3 were detected for Pertuzumab, these were only present in minimum rates and with high deviation between triplicates and were therefore not included in the evaluation.

3. **Enzyme comparison:** Trypsin, Lys-C, and a combination of both enzymes were used for the digestion of Pertuzumab. Table 3 shows that digestion with Lys-C yielded low deamidation rates at the PENNY peptide regardless of the pH conditions. Protocols D and E applied to the digestion of Pertuzumab can serve as an example (0.98% and 1.97% deamidation rate, respectively, for deamidation at N386). In contrast to that, digestion of Pertuzumab with solely trypsin led to significantly higher deamidation rates. Protocols A and B applied to the digestion of Pertuzumab can serve as an example here (2.03% and 2.17% deamidation rate, respectively, for deamidation at N386).

PERTUZUMAB

		Protocol A (trypsin pH 7.0)			Protocol B (trypsin pH 7.8)			Protocol C (trypsin + Lys-C pH 5.5)		
Peptide Sequence	**Type of modification**	**Modifi-cation**	**STD**	**RSD (%)**	**Modifi-cation**	**STD**	**RSD (%)**	**Modifi-cation**	**STD**	**RSD (%)**
SGTASVVCLLNN FYPR	Ammonia Loss N134	NA	NA	NA	NA	NA	NA	0.51%	0.0005	10.18
VVSVLTVLHQDW LNGK	Ammonia Loss N317	1.00%	0.0126	125.08	5.21%	0.0277	53.12	2.05%	0.0011	5.42

CKVSNK	Ammonia Loss N327	0.67%	0.0063	93.88	7.58%	0.0742	97.98	0.18%	0.0001	2.99
GFYPSDIAVEWESNGQPENNYK	Ammonia Loss N436	NA	NA	NA	NA	NA	NA	0.56%	0.0003	4.70
GFYPSDIAVEWESNGQPENNYK	Ammonia Loss N386	NA	NA	NA	0.21%	0.0007	33.33	0.35%	0.0001	2.02
EVQLVESGGGLVQPGGSLR	Deamidation Q	0.09%	NA	NA	0.19%	0.0015	79.83	NA	NA	0.00
GQPREPQVYTLPPSR	Deamidation Q	NA	NA	NA	NA	NA	NA	0.97%	0.0006	6.01
SGTASVVCLLNNFYPR	Deamidation N	NA	NA	NA	NA	NA	NA	0.31%	0.0008	26.39
GFYPSDIAVEWESNGQPENNYK	Deamidation N386	2.03%	0.0016	7.87	2.17%	0.0018	8.28	1.05%	0.0011	10.61
DTLMISR	Oxidation M254	56.57%	0.1641	29.01	29.01%	0.0446	15.37	25.89%	0.0053	2.03
LSCAASGFTFTDYTMDWVR	Oxidation M34	56.09%	0.2046	36.48	39.00%	0.1323	33.93	12.57%	0.0026	2.06
EEMTKNQVSLTCLVK	Oxidation M360	39.64%	0.1313	33.11	28.97%	0.0451	15.58	27.15%	0.0065	2.41
DIQMTQSPSSLSASVGDR	Oxidation M4	39.13%	0.1457	37.24	30.42%	0.0816	26.83	18.08%	0.0035	1.94
WQQGNVFSCSVMHEALHNHYTQK	Oxidation M430	23.16%	0.0707	30.52	15.42%	0.0305	19.78	13.63%	0.0051	3.70
NTLYLQMNSLR	Oxidation M83	42.63%	0.1711	40.15	28.37%	0.0716	25.25	16.68%	0.0074	4.44

		Protocol D (Lys-C PH 5.5)			Protocol E (Lys-C pH 7.0)		
Peptide Sequence	**Type of modification**	**Modifi-cation**	**STD**	**RSD (%)**	**Modifi-cation**	**STD**	**RSD (%)**
SGTASVVCLLNNFYPREAK	Ammonia Loss N134	0.04%	0.0000	3.35	0.09%	0.0001	14.88
SRWQQGNVFSCSVMHEALHNHYTQK	Ammonia Loss N436	0.43%	0.0003	7.49	0.21%	0.0002	9.84
GFYPSDIAVEWESNGQPENNYK	Ammonia Loss N386	0.18%	0.0001	5.99	0.75%	0.0001	1.25
DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	Deamidation N	0.03%	0.0002	50.93	NA	NA	NA
GFYPSDIAVEWESNGQPENNYK	Deamidation N386	0.98%	0.0101	103.71	1.97%	0.0006	2.82
SRWQQGNVFSCSVMHEALHNHYTQK	Deamidation Q	0.08%	0.0002	27.11	0.21%	0.0002	10.48
DTLMISRTPEVTCVVVDVSHEDPEVK	Oxidation M254	23.16%	0.0015	0.66	30.75%	0.0074	2.42
EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGK	Oxidation M34	24.73%	0.0102	4.11	37.98%	0.0114	3.00

GQPREPQVYTLPPSREEMTK	Oxidation M360	31.91%	0.0216	6.76	34.11%	0.0025	0.73
DIQMTQSPSSLSASVGDRVTITCK	Oxidation M4	19.61%	0.0059	3.01	26.57%	0.0156	5.87
SRWQQGNVFSCSVMHEALHNHYTQK	Oxidation M430	14.03%	0.0051	3.64	13.28%	0.0091	6.88
NTLYLQMNSLRAEDTAVYYCARNLGPSFYFDYWGQGTLVTVSSASTK	Oxidation M83	12.30%	0.0098	7.9	11.39%	0.0560	49.21

Table 3. Relative quantification of spontaneous ammonia loss, deamidation, and oxidation reactions occurring during the digestion of Pertuzumab. Rates of ammonia loss, deamidation and oxidation are derived from different tryptic digestion protocols and quantified for different positions of the modified amino acid. The data was obtained from an HPLC-MS/MS peptide mapping analysis as explained in the Material and Methods section I, 2. Columns are assigned as follows: peptide amino acid sequence, type of modification with position of the modified amino acid in the sequence of the mAb, average modification rate (in percentage) present in the samples, absolute and relative standard deviation between triplicates. The results are separated per tested protocol (A – E).

Just like quantifying the rate of ammonia loss, deamidation, and oxidation reactions occurring during digestion of Pertuzumab (Table 3), their occurrence during proteolytic digestion of Trastuzumab was analyzed. Similar results as for the digestion of Pertuzumab were observed: Digestion with solely trypsin yielded significantly higher deamidation rates than digestion with Lys-C. As an example, applying protocols A and B to the digestion of Trastuzumab led to deamidation rates of 4.89% and 8.95% at N30, respectively (Table 4). In contrast to that, a deamidation rate of 0.00 % and 0.59% at N30 was observed when applying protocols D and E respectively. Asparagine N30 in Trastuzumab is a particularly interesting position of the modified amino acid to consider, since it is known as a "HotSpot" for deamidation, as described by Bults et al.[59]

TRASTUZUMAB

		Protocol A (trypsin pH 7.0)			Protocol B (trypsin pH 7.8)			Protocol C (trypsin + Lys-C pH 5.5)		
Peptide Sequence	**Type of modification**	**Modifi-cation**	**STD**	**RSD (%)**	**Modifi-cation**	**STD**	**RSD (%)**	**Modifi-cation**	**STD**	**RSD (%)**
ASQDVNTAVAWYQQKPGK	Ammonia Loss N30	0.20%	0.0020	102	1.50%	0.0022	14.86	0.99%	0.0024	24.43
IYPTNGYTR	Ammonia Loss N55	0.34%	0.0002	4.60	0.96%	0.0010	10.75	NA	NA	NA
VVSVLTVLHQDWLNGKEYK	Ammonia Loss N318	0.20%	0.0001	4.71	0.76%	0.0004	5.33	NA	NA	NA

CKVSNK	Ammonia Loss N328	2.62%	0.0003	0.96	1.24%	0.0108	86.94	66.42%	0.1518	22.86
EEMTKNQVSLTCLVK	Ammonia Loss N364	25.78%	0.0606	23.50	NA	NA	NA	NA	NA	NA
IYPTNGYTR	Deamidation N55	0.10%	0.0001	8.01	0.17%	0.0008	45.17	NA	NA	NA
ASQDVNTAVAWYQQKPGK	Deamidation N30	4.89%	0.0366	74.90	8.95%	0.0053	5.91	NA	NA	NA
WGGDGFYAMDYWGQGTLVTVSSASTK	Oxidation M107	39.75%	0.0745	18.74	51.22%	0.0470	9.17	53.32%	0.0269	5.05
DTLMISR	Oxidation M254	54.59%	0.0845	15.48	62.52%	0.0608	9.72	35.85%	0.0271	7.57
EEMTKNQVSLTCLVK	Oxidation M360	31.21%	0.0410	13.14	34.44%	0.0184	5.33	31.67%	0.0320	10.11
DIQMTQSPSSLSASVGDR	Oxidation M4	28.46%	0.0542	19.04	35.14%	0.0268	7.64	30.27%	0.0745	24.62
WQQGNVFSCSVMHEALHNHYTQK	Oxidation M430	32.32%	0.2789	86.30	15.71%	0.0177	11.29	11.33%	0.0100	8.79
NTAYLQMNSLR	Oxidation M83	32.23%	0.0528	16.38	37.97%	0.0291	7.67	24.42%	0.0248	10.16

		Protocol D (Lys-C PH 5.5)			Protocol E (Lys-C pH 7.0)		
Peptide Sequence	**Type of modification**	**Modification**	**STD**	**RSD (%)**	**Modification**	**STD**	**RSD (%)**
GFYPSDIAVEWESNGQPENNYK	Ammonia Loss N387	NA	NA	NA	0.79%	0.0003	4.21
GLEWVARIYPTNGYTRYADSVK	Ammonia Loss N55	NA	NA	NA	1.64%	0.0004	2.27
SRWQQGNVFSCSVMHEALHNHYTQK	Ammonia Loss N437	0.19%	0.0010	52.77	0.19%	0.0002	12.28
IYPTNGYTR	Deamidation N55	NA	NA	NA	0.14%	0.0007	52.35
ASQDVNTAVAWYQQKPGK	Deamidation N30	NA	NA	NA	0.59%	0.0004	6.62
NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSSASTK	Oxidation M107	20.86%	0.0132	6.33	13.28%	0.0563	42.40
DTLMISRTPEVTCVVVDVSHEDPEVK	Oxidation M254	36.53%	0.0131	3.58	28.96%	0.0114	3.95
GQPREPQVYTLPPSREEMTK	Oxidation M360	38.67%	0.0098	2.54	32.84%	0.0079	2.42
SRWQQGNVFSCSVMHEALHNHYTQK	Oxidation M430	18.14%	0.0009	0.50	16.81%	0.0041	2.45

Table 4. Relative quantification of spontaneous ammonia loss, deamidation, and oxidation reactions occurring during the digestion of Trastuzumab. Rates of ammonia loss, deamidation and

oxidation are derived from different tryptic digestion protocols and quantified for different positions of the modified amino acid. The data was obtained from an HPLC-MS/MS peptide mapping analysis as explained in the Material and Methods section I, 2. Columns are assigned as follows: peptide amino acid sequence, type of modification with position of the modified amino acid in the sequence of the mAb, average modification rate (in percentage) present in the samples, absolute and relative standard deviation between triplicates. The results are separated per tested protocol (A – E).

As seen in Tables 2 and 3, a high deamidation propensity could be observed when using protocols A and B for the digestion of Pertuzumab and Trastuzumab. This is depicted in Figure 5 for three particularly interesting positions of the modified asparagine residues:

- N386 in Pertuzumab: PENNY peptide known as propense to deamidation[90,91]
- N30 in Trastuzumab: known as "HotSpot" for deamidation[1]
- N55 in Trastuzumab: located in CDR and with possible effect on binding activity to HER2 receptor if deamidated[20,28,59]

It was observed that neither N30 nor N55 showed any deamidation reaction when using protocols C and D (low pH of 5.5) for the digestion of Trastuzumab. In contrast to that, the use of solely trypsin at high pH levels (protocols A and B) led to high deamidation rates for the digestion of Trastuzumab, especially at N30 (see Figure 5).

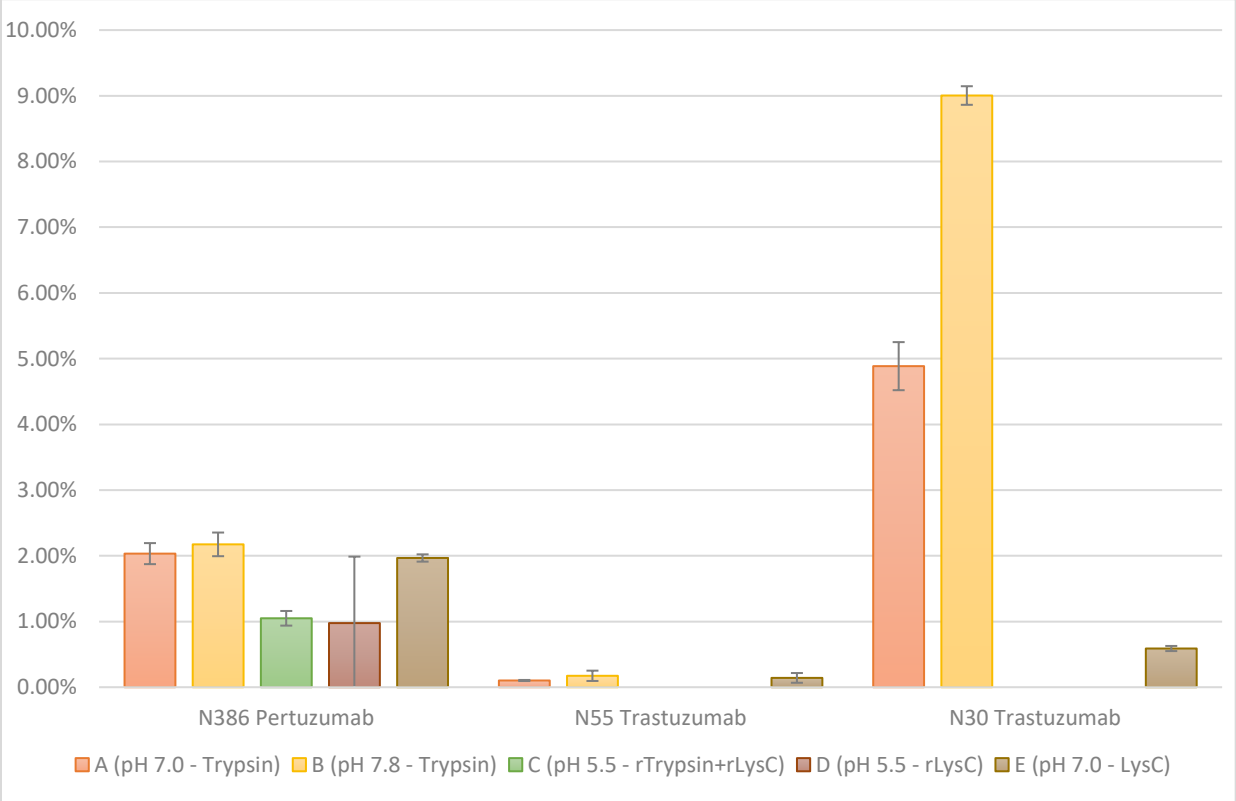


Figure 5. Relative quantification of spontaneous deamidation reactions occurring during the digestion of Pertuzumab and Trastuzumab. The rates of deamidation are derived from different tryptic digestion protocols and quantified for different modified amino acids. The data was obtained from an HPLC-MS/MS peptide mapping analysis as explained in the Material and Methods section I, 2. Standard deviation between triplicates is shown by grey whiskers. Y axis: Relative deamidation rate. X axis: Different positions of the modified amino acid: N386 in Pertuzumab, N30 in Trastuzumab, N55 in Trastuzumab. Legend: A) Trypsin at pH of 7.0. B) Trypsin at pH of 7.8. C) Lys-C and trypsin at pH 5.5. D) Lys-C at pH of 5.5. E) Lys-C at pH of 7.0.

After showing selected deamidation rates for different positions of modified amino acids (Figure 5), a similar presentation was chosen for oxidation reactions occurring during the digestion of Pertuzumab (Figure 6). It could be observed that digestion using protocol A yielded the highest average oxidation rate for all evaluated modified amino acids. Besides that, no confident conclusions could be drawn from the measured oxidation rates due to high standard deviations between triplicates. Especially the protocols with high pH value (protocols A, B, and E) showed a relative standard deviation of up to 49%. Protocol C was the only of the five protocols that showed both acceptably low average oxidation rates and acceptably low standard deviations between triplicates. For all the assessed positions of oxidation reactions occurring during the digestion of Pertuzumab, relative standard deviations for digestions using protocol C did not exceed 5%. Hence, only protocol C allowed higher reproducibility and degree of confidence compared to the other protocols.

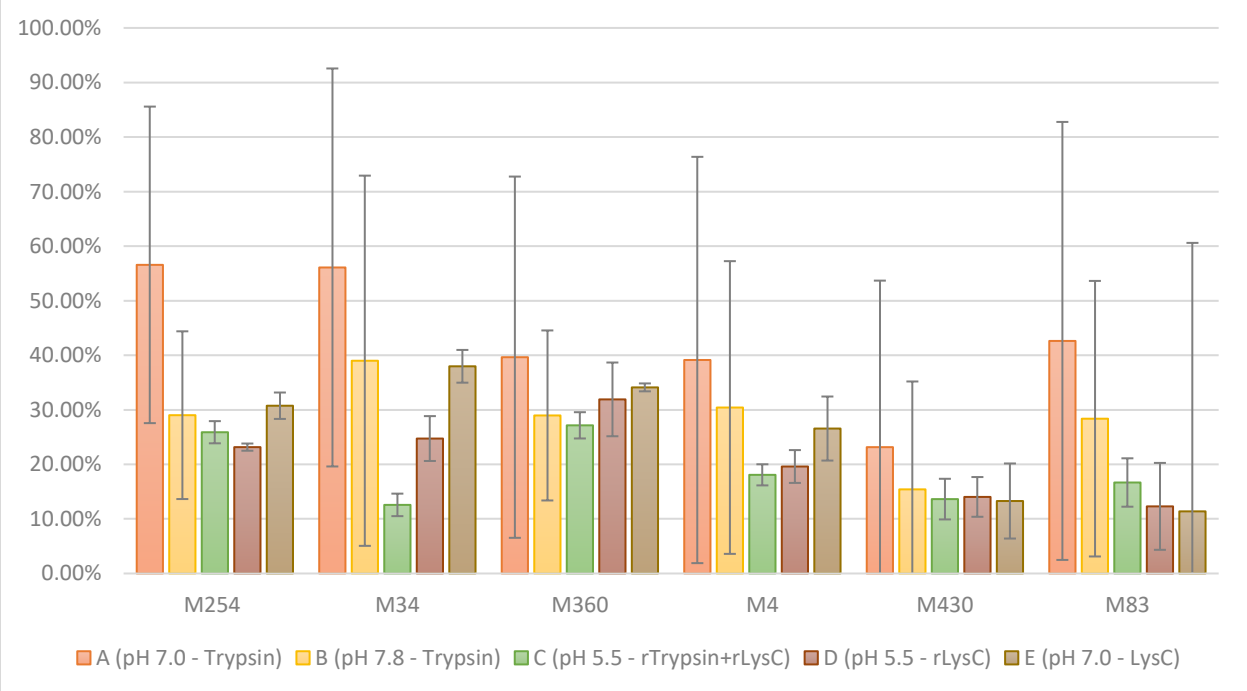


Figure 6. Relative quantification of spontaneous oxidation reactions occurring during the digestion of Pertuzumab. The rates of oxidation are derived from different tryptic digestion protocols and quantified for different modified amino acids. The data was obtained from an HPLC-MS/MS peptide mapping analysis as explained in the Material and Method section I, 1. Standard deviation between triplicates is shown by grey whiskers. Y axis: Relative oxidation rate. X axis: Different positions of the oxidized methionine in Pertuzumab: M254, M34, M360, M4, M430, M83. Legend: A) Trypsin at pH of 7.0. B) Trypsin at pH of 7.8. C) Lys-C and trypsin at pH 5.5. D) Lys-C at pH of 5.5. E) Lys-C at pH of 7.0.

Discussion

Sensitive, accurate, and fast scanning of the underlying biotherapeutics is often crucial in proteomic workflows. In specific, therapeutic monoclonal antibodies (mAbs) are subject of the measurements within this study. The occurrence of modifications such as deamidation and oxidation may decrease the efficacy of the mAbs. Hence, quantification of such deamidation and oxidation products in mAbs is essential. One method of choice for that is the enzymatic digestion of proteins followed by a peptide mapping through LC-MS/MS. The peptides generated through tryptic digestion allow to create a sequence and a modification coverage library for the analyzed protein. Such a library enables to search and identify peptides with or without specific modifications and quantify them to generate multi-attribute data. The proteolytic digestion of the underlying mAbs prior to analysis through using enzymes must be performed at conditions that are favorable for the induction of deamidation and oxidation side reactions. These conditions include prolonged incubation times at high temperature and pH values of around 8.0.

In the Results section of this chapter, different digestion conditions were tested against each other. For that, different digestion protocols differing in the used enzymes and the digestion conditions (temperature, pH) were tested. The protocols were then compared in terms of obtained sequence coverage and observed modification rates. One result was that using solely Lys-C as enzyme had a lower sequence coverage than protocols using also or solely trypsin. This may be explained by the lower abundance of cleavage sites (lysin amino acids) of Lys-C in the mAbs compared to trypsin (arginine and lysine amino acids). A lower amount of possible cleavage sites means that larger peptides are obtained, which are less optimal for ionization in MS analysis and result in a lower sequence coverage. In contrast, trypsin allows to obtain peptides of smaller length and to retain the amino acids of basic charge at the C terminus. This provides an advantage for LC-MS/MS analysis.

However, regardless of the enzyme of choice, a disadvantage of enzymatic digestion is the introduction of modifications such as deamidation and oxidation. Main reasons for deamidation and oxidation are elevated temperature, alkaline pH buffers, and long digestion times. Such conditions are usually required for the peptides to be completely solvent-exposed for proteolytic digestion. As observed from the obtained results, the use of recombinant trypsin (resistant to low pH values) allowed to carry out the enzymatic digestion at a low pH value of 5.5, which minimized the induction of deamidation and oxidation.

Deamidation was observed in very low rates when using recombinant trypsin at low pH values. Some of the deamidated peptides did not present deamidation rates in all replicas. The lack of detection of deamidation in some of the replicas was possibly influenced by the sequence coverage or by the extremely low abundance of deamidation. As the occurrence of deamidation on these peptides would not influence Pertuzumab's pharmacokinetic effect in the cases they occurred, they were not further investigated. Instead, deamidation seen at the PENNY peptide (iso-aspartic acid formation of immunogenic concern) and at the GLEW peptide (deamidation with a possible impact on drug-receptor binding) in the CDR region was the focus of this study. The goal was to choose the most suitable protocol that minimizes the induction of deamidation at these two peptides. For further information on why these two peptides were selected, the reader may be referred to the Discussion section of chapter II, 1.

Within the scope of this study, using Lys-C and trypsin together at low pH value was concluded to cause the lowest deamidation rates derived from sample preparation. Deamidation obtained at the PENNY peptide was up to 50% lower compared to the other protocols carried out at higher pH values (7.0 and 7.8). As observed for the mAb Trastuzumab, even deamidation at reported hotspots mentioned in the literature ("highly propense to deamidation")[92,93] was completely avoided.

The study of oxidation yielded very high standard deviations between experiments. This was to be expected as oxidation may occur spontaneously not only during sample preparation, but also during analysis. For example, a peptide may be subject to oxidation during ionization in an LC-MS/MS analysis. However, lower variability was obtained through experiments using recombinant enzymes resistant to low pH value. This could be influenced using L-Methionine in such protocols with recombinant enzymes. L-Methionine inhibits oxidation of the Methionine residues in the polypeptide chain. Although high standard deviation values were obtained between replicas, a higher overall reproducibility was reached for the experiments performed with recombinant enzymes resistant to low pH value.

Based on this comparison, it was concluded that the protocol using recombinant enzymes at a low pH value of 5.5 represented the best option for further optimization. The best results were obtained when using both Lys-C and trypsin together. With this combination, high sequence coverage and lower standard deviation between samples were achieved. In sum, this combination of Lys-C and trypsin at low pH value yielded the best reproducible sample preparation method.

Digestion efficiency is highly dependent on several factors including the type of antibody, the protein structure (primary, secondary, tertiary), solubility, accessibility of the enzyme to the protein for hydrolyzation, buffer, temperature, type of enzyme used, etc. Several studies available in the literature have focused on assessing the effect of various digestion parameters in terms of digestion efficiency and induction of side reactions. For example, deamidation rates induced by optimal conditions for enzymatic protein cleavage have been previously explored[62,63,66,90]. In a study performing a digestion at 37°C for 12 hours, 70% - 80% of Asn deamidation was detected[94]. Similarly, another study reported a 55% N-terminal glutamine cyclization during a tryptic digestion of 24 hours[95]. Given the fact that glutamine is the main N-terminal residue in recombinant human proteins, cyclization is one of the heterogeneities that requires to be identified and quantified within the biopharmaceutical industry, as it may lead biologically to the protection

of cyclized secreted proteins from degradation by extracellular aminopeptidase. Other strategies had been explored involving lower temperatures and lower pH values during digestion, although these conditions were observed to affect the activity of used proteases[63].

The impact of different buffer type, pH, and temperature conditions on the deamidation rate of a mAb has been investigated by Pace et al. In their studies, different storage temperatures (5°C to 40°C), acidic and basic buffering species (Tris/Tris-HCl, Histidine chloride, Sodium phosphate, Sodium acetate), and pH ranges were compared and their impact on deamidation rate was assessed. The observed differences were attributed to the change of hydroxide ion concentration influenced by buffer specie and temperature and to the lower activation energy rates in acidic buffer[90].

Besides different methodologies, the influence of utilized buffers during enzymatic digestion has also been questioned. Kori et al. addressed through their studies the impact of several commonly used buffers (optimal for maintaining trypsin activity) on inducing or reducing Asn deamidation[67]. This was based on the premise that organic solvents have previously shown to decrease induced deamidation rates due to the decreased dielectric strength and to enhance trypsin activity[68,69]. The addition of such organic solvents (methanol, ethanol, and acetonitrile) to commonly used buffers (Tris, HEPES, sodium phosphate, and ammonium bicarbonate) was studied. A considerably lower induced deamidation rate was demonstrated when Tris buffer at low concentrations (10mM and 20 mM) was used with an addition of 10% acetonitrile[67].

Finally, different digestion approaches have been explored to increase sequence coverage. For example, Pang et al. proposed a novel approach using a pepsin-containing nylon membrane as a controlled proteolysis reactor for digestion prior to MS analysis[96]. The procedure employed an enzyme-containing membrane. The calculation of the relation between the membrane thickness, volumetric flow rate, and the exposed area at the faces of the membrane would provide different peptide sizes. The pepsin-modified membranes acted as controlled reactors for mAb proteolysis under acidic conditions in a matter of minutes. The proposed method was applied to a 3 min digestion of Trastuzumab, showing a 100% yield of peptide coverage for both the light and the heavy chain of the mAb. Additionally, the presence of induced deamidation rates could be avoided by applying a prior reduction step under acidic conditions (summed over the short time for the entire preparation).

No protocol is optimal for all proteins and there will always be a need of optimization depending on the sample. An equilibrium and compromise between digestion efficiency, maintenance of solubility and denaturized state, high enzyme activity, induction of modifications, and protein degradation/aggregation must be found. Nevertheless, the optimization of sample preparation to reduce the introduction of modifications derived from digestion conditions can be very complex and time lengthy. It must be carefully assessed which modifications are aimed to be avoided during the selection process of preparation conditions. Oxidation and deamidation rates must be carefully interpreted as they could be the product of chemical pathways occurring during sample preparation.

Different sample digestion parameters for the digestion of therapeutic mAbs such as Pertuzumab and Trastuzumab were compared in this chapter. The impact of these parameters on digestion efficiency and side reactions including deamidation and oxidation was to be tested. Digestion parameters could be selected to achieve higher efficiency, higher reliability of the data, and lower deamidation and oxidation rates. It was found that protocol C using a combination of Lys-C and trypsin at a pH value of 5.5 provided optimal digestion conditions within this setup ("quantification of low-abundant deamidation and oxidation species in a complex biological matrix"). Hence, those optimal digestion conditions were selected and chosen for further method developments. Chapter II tackles the question whether such conditions can be used for developing an LC-MS/MS method and potentially further optimized along the single digestion steps.

II. Development of an LC-MS/MS method for the quantification of side reactions including deamidation and oxidation during digestion of Pertuzumab in a biological matrix

For the success of mAb-based therapies in the clinic, it is crucial to understand how fast and to which extent the protein is modified after administration to the patient. For answering this question, a reliable strategy for the quantification of non-modified mAbs and their modified variants is required. The aim of this chapter was to develop a targeted bottom-up LC-MS/MS method that allowed to quantify low-abundant modifications such as deamidation and oxidation at peptide level in biological matrices (e. g. animal serum). For this purpose, a sensitive LC-MS/MS method should be developed through the following steps (performed sequentially):

- Peptide selection
- Optimization of chromatographic separation and development of mass spectrometry method
- Determination of the method's LLOQ (lower limit of quantification) for tryptic peptides of Pertuzumab in animal serum
- Optimization of the protein digestion efficiency for quantifying tryptic peptides of Pertuzumab at the LLOQ

1) Peptide selection

Results

To quantify low-abundant modifications of clinical relevance (such as deamidation and oxidation) at peptide level, it is crucial to select proper peptides to be monitored. The candidate peptides to be monitored were selected based on two criteria:

- Propensity to degradation
- Pharmacokinetic relevance

To better assess propensity to degradation, samples of the Pertuzumab originator (Perjeta®) were incubated in vitro and analyzed through an LC-MS/MS peptide mapping (see Material and Methods section II, 1). The following incubation conditions were selected to induce high rates of degradation such as deamidation and oxidation:

- pH stress (8.5)
- oxidative stress in presence of H_2O_2 (0.1%)
- temperature stress (25 °C, 40 °C)

Details on the incubation conditions can be found in the Material and Methods section II, 1. The proteolytic digestion of Pertuzumab was then performed for all samples at the same time and by applying digestion protocol C from Chapter I. After that, the modification rates were relatively

quantified for each variant of incubation conditions at peptide-level. For each of the evaluated peptides, the modification rate before stress (kept at -80 °C) was compared to the modification rate after stress. This comparison was done for the two modification types: deamidation (Table 5) and oxidation (Table 6). In both cases, the peptides with highest increase in modification rate (before vs. after stress) were pre-selected for further screening. The pre-selected peptides were assessed on their suitability for MS analysis (unique sequence, ionization properties, digestion efficiency, etc.) and on their pharmacokinetic relevance. This assessment will be further presented in the Discussion section of Chapter II among with the selection of the top peptide candidates for further monitoring.

Peptide	*Modification*	*Before stress (%)*	*STD*	*RSD (%)*	*Type of stress*	*After stress (%)*	*STD*	*RSD (%)*	*Increase due to stress (%)*
GFYPSDIAVEWES NGQPENNYK	N386, N391 (Deam)	1.07	0.0107	16.85	40 °C / 2 weeks	2.15	0.0014	2.50	99.98
					pH 8.5 / 4 days	3.72	0.0028	13.53	246.58
					pH 8.5 / 7 days	4.84	0.0011	2.23	350.52
GLEWVADVNPNS GGSIYNQR	N54 (Deam)	0.00	NA	NA	pH 8.5 / 4 days	3.72	0.0028	7.39	NA
					pH 8.5 / 7 days	1.35	0.0006	4.12	
NLGPSFYFDYWG QGTLVTVSSASTK	N99 (Deam)	0.00	NA	NA	pH 8.5 / 4 days	2.29	0.0052	4.40	NA
					pH 8.5 / 7 days	2.12	0.0039	18.23	

Table 5. Relative deamidation rates for the digestion of Pertuzumab before and after stress (temperature: 40 °C for 2 weeks, pH: 8.5 for 4 and 7 days, respectively). “Deam” stands for deamidation. Columns are assigned as follows: peptide amino acid sequence, type of modification with position of the modified amino acid in the mAb sequence, average deamidation rate with absolute and relative standard deviation between triplicates before stress, type of stress performed, average deamidation rate with absolute and relative standard deviation between triplicates after stress, relative increase of deamidation rate due to stress conditions.

Peptide	*Modification*	*Before Stress (%)*	*STD*	*RSD (%)*	*Type of stress*	*After Stress (%)*	*STD*	*RSD (%)*	*Increase due to stress (%)*
DTLMISR	M254(Ox)	41.97	0.0520	8.07	25 °C / 4 weeks	44.22	0.0436	9.70	5.35
					40 °C / 2 weeks	42.92	0.0175	4.14	2.25
					H_2O_2 0.1%	73.00	0.0120	1.63	74.75
NTLYLQMNSLR	M83 (Ox)	17.85	0.0319	5.59	25 °C / 4 weeks	23.37	0.0360	15.25	30.90

					40 °C / 2 weeks	20.75	0.0125	6.01	16.22
					H_2O_2 0.1%	53	0.0435	8.22	196.39
EEMTK	M360 (Ox)	28.52	0.0179	15.94	25 °C / 4 weeks	35.09	0.0241	6.83	23.02
					40 °C / 2 weeks	32.11	0.0106	3.32	12.57
					H_2O_2 0.1%	43.00	0.0098	2.27	51.72
WQQGNVFSCS VMHEALHNHY TQK	M430 (Ox)	9.96	0.0076	13.18	25 °C / 4 weeks	15.12	0.0128	8.45	51.76
					40 °C / 2 weeks	13.64	0.0099	7.27	36.91
					H_2O_2 0.1%	30.00	0.0127	4.19	204.74
DIQMTQSPSSLS ASVGDR	M4 (Ox)	18.80	0.0233	8.07	25 °C / 4 weeks	31.96	0.0284	8.82	220.83
					40 °C / 2 weeks	27.00	0.0151	5.60	43.60
					H_2O_2 0.1%	25.00	0.0273	10.93	32.96
LSCAASGFTFT DYTMDWVR	M34 (Ox)	0.00	NA	NA	40 °C / 2 weeks	40.10	0.0342	8.52	NA
					H_2O_2 0.1%	53.00	0.0435	8.22	NA

Table 6. Relative oxidation rates for the digestion of Pertuzumab before and after stress (temperature: 25 °C for 4 weeks, 40 °C for 2 weeks, oxidative: 0.1% H_2O_2 for 24 hours). "Ox" stands for oxidation. Columns are assigned as follows: peptide amino acid sequence, type of modification with position of the modified amino acid in the mAb sequence, average oxidation rate with absolute and relative standard deviation between triplicates before stress, type of stress performed, average oxidation rate with absolute and relative standard deviation between triplicates after stress, relative increase of oxidation rate due to stress conditions.

Besides looking at the obtained changes in modification rate on peptide-level, a more general comparison between different types of stress could be performed. This comparison was done for the two modification types: deamidation (Figure 7) and oxidation (Figure 8). It could be observed as expected, that high degradation conditions as in both oxidative and pH stress led to significantly higher rates of modification than temperature stress conditions.

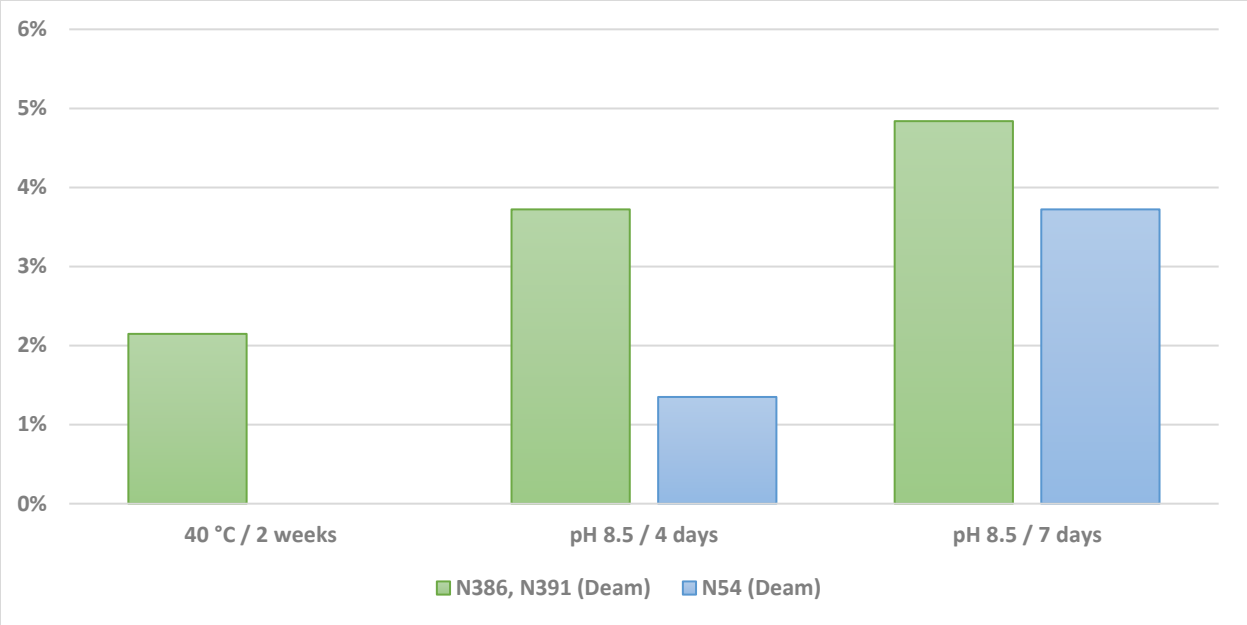


Figure 7. Relative deamidation rate for asparagine deamidation in Pertuzumab after temperature stress (temperature: 40 °C for 2 weeks) and basic pH stress (pH: 8.5 for 4 and 7 days, respectively). N386, N391 moiety peptide at the Fc region of Pertuzumab and N54 peptide at the CDR of Pertuzumab are presented. Absolute and relative standard deviations on the deamidation rates between triplicates can be obtained from Table 5. "Deam" stands for deamidation.

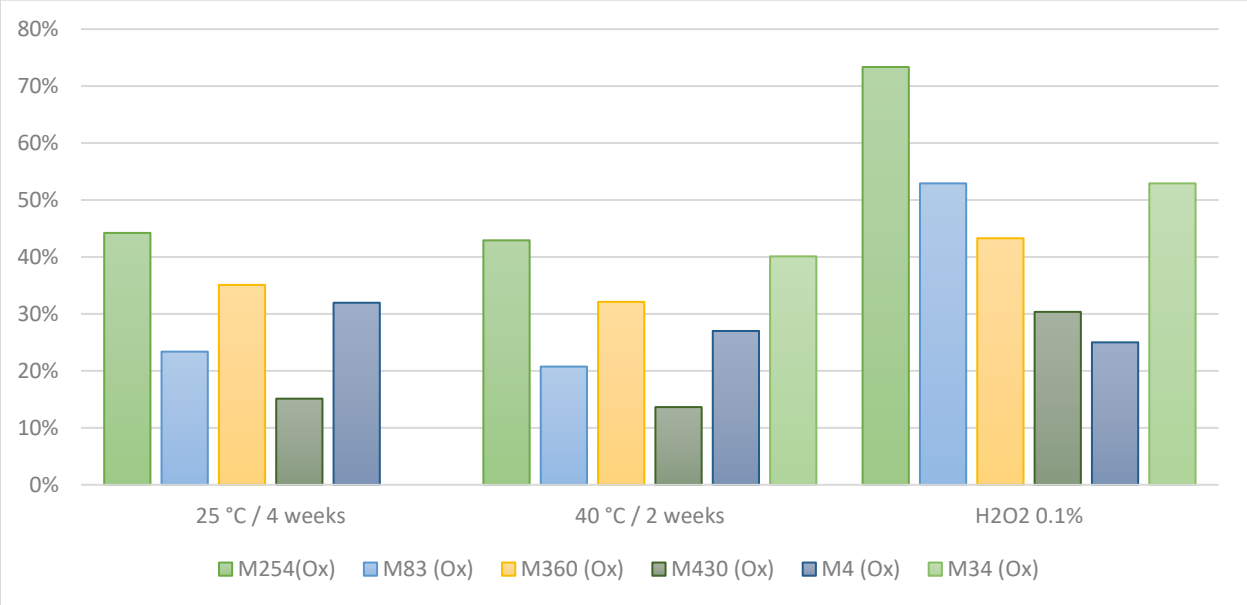


Figure 8. Relative oxidation rate for methionine oxidation in Pertuzumab after temperature stress (temperature: 25 °C for 4 weeks, 40 °C for 2 weeks) and oxidative stress (H_2O_2: 0.1% for 24 hours). Absolute and relative standard deviations on the oxidation rates between triplicates can be obtained from Table 6. "Ox" stands for oxidation.

Discussion

For the quantification of low-abundant modifications of clinical relevance (such as deamidation and oxidation) at peptide level, proper peptides needed to be selected. The results in this subchapter allowed to compare the peptides with highest propensity to oxidation and deamidation and to select those of interest for further analysis. In the case of oxidation, high modification rates could be observed even under non-stressed conditions in certain peptides. This result was to be expected, as the introduction of spontaneous oxidation both during sample preparation and during LC-MS/MS analysis has been well reported and studied in the literature[97]. The spontaneous occurrence of oxidation may derive from sample preparation and from analysis (by electrospray mass spectrometry due to the increase of the potential applied to the electrospray needle)[64,65]. Its spontaneous occurrence hampers the accurate analysis both in vivo and in vitro. However, in this study peptides with a higher oxidation rate derived from the applied stress incubation conditions could be identified. This was possible through an estimation approach as all samples were prepared and analyzed simultaneously.

Six top peptide candidates were selected for oxidation study as observed in the results. However, this selection was narrowed down to only one peptide to be used as proof of concept. The other peptides showed rather low suitability for enzymatic digestion or for LC-MS/MS analysis. The arguments against the other peptides were as follows:

1) EEMTK (M360) is a peptide composed of only five amino acids. Its sequence was therefore too short for being a suitable candidate for ionization in LC-MS analysis.

2) DTLMISR (M254) is located within the Fc region of IgG1 in humans. However, it was found through a BLAST (basic local alignment search tool) search that this peptide is also present in the Fc region of IgG in white New Zealand animals. As the peptide is hence endogenous to the organism and biological matrix of use (animal serum), it was assessed as unsuitable for the study.

3) WQQGNVFSCSVMHEALHNHYTQK (M430) contained three potential sites of modification, two of oxidation (Tryptophan, Methionine) and one of carbamidomethylation (Cysteine). As it may not be assumed that 100% of carbamidomethylation in Cysteine is achieved during sample preparation through treatment with iodoacetamide, carbamidomethylation needs to be included as possible modification together with oxidation at Trp and Met. Taking these three potential sites of modification into account, this yields a total number of $2^3 = 8$ possible peptide variants to be studied. If all these variants were to be included in the assay, eight different internal standards would be required. This would increase the assay to 16 different product ions (8 from heavy-labeled peptides, 8 from the tryptic peptides of Pertuzumab) to be quantified and monitored just to study one single peptide. This would not only increase the complexity of the assay but also hamper the possibility of reaching an accurate quantitation of all peptide variants. Therefore, WQQGNVFSCSVMHEALHNHYTQK (M430) was excluded from the study.

In general, the presence of cysteines within the selected peptides studied for oxidation was avoided. This decision can be supported by reports already available in the literature[97]. In these sources, it has been observed within quantification analyses of oxidation residues, that both methionine and cysteine show a significantly higher modification rate when treated with hydrogen peroxide treatment compared to other amino acids in a peptide.

4) LSCAASGFTFTDYTMDWVR (M34) is a peptide in which a similar problem was encountered. This peptide contains a Methionine and a Cysteine respectively prone to oxidation and carbamidomethylation. This peptide was initially of interest due to its location close to the CDR region. Such local proximity could potentially imply that a modification within the peptide has a stoichiometric effect on the CDR region. In the end, it was decided to also exclude it from the assay due to the increased analysis complexity from carbamidomethylation sites.

Moreover, when studying the folding structure of Pertuzumab, LSCAASGFTFTDYTMDWVR (M34) was assessed to form a disulfide bridge between Cys 22 and Cys 96 (Figure 9). This fact would require a more detailed optimization of reduction and alkylation conditions to reach an efficient digestion for peptide detection. Therefore, the peptide under study was excluded from the assay.

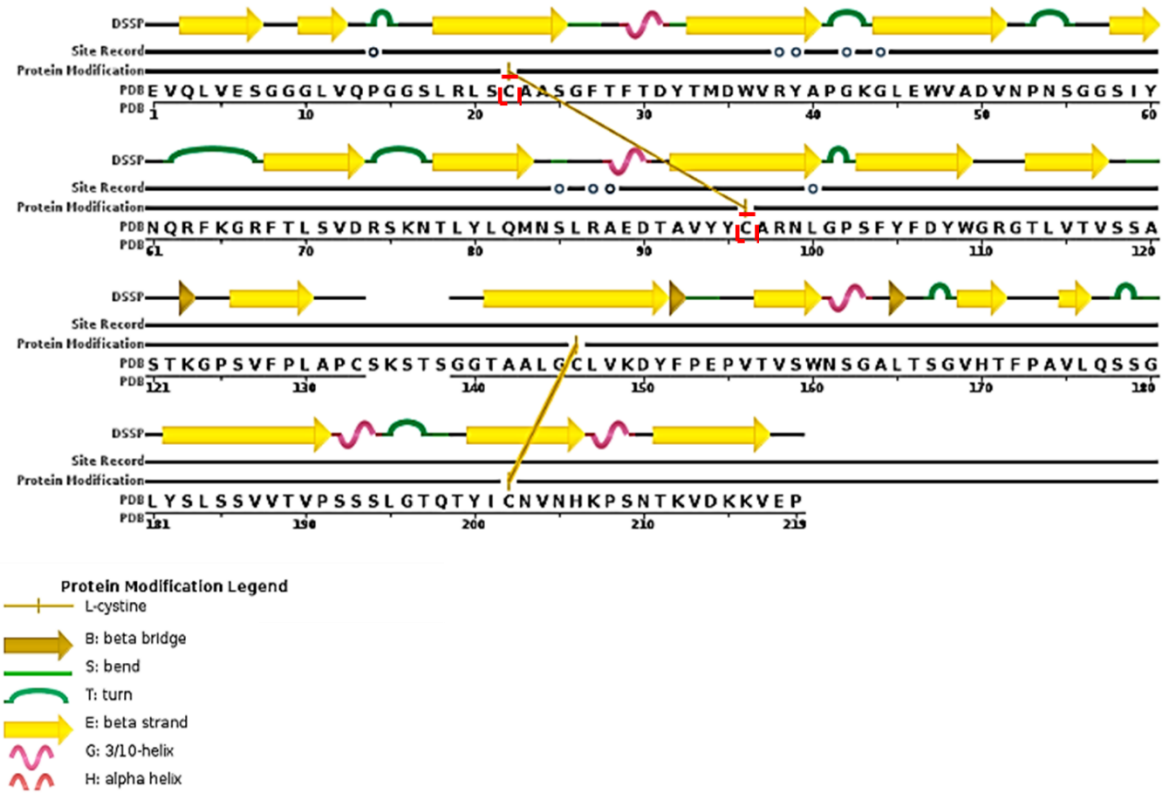


Figure 9. Scheme from the variable region of Pertuzumab's heavy chain depicting its folding structure within the sequence. The disulfide bond between Cys22 and Cys96 is indicated in red (retrieved from Protein Data Bank).

5) DIQMTQSPSSLSASVGDR (M4) was the fifth peptide to be excluded. During MS/MS analysis of this peptide, it was observed that low signal intensities were obtained even at high concentrations of digested Pertuzumab in PBS. However, the heavy- and non-heavy-labeled version of this synthesized peptide yielded high MS1 and MS2 signal intensities even at low concentrations. From these observations, a problem during ionization or fragmentation of the peptide was discarded, as the synthesized versions showed good MS1 and MS2 signals. It was hypothesized that a hampered digestion efficiency was the main cause of this effect. The impairment of digestion for this peptide would yield a low signal intensity detection during MS analysis. This hypothesis was confirmed by reports from the literature (Figure 10), where it has been stated that the presence of certain amino acid motifs at the cleavage site results in lower kinetics in the enzymatic digestion and a cleavage with 3 orders of lower magnitude of speed[98]. In the case of the DIQMT peptide, the presence of an aspartic acid on the N-terminal site of the cleavage site (DR motif) was causing a reduced hydrolysis in the enzymatic reaction. As the presence of these motifs does not only lead to slower enzymatic activity, but also to missed cleavage peptides even at prolonged digestion times, its suitability for a bottom-up based analysis was negated.

It can be further noted that the DIQMT peptide is the first trypsin-cleaved amino acid sequence from the heavy chain N-terminal site of Pertuzumab. Noting that, it is furthermore adjacent to the leader peptide sequence. The leader peptide sequence targets the mature antibody for export from the cell in which it is produced. It is also cleaved after translocation to the endoplasmic reticulum and prior to secretion[99]. While in Perjeta (originator) the leader peptide sequence is an amino acid sequence of length 3 incorporated in the light chain, in Pertuzumab (biosimilar) the leader peptide is an amino acid sequence of length between 5 and 7 incorporated in the heavy chain. Inclusion of peptide DIQMT in the assay would represent a complication during analysis, as miscleavage of this leader sequence may occur resulting in truncation or elongation at the N-terminus. If such protein heterogeneity even at minimal rates was to be obtained, the accurate analysis and quantification of this peptide may not be assured.

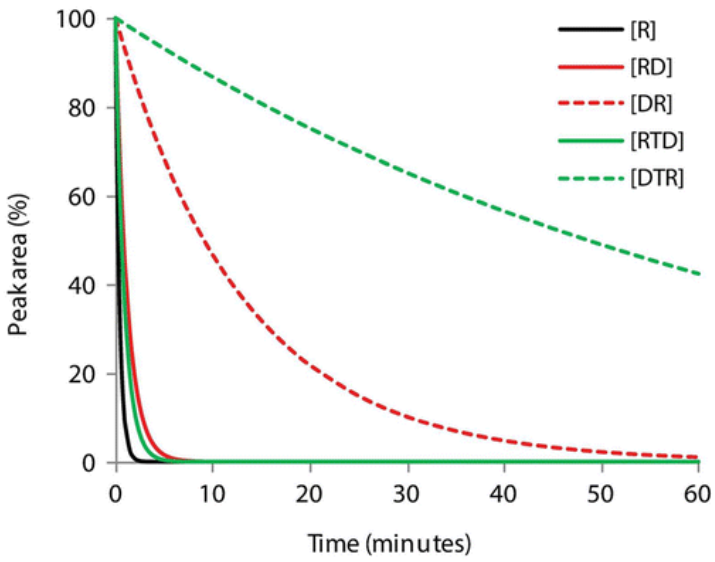


Figure 10. Dependencies of digestion from peptides containing the represented sequence motifs. DR motif as contained in the peptide of interest in this study (DIQMT) is shown in dotted red lines. The decay rate of the peptides was calculated as peak area (in %) by the formula $100 \times \frac{10}{k}$where k is the kinetic constant (Šlechtová T, 2015, Anal Chem).

The only peptide selected for further study and to be used as proof of concept for the quantification of oxidation in presence of serum was NTLYLQMNSLR (M83). The need of including a peptide to monitor oxidation relied on the premise that incubation in presence of a biological matrix is needed to reproduce results compared to incubation in PBS[39]. The NTLYL peptide was selected as it showed good analytical properties: high sensitivity in MS, uniqueness for the protein analyte, and proper size in the range of 8 to 20 amino acids in length.

Regarding deamidation, two peptides were selected for study. The GFYPSDIAVEWESNGPENNYK (N386, N391 N392 moiety) peptide, commonly denominated as the PENNY peptide, is known in the literature for its propensity to deamidation. Although it is present in the Fc region, the importance of studying its rates of iso-aspartic acid formation relies on the fact that high rates of iso-aspartic acid have been detected in patients (systemic lupus erythematosus) and raised immunogenicity and safety concerns of the health authorities as the FDA (Food and Drug Administration) [100]. Iso-aspartic acid is an unnatural amino acid; therefore, it may trigger an immune response in the clinic when present in high rates. Consequently, this peptide was assessed as a good candidate for monitoring iso-aspartic rates over time.

The second peptide selected was GLEWVADNPNSGGSIYNQR (N54) being highly relevant due to its location in the CDR region. Degradation at this site could cause a pharmacokinetic impact through an impairment of drug receptor binding[18,31]. Although its deamidation was observed in minimal rates during sample preparation, its potential occurrence in vivo should be closely monitored. This high stability (low rate of deamidation) seen during sample preparation may be explained with the fact that Pertuzumab belongs to a newly designed generation of therapeutic

mAbs with CDR regions optimized for stability. However, deamidation of this mAb in vivo has not been thoroughly studied, and its occurrence over time (after dosage) at physiological conditions should still be carefully assessed. Other deamidated peptides seen within stress conditions were not selected due to their absence of pharmacokinetic relevance and minimal detected modification rates.

Finally, the stable peptide DSTYSLSSTLTLSK located in the light chain was included in the assay to quantify in exploratory animal study samples Pertuzumab's concentration over time after dosage.

The final selected peptides to be monitored are listed below (see Figure 11 for location and domain of regions):

1. NTLYLQMNSLR: Heavy chain, Fab region, VH3 domain
2. GFYPSDIAVEWESNGQPENNYK: Heavy chain, Fc region, CH3 domain
3. GLEWVADVNPNSGGSIYNQR: Heavy chain, Fab region, VH3 domain, CDR2
4. DSTYSLSSTLTLSK: Light chain, Fab region, Ck Domain

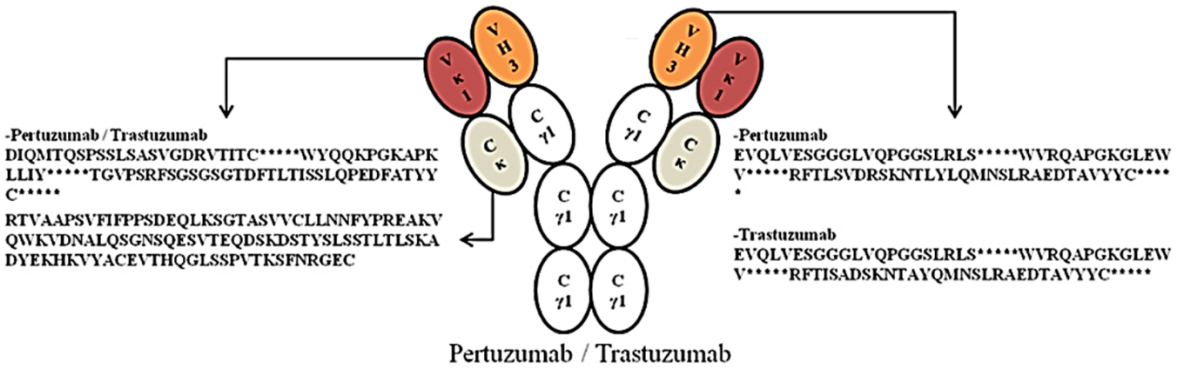


Figure 11. Framework regions for Pertuzumab and Trastuzumab. Sequence for variable regions are shown. *Denotates complementary determining regions (CDR) (Ling W. 2018, Front. Immunol).

Targeted proteomic methods rely on the analysis of a limited number of peptides per protein. As only few peptides are used for protein quantitation, their selection becomes crucial as the wrong peptide selection might lead to biased results[101]. Although rules for peptide selection guidance (based on amino acidic composition, ionization, stable fragmentation pattern, etc.) have been proposed. These rules do not imply that missed cleavages, chemical or post-translation modifications, single point mutations, or variability under different conditions may occur[101]. Available computational platforms may serve as guide for peptides candidate selection.

For instance, enhanced signature peptide predictor is a computational method to predict high-responding peptides of target proteins without experimental data[102]. The software PeptidePicker provides a scientific workflow to process and integrate information from different online data sources for selecting the optimal signature peptides[103]. Additionally, selection of optimal peptides relying on experimental data has been developed using in-vitro-synthesized proteins and Skyline software[104,105].

However, the ultimate choice of the peptides of interest will be based not only on their LC-MS analysis suitability and proteolytic digestion, but also on the method's scope and particular interest in certain peptides/modifications.

2) Optimization of chromatographic separation and development of mass spectrometry method

Results

For the quantification of low-abundant modifications of clinical relevance (such as deamidation and oxidation) at peptide level, it was crucial to select the peptides of highest interest to be monitored in the first step. As next step, the optimization of chromatographic separation and the development of a mass spectrometry method was needed. Optimization of the chromatographic separation is next presented as the first sub-goal of this chapter.

The optimization of chromatographic separation is a crucial step due to one characteristic of the PTMs under study (deamidation). Both oxidation and deamidation introduce mass changes in the peptide of modification occurrence. While oxidation reaction increases the mass by 16 Da, the mass change caused by a deamidation reaction is less than 1 Da (0.984 Da)[40]. This low mass change presents a challenge for detection and separation of the modification within LC-MS/MS analysis. Due to that, the peptide variants (deamidated and non-deamidated species) may coelute in a chromatographic separation and their generated mass spectra in MS analysis may overlap. To overcome these challenges, a strategy focusing on optimizing the chromatographic separation is needed.

To set up an optimization strategy for chromatographic separation of deamidated species, several chromatographic separation methods were designed and compared. To improve separation for the peptides of interest in reversed-phase chromatography, steeper gradient slopes (designed by reducing the content of organic solvent) in the chromatographic method were implemented for the elution times of the peptides of interest. Observations in two compared setups were the following:

- Figure 12A shows the co-elution of iso-aspartic and aspartic acid formation with the non-deamidated specie of the PENNY peptide in Pertuzumab. The obtained ion chromatograms were not baseline separated and presented a shouldering visual effect with the non-deamidated specie. For this analysis, an incubated sample at pH stress conditions (pH of 8.5 for 7 days) known to present high levels of deamidation at PENNY peptide was used.

- Figure 12B shows the chromatogram obtained from a gradient after optimization and where the same sample was analyzed. In here, a baseline separation between deamidated and non-deamidated peptides was achieved.

Details on the tested chromatographic methods are described in the Methods and Materials section II, 2. The developed chromatographic method allowed a sufficient baseline separation of 0.5 to 1.0 minutes between species. This baseline separation was achieved in a final chromatographic method with a total run time of 45 minutes (see Material and Methods section II, 2). This runtime allowed sequenced measurements of up to 64 samples for a 48-hour stability in autosampler (total run time aimed for method validation and in vivo and in vitro samples analysis).

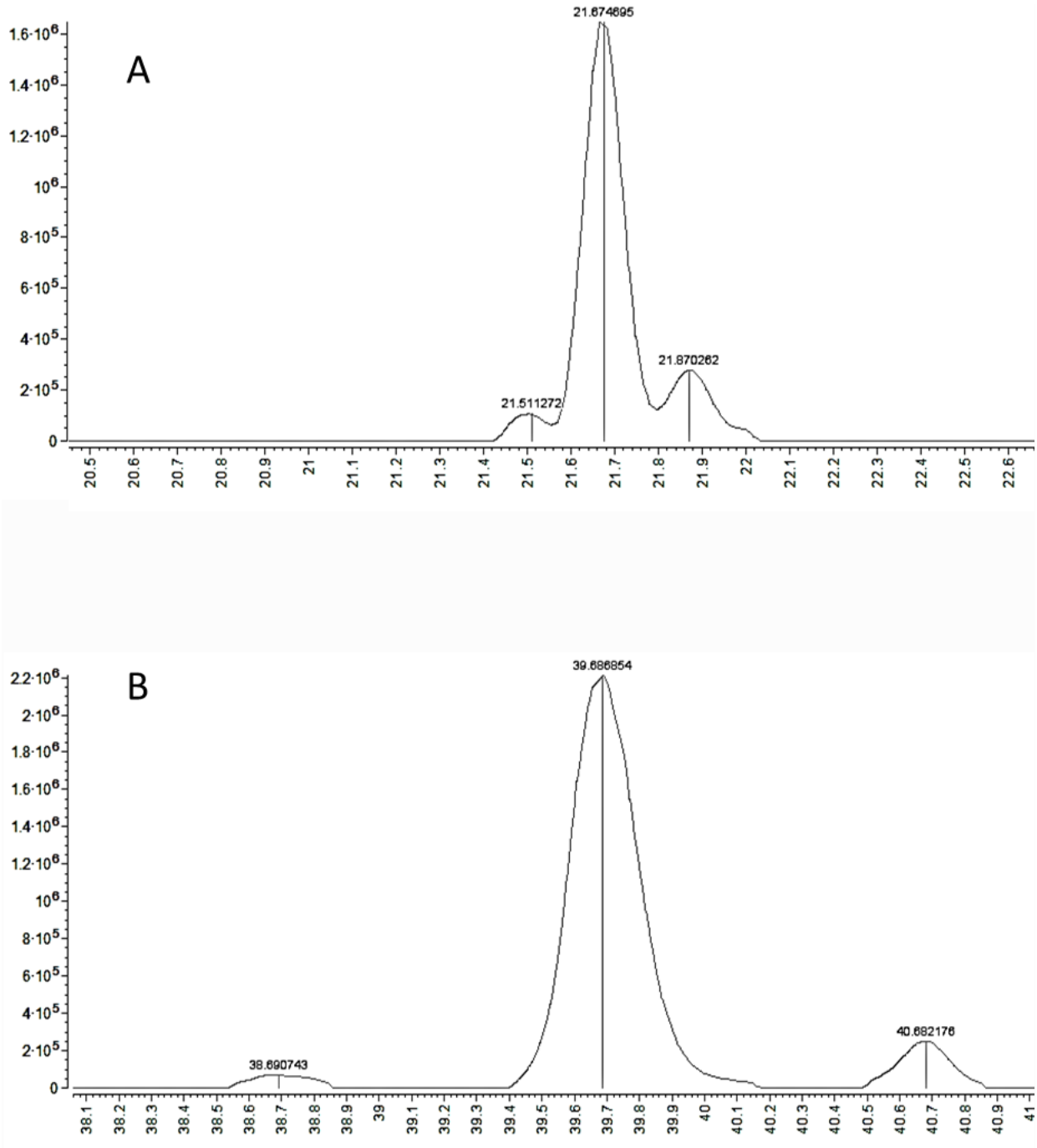


Figure 12. Chromatograms of the PENNY peptide in Pertuzumab samples after tryptic digestion. Analysis performed through reversed-phase liquid chromatography coupled to mass spectrometer and peptide mapping analysis. Different degradation species (iso-aspartic acid formation, non-deamidated peptide, aspartic acid formation) of the PENNY peptide are visible in that order of elution. A) Before chromatographic resolution optimization. B) After chromatographic resolution optimization. Y axis: Signal intensity in the ion chromatogram. X axis: Retention time (in minutes).

After an optimized chromatographic separation was obtained, the development of a suitable mass spectrometry method was needed. For that, the assignation and characterization of predominant deamidated species had to be confirmed at MS/MS level. This was achieved through the analysis of b and y ions generated from fragmentation during peptide mapping analysis. Ions y4, y6, and y10 were used to confirm the assignation of the deamidated species. Figure 13A depicts the MS/MS spectrum generated from the non-deamidated PENNY peptide, while Figure 13B shows the spectrum corresponding to the N386G-Iso-aspartic formation eluting earlier at a reversed-phase separation. In Figure 13C, the spectrum generated from aspartic acid formation on the N391, N392 moiety can be seen.

Mass changes could be observed at the following y ions on the obtained spectra:

- y10 (SNGPENNYK fragment) in spectrum generated from N386G-Iso-aspartic formation: 1150.51 Da (theoretical mass) → 1151.49 Da (Figure 13B)
- y6 (PENNYK fragment) in spectrum generated from N391, N392 moiety: 764.36 Da (theoretical mass) → 765.34 Da (Figure 13C)
- y4 (NNYK fragment) in spectrum generated from N391, N392 moiety: 538.26 Da (theoretical mass) → 539.24 Da (Figure 13C)

No mass changes however were observed at the following positions of the PENNY peptide:

- y6 (PENNYK fragment) in spectrum generated from N386G-Iso-aspartic formation: 764.35 Da (Figure 13B)
- y4 (NNYK fragment) in spectrum generated from N386G-Iso-aspartic formation: 538.26 Da (Figure 13B)

From the observed mass increases, iso-aspartic formation could be concluded at N386G (mass increase in y10 but no mass increase in y6 and y4) and aspartic formation at N391, N392 moiety (mass increase in y4 and y6)

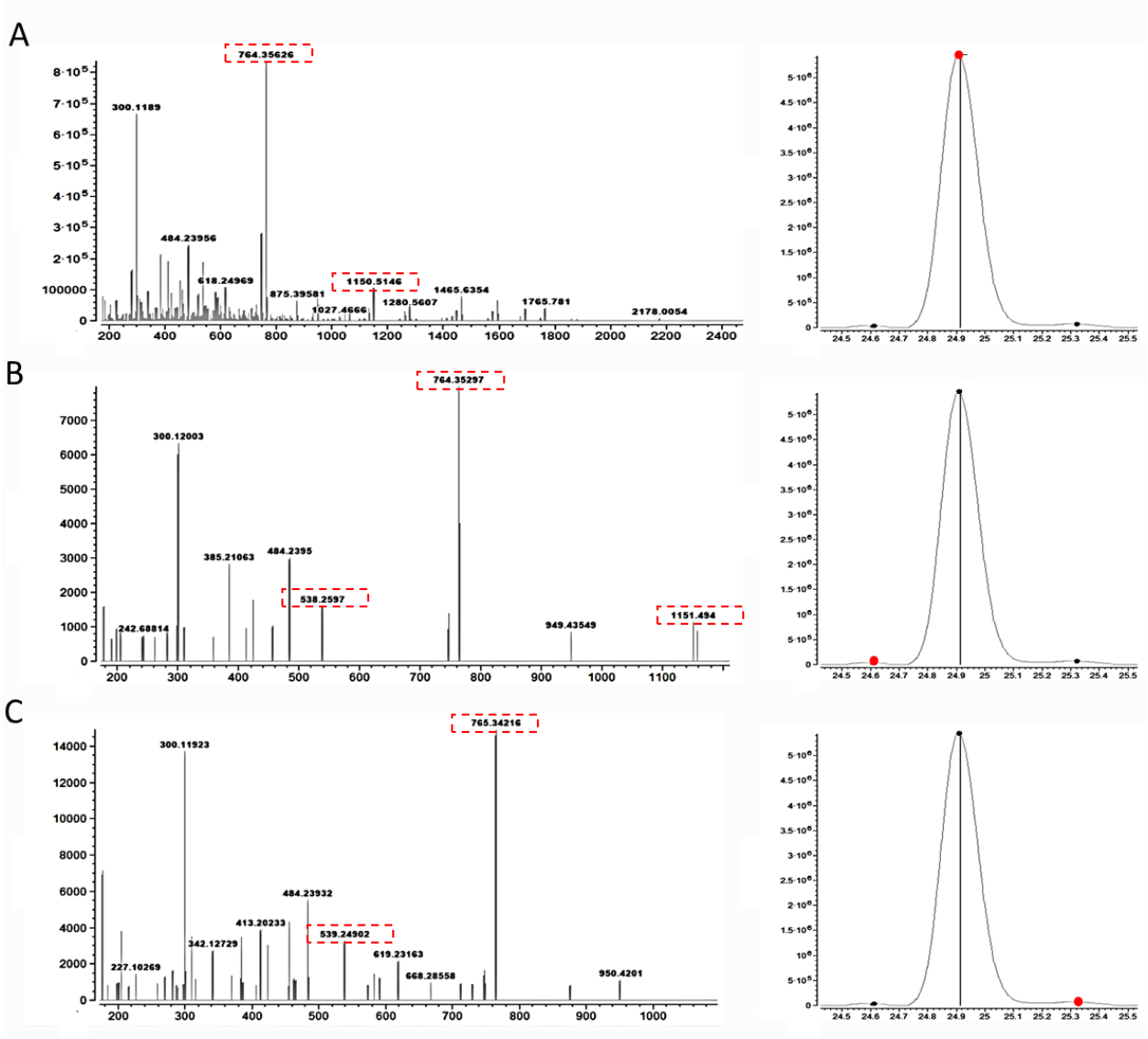


Figure 13. MS/MS spectra and extracted ion chromatograms (EIC) obtained from fragmentation and analysis of the doubly charged precursor of the PENNY tryptic peptide. Left panels: Generated MS/MS spectra from the corresponding ion chromatogram of the corresponding right panel. y ions (y4, y6, and y10) are marked in dashed red boxes used to confirm the site of mass change and modification. Y axis (left): MS2 signal intensity. X axis (left): m/z ratio. Right Panels: EIC where red dots indicate the peak that the MS/MS spectra on the left panel corresponds to. Y axis (right): Ion chromatogram signal intensity. X axis (right): Retention time (in minutes). A) Non-deamidated PENNY peptide. B) Iso-aspartic acid formation at N386. C) Aspartic acid formation at N391, N392 moiety.

Once a suitable chromatographic method and a characterization of deamidated species (iso-aspartic and aspartic acid formation) were obtained, a PRM (parallel reaction monitoring) method at a quadrupole orbitrap mass spectrometer instrument was developed. For details on this type of

targeted MS method see the Introduction and the Discussion section of Chapter III. For quantitation purposes, the charge state of each peptide with the highest signal intensity was selected among with the product ion that had the highest signal intensity after fragmentation of the precursor (see Figure 14). For confirmation purposes, the two product ions with next highest signal intensity were also selected (y8 and y10).

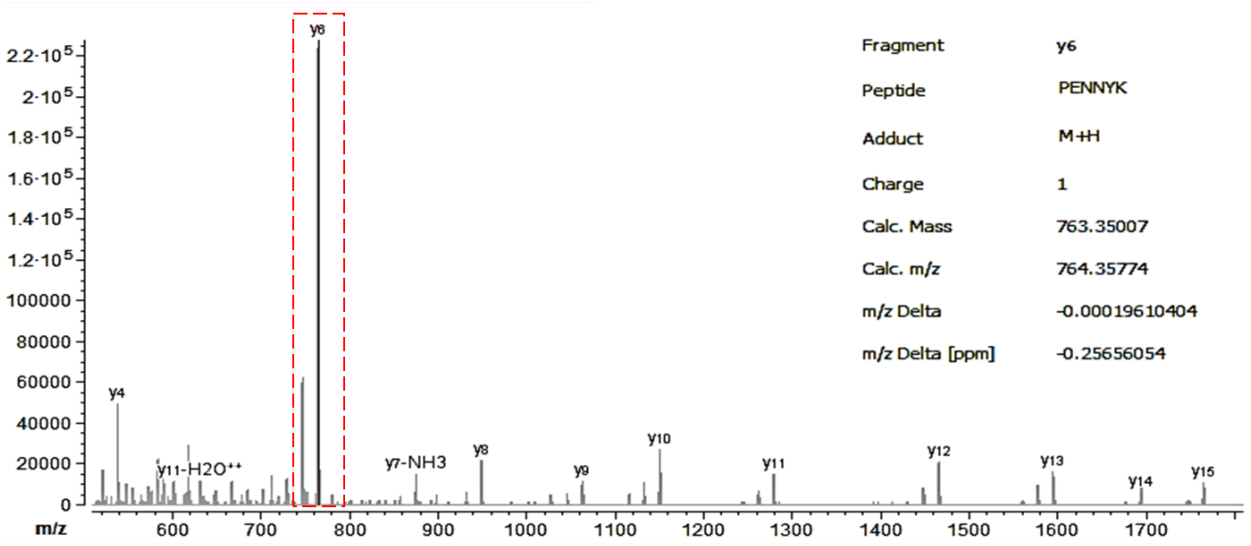


Figure 14. MS/MS spectrum of GFYPSDIAVEWESNGPENNYK (PENNY peptide) after fragmentation of the doubly charged precursor. Red dashed box: Fragment with the highest signal intensity (y6). Y axis: MS2 signal intensity. X axis: m/z ratio.

Discussion

During the development of MAM methods in the industry, several attributes are tracked simultaneously. Between these attributes, deamidation represents a special challenge in terms of monitoring difficulty. This is caused by the usual coelution of deamidated and non-deamidated species. The coelution is derived from the small mass difference between these species and lack of baseline separation in the obtained chromatograms. The ion chromatograms (even if baseline separation is achieved) may hence require extra peak editing and manual selection during data analysis. By this, MAM implementation within regulated automated procedures may be impaired.

Within LC-MS/MS analysis, also the chosen MS instrumentation such as a triple quadrupole (QQQ) may present difficulties in the power of resolution to discriminate between deamidated and non-deamidated species. The reason for that is that deamidation causes less than 1 Da mass change and the mass window range in a QQQ itself is of 1 Da already. Although this problem for monitoring deamidation may be addressed using a quadrupole orbitrap MS instrument (which offers a higher resolution), the higher sensitivity obtained from instrumentations such as a QQQ must be considered. The requirements of resolution and sensitivity should hence be carefully assessed depending on the biological question and goal of the assay. A broader comparison of

these two types of instruments in a technical way and the difference between SRM and PRM analysis will be presented in Chapter III. Nevertheless, regardless of the instrumentation of choice, deamidation monitoring represents a challenge in chromatographic separation. Optimization of the chromatographic separation method is crucial to obtain a baseline separation and a confident assignment of deamidated and non-deamidated species.

Within this subsection, the optimization of a chromatographic separation for monitoring deamidated species was presented and an adequate baseline separation of deamidated species was achieved. It was of major importance for the study to acquire a complete baseline separation of species to relatively quantify the species with confidence. This optimization of chromatographic separation had to be developed in an LC gradient (being as short as possible) whose total run time would also allow the analysis of multiple samples derived from preclinic and clinic. The retention time of the PENNY peptide would be situated at the later segments of the run length due to its high hydrophobicity and therefore its high interaction with a reverse-phase analytical column packing. Lengthy runs of up to 100 minutes during the optimization phase were first assessed. This was done to determine the appropriate percentage of organic solvent at the elution point of the PENNY peptide and hence to achieve a proper separation. The entire cohort of monitored peptides differed highly in hydrophobicity (PENNY peptide being highly hydrophobic, while NTLYL peptide being highly hydrophilic). This required first, that in the developed gradient a proper separation of deamidated species was evaluated. Second, it was crucial that all peptides of interest were detected in the elution range (not pre-eluting or in the washing steps of the analytical column). And third, that all peptides of study were separated from each other. As result, a chromatographic method with total run time of 45 minutes (including steps of washing and equilibration of the analytical column) with baseline separation between peptides and modified variants was achieved.

The observed baseline separated peptide species in the PENNY peptide matched the reports in existing literature for the deamidation species (Figure 15). It was observed that N386 iso-aspartic acid formation eluted before the non-deamidated peptide and N391, N392 moiety aspartic acid formation eluted after. Elution pattern to be expected when the separation is performed on a reverse-phase column.

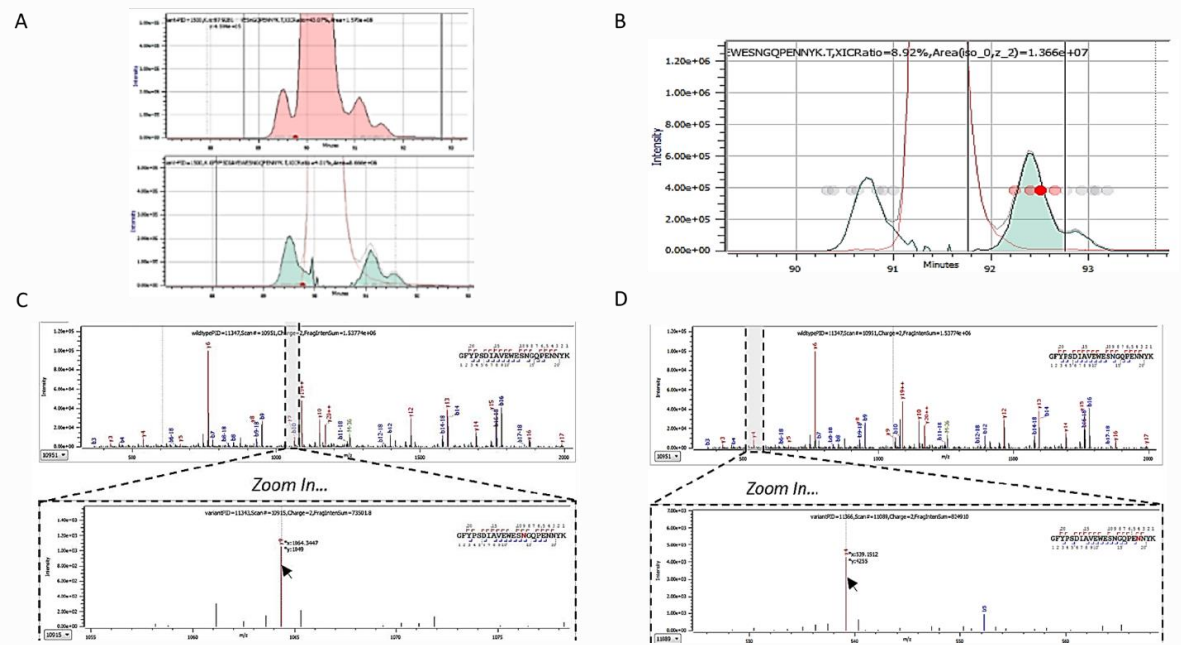


Figure 15. A) Total extracted ion chromatogram of …NGQPENNY peptide, both deamidation sites are highlighted in green. B) Deconvolution of the deamidated form of the N391, N392 moiety in the PENNY peptide. C) Annotated MS2 spectra of N386. D) Annotated MS2 spectra of N391, N392 moiety (Carlson E, 2015, Protein Metrics).

Deamidation study has been reported in the literature using different analytical techniques, such as isoelectric focusing, capillary electrophoresis, and a variety of LC-MS/MS techniques. All of them have limitations that make their analysis a challenge[47,54,106–109]. The greatest challenge for LC-MS based methods is the mass shift of only 1 Dalton between the modified and native forms of the peptides. This causes the deamidated species to overlap with the mass-to-charge (m/z) ratios of the unmodified species. Without a separation technique that allows to fully distinguish the modified and the unmodified versions, mass-spectrometric analysis of deamidation can be highly challenging. Badgett et al. developed a HILIC-MS-based method for separation and quantification of deamidated peptides[110]. Although they achieved a baseline separation through HILIC-MS, they reported to not be able to separate deamidated from non-deamidated species of the PENNY peptide through reverse phase chromatography, in contrast to what was achieved in the work presented within this study.

Other novel methodologies for achieving separation and quantification of deamidated species have been proposed. For instance, an approach using a reverse phase as first dimensional separation and an electrostatic repulsion-hydrophilic interaction chromatography as second one coupled to MS analysis[111], was developed by Hao et al. Within the first dimension, coelution of the deamidated and non-deamidated species is not considered a disadvantage, as they are collected in the same fraction, separated, and identified in the second dimension based on pI and GRAVY values.

Deamidated and non-deamidated peptides (containing an Asn, n-Asp, or iso-Asp amino acid at the same position) originating from the same protein can (in principle) be identified and quantified by LC−MS/MS if previously separated by HPLC[111]. However, this would not be a likely scenario in a shotgun proteomics approach. The typical coelution of the isomeric Asp and iso-Asp peptides from reverse phase columns is a great challenge encountered in RPLC-MS/MS for deamidation analysis. Since the isomers have almost identical fragmentation patterns, they interfere with each other in identification of the reporter ions[111]. Consequently, it is extremely challenging that deamidated peptides of low abundance are identified on a proteome-wide scale. These are only some of the reasons why previous lengthy method optimization will be inevitably required within the analysis of deamidated peptides.

3) Determination of the method's LLOQ for tryptic peptides of Pertuzumab in animal serum

Results

Once a method at both LC and MS level was developed, the required range of quantification to be applied in calibration curves for method validation was defined. This was based on exploratory animal study data obtained through ELISA analysis (ELISA full data set not available, samples based on PK profile but data cannot be disclosed). Pertuzumab concentrations (after administration) in individual animals were determined over time and a pharmacokinetic profile (PKP) curve was built for each animal (Table 7 and Figure 16). Only animals with sufficient concentration levels after 28 days were included for further analysis due to the need for sufficient sensitivity. In this case, only animals 1, 2, and 3 were selected. In turn, animals 4, 5, and 6 presenting the lowest levels of concentration after 28 days were discarded due to potential sensitivity problems in LC-MS/MS analyses. Based on the same rationale, animal 7 was discarded.

Pertuzumab concentration (ug/ml) in individual animals.							
Animal	**50 minutes**	**24 hours**	**5 days**	**16 days**	**22 days**	**25 days**	**28 days**
4	-	-	-	-	-	-	-
1	553.8175	323.9392	145.4515	66.4390	29.0445	23.4169	16.0223
5	-	-	-	-	-	-	-
6	-	-	-	-	<LLOQ (0.025)	<LLOQ (0.025)	<LLOQ (0.025)
7	-	-	-	-	-	-	-
2	477.8546	231.0665	134.0732	56.2922	26.3020	11.3701	11.9457
3	483.3383	NA	169.7531	80.4569	54.2480	41.8413	27.2579

Table 7. Pertuzumab concentration determined through ELISA analysis on 7 different animals after intravenous infusion. Time points for collection of blood samples ranged from 50 minutes to 28 days. Full ELISA data not available (data cannot be disclosed). The LLOQ used in ELISA analysis was at 0.025 ug/ml. Samples based on PK profile but data cannot be disclosed

The determination of the needed LLOQ for the method to be developed was based on two key factors:

- Expected deamidation and oxidation rates in Pertuzumab under physiological conditions (37 °C, pH ~8.0) after 28 days
- Concentration level of Pertuzumab observed after 28 days (after administration) in exploratory animal study samples

Note that the needed LLOQ does not correspond to the total concentration level of Pertuzumab at day 28 seen in animals. Instead, the estimated deamidation and oxidation rate in Pertuzumab at day 28 was considered. For which the total concentration level of Pertuzumab was corrected by a proper factor. The estimated deamidation and oxidation rates were calculated based on the rate of degradation seen during incubation of Pertuzumab in PBS close to physiological conditions (40 °C for 2 weeks) in Section II, 1 "Peptide Selection".

From the results for peptides that are most prone to degradation, a value between 10% - 15% of degradation after 4 weeks was estimated. The total Pertuzumab concentration level after 28 days of the animals selected above was between 12 ug/ml and 27 ug/ml. Correcting with the estimated degradation rate of 10 - 15% yields an LLOQ value of ~2 ug/ml. This value can be interpreted as the minimum Pertuzumab concentration level to assess rates of deamidation and oxidation after 28 days at least in two (animals 3 and 3) of the seven animals. In addition, it would also allow to confidently quantify deamidation and oxidation values within after 25 days in all individuals and the total concentration of non-modified Pertuzumab over all time points of the study (until 28 days). By that, a comparison of pharmacokinetic curves between the LC-MS/MS method and results obtained from ELISA would be possible.

After deriving a suitable value for the LLOQ of the method, it was aimed to further improve sensitivity and fulfill certain signal-to-noise-ratio criteria. This was addressed through strategies for improving the digestion efficiency of all peptides in presence of serum as biological matrix. Such strategies that will be described in the following Chapter II, 4 led to further protocol optimization through tests of different denaturation, reduction, and digestion conditions.

4) Optimization of the protein digestion efficiency for quantifying tryptic peptides of Pertuzumab at the LLOQ in animal serum

Results

In Chapter I, different incubation conditions were tested to optimize digestion efficiency and their impact on side reactions such as deamidation and oxidation. The optimal conditions in chapter I were selected independently of the LLOQ required in the method. To quantify the peptides at the required LLOQ concentration with higher reproducibility, robustness, and better signal-to-noise ratio, a higher sample digestion efficiency was needed. For that purpose, further optimization steps of the proteolytic digestion in presence of the proper matrix (animal serum) were designed (see

Table 8 and Methods and Material section II, 3). For optimizing the whole process of digestion, it seemed reasonable to consider the single digestion steps. These are:

- Denaturation
- Reduction
- Pre-Digestion
- Digestion

The effect of varying conditions through each of the digestion steps was evaluated per peptide. The evaluation was performed at a fixed Pertuzumab concentration level of 10 ug/ml for all samples to assure detection of all peptides in the presence of serum.

	Denaturation	Reduction	Pre-Digestion	Digestion
Test 1	no denaturation	use of 100mM TCEP	no pre-digestion	3 hours digestion
Test 2	denaturation solution from vendor (Promega) for 30min	use of 200mM TCEP	pre-digestion with Lys-C (at pH 5.5)	4 hours digestion
Test 3	conditions of test 2 + guanidine HCL addition	use of 300mM TCEP	-	5 hours digestion
Test 4	conditions of test 3 + increase of time (30 min to 45 min)	-	-	-
Test 5	conditions of test 4 + increase of time (45 min to 90 min)	-	-	-

Table 8. Conditions tested per protein digestion step during proteolytic digestion of Pertuzumab (concentration: 10 ug/ml). Optimal conditions per step were assessed subsequently, e. g. after choosing optimal conditions for protein denaturation, different reduction conditions were explored based on the optimal selected denaturation conditions.

Starting with the denaturation step, for each of the four peptides selected in chapter II, 1 (see Discussion section) the impact of varying denaturation conditions on the obtained peptide quantification was assessed (Figure 16). All peptides were quantified based on their MS2 spectra (developed PRM method). The obtained signal intensities for each peptide were corrected by the addition of an internal standard (heavy-labeled IgG1 and heavy-labeled synthetic peptides) at the beginning of the digestion protocol.

From Figure 16, it can be observed that Test 1 of not denaturizing the protein had a particularly high impact on the quantification of peptide DSTYSLSSTLTLSK, whose signal was completely

lost. The subsequently performed Tests 2 to 5 showed increased intensities of the MS2 signal for all peptides, while the degree of improvement was dependent on the peptide itself. The best results over all peptides were achieved with Test 5.

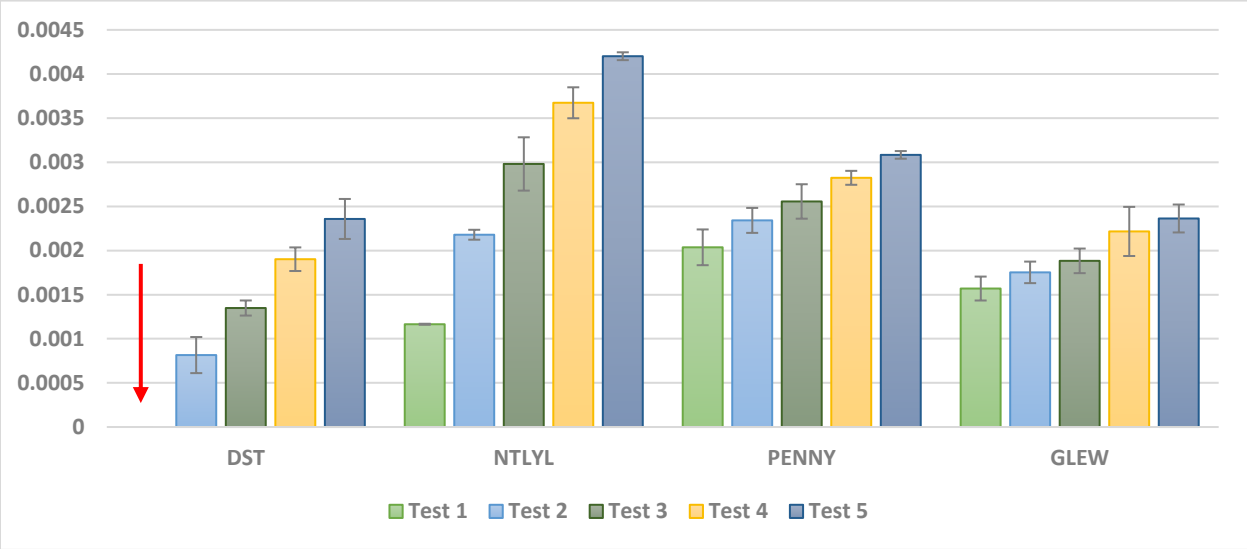


Figure 16. Impact of different denaturation conditions on the quantification of selected tryptic peptides after digestion of 10 ug/ml Pertuzumab in animal serum. The red arrow illustrates the complete loss of DST tryptic peptide signal without denaturation. Standard deviation between triplicates is depicted by grey whiskers. Y axis: Intensity of MS2 signal corrected with MS2 signal for the corresponding peptide of the IS. The IS was spiked at the beginning of the digestion protocol. X axis: Different tryptic peptides with different denaturation conditions per peptide. Test 1: No denaturation performed. Test 2: Denaturation solution from vendor (Promega). Test 3: Addition of guanidine HCL. Test 4: Prolonged time from 30 minutes to 45 minutes. Test 5: Prolonged time from 45 minutes to 90 minutes.

Next, the impact of varying reduction conditions on the obtained peptide quantification was assessed per peptide. Increasing concentrations of TCEP (Figure 17) were tested based on the previously selected best denaturation conditions (Test 5 of denaturation step). By comparing Figures 13 and 14, one can observe that in contrast to the impact of denaturation, all TCEP concentration levels had a depreciable effect on the detected intensities of the MS2 signal for all peptides.

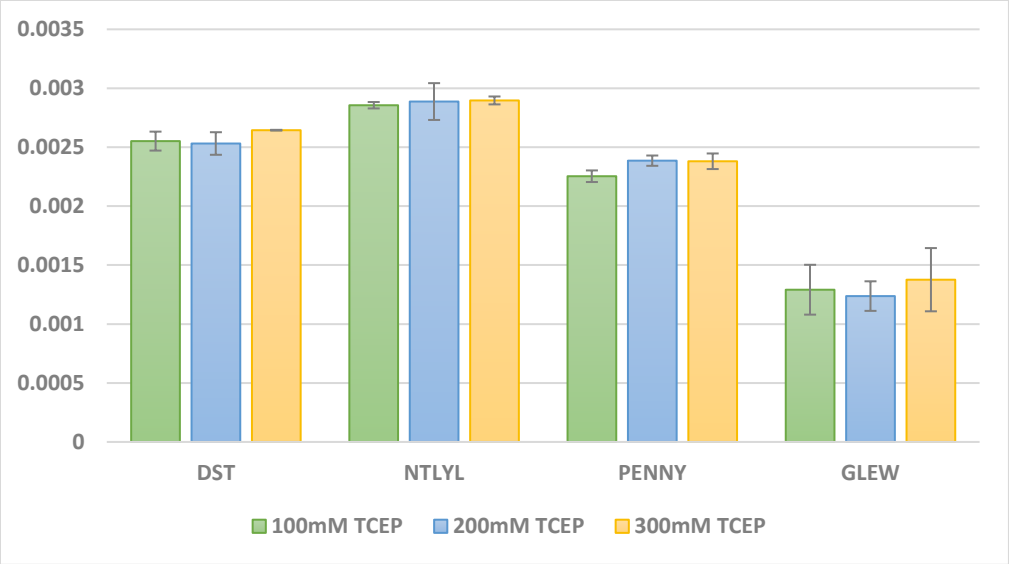


Figure 17. Impact of different reduction conditions on the quantification of selected tryptic peptides after digestion of 10ug/ml Pertuzumab in animal serum. Standard deviation between triplicates is depicted by grey whiskers. Y axis: Intensity of MS2 signal corrected with MS2 signal for the corresponding peptide of the IS. The IS was spiked at the beginning of the digestion protocol. X axis: Different tryptic peptides with different reduction conditions (TCEP concentrations) per peptide.

As final optimization steps, the following parameters were tested:

- Pre-digestion: with Lys-C (at pH 5.5) for one hour vs. no pre-digestion (Figure 18)
- Digestion: increasing times of digestion with trypsin and Lys-C (Figure 19)

By these tests, the benefit of a potential pre-digestion step and an increasing incubation time was explored.

It could be observed that pre-digestion with Lys-C yielded higher intensities of the MS2 signal compared to the case when no pre-digestion was performed. In terms of increased incubation time, the GFYP peptide (commonly referred to as PENNY peptide) showed a steeper increase in intensity of MS2 signal (38% relative increase from 3 to 5 hours incubation time) than the other peptides (20% on average between DST, GLEW, and NTLYL). It was furthermore checked and proven that the increased incubation time did not induce additional deamidation rates in the samples. From that, it could be concluded that increasing the incubation time up to 5 hours had a beneficial impact on sensitivity when quantifying peptides at the required LLOQ.

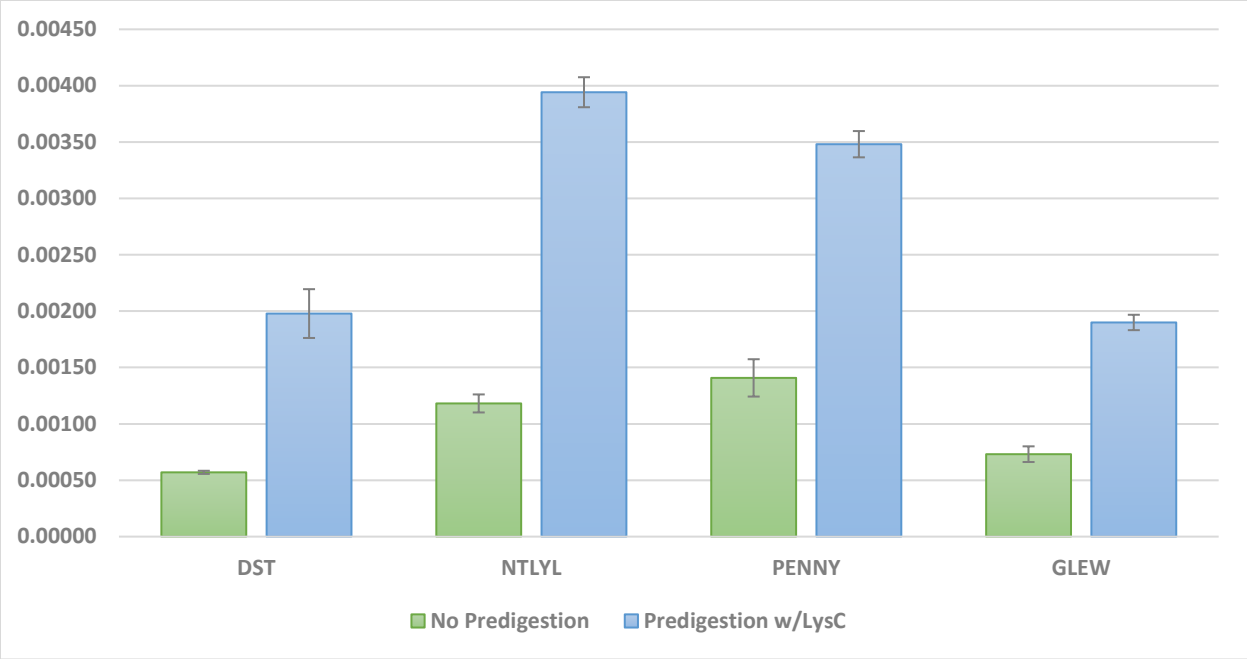


Figure 18. Impact of a pre-digestion step with Lys-C at low pH (5.5) on the quantification of selected tryptic peptides after digestion of 10 ug/ml Pertuzumab in animal serum. Standard deviation between triplicates is depicted by grey whiskers. Y axis: Intensity of MS2 signal corrected with MS2 signal for the corresponding peptide of the IS. The IS was spiked at the beginning of the digestion protocol. X axis: Different tryptic peptides with different pre-digestion conditions per peptide.

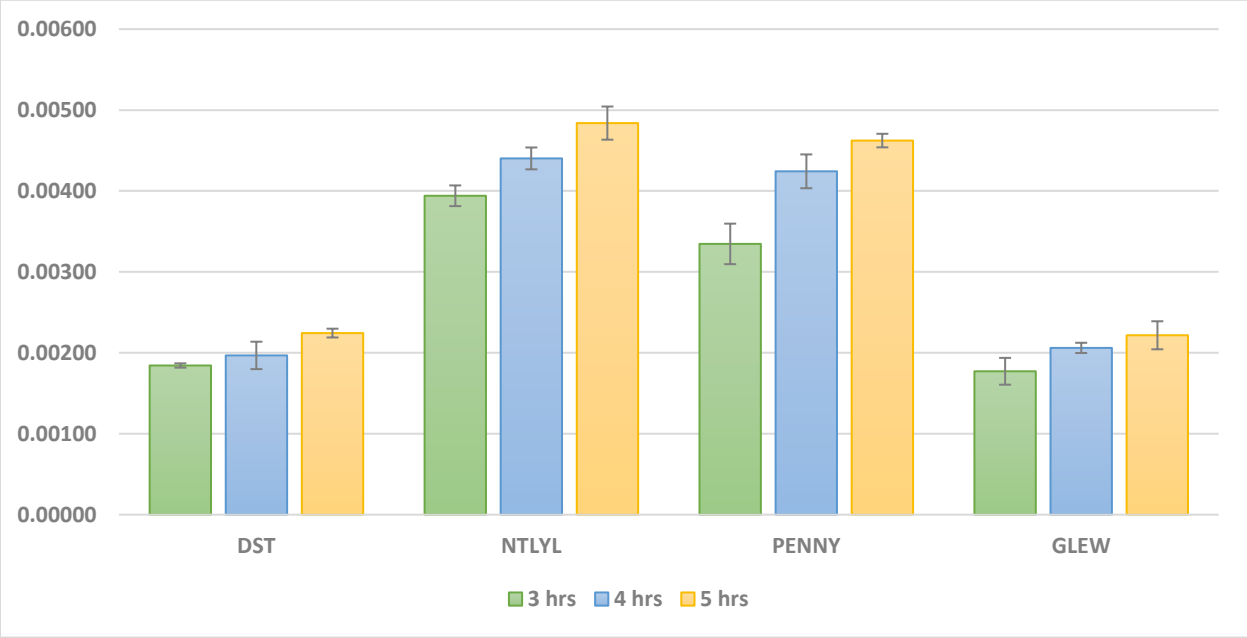


Figure 19. Impact of increasing digestion time with trypsin and Lys-C at low pH (5.5) on the quantification of selected tryptic peptides after digestion of 10 ug/ml Pertuzumab in animal serum. Standard deviation between triplicates is depicted by grey whiskers. Y axis: Intensity of MS2 signal corrected with MS2 signal for the corresponding peptide of the IS. The IS was spiked at the beginning of the digestion protocol. X axis: Different tryptic peptides with different digestion conditions per peptide.

Discussion

In this subchapter, varying digestion conditions for each digestion step were compared per monitored peptide. From the tested alternatives, the best conditions were identified and selected for further analysis and development.

For the denaturation step, the use of Guanidine HCL within sample preparation was explored. Although Guanidine HCL is commonly avoided in LC-MS analysis due to its signal suppression effect during ionization, the implementation of a solid phase extraction cleaning step prior to LC-MS/MS analysis was performed within this study (see Material and Methods section II, 3). This allowed Guanidine HCL to be included in the comparison of different denaturation conditions. The effect on digestion efficiency varied per peptide when comparing different denaturation conditions. While only a slight improvement in digestion efficiency was observed for peptides GFYP (PENNY peptide) and GLEW, the effect was more evident for peptides NTLYL and DST.

Moreover, the signal intensity of DST was completely lost when no protein denaturation step was performed. The signal intensity improved significantly using Guanidine HCL and longer denaturation times. This effect may be explained based on the peptide's location in the tertiary structure of the protein. The DST peptide is located in the light chain of Pertuzumab. Its enzymatic cleavage site (K/DST…) is in a protein loop (Figure 20). The presence of this amino acid sequence in a loop requires a step of protein unfolding for better accessibility of trypsin and Lys-C. Consequently, the lack of protein denaturation makes the site inaccessible for enzymatic activity.

Similarly, NTLYL peptide (located in the heavy chain) was identified to be next to a protein loop in the tertiary protein structure. Accordingly, this peptide also showed an improved digestion efficiency at longer times for protein denaturation. However, this effect was not as pronounced as for the DST peptide as its detection was achieved even without a denaturation step. From this, it was hypothesized that complete unfolding of the protein may not be necessary for NTLYL due to its probable higher exposure to the protein's surface (Figure 20).

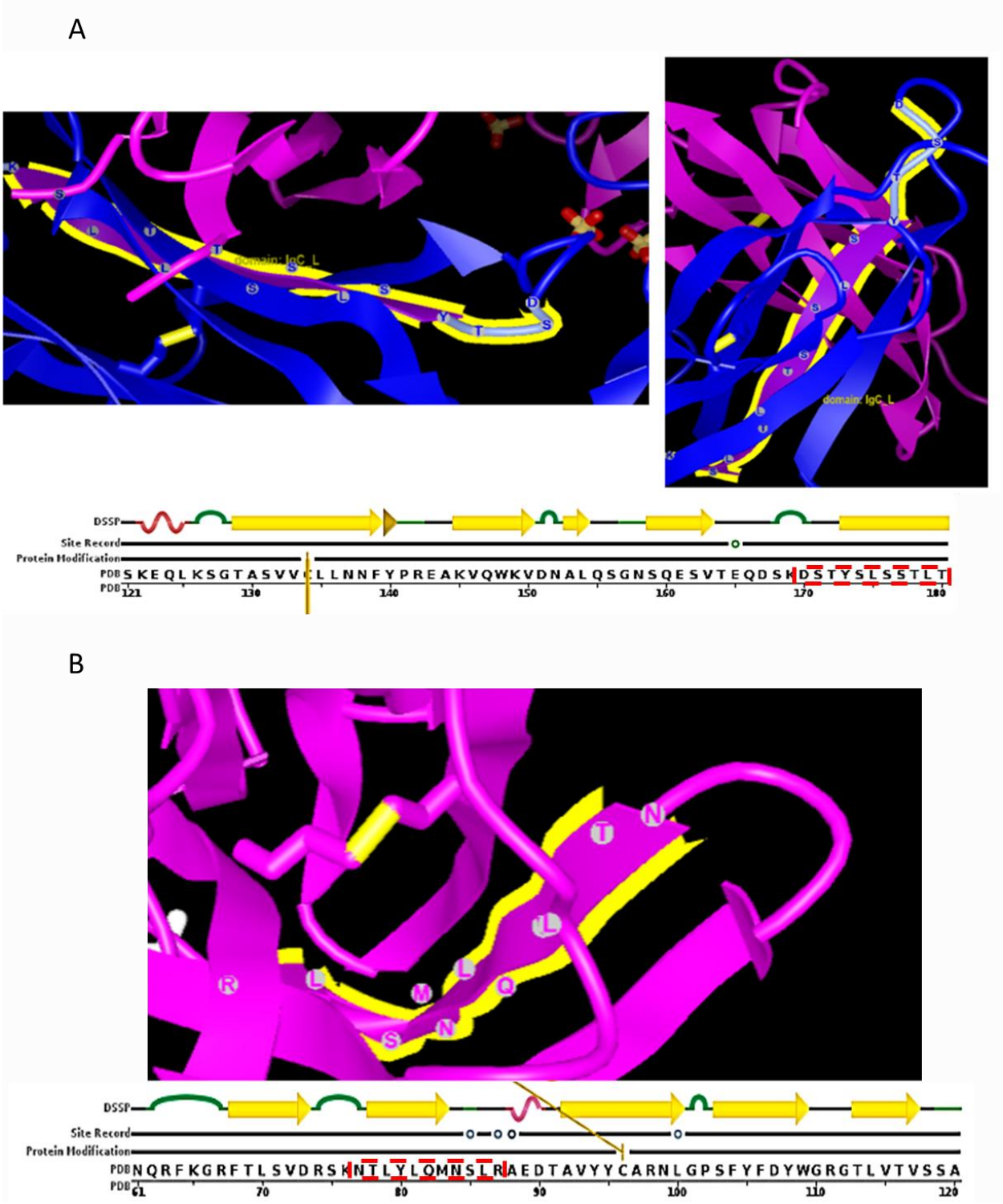


Figure 20. Scheme showing the tertiary structure of Pertuzumab. A) Location of the DST peptide in the tertiary structure in loops of the LC. B) Location of the NTLYL peptide in the tertiary structure in loops of the heavy chain.

The protein reduction step was analyzed after the denaturation step. For that, different concentrations of TCEP during the reduction step were compared. No significant impact of the different TCEP concentrations on the detected signal intensity was observed. This may be explained by the fact that none of the monitored peptides contained cysteine amino acids. That means that a disruption of disulfide bonds through protein reduction was not crucial. The absence of cysteines was beneficial for achieving a more complete proteolytic digestion. This is due to the fact that a higher presence of cysteines may cause multiple disulfide bonds formations. That, in turn, gives the protein a constraint conformation, makes it thermally more stable, and compromises complete enzymatic digestion. Such incomplete digestion diminishes the accuracy and reproducibility of the assay when an internal standard is not present.

Regardless of the minimum impact of different TCEP concentrations seen within the reduction step, it was decided to maintain the step to achieve a better overall digestion of the molecule. An alkylation step with iodoacetamide was also kept within the sample preparation to avoid possible side reactions of cysteines.

A potential pre-digestion step was assessed after the protein reduction step. For that, Lys-C was used as the enzyme of choice. It was found that its inclusion had a beneficial impact on digestion efficiency for all peptides. Similarly, the use of trypsin plus Lys-C was observed to yield a slight increase in signal intensity with increasing digestion times for all peptides. However, this increase was not very pronounced, which allowed to conclude that longer incubation times might provide a milder benefit compared to the used rate of enzyme concentration.

On the other hand, an increase in enzyme concentration may lead to a signal suppression during MS analysis. This is because trypsin and Lys-C show auto-proteolytic activity and the generated peptides from this auto-proteolytic digestion might coelute with the peptides of interest from Pertuzumab. This coelution might influence the assay's sensitivity and lead to signal suppression. Additionally, an incremented enzyme concentration leads to increased costs per sample.

Moreover, a digestion of five hours length with a fixed amount of enzyme was chosen as final setting. Together with the pre-digestion step of one-hour length, the total incubation period for digestion summed up to six hours. It was proven within the Results section for method validation that this incubation period does not yield artificial deamidation (see Table 10 of Chapter IV).

The final sample preparation conditions were assessed as the best combination to

- acquire an acceptable digestion efficiency for all peptides
- achieve a proper sensitivity at the LLOQ with a minimized signal-to-noise ratio in serum
- not cause extra rates of deamidation due to incubation times

From the tests per digestion step described above, the final optimal conditions to achieve higher intensity of MS2 signal and hence higher digestion efficiency in serum were selected. These were:

- Denaturation: solution from vendor (Promega) + guanidine HCL addition for 90 minutes
- Reduction: 100mM TCEP

- Pre-digestion: Lys-C at pH 5.5 for 60 minutes
- Digestion: Lys-C and trypsin at pH 5.5 for 5 hours

Strategies with varying enzymatic digestion conditions have previously been explored to optimize digestion efficiency while reducing deamidation rates. One approach by Da Ren et al. sought to maximize trypsin activity[66]. This optimization was attempted through complete removal of guanidine from the digestion buffer to avoid its inhibition activity on trypsin. Through this implementation, a complete digestion of immunoglobin gamma molecules was achieved in only 30 minutes. Their results showed diminished rates of induced deamidation, cleaner tryptic maps, and fewer nonspecific cleavages due to less trypsin self-digestion[66]. However, the main disadvantage of this approach was that the protein recovery was free of guanidine after a desalting step, as it was reported as only of 70%[66].

Other types of approaches for increasing digestion efficiency while avoiding induced side reactions have been proposed. One example is the use of controlled microwave irradiation to achieve a protein digestion in a matter of minutes[71]. This methodology is based on the premise that microwave irradiation has the potential to accelerate organic reactions due to energy transfer. Formolo et al. applied this approach to address the impact on digestion efficiency and method-induced deamidation when using microwave-assisted hydrolysis techniques[22]. However, this approach requires extra devices under very controlled conditions that could not be the ideal choice in terms of reproducibility and robustness. Similarly, another methodology that has been explored is the use of pressure cycling technology to optimize digestion efficiency in a rapid way[112]. However, these approaches require extra equipment and might not be easy to be applied.

With the findings of this chapter, a suitable sample preparation for the required LC-MS/MS analysis could be optimized. Increased digestion efficiency and lower signal-to-noise ratio in presence of a serum matrix were obtained through the steps described above as bullet points. By that, an acceptable sensitivity of the method was achieved. As next step, it seemed reasonable to check if sensitivity results could be further improved by using a different instrument and method. Hence, a comparison between a PRM analysis in a quadrupole orbitrap instrument and an SRM analysis in a triple quadrupole mass spectrometer was performed.

III. Comparison between SRM and PRM approaches in terms of sensitivity and specificity achieved in complex biological matrices

Results

1) Comparison between SRM and PRM methods

During the digestion process of therapeutic proteins, the protein can be subjected to conversion reactions such as deamidation and oxidation. These degradations are usually present in low abundancy. Therefore, the required LLOQs in complex biological matrices are very low. Consequently, high sensitivity in LC-MS/MS methods is required to confidentially quantify such modifications. In Chapters I and II, several optimization steps performed to increase signal detection were presented. In this chapter, an additional optimization tested parameter is presented: the used method in the corresponding instrument. Hence, a comparison between an SRM method in a triple quadrupole instrument and a PRM method in a quadrupole orbitrap instrument was performed. The different methods were compared in terms of sensitivity, lower limits of detection, selectivity, and resolution, among others. Through this comparison, the most suitable instrumentation for the quantification of low-abundant deamidation and oxidation modifications would be selected.

As first step, a transfer from the PRM method developed in Chapter II to an SRM method was performed. Therefore, LC and MS parameter optimization was performed in a triple quadrupole coupled to an LC in the same way as described for a quadrupole orbitrap in Chapter II, 2. A proper chromatographic baseline separation between deamidated and non-deamidated species was reached by this. A difference in retention times (RT) of ~0.5 minutes was obtained (see Figure 21 for ion chromatogram of the PENNY peptide).

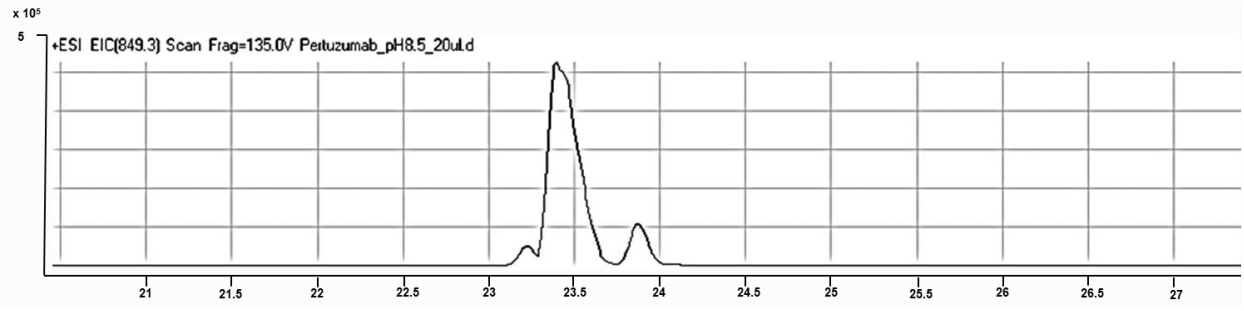


Figure 21. Ion chromatogram of the PENNY peptide. Consecutive elution of iso-aspartic acid formation, non-deamidated peptide, and aspartic acid formation in the mentioned order. Sample contained Pertuzumab after tryptic digestion. Analysis through reverse phased liquid chromatography (RPLC) coupled to a triple quadrupole instrument. Y axis: Ion chromatogram signal intensity. X axis: Retention time (in minutes).

To develop and optimize the SRM method introduced above, a non-incubated sample (before stress) containing Pertuzumab at 0.5 mg/ml spiked and digested in PBS was analyzed in a full scan mode analysis. First, the precursor with highest signal intensity per peptide was selected. Second, an MS2 analysis with varying collision energies (CE) was performed to select the product ion with highest signal intensity. The selected product ion per peptide was then used for quantification of the corresponding peptide present in Pertuzumab in the serum matrix. In addition, the two product ions with the next highest signal intensity were included for confirmatory purposes for all peptides and heavy-labeled standards. Furthermore, the optimal energy values for a complete fragmentation of the precursor could be selected per peptide from this test with varying collision energies (see Material and Methods section III, 1 and Table S3 in Supplemental Material).

After the development and optimization of the SRM method as described above, a sample containing Pertuzumab spiked into the biological matrix (animal serum) and digested was analyzed through the developed SRM (Figures 19 and 20).

Figure 22 shows the fragmentation of the doubly charged precursor of Pertuzumab's tryptic NTLYL peptide (m/z: 676.8). The y6 fragment QMNSLR (m/z: 748.5) was found to be the product ion with highest signal intensity in PBS (Figure 22A). Figure 22B shows the peak of interest (ion chromatogram of the product ion y6 at a retention time of 19.473 minutes) in presence of serum. It can be observed that close peaks with a matching precursor mass value surrounding the peak of interest are present due to the complex biological matrix. Figure C shows the overlay of the ion chromatograms of the product ions used for quantification and confirmatory purposes in presence of serum.

Figure 23 shows the fragmentation of the triple charged precursor of Pertuzumab's non-deamidated and deamidated tryptic PENNY peptide (m/z: 848.7 and 849.0, respectively) spiked in animal serum. Both in Figure 23A and Figure 23B a sample of digested non-stressed Pertuzumab (kept at -80 °C and digested immediately after thawing) was analyzed. It can be observed in Figure 23A and B that in both extracted ion chromatograms (non-deamidated and deamidated variants) the peak selected for integration showed the same retention time. However, as this sample was of non-stressed nature no deamidation was expected to be observed.

In Figure 23C a sample incubated at temperature stress (40 °C for 4 weeks) was analyzed. It was observed that both peptide variants (non-deamidated and deamidated) were detected and quantified under the same mass value of product ion y6 from the deamidated variant (m/z: 765.3). This made a manual peak selection necessary for both variants and required delimitation/editing of the integrated area under the peaks.

Further remarks on the results of Figure 23 and their linkage to the resolution obtained from a triple quadrupole instrument will be presented in the Discussion section of this chapter.

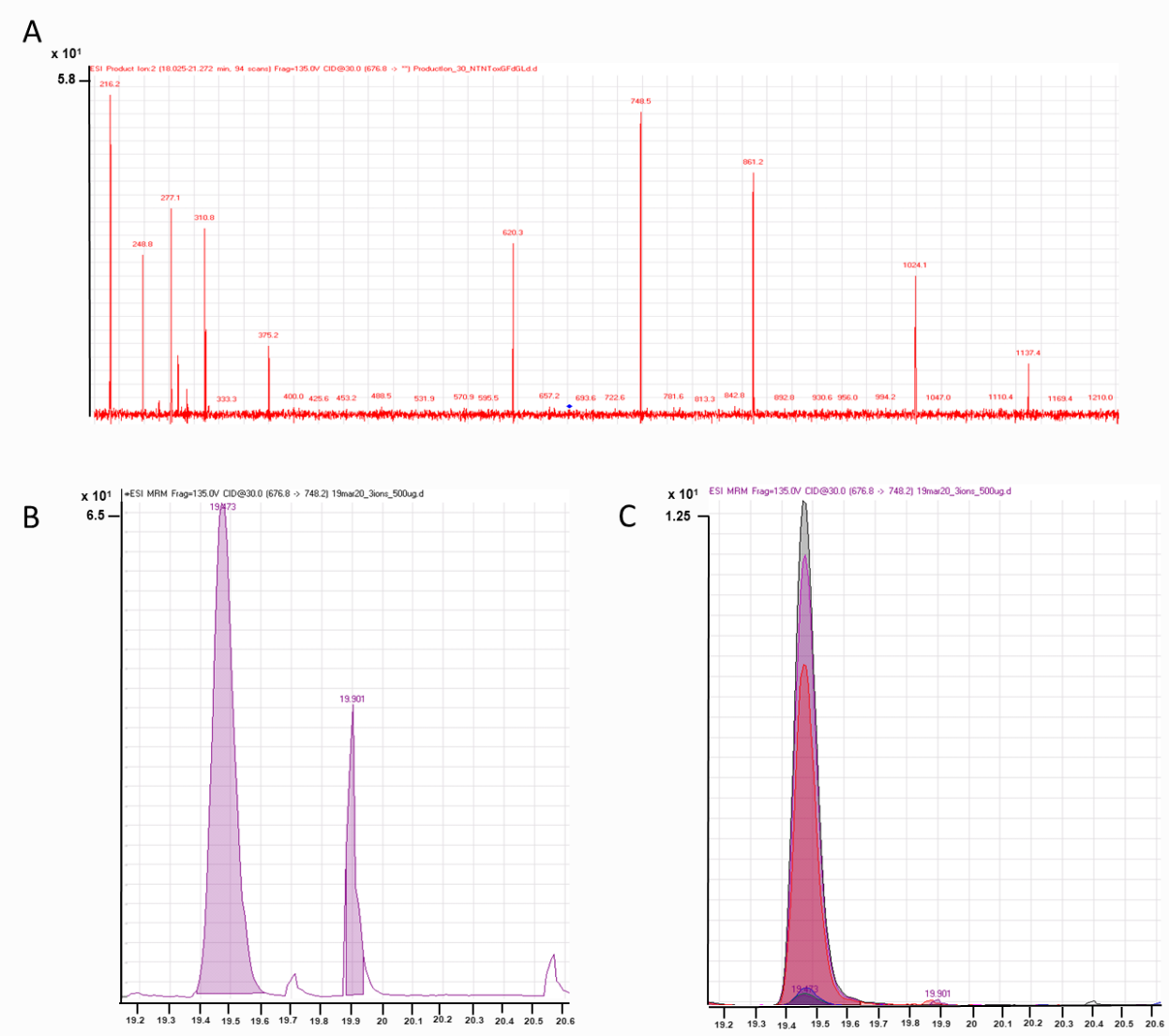


Figure 22. MS/MS spectra and EIC obtained from fragmentation of the doubly charged precursor of Pertuzumab's tryptic NTLYL peptide (m/z: 676.8) spiked in PBS and in animal serum. Analysis performed through SRM in a QQQ instrument. A) Fragmentation of doubly charged tryptic NTLYL peptide at the determined optimal collision energy in PBS. Blue diamond indicates the complete fragmentation of the precursor. Y axis: Spectrum signal intensity. X axis: m/z ratio. B) Chromatogram of the y6 product ion of the NTLYL peptide in serum. Y axis: Signal intensity in the ion chromatogram. X axis: Retention time (in minutes). C) Overlay of chromatograms for the quantification of the product ion with highest signal intensity (y6) and confirmatory product ions (y8 and y7) in presence of animal serum as biological matrix. Y axis: Signal intensity in the ion chromatogram. X axis: Retention time (in minutes).

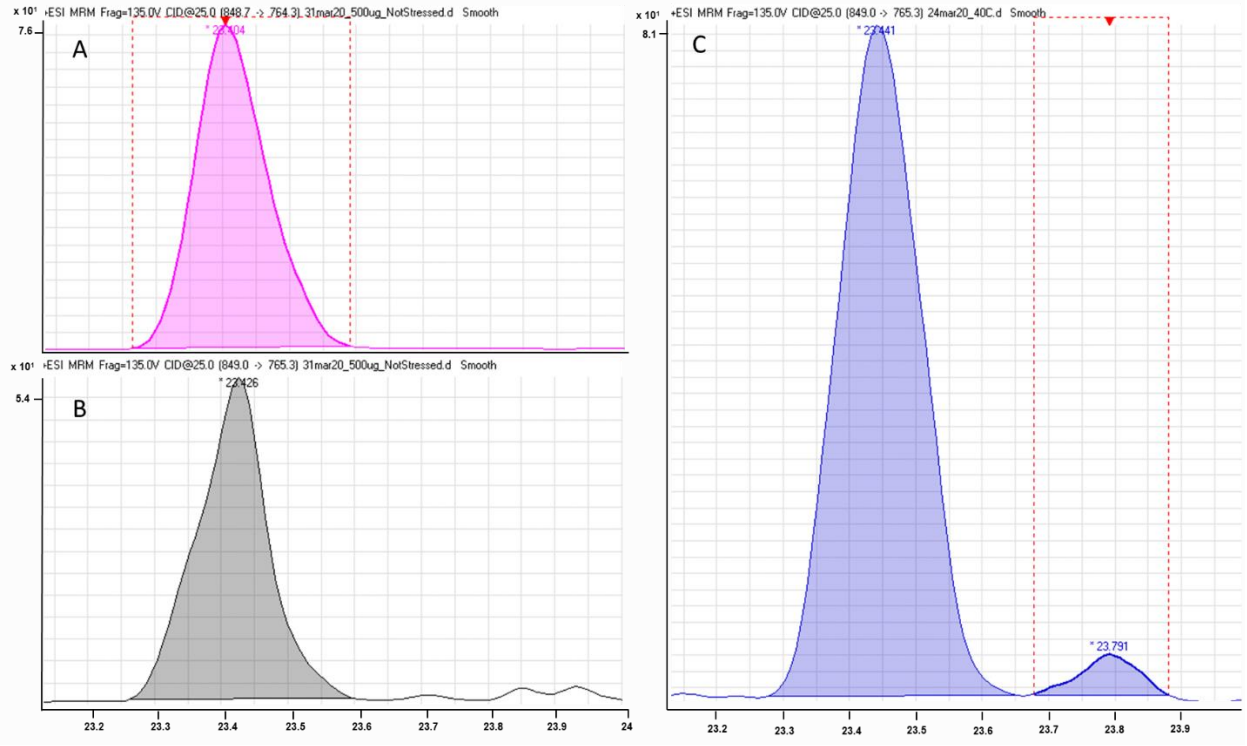


Figure 23. EICs obtained from fragmentation of the triple charged precursor of Pertuzumab's non-deamidated and deamidated tryptic PENNY peptide (m/z: 848.7 and 849.0, respectively) spiked in animal serum. In A and B, a sample containing non-incubated Pertuzumab (kept at -80 °C and digested immediately after thawing) was analyzed. In C, a sample of Pertuzumab digested after being incubated at temperature stress (40 °C for 4 weeks) was analyzed. Y axis: Signal intensity in the ion chromatogram. X axis: Retention time (in minutes). A) Product ion y6 corresponding to non-deamidated species (m/z: 764.3). B) Product ion y6 corresponding to deamidated species (m/z: 765.3) falsely detected and integrated in the same sample. C) Both species (deamidated and non-deamidated) detected and quantified under the same fragment value extraction that corresponds to the product ion y6 of a deamidated species (m/z: 765.3) and requires manual peak selection and/or editing.

This analysis allowed a comparison between results obtained from SRM and previous results obtained from PRM in terms of sensitivity in complex biological matrices (pooled animal serum). While the LLOQ obtained for PRM on a quadrupole orbitrap was at 2 ug/ml, it was possible to achieve a significantly lower limit of detection (150 ng/ml) for SRM in a QQQ, yielding a 13 times higher sensitivity (Figure 24).

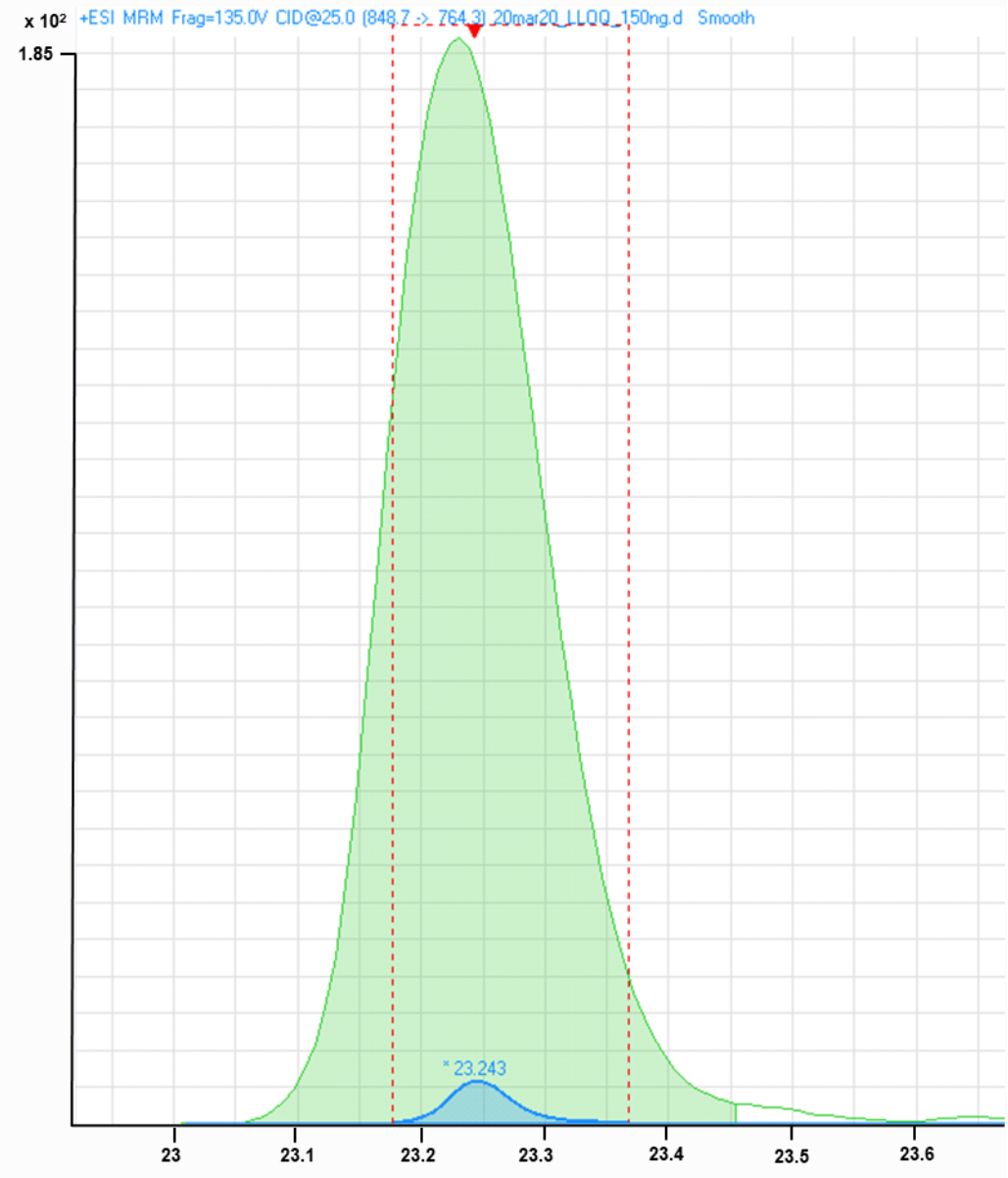


Figure 24. Overlay of ion chromatograms obtained from fragmentation of the triple charged precursor of the tryptic PENNY peptide for both Pertuzumab (blue) and heavy-labeled IS (green) in animal serum. The fragment y6 (m/z: 764.3) with highest signal intensity is shown. The level of sample concentration was at 150 ng/ml for Pertuzumab in animal serum. Y axis: Signal intensity in the ion chromatogram. X axis: Retention time (in minutes).

However, although a higher sensitivity was confirmed in a QQQ setup, selectivity was lower. During analysis of the peptides of interest, endogenous peptides of the biological matrix (animal serum) were also detected. These incremented the obtained noise (Figure 22) as their precursor mass matched the precursor masses of the peptides of interest. In summary, a PRM method in a

quadrupole orbitrap yielded the following advantages compared to an SRM method in a QQQ setup:

- higher resolution
- higher specificity
- lower background noise

This observation played a decisive role in the comparison of instruments at a technical level. Further technical considerations are presented in the Discussion section of this chapter and can be seen as the basis for optimized instrumentation choice.

2) Comparison between PRM and targeted SIM/dd-MS2 methods

In addition to the comparison between SRM and PRM methods, a targeted SIM/dd-MS2 method was compared to a PRM method. In this case, both methods were performed on a quadrupole orbitrap (Figure 25).

Note: For a "targeted SIM/dd-MS2 method" the reader should think of a peptide quantification based on MS2 scans that are dependent on MS1 data, where precursors are given in an inclusion list and scanned through the whole run (scan time segments are not possible to be defined). On the other side, in PRM methods peptide quantification is also based on MS2 scans dependent on MS1 data and precursors are also given in an inclusion list. However, precursors are just scanned in specified time segments and not through the whole run.

The same sample (tryptic digested Pertuzumab spiked in animal serum) was analyzed through both methods (see Material and Methods section III, 2 and III, 3) on the same day. Both methods contained an inclusion list with the same peptides' precursor mass values (doubly charged precursors of the peptides of interest NTLYL, DST, GLEW, and PENNY). In the analysis obtained from the PRM method, insignificant background noise was obtained and only the precursors of the peptides of interest were observed (see upper panel of Figure 25). In contrast, additional signals to those of interest were observed when the same sample was analyzed through a targeted SIM/dd-MS2 method (see lower panel of Figure 25). The additional signals observed in the targeted SIM/dd-MS2 method were derived from serum peptides with a matching precursor mass to the peptides of interest. As a second disadvantage of the SIM/dd-MS2 method, a lower signal intensity was observed compared to the PRM method with the same sample volume injection. A discussion on these two types of targeted methods at a technical level will be further presented in the Discussion section of this chapter.

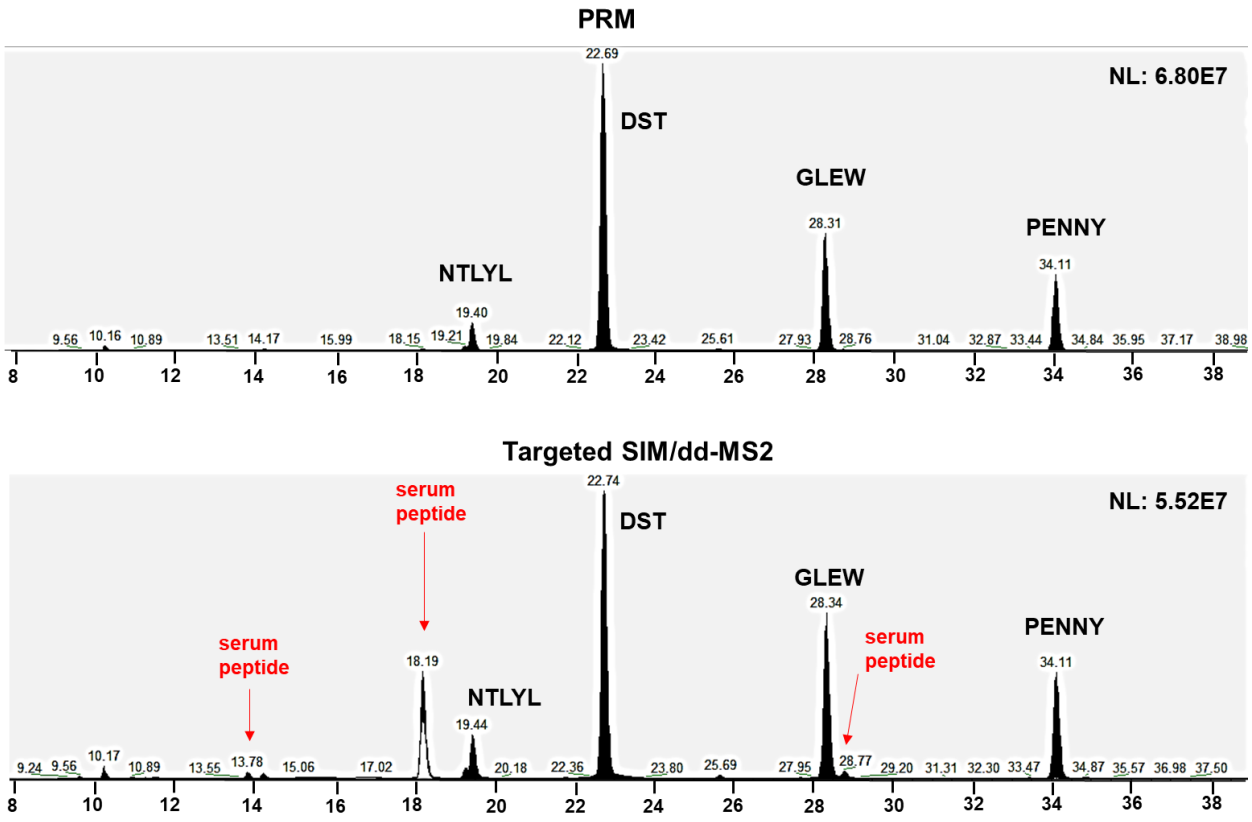


Figure 25. Extracted ion chromatograms of a sample containing tryptic digested Pertuzumab spiked in animal serum analyzed through two different targeted methods. For both method the same instrument (quadrupole orbitrap) and the same inclusion list containing the peptides' precursor mass values: m/z 676.85323 (NTLYL peptide), 684.85323 (oxidized NTLYL), 1088.5245 (GLEW), 1089.02457 (deamidated GLEW), 1272.5693 (PENNY), 1273.0621 (deamidated PENNY), 751.8829 (DST) were used. Upper panel: EIC obtained through a PRM method, where precursors given in an inclusion list are scanned in specified time segments. Lower panel: EIC obtained through a targeted SIM/dd-MS2 method, where precursors given in an inclusion list are scanned through the whole run (no scan time segments possible to be specified). The red arrows point at those signals not observed in the PRM method when analyzing the same sample. Y axis: Signal intensity in the ion chromatogram. X axis: Retention time (in minutes).

Discussion

In this chapter, the method of preference in the corresponding instrument was selected. The goal was to further optimize the method developed in Chapters I and II to reach high sensitivity by comparing different methods in different instruments.

In contrast to shotgun proteomics methods (e. g. data-dependent acquisition), where hundreds of thousands of spectra are generated to obtain full peptide or protein sequence coverage, targeted

proteomics methods offer a higher sensitivity and reproducibility. Targeted proteomics using PRM (parallel reaction monitoring) and SRM (selected reaction monitoring) methods have arisen as relative or absolute quantification methods for a broad spectrum of applications[77,113]. These types of methods usually offer consistency, robustness, and accuracy. However, technical differences may be found between these two types of analysis. That makes it crucial to carefully assess the proper method and instrumentation to be used (depending on the biological question). Even in the case that the instrument (here: mass spectrometer) is already predefined by the nature of the study, there are still distinct fragmentation mechanisms (ion trap, orbitrap, or triple quadrupole mass spectrometers) that may be used. Each of them will yield a different resolution, specificity, and sensitivity. A discussion focusing on the technical comparison, advantages, and constraints of SRM, PRM, and the MS instrumentation used in both types of method will be now presented.

Comparison between SRM and PRM methods

An SRM method is generally performed on a triple quadrupole mass spectrometer. A predefined series of precursors is monitored over time for precise quantification when using an internal standard. The intensities of individual fragment product ions derived from a single precursor ion can differ substantially for an SRM. Hence, optimization can be very time-consuming as it is needed to select the most intense product ions for higher sensitivity. It can be also challenging to acquire robustness between all analyses. The process of targeting a peptide in SRM involves two stages of mass filtering in different quadrupoles with tight tolerance for both precursors and product ions (selection of the precursor at Q1 and product ions at Q3). In the literature, it is stated that the low resolution of a quadrupole and the two stages of mass filtering do not prevent concomitant detection of interfering ions[114,115].

PRM methods in contrast, are performed in a high-resolution hybrid quadrupole orbitrap instrument. All product ions of a targeted peptide are simultaneously monitored. Only one stage of quadrupole mass filtering with the given precursor is done, proceeding then with a mass analysis in an orbitrap. The precursor ion is isolated by the quadrupole and fragmented by the high energy collisional dissociation cell. All fragment ions are analyzed with an orbitrap mass analyzer and no pre-selection of the target peptide product ions is required. In other words, the third quadrupole from a QQQ is substituted by a high resolution and accurate MS/MS analyzer. This allows for parallel detection of all product ions in one single analysis. That characteristic offers the possibility to monitor all product ions of one target peptide in parallel with one injection and full mass range instead of serially monitoring the target product ions over several ion injections and low-resolution mass measurement periods. That yields a lower likelihood of the analysis being affected by interfering ions[113,114], as it may effectively separate ions of interest from background ions.

However, there are also some disadvantages of the PRM method to be evaluated. First, the dependence of the number of precursor ions that can be monitored by the duty cycle of the mass analyzer should be considered. Although a higher number of precursor ions can be monitored in a PRM compared to an SRM method, this number is still limited.

Second, PRM (LLOQ = 2 ug/ml for Pertuzumab in animal serum) could not provide the same sensitivity as an SRM approach (LLOQ = 150 ng/ml for Pertuzumab in animal serum). While

enhanced selectivity can be seen in PRM when complex samples are analyzed, the SRM method is limited in selectivity. This is because the resolution of the quadrupole used for mass selection is low. Therefore, interferences within the mass selection windows and tolerances of both quadrupoles used in the two stages of mass filtering may occur. Especially presence of interferences must be considered when using complex matrices and potential peptides of interest at trace rate. This is due to the similarity in peptide nature of both analytes and interferences in proteomic assays. However, a third quadrupole instrument (as seen for SRM) possesses a duty cycle approaching 100% and uses electron multiplier-based detection[113]. That offers a higher sensitivity than the current-based detection of an orbitrap and hence represents an advantage of the SRM compared to the PRM method.

Third, SRM approaches may also provide a better measurement precision in the presence of a matrix. The reason for that is the higher sampling rate of a third quadrupole compared to a PRM method. As a QQQ is a beam-type instrument (opposed to a scanning instrument as the orbitrap), there is very few downtimes in which no data is acquired. In contrast, the inter-scan time, in which no mass analysis is performed, will be higher in the orbitrap's design. The two reasons for that are the low sample concentrations used in the orbitrap and the fact that its cycle time is based on ion accumulation times. Although SRM and PRM may show similar linearity, accuracy, and sensitivity in empirical studies[115], SRM may allow a lower limit of detection in complex matrices.

Despite of disadvantages inherent to a PRM method, also important advantages over SRM methods may be observed. One advantage of the PRM over the SRM approach is the higher resolution obtained from an orbitrap mass analyzer. The higher resolution was beneficial in specific for the detection of deamidated species in complex biological matrices. Analyzing digested proteins in serum is expected to give rise to many more interfering peaks in the chromatograms. As the digested serum proteome has very similar masses and properties as the digested protein of interest, the sample results are composed of many background peptides in an extremely complex solution. A triple quadrupole shows an insufficient discrimination between the background peptides and the signature peptide of interest as the unit mass resolution and mass extraction window lies at 1 Da. Deamidated and non-deamidated peptides differ only in very low mass increase (< 1 Da). Therefore, their product ions would not be mutually exclusive, which yields a disadvantage of an SRM method.

Decreasing the mass extraction window (from 0.7-1 Da the typical value for a QQQ to for instance 0.01 Da) is only possible in instruments of higher resolution such as a quadrupole orbitrap. Only like that, a lower background noise can be achieved as a major part of the interferences from digested plasma proteins is no longer selected for detection, and the calculation of the signal-to-noise ratio can be improved. The latter is almost impossible with SRM applications, as the peaks in the background technically do not represent noise, but endogenous components of serum and hence interfering ions. For the mentioned reasons, high resolution represents a key factor of consideration when evaluating both instruments in a choice decision. This better selectivity may, however, not always lead to better quantification limits, because the absolute instrument sensitivity of QQQ still is superior to that of high-resolution mass spectrometry[116].

A second advantage of a PRM method inherent to the above discussed higher resolution, is the high specificity of the spectrum. This is the case thanks to the increased number of peptides to be monitored in one experiment. A high degree of selectivity is ensured due to a high resolution and an accurate mass measurement in the PRM method. Additionally, these ions available for identification and quantification reduce the disruption in spectral quality that interfering ions cause. Therefore, the targeted peptides may be discriminated from background interferences providing high selectivity and a dynamic range. This was confirmed in the performed analysis within digested serum, in which a lower background noise was obtained through PRM.

In addition to the arguments above, a loss of optimal peak shape was observed within SRM analysis for samples whose concentration was close to the LLOQ. This effect is derived from the number of data points acquired per peak. As previously mentioned as advantage, the used dwell time and overall MS cycle time in SRM enable to reach higher sensitivity limits. However, also a lower amount of data points per peak for each scan are obtained. In a dynamic SRM, dwell times are adjusted based on the specified cycle time, retention times, and retention time windows that are given to keep the scan time constant for best quality of quantification data. This allows a much faster MS cycle time than when using segment methods. However, at such narrow time windows, the MS duty cycle is occupied by several compounds being monitored at the same time during each MS scan. This leads to a loss of optimal peak shape in low concentration samples.

Several reports may be found in the literature aiming to compare MS/MS quantification of surrogate peptides between instruments. For instance, Plumb et al. reported a ten-times smaller sensitivity on a Q-TOF compared to a QQQ instrument[117]. However, a posterior publication by Bults et al. showed a four-fold improvement of the achievable LLOQ on a Q-TOF compared to a QQQ[118]. Another example of comparison between PRM and SRM methods was performed by Ronsein et al. Through a method focused on the quantification of high-density lipoprotein, their study showed comparable linearity, dynamic range, precision, and quantification repeatability between both methods[114].

In the end, the comparison between SRM and PRM methods may be concluded as follows: Although PRM may provide greater specificity and nearly unambiguous target confirmation from the background, this does not imply a greater overall performance. This will rather depend on more factors such as the capability of both analyzers (current or electron-multiplier based) to detect the target species with reproducibility and accurate measurement of the abundance of the targets[113]. As both methods have their advantages and disadvantages (sensitivity/specificity, resolution, etc.) as shown above, their choice will depend on the biological question under study and the underlying interest of the assay. Furthermore, also the selection of the best surrogate peptide and the nature of the matrix (biological fluid or non-complex matrix) have an impact on the choice of the right method.

Comparison between PRM and targeted SIM/dd-MS2 methods

As discussed in the previous section ("Comparison between SRM and PRM methods"), specificity and high resolution were of crucial interest for the analysis of deamidates species in complex matrices. It was assessed that the use of a quadrupole orbitrap instrument can help to optimize on

these two dimensions, and hence to discriminate between deamidated and non-deamidates species. Hence, an additional targeted method in this instrument (targeted SIM/dd-MS2) was evaluated and compared to the developed PRM method. A targeted SIM/dd-MS2 method performs MS2 scans from targeted MS1 precursors, where the precursors are scanned through the whole run and fragmented when being found. The quadrupole is operated in survey scan mode allowing only a narrow mass range to enter to the C-trap. Afterwards, the product ion spectra are obtained. In other words, in a SIM/dd-MS2 method the given inclusion list is always activated. In contrast, MS2 scans in a PRM method are divided by specific given time segments in the precursor inclusion list, resulting in a higher selectivity and specificity for complex samples.

When comparing the two methods with each other, the specificity was assessed as the main decisive factor. It could be confirmed that the PRM method had a higher specificity than a targeted SIM/dd-MS2 method when analyzing digested Pertuzumab in the presence of serum. For the PRM method, a cleaner noise background was observed in comparison to the targeted SIM/dd-MS2 method, where additional peaks were observed. This may be explained by the fact that endogenous components of the matrix (serum peptides) were selected for fragmentation through the whole run when their precursor mass matched the ones of the analytes within the given inclusion list. In a PRM, this effect is diminished thanks to the defined narrow scan time windows for the precursor. By that, selectivity and specificity in complex matrices are enhanced, lower background interference is obtained, and higher signal-to-noise ratios are achieved.

Summary of the comparisons

Different methods in different instruments were compared in this chapter to present advantages and disadvantages of each when quantifying low-abundant deamidation and oxidation modifications in biological matrices. Since every method has advantages and disadvantages, a trade-off decision must be done to select the proper instrument for the scope of the study. In this case, a PRM method in a quadrupole orbitrap instrument was selected due to its higher resolution and its higher selectivity (achieving so a better signal-to-noise ratio). The required LLOQ for the study was also achieved with the chosen PRM method (although SRM reached a 13-fold lower LLOQ).

However, it should be mentioned again that the compromise between sensitivity, resolution, and specificity must be carefully assessed depending on the main objective of the scientific question and the mAb under study. This may sometimes not be so straightforward as it is the case for the quantification of low-abundant deamidated species in complex biological matrices, where high sensitivity, high resolution, and high specificity are all equally relevant and required for confident analysis. Although a triple quadrupole instrument is sometimes seen as the gold standard for quantification, other applications might require a different approach. The best available types of analysis and instruments must be investigated to explore the best performance and to obtain the highest quality and confidence in the results.

Within this study, the method of choice was concluded to be a PRM method. As final step before this method could be applied to the samples under study, a method validation according to regulatory guidelines must be performed. This process will be presented in Chapter IV.

IV. LC-MS/MS (PRM) method validation according to FDA guidelines

Results

To report modification rates for deamidation and oxidation reliably and confidently in in vitro and in vivo samples, a method validation per industry standards was required. The developed LC-MS/MS method was aimed to be validated according to the FDA guidelines and to determine during method validation if the matrix influenced the quantification of modifications. To achieve these aims, the following strategy was developed:

- follow the FDA guidelines for bioanalytical LC-MS/MS-based methods validation
- compare two different quantification strategies (presented in detail in subsection 5)

The results on the method validation of the developed LC-MS/MS method are presented in the following order:

- Generation of a stressed reference standard and an internal standard through incubation conditions
- Characterization of the stressed reference standard and the internal standard for validation
- Reproducibility and sensitivity assessment for the quantification of Pertuzumab's tryptic peptides at the LLOQ in animal serum as matrix
- Method validation according to FDA guidelines
- Assessment of the matrix effect on the quantification of deamidations and oxidations

1) Generation of a stressed reference standard and an internal standard through incubation conditions

In a multi-attribute method (MAM), several quality attributes, post-translational modifications (PTMs), and products of degradation are aimed to be monitored, quantified, and reported within the same assay. The challenge of monitoring several attributes from one tryptic peptide is a major bottleneck in terms of validation within the biopharma industry. By doing so, not only the assay's complexity rises, but also the cost derived from the required synthesized peptides per modification. Additionally, modification products are usually present in very low rates. When modifications are present in the sample in low abundancy, the accurate quantification of modified species becomes challenging.

As an alternative to overcome these restraints, stressed materials for both the reference standard (Pertuzumab) and a heavy-labeled internal standard were created. Analyzing the modifications present at high rates and in presence of the biological matrix would allow to:

- assess MS response similarity/difference due to the modifications when compared to non-stressed reference material
- assess if a response is dependent on the mAb/protein concentration
- assess a matrix effect

- propose quantification strategies based on the correction through the stressed IS to overcome the matrix effect
- assess if the use of a stressed protein and a correction per modification is necessary for an accurate quantification

MAMs have been developed for formulated products where analysis is manageable, as there is no interference of components that may exacerbate a matrix effect on the ionization efficiency and reproducibility of the ionization source[119–121]. However, so far MAMs have not been explored for their application in exploratory animal study or clinical samples, where complex matrices may impact the accuracy of the data. In addition, analysis of in vivo samples increases the complexity of the assay because endogenous compounds are present and complex sample preparations are needed in this case. This requires the use of internal standards (IS) to correct fluctuations in the analytical response caused by variations in experimental conditions[122]. However, this is often not easy as internal standards are required per every individual cQA (critical quality attribute) under study. Therefore, the costs of a MAM increase as the synthesis of multiple peptides is required due to the lack of intact heavy-labeled mAbs in the market. The strategy presented within this work proposed the use of an IS subject to stressed conditions (stressed IS) to reduce costs derived from the multiple purchase of synthetic peptides. The proper stress conditions would allow to obtain all degradation sites of interest.

Similarly, calibration curves and quality control samples built both with stressed and non-stressed mAbs were compared. Both materials, Pertuzumab and the IS (a combination of intact SIL (stable isotope labeled) IgG1 plus 2 synthesized peptides for CDR and Fab region of Pertuzumab) were stressed under the same incubation conditions (see Material and Methods section IV, 1). In Pertuzumab, it was required that the rate of degradation surpasses a 20% method variability threshold. In the IS, a rate lower than the required 20% was accepted for correction purposes.

Aliquots were taken at different time points during the incubation period (see Material and Methods section IV, 1) for comparison purposes (Figure 26). Deamidation rates in Pertuzumab after 44 days under the incubation conditions of pH stress (pH 9) and temperature stress (40 °C) reached up to 43% in the PENNY peptide at N391. In contrast, the IS yielded significantly higher deamidation rates of up to 82% for the same peptide and under the same incubation conditions. In the IS, already after 7 days a deamidation rate of 29.6% could be seen at N391. In comparison, it took more than 21 days to reach the same deamidation rate at N391 in Pertuzumab. As final observation on this matter, stress conditions over 28 days led to 37% deamidation rate at N391 in the PENNY peptide in Pertuzumab. On the other hand, the deamidation rate reached up to 82% for SIL IgG1 in the same period. In all cases, deamidation rates for aspartic acid formation at the Asparagine N386 were significantly smaller than deamidation rates for iso-aspartic formation at the same site.

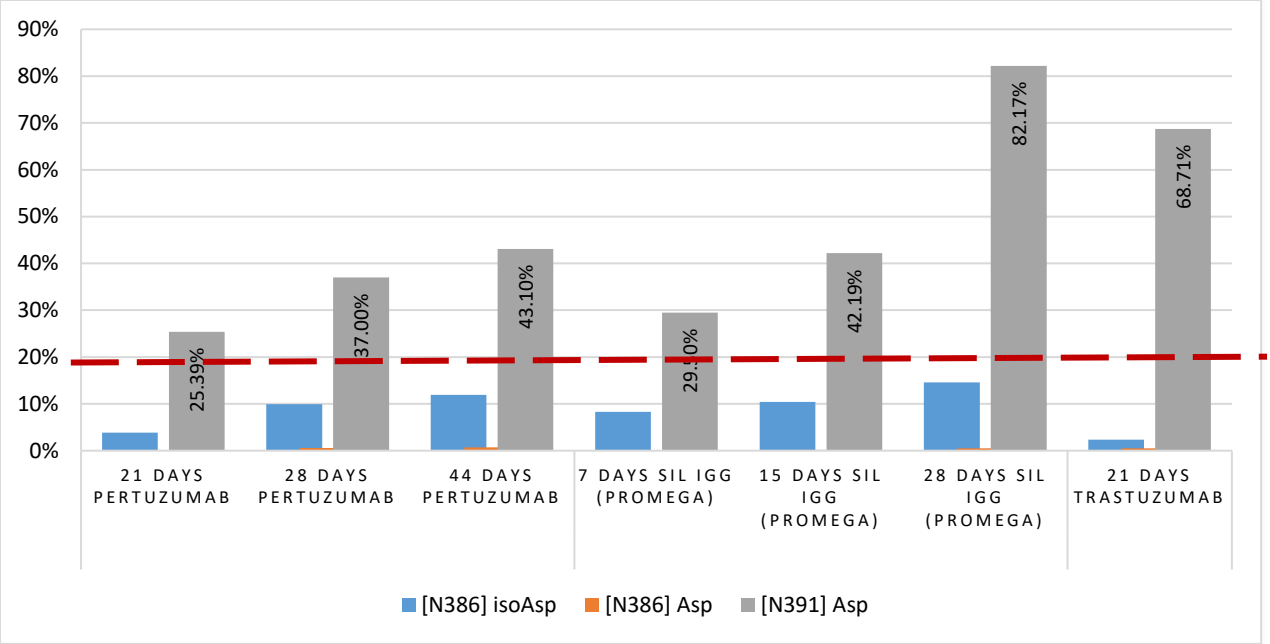


Figure 26. Deamidation rates for the PENNY peptide (Fc region) present in Pertuzumab, heavy-labeled IgG1 (Promega), and Trastuzumab incubated under the same conditions of pH stress (pH value of 9) and temperature stress (temperature of 40 °C) over a period of between 7 and 44 days. Samples analyzed after tryptic digestion. Deamidation rates are calculated as the ratio of the area under the peak over the sum of all areas under the peaks of all modified and non-modified species of the peptide. No correction with IS was performed. The minimum rate needed to overcome the method variability in the validation process is shown as a dashed line in red. Y axis: Relative deamidation rate. X axis: Time of incubation per mAb.

The PENNY peptide is present in the Fc part of both Pertuzumab and the heavy-labeled IgG1. It was considered of interest to further compare the deamidation rates of this peptide with the rate in another mAb of the IgG1 subtype (e. g., Trastuzumab) under the same incubation conditions as mentioned above. A significant difference in deamidation rate after 21 days between Trastuzumab (69%) and Pertuzumab (25%) could be observed (Figure 26). This difference was of similar magnitude as the one observed above between Pertuzumab and heavy-labeled SIL IgG1. Based on this result, a difference in stability in terms of deamidation propensity under the same conditions could be observed. Pertuzumab was assessed to be the most stable molecule as it presented the lowest deamidation rate (43%) even after 44 days of incubation compared to SIL IgG1 (42% at 15 days) and Trastuzumab (69% at 21 days). A hypothesis based on these results will be further presented in the Discussion section of Chapter 4 and Chapter 5. From the observed deamidation rates, the appropriate incubation times per molecule could be defined to obtain the desired deamidation rates in the reference standard and the IS. These incubation times were then used afterwards in the method validation.

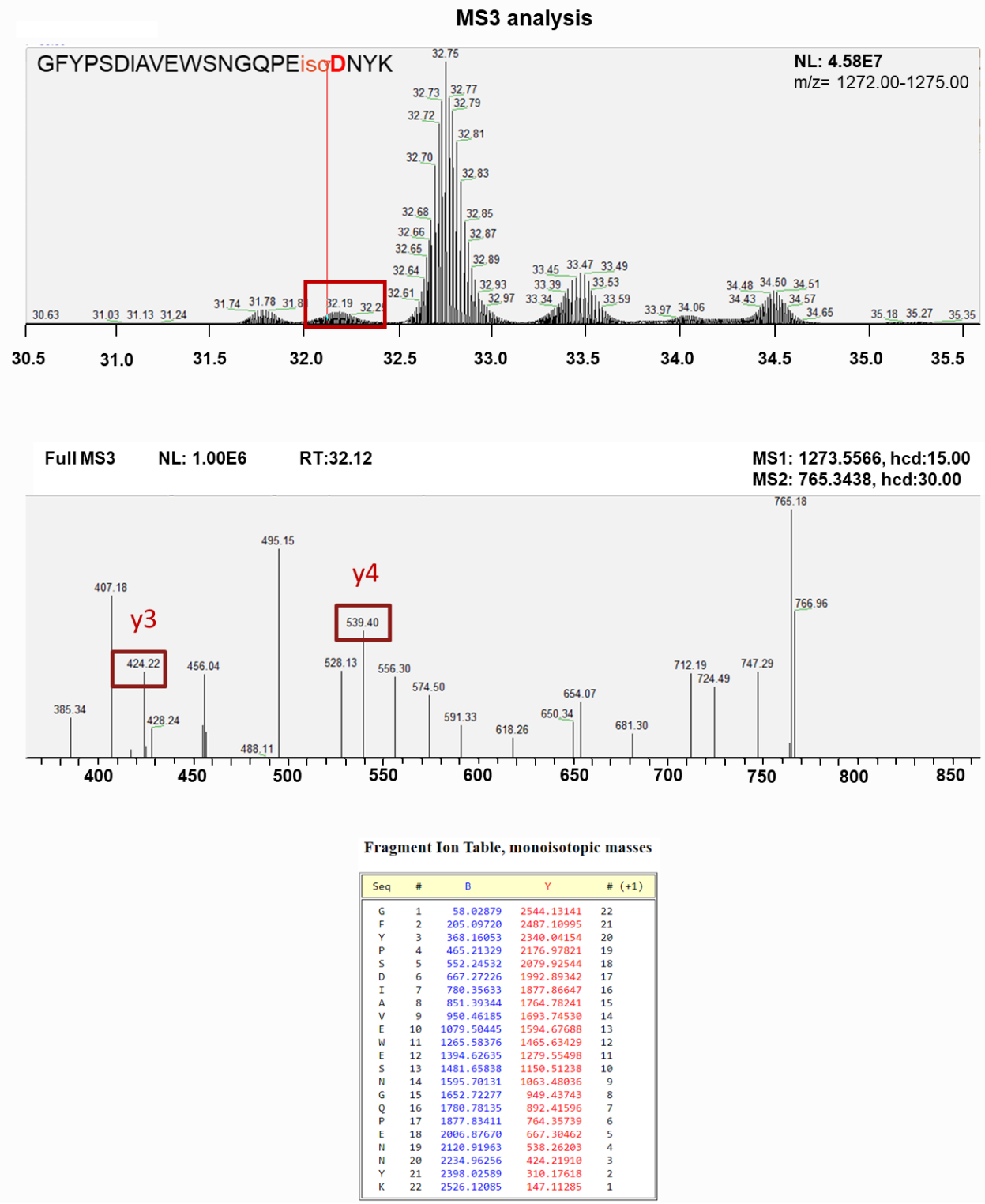
2) Characterization of the stressed reference standard and the internal standard for validation

The stress incubation conditions led to several degradation products in both Pertuzumab and the internal standard. These degraded species were now aimed to be characterized. The fragmentation quality acquired through HCD (higher energy collisional dissociation) at a quadrupole orbitrap was not sufficient to provide the needed spectra for assigning the fragmentation pattern to the modified amino acids. Hence, two approaches were used here to characterize the stressed molecules:

- Data acquisition through MS3 methods in an orbitrap fusion instrument using both HCD and ETD (electron-transfer dissociation) fragmentation
- Analysis (PRM method) of synthetic peptides to confirm the proposed characterization obtained from MS3 methods

Through the first approach (MS3), a list of product ions derived from MS2 was created to be further fragmented (see Material and Methods section IV, 2 and Table S4 in Supplemental Material for full information and inclusion list of fragments). The quality of fragmentation obtained from the use of ETD allowed to obtain better spectra and partial assignment of the modification sites (Figure 27). In the shown example of Figure 27, the product ion y6 from a deamidated species (possibilities: PEDNYK/PENDYK/PEisoDNYK/PENisoDYK) with an m/z of 765.3438 was fragmented. Mass gains of 1 Da observed between product ions in the spectrum at MS3 fragmentation allowed an assignation of iso-aspartic and aspartic acid formation to specific amino acids.

In the middle panel of Figure 27, the obtained spectrum generated from a selected species of the ion chromatogram in the upper panel is shown. A mass gain of 1 Da at the product ion y4 (538.26 in silico vs. 539.40) could be observed, while product ion y3 showed a mass that matched its mass for theoretical in silico fragmentation. From this observation, the site of change could be identified as isoDNYK in the shown example. As the spectrum generated from the ion chromatogram corresponding to isoDNYK, a similar spectrum analysis of the ion chromatogram was performed for all deamidated species. From these spectra analyses, first hypotheses for a preliminary assignation of modified amino acids could be formulated for all observed species.



Seq	#	B	Y	# (+1)
G	1	58.02879	2544.13141	22
F	2	205.09720	2487.10995	21
Y	3	368.16053	2340.04154	20
P	4	465.21329	2176.97821	19
S	5	552.24532	2079.92544	18
D	6	667.27226	1992.89342	17
I	7	780.35633	1877.86647	16
A	8	851.39344	1764.78241	15
V	9	950.46185	1693.74530	14
E	10	1079.50445	1594.67688	13
W	11	1265.58376	1465.63429	12
E	12	1394.62635	1279.55498	11
S	13	1481.65838	1150.51238	10
N	14	1595.70131	1063.48036	9
G	15	1652.72277	949.43743	8
Q	16	1780.78135	892.41596	7
P	17	1877.83411	764.35739	6
E	18	2006.87670	667.30462	5
N	19	2120.91963	538.26203	4
N	20	2234.96256	424.21910	3
Y	21	2398.02589	310.17618	2
K	22	2526.12085	147.11285	1

Figure 27. Upper panel: Ion chromatogram and spectra from the PENNY peptide using an MS3 method through ETD fragmentation. Product ion y6 from a deamidated species (PEDNYK/PENDYK/PEisoDNYK/PENisoDYK) with a m/z of 765.3438 was fragmented. X axis: Retention time (in minutes). Middle panel: Generated mass spectrum from selected species

of the ion chromatogram in the upper panel. Product ions y3 (NYK) and y4 (NNYK) are highlighted in red boxes. X axis: m/z ratio. Bottom table: In silico fragmentation of the PENNY peptide.

Through the second approach, assignation of species was then confirmed by the analysis of synthetic peptides obtained as a donation (see Material and Methods section IV, 2). In these tests, two different species with a highly similar retention time were identified (see red boxes in Figure 28). The main difference between these two species was the shape of the peak: When analyzing all synthetic peptides at once (deamidated and non-deamidated), a higher and slightly broader peak shape was observed for the ion chromatogram (see Figure 28 B) compared to the ion chromatogram obtained from the analysis of only deamidated peptides (see Figure 28 A). Based on this observation, all peptides were analyzed individually to assess which deamidated species was coeluting with the non-deamidated peptide. From this analysis, a coelution of the PENisoDNY peptide (iso-aspartic formation at the N-terminal Asparagine) and the non-deamidated PENNY peptide could be verified through comparison of their retention times (Figure 28 C and D).

To verify that both peptides could be quantified separately and that the reported rates of the different peptides were neither biased nor overestimated despite their coelution, the influence of the proper mass extraction windows was assessed. As the non-deamidated PENNY peptide and the PENisoDNY peptide have a y8 fragment mass value of 949.44 and 950.44 respectively, the two mass extraction windows [948.5 – 949.7] and [950.3 – 952.3] were evaluated. With the mass extraction window [948.5 – 949.7], species isoDG, PENNY (non-deamidated), and DG (all three not showing a mass addition at the y8 product ion) were observed. In turn, species GisoEP, PEisoDNY, PENisoDY, PEDNY/PENDY moiety, and GEP were observed when using a mass extraction window of [950.3 – 952.3] as they present the mass increase of 1 Da already by product ion y8. Therefore, coeluting species could be separately observed and quantified both by the confirmatory product ion y8 and the product ion y6 (mass value of 764.36) following the same principle as before described for product ion y8.

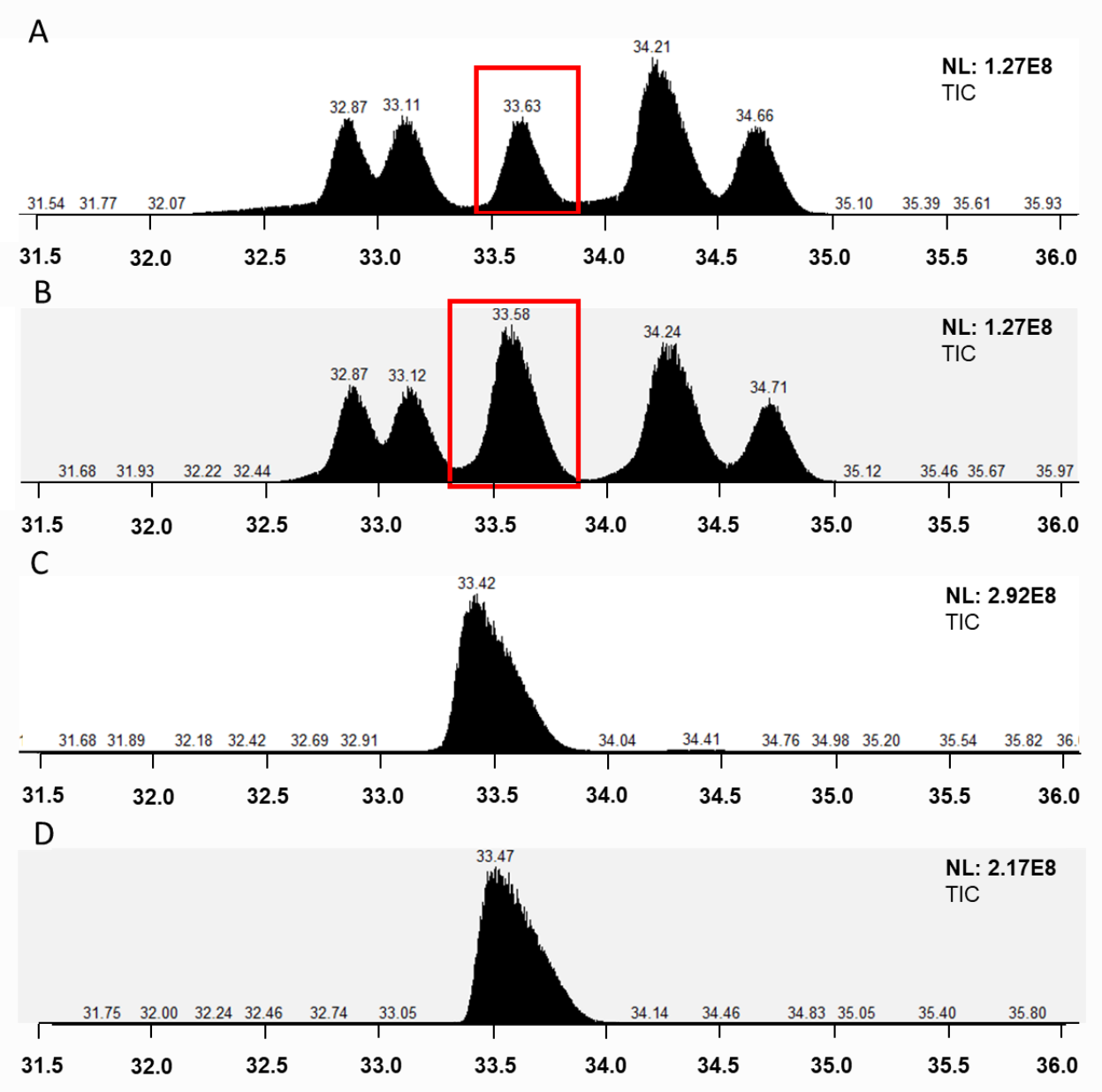


Figure 28. Ion chromatograms from the analysis of synthetic peptides for all different iso-aspartic and aspartic acid formations of the PENNY peptide. A) Sample: All deamidated peptides (containing an aspartic or iso-aspartic acid formation) are mixed and analyzed together. B) Sample: All deamidated peptides (containing an aspartic or iso-aspartic acid formation) plus the non-deamidated peptide are mixed and analyzed together. In red boxes, the species that coelute are highlighted (PENisoDY iso-aspartic acid formation and non-deamidated peptide). C) Sample: Only the non-deamidated peptide is analyzed. D) Only the iso-aspartic acid formation at the first asparagine at the N-terminus (PENisoDY peptide) is analyzed. X axis (for all panels): Retention time (in minutes).

To prove that the approach for synthesized peptides described above was valid and that there was no tolerance bias given to the entered values from the software within the mass extraction windows, the GLEW peptide was analyzed as assurance measure (Figure 30). No peptides were observed to coelute and the non-deamidated species (m/z: 1192.5 – 1192.8) was not observed at all within the mass extraction window used for deamidated species (m/z: 1193.5 – 1994.0). From these observations, it could be concluded that the proposed approach for synthesized peptides was valid.

Based on the two approaches described above (MS3 methods and synthesized peptides), a characterization of deamidation modifications in the stressed materials was obtained (Figure 29 and Figure 30). Like the previous comparison in Figure 26 for 3 deamidation sites, rates of all degradation sites of the PENNY peptide in Pertuzumab were compared to a second mAb (Trastuzumab). It was observed that a total deamidation rate of 93.50% was reached in Trastuzumab after 21 days of pH stress incubation. Compared to that, the deamidation rate reached 55.19% under the same conditions in Pertuzumab (Figure 29).

After characterization of the deamidated species in stressed Pertuzumab, the next step was to digest the stressed Pertuzumab in triplicates. This step was needed to confirm the proper chromatographic peptide separation and the minimum standard deviation in modification rates (see Figure 29, upper panel). As both requirements were confirmed, stressed Pertuzumab could be later used as the final reference standard in the method validation. In this reference standard, total degradation rates were obtained as follows:

- PENNY peptide (Fc region): 55.19% deamidation (see Figure 29)
- GLEW peptide (CDR region): 31.49% deamidation (see Figure 30),
- NTLYL peptide (Fab region): 36.78% oxidation (see middle panel of Figure 31)

The presence of double oxidation derived from oxidative stress with H_2O_2 (hydrogen peroxide) was not observed in triplicates and can hence be considered to equal 0% (see lower panel of Figure 31).

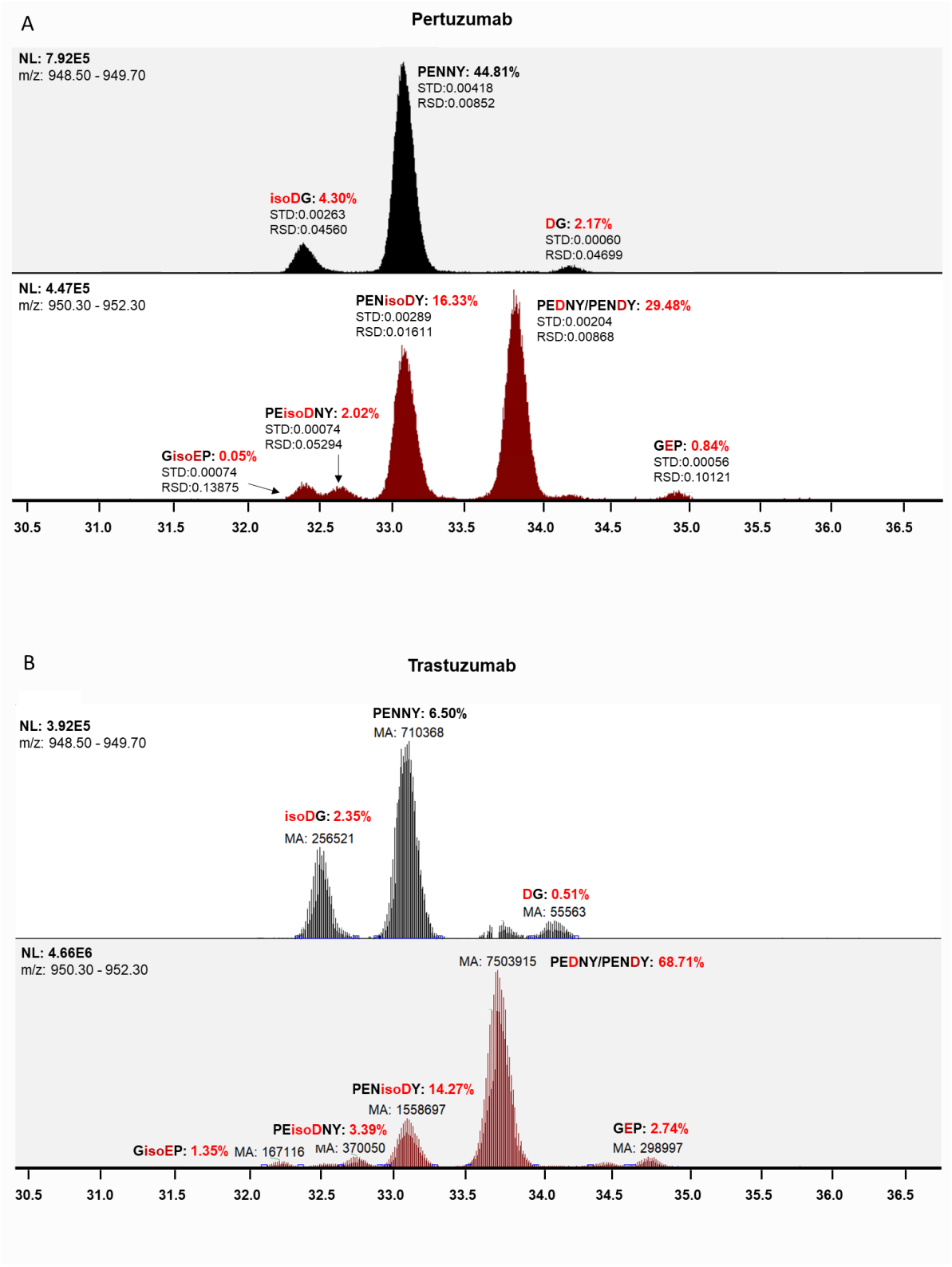


Figure 29. Two ion chromatograms of the PENNY peptide. A) From Pertuzumab: tryptic digested and analyzed after incubation at pH of 9 over 44 days. B) From Trastuzumab: tryptic digested and analyzed after incubation at pH of 9 over 21 days. Upper panels of both A and B show a mass

extraction window of [948.5 – 949.7]. Lower panels of both A and B show a mass extraction window of [950.3 – 952.3]. Several deamidation species are characterized and deamidation rates are reported for each mAb. X axis: Retention time (in minutes).

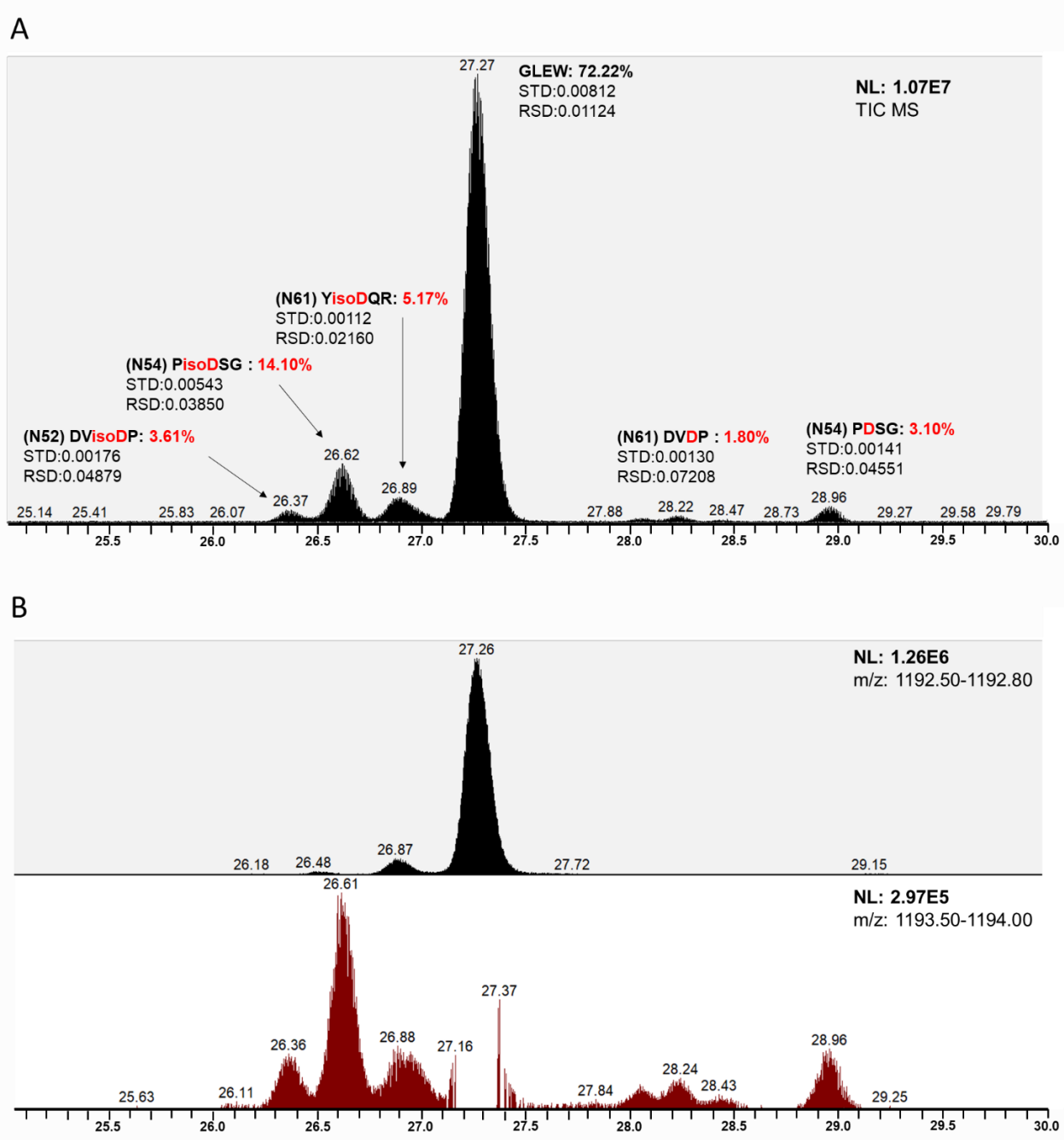


Figure 30. Ion chromatograms of tryptic GLEW peptide located in the CDR region of Pertuzumab and its degradation products after incubation at pH 9 for 45 days. A) Total ion chromatogram: Standard deviation between triplicates is depicted in absolute and relative values. B) Extracted ion chromatogram: Restricted extraction windows for quantification of product ions y11. Upper panel

(of B): Generated from non-deamidated peptides (theoretical mass: 1192.57 Da) with mass extraction window [1192.5 – 1192.8]. Lower panel (of B): Generated from deamidated peptides (theoretical mass: 1193.55 Da) with mass extraction window [1193.5 – 1194.0]. X axis: Retention time (in minutes).

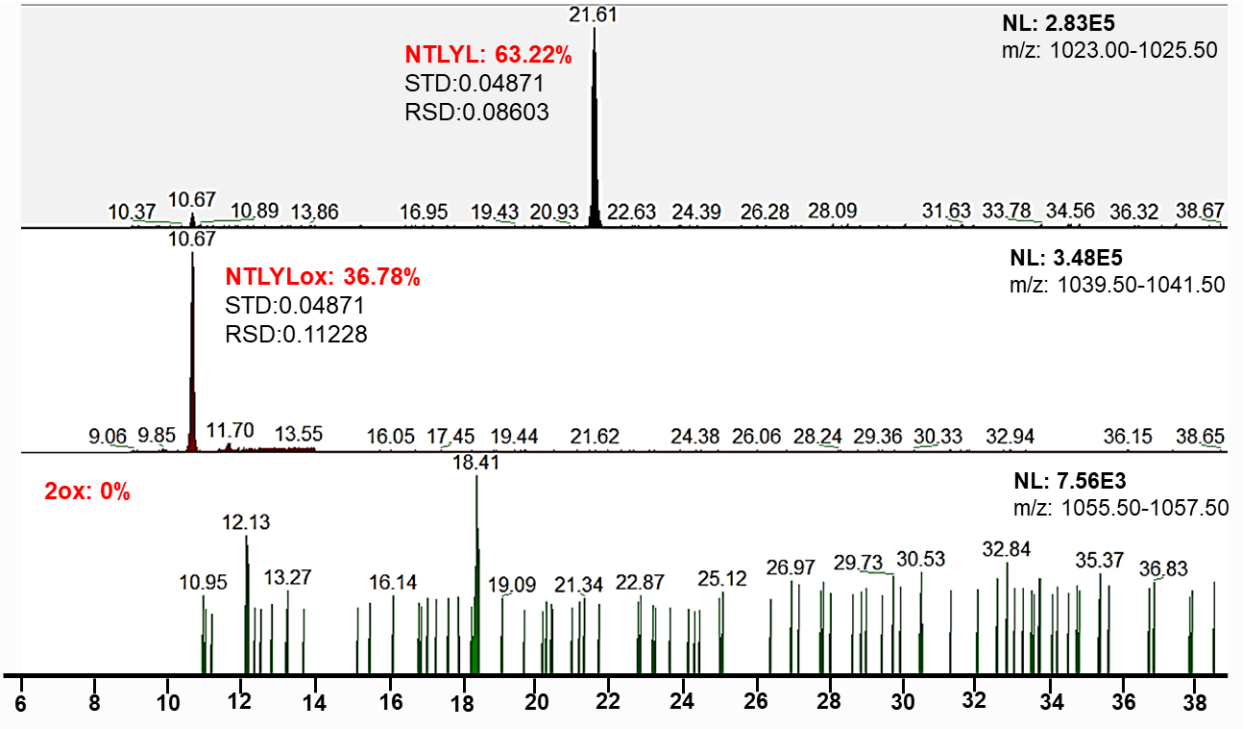


Figure 31. Extracted ion chromatogram of NTLYL peptide in the Fab region of digested stressed Pertuzumab after incubation at pH 9 over 45 days. Upper panel: non-oxidized peptide. Middle panel: Oxidized peptide. Lower panel: Double oxidation. Corresponding oxidation rates are depicted in red color. Standard deviations between triplicates are shown in absolute and relative values for the upper and middle panel. X axis: Retention time (in minutes).

Total degradation in the final reference standard to be used in calibration curves and QC (quality control) samples during validation was confirmed to be in rates over 20%. Proper separation of peptides and degradation products allowed to restrict time segments for each peptide within each MS scan enhancing sensitivity and specificity (Figure 32).

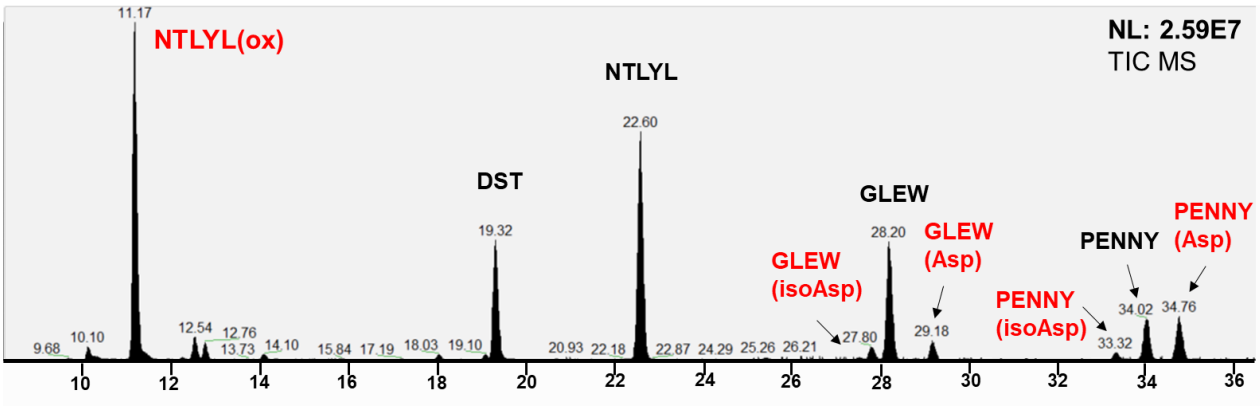


Figure 32. TIC of stressed Pertuzumab digested after pH (9-10), oxidative (H_2O_2), and temperature (40 °C) stress over 45 days. A proper separation between all peptides and an optimal chromatographic resolution between iso-aspartic formation, aspartic formation, and non-deamidated species is seen. X axis: Retention time (in minutes).

To get a first indication for the concentration of the final reference standard, a BCA (Bicinchoninic acid assay) test was performed. This test allowed a first estimation for the concentration of stressed Pertuzumab in the final dilution. The estimated value was used to prepare triplicate samples at a stressed Pertuzumab concentration of 2 mg/ml in PBS. These samples were then analyzed through the developed LC-MS/MS method. Within the same run, samples with non-stressed Pertuzumab in PBS (ranging from 0 mg/ml to 3 mg/ml) were also measured to be used as a calibration curve. Through quantification of product ion y8 from the stable tryptic peptide DST in the Fc region, an equation of the form $y = \mathrm{mx} + \mathrm{t}$ for the calibration curve was obtained from non-stressed Pertuzumab (Figure33). The variables of the curve are used as follows:

- y - area under the curve (AUC) for the EIC of product ion y8 from the DST peptide
- x - concentration of Pertuzumab to be determined

This linear equation of the form would be later used to determine the real concentration for the triplicates. For this, y needed to be substituted by the average AUC values of the product ion y8 from the stressed Pertuzumab triplicates. Solving the equation for x allowed to determine the needed concentration value. This was then extrapolated to the overall Pertuzumab concentration in the original dilution used for the sample preparation of the triplicates. By that, the Pertuzumab concentration was determined to be 10.48 mg/ml.

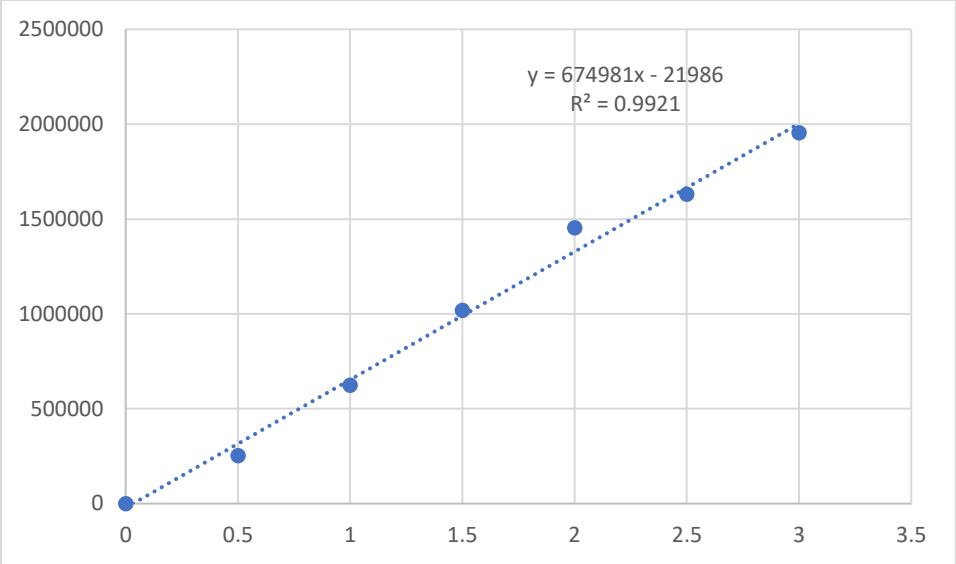


Figure 33. Calibration curve from digested Pertuzumab (kept at -80 °C) used to determine the concentration of stressed Pertuzumab incubated at a pH value of 9 and a temperature of 40 °C for 45 days. The stable tryptic peptide DST was monitored and quantified based on the product ion y8 (m/z: 836.47) with the most intense signal. The calibration curve was built in a range from 0 to 3 mg/ml. No IS was added to the samples. Y axis: Total area under the peak. X axis: Level of concentration (in mg/ml).

The characterization of Pertuzumab described within this subchapter was also performed for the stressed heavy-labeled internal standard (characterization and determination of Pertuzumab concentration). Exact degradation rates for deamidation and oxidation sites of interest within the internal standard could be determined like that (see Figure 36 for deamidation and oxidation rates in the IS).

3) Reproducibility and sensitivity assessment for the quantification of Pertuzumab's tryptic peptides at the LLOQ in animal serum as matrix

After characterization of the stressed reference standard, sensitivity and reproducibility were to be confirmed at the LLOQ. This was reached by performing analyses on five different samples whose Pertuzumab concentration was at the LLOQ (Figure 34). The tests were run within three subsequent days to assess the robustness of the obtained result. Sensitivity at the LLOQ was assessed through the signal-to-noise ratio within the sample as per FDA guidelines and United States Pharmacopeia definition[123]:

$$\frac{S}{N} = \frac{2 * height\ of\ the\ peak}{maximum\ height\ around\ the\ peak} \geq 10$$

All peptides under consideration passed this ratio threshold and hence allowed to classify all peaks as quantifiable (Table 9). This observation held within all tested samples and days, confirming the required robustness, reproducibility, and sensitivity at the LLOQ.

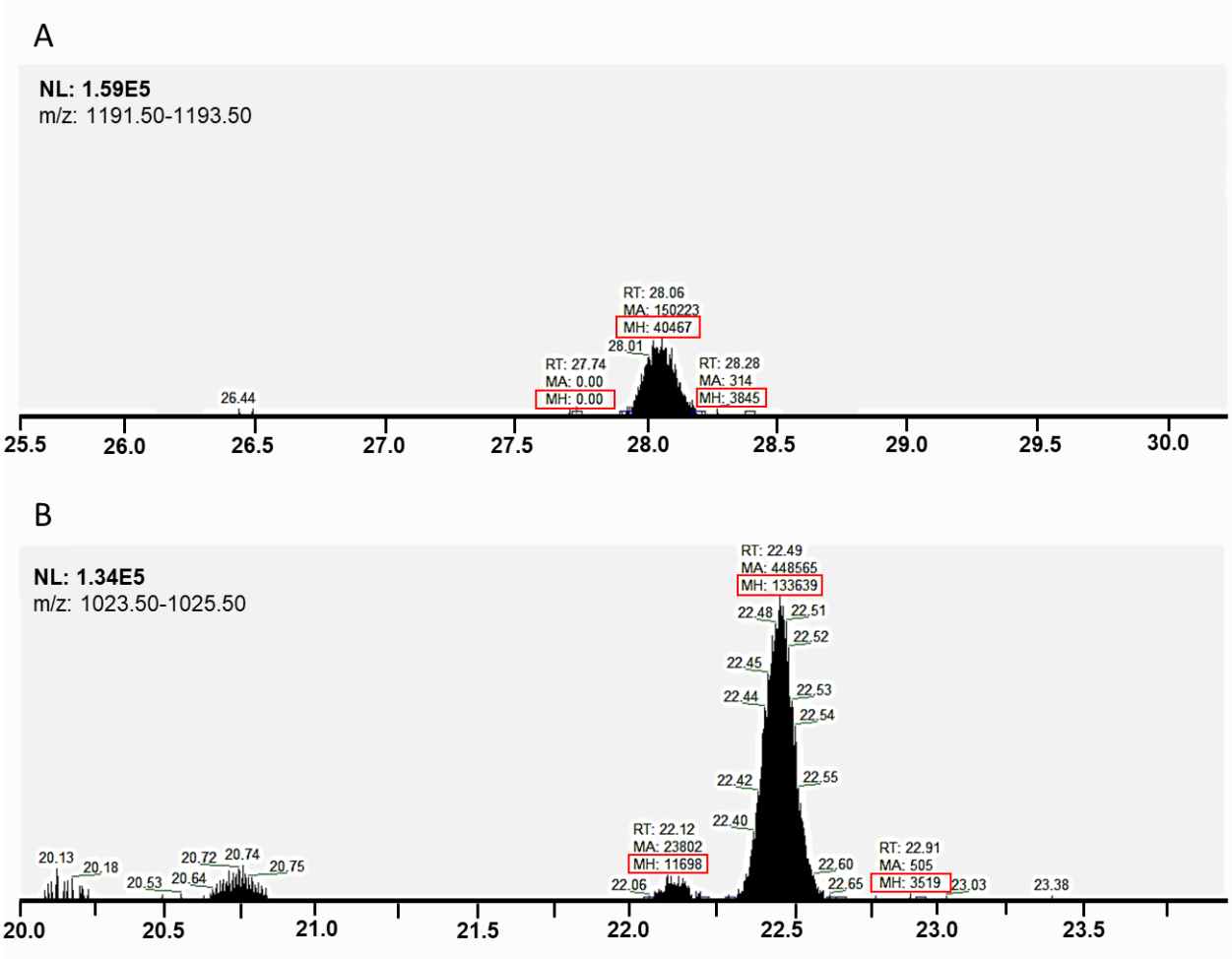


Figure 34. Extracted ion chromatograms of two tryptic peptides from Pertuzumab in animal serum. Pertuzumab concentration in the sample was at the LLOQ. Heights of the peaks of interest and of the noise area surrounding them are marked in red boxes. Sensitivity criteria are defined as per Unites States pharmacopeia (USP) definition of the (S/N) signal-to-noise ratio (Unites States Pharmacopeia 34 NF 29 effective date May 1, 2011). Y axis: Signal intensity. X axis: Retention time (in minutes). A) GLEW peptide with an S/N ratio of 21. B) NTLYL peptide with an S/N ratio of 23.

Signal-to-noise ratio (area around analyte peak in LLOQ)					
	Analysis 1 / Day 1	*Analysis 2 / Day 1*	*Analysis 3 / Day 2*	*Analysis 4 / Day 2*	*Analysis 5 / Day 3*
NTLYL	12.86	12.04	11.19	12.03	12.34
DST	14.58	13.87	14.96	12.25	16.99

GLEW	28.21	26.13	21.05	25.13	26.89
PENNY	29.06	22.07	22.85	24.66	25.78

Table 9. S/N ratios calculated for all peptides under consideration in a sample of stressed Pertuzumab in animal serum. Pertuzumab concentration in the sample was at the LLOQ. Results for five different samples performed on 3 subsequent days are shown.

In addition to the signal-to-noise ratio definition used above within the sample, a second definition of the signal-to-noise ratio was assessed. The purpose of it was to assess the signal intensity in the sample at LLOQ Pertuzumab concentration against the baseline in the blank. The blank consisted in a sample of solely animal digested serum. The corresponding assessment is as follows:

$$\frac{S}{N} = \frac{2 * maximum\ height\ of\ peak\ in\ the\ analyte}{height\ of\ noise\ signal\ in\ the\ blank} \geq 10$$

Note that the noise signal in the blank should be assessed within the product ions' retention time ranges used for quantification per peptide. It was observed that the obtained total ion chromatogram for LC-MS/MS did not contain interfering peaks where the retention time of the blank matched the retention time of the analytes of interest (see Figure 35). Furthermore, it was observed that for all peptides under consideration the S/N ratio as defined above was above 10 (see Table 10).

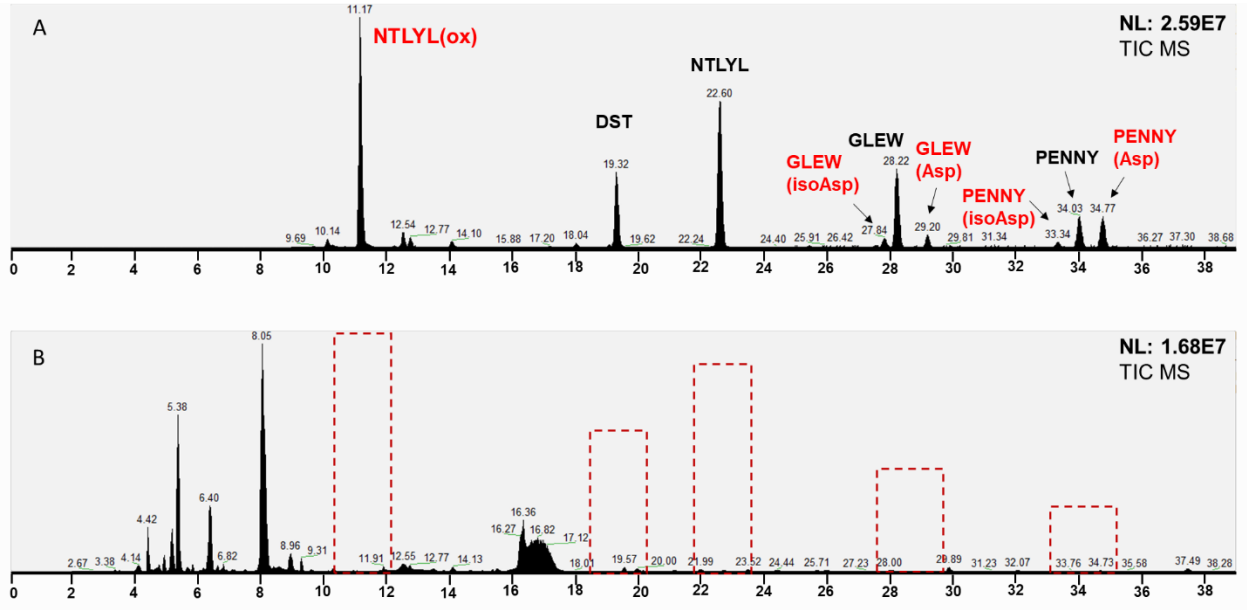


Figure 35. Total ion chromatograms obtained from analysis of: A) Digest of stressed reference standard (Pertuzumab) in PBS. B) Digest of blank sample (digested animal serum). Expected

retention times of analytes of interest are marked in the blank sample with red dashed boxes. Y axis: Signal intensity. X axis: Retention time (in minutes).

Signal-to-noise ratio (against blank sample)	
NTLYL (ox)	12.85
NTLYL	93.66
DST	24.19
GLEW/GLEW variants	30.48
PENNY/PENNY variants	131.92

Table 10. S/N ratios for all peptides under consideration in a sample of stressed Pertuzumab in animal serum. Pertuzumab concentration in the sample was at the LLOQ.

4) Method validation according to FDA guidelines

The method developed in the previous chapters was then validated according to FDA guidelines[124]. A summary of the main results and the fulfilled criteria is presented in the following. For the full obtained data, the reader is referred to the Supplemental Material (Tables S5 – S11).

Method validation included demonstrations of:

- selectivity
- accuracy and precision
- quality control samples
- linearity
- sensitivity
- reproducibility
- stability of analyte in spiked samples
- stability of samples in autosampler

Main results of the performed validation analyses are shown in Figures 33-35. Two types of Pertuzumab (non-stressed and stressed) were used for creation of the calibration curves and the quality control samples. Non-stressed Pertuzumab had depreciable rates of spontaneous deamidation, and oxidation reactions caused by sample preparation (Table 11). In contrast to that, stressed Pertuzumab led to an overall deamidation and oxidation rate of more than 20%.

According to the FDA[124], the detected analyte response should not deviate more than +/- 20% from the nominal concentration in each sample. Hence, this percentage value represents the method's variability. This means that the minimum total deamidation and oxidation rate needed to overcome this variability would be 20%. In the case of stressed Pertuzumab, this threshold was overcome. For that reason, calibration curves were aimed to be built for deamidation and oxidation using the stressed material.

Following, the needed criteria to validate a method according to FDA guidelines and which were fulfilled within this study are presented in detail:

- **Selectivity** was confirmed through the analysis of blank samples from 7 sources of the appropriate biological matrix (7 different white NWZ animals). The ability to differentiate and quantify the analyte in presence of other components in the sample was proven. Each blank sample was tested and no interference of endogenous components of the biological matrix with the analytes of interest was observed. This was shown by the fact that none of the 7 obtained chromatograms in LC-MS/MS contained any interfering peaks in the same retention time as the analyte of interest (see Supplemental Material, Table S10).

- **Accuracy and precision** were confirmed through quality control samples (QC). Three different levels of concentration within the expected range of the investigated sample were analyzed. The determined Pertuzumab concentrations of the samples were within a 15% deviation range around the nominal concentration value. At the LLOQ, the determined Pertuzumab concentrations of the samples were within a 20% deviation range around the nominal value. This was determined by triplicate analysis of high, medium, and low QC samples. The concentration levels the QC samples were distinct from the concentration levels representing the points of the calibration curve. Moreover, they were prepared from a different reference standard stock than the samples of the calibration curves (See Material and Method section IV, 5).

- **Quality control samples** were analyzed in duplicates at three different Pertuzumab concentrations in every run: one close to 10x the LLOQ (low QC), one in the midrange of the calibration curve (middle QC), and one approaching the high end of the calibration curve (high QC). The following criteria of acceptance were fulfilled:
    1. At least 67% of the QC concentration results were within their respective nominal values (theoretical values).
    2. At least 50% of QCs at each level were within a 15% deviation range of their nominal concentrations.
    3. The minimum number of total QCs was at least six.
    4. Calibration standards and QCs were prepared from separate stock solutions (see Material and Methods section of Chapter IV, 5 for details).

- **Linearity** was proven by the analysis of six calibration curves (three from stressed and three from non-stressed Pertuzumab). Each curve was built through eight different samples: a blank sample (just matrix), a zero sample (matrix sample with internal standard), and six non-zero samples (matrix samples with analyte and internal standard). Like that the expected range of Pertuzumab concentration in exploratory animal study samples was covered (see Material and Methods section of Chapter IV, 5). The validation included the minimum requirement of six runs conducted over several days. The concentrations in all points of the calibration curves did not deviate by more than 15% from nominal concentration levels and not more than 20% at the LLOQ[124]. In total, at least 75% of non-zero standards met the above deviation criteria including the LLOQ. By that, the

acceptance criteria for the stressed and non-stressed reference standard curves were fulfilled.

It was observed that the stable tryptic peptide DST showed a slope that was almost identical for stressed (pH 9, 40 °C, H_2O_2, 45 days) and non-stressed (kept at -80 °C) Pertuzumab. However, divergent slopes between stressed and non-stressed samples were observed for the non-modified variants of those peptides prone to degradation (Figure 37). The GLEW peptide located in the CDR region showed a deviation of 0.9-fold between reference standards, while for the NTLYL and PENNY peptides a 1.31-fold and 1.83-fold deviation between slopes was observed. Details on this effect can be found in the Discussion section of this chapter. Details on the concentration corrections needed to plot the calibration curves for the stressed Pertuzumab can be found in the Material and Methods section of Chapter IV, 5.

Calibration curves were also obtained for all modified variants of the peptides under study (Figure 38). Proper linearity was proven for all samples and peptides while observing a sufficiently small standard deviation between triplicates.

- **Sensitivity** was proven through confirming accuracy and precision at the lowest concentration level within all calibration curves (non-zero sample 1). The measured concentration value did not deviate more than 20% from the theoretical spiked concentration level. The lowest concentration value presented through the calibration curve was accepted as the LLOQ, since the following conditions were met[124]:

    - The following S/N ratio criteria should be fulfilled:

$$\frac{S}{N} = \frac{Analyte\ response\ level\ at\ LLOQ}{Response\ level\ of\ blank\ sample} \geq 5$$

      In this method, an S/N ratio of more than 10 for all peptides comparing the first non-zero sample to blanks was observed.

    - The analyte peak response was identifiable, discrete, and reproducible. Both precision and accuracy did not deviate more than 20% from the nominal concentration value.

  By this, the proper sensitivity was validated.

- **Reproducibility** within the run was assessed by performing replicate measurements. Three points of each of the six calibration curves were reanalyzed at the end of every run. Both signal intensities, determined concentration values and deamidation/oxidation rates in the peptides under consideration were consistent with the first time of injection.

- The **chemical stability** of both the reference standard (stressed Pertuzumab) and the internal standard was proven for the entire validation time. This was achieved through the

comparison of six-fold analysis before and at the end of the study. Deamidation and oxidation rates between both time points were compared and confirmed to not deviate from each other (<10%).

- The **chemical stability of the analyte in autosampler** in the biological matrix was proven for the given time interval of analysis. This was assessed through one analysis at the beginning and one at end of the sequence within the same set of samples (3 points of the calibration curve). By that, stability during the run length of 48 hours could be confirmed.

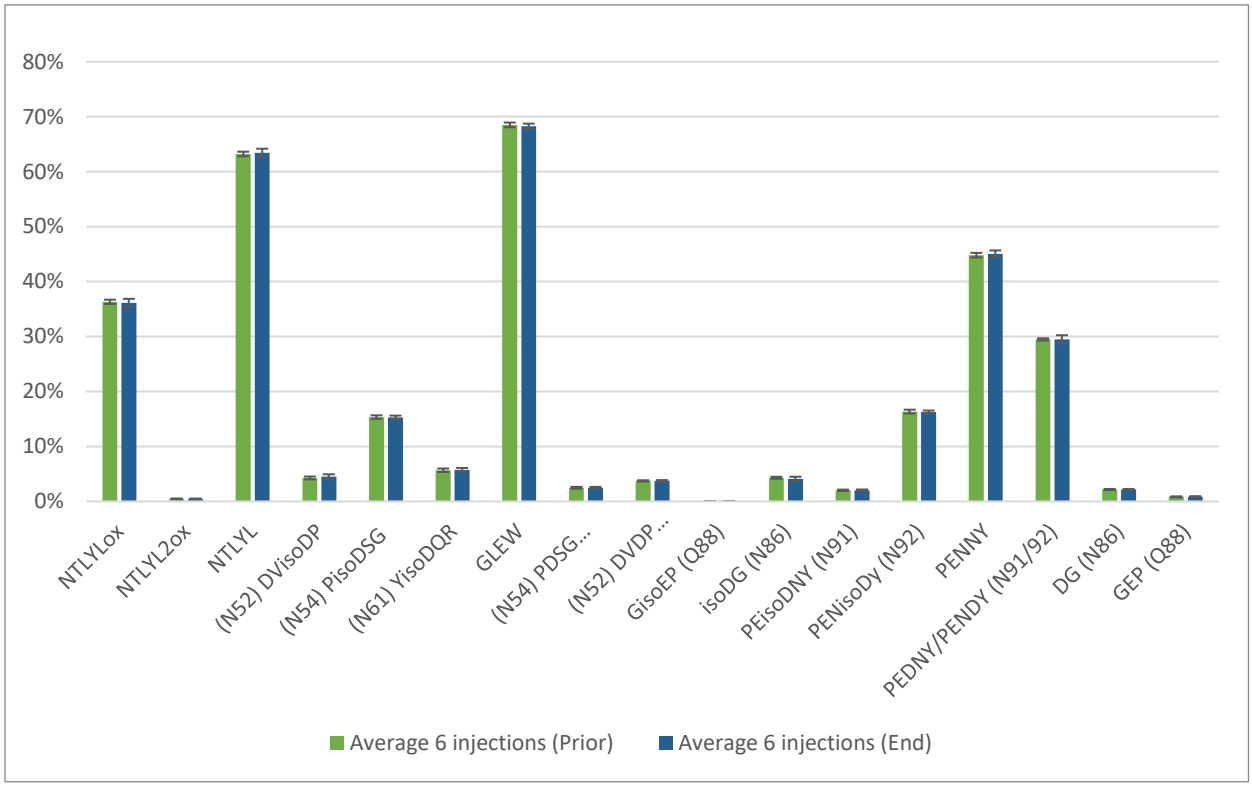
Non-stressed Pertuzumab

	NTLYL ox	*NTLYL*						
Oxidation	**2.46%**	**97.54%**						
STD	0.004655	0.004655						
RSD	18.90%	0.48%						
	(N52) iso-Asp	*(N54) iso-Asp*	*(N61) iso-Asp*	*GLEW*	*(N54) Asp*	*(N52) Asp*		
Deamidation	**0.00%**	**0.00%**	**0.00%**	**100.00%**	**0.00%**	**0.00%**		
STD	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000		
RSD	NA	NA	NA	NA	NA	NA		
	(Q88) iso-Glu	*(N86) iso-Asp*	*(N91) iso-Asp*	*(N92) iso-Asp*	*PENNY*	*(N91/92) Asp*	*(N86) Asp*	*(Q88) Glu*
Deamidation	**0.00%**	**0.00%**	**0.55%**	**0.00%**	**98.78%**	**0.67%**	**0.00%**	**0.00%**
STD	0.000000	0.000000	0.001607	0.000000	0.003592	0.002147	0.000000	0.000000
RSD	NA	NA	29.31%	NA	0.36%	31.88%	NA	NA

Stressed Pertuzumab

	NTLYL ox	*NTLYL 2ox*	*NTLYL*	**Total Ox.**					
Oxidation	**36.32%**	**0.46%**	**63.22%**	**36.78%**					
STD	0.003860	0.000764	0.004190						
RSD	1.06%	16.72%	0.66%						
	(N52) iso-Asp	*(N54) iso-Asp*	*(N61) iso-Asp*	*GLEW*	*(N54) Asp*	*(N52) Asp*	**Total Deam.**		
Deamidation	**4.27%**	**15.35%**	**5.67%**	**68.51%**	**2.48%**	**3.73%**	**31.49%**		
STD	0.002586	0.003209	0.003182	0.004283	0.001685	0.001158			
RSD	6.06%	2.09%	5.62%	0.63%	6.79%	3.10%			
	(Q88) iso-Glu	*(N86) iso-Asp*	*(N91) iso-Asp*	*(N92) iso-Asp*	*PENNY*	*(N91/92) Asp*	*(N86) Asp*	*(Q88) Glu*	**Total Deam.**
Deamidation	**0.05%**	**4.30%**	**2.02%**	**16.35%**	**44.81%**	**29.48%**	**2.17%**	**0.84%**	**55.19%**
STD	0.000168	0.001859	0.001319	0.003545	0.004121	0.001999	0.000287	0.000856	
RSD	37.23%	4.32%	6.53%	2.17%	0.92%	0.68%	1.32%	10.24%	

Table 11. Deamidation and oxidation rates from monitored variants of Pertuzumab's tryptic peptides. Upper table: non-stressed Pertuzumab stored at -80 °C and digested immediately after thawing. Lower table: stressed Pertuzumab digested after incubation at stressed conditions for 45 days (pH: 9-10, temperature: 40 °C, oxidative: H_2O_2). "Ox" and "Deam" stand for oxidation and deamidation, respectively.



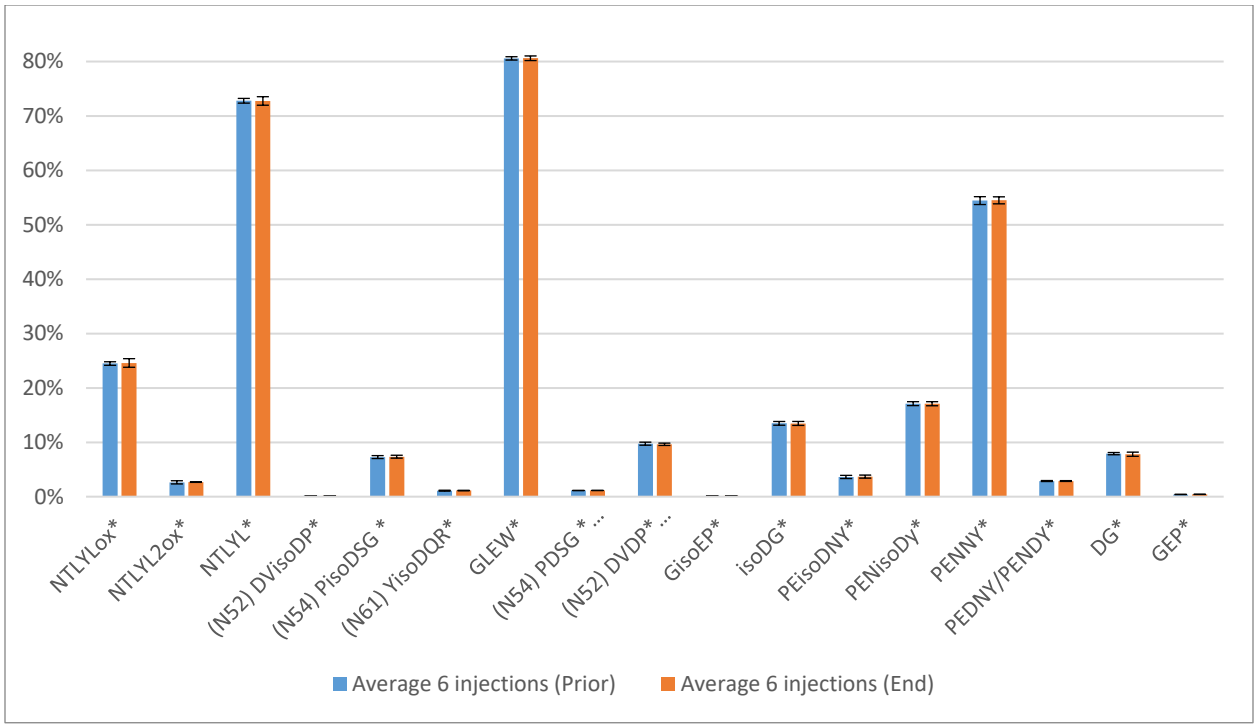
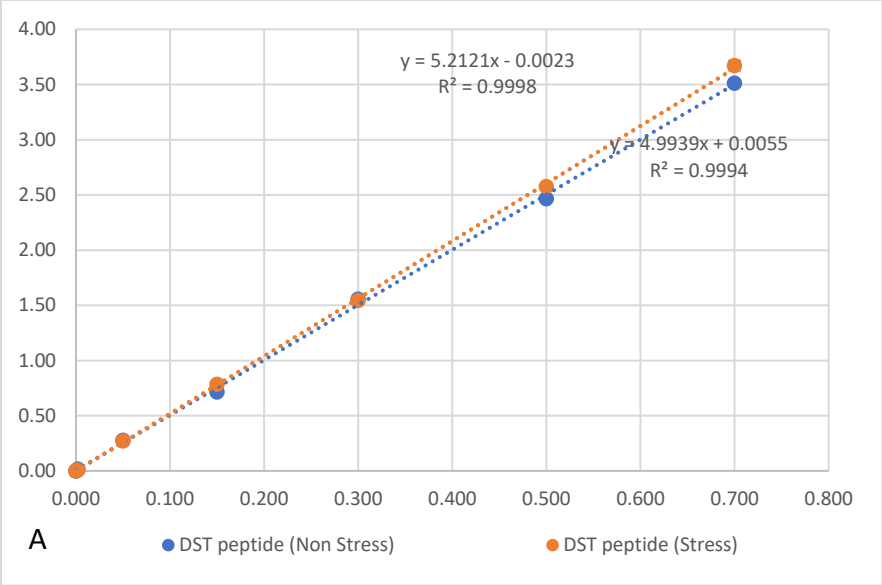
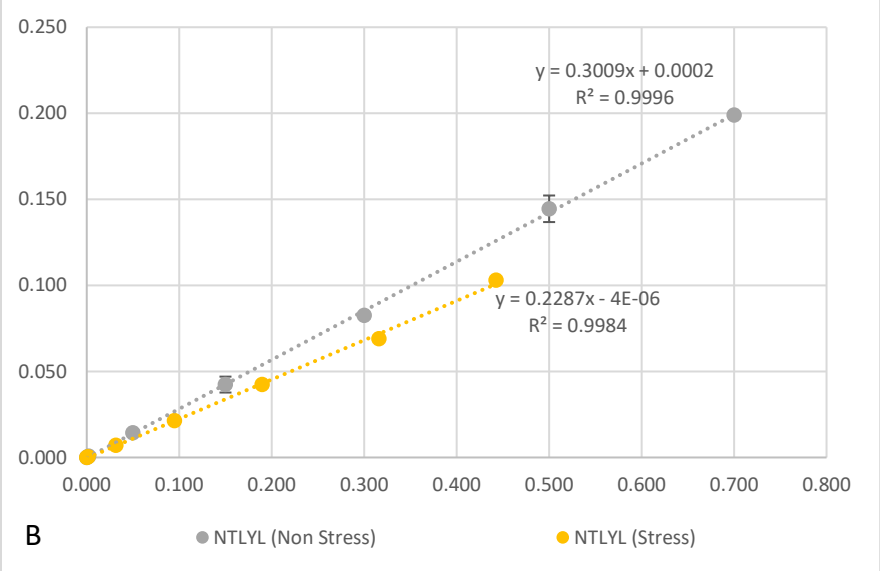


Figure 36. Modifications (deamidation and oxidation) rates present in both Pertuzumab as stressed reference standard (upper panel) and the internal standard (lower panel) after being incubated at stressed conditions (pH: 9-10, temperature: 40 °C, oxidative: H_2O_2) for 45 and 28 days, respectively. Standard deviations between the 6 replicas are depicted by grey whiskers for each mAb. Y axis: Modification rate. X axis: Specific modified peptide variant.


y = 5.2121x - 0.0023
R² = 0.9998
y = 4.9939x + 0.0055
R² = 0.9994
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.000
0.100
0.200
0.300
0.400
0.500
0.600
0.700
0.800
A
DST peptide (Non Stress)
DST peptide (Stress)


y = 0.3009x + 0.0002
R² = 0.9996
y = 0.2287x - 4E-06
R² = 0.9984
0.250
0.200
0.150
0.100
0.050
0.000
0.000
0.100
0.200
0.300
0.400
0.500
0.600
0.700
0.800
B
NTLYL (Non Stress)
NTLYL (Stress)

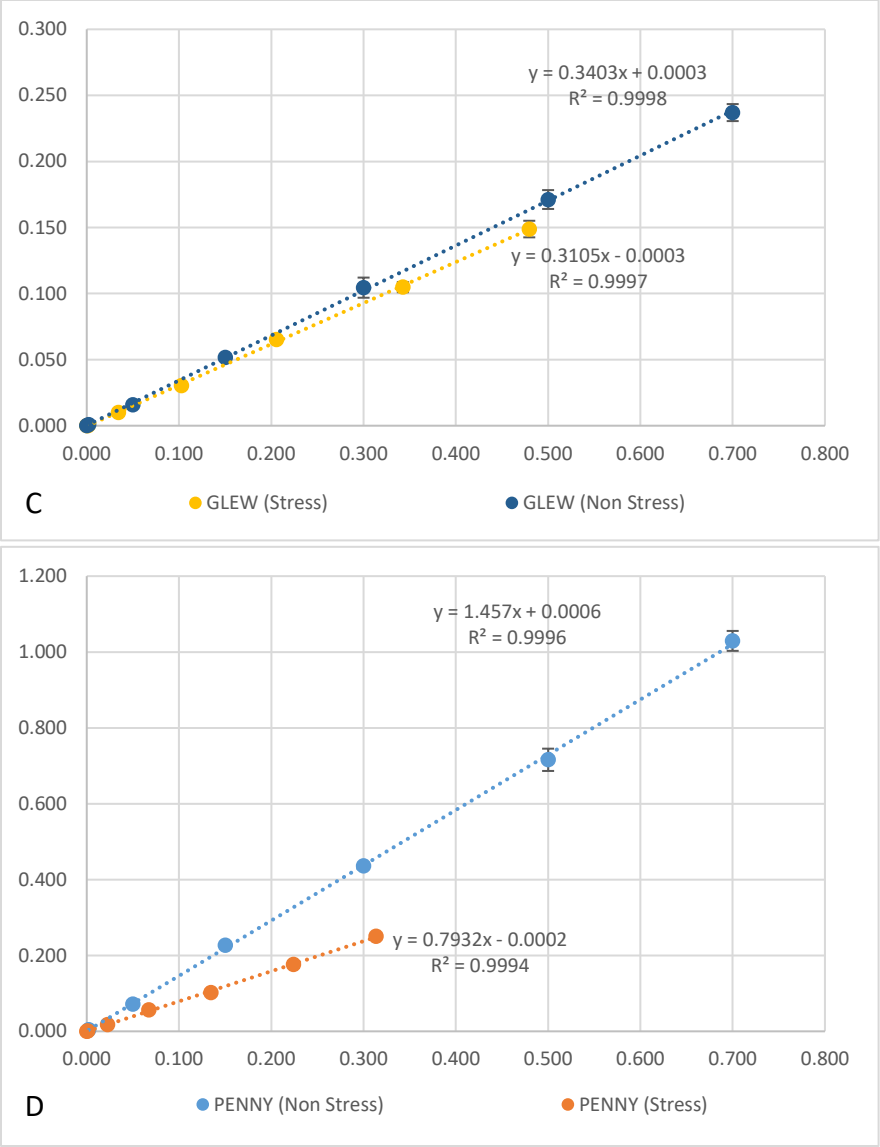
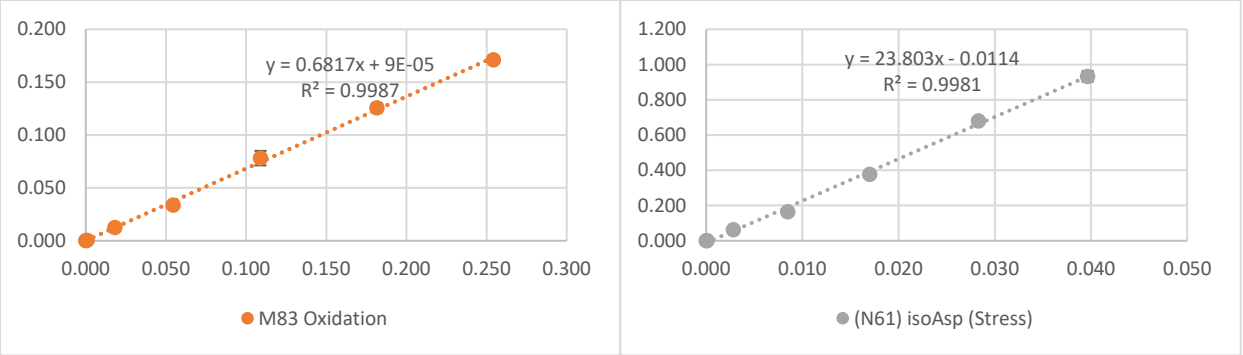
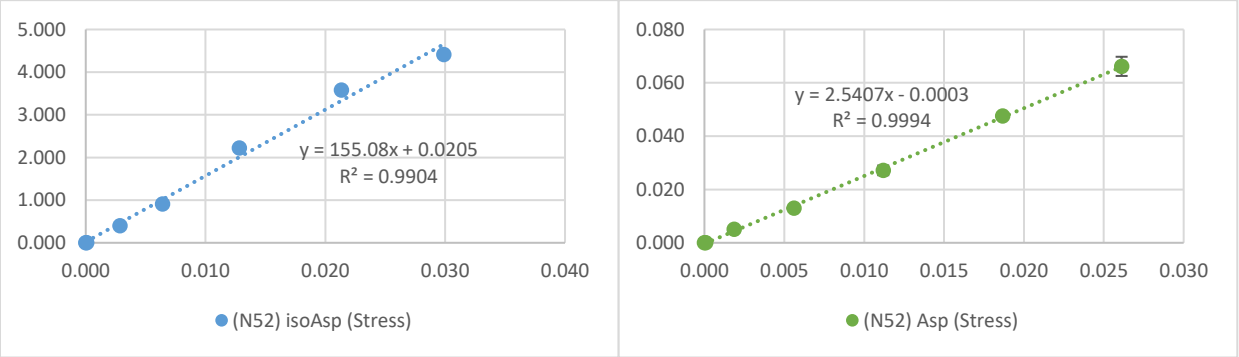
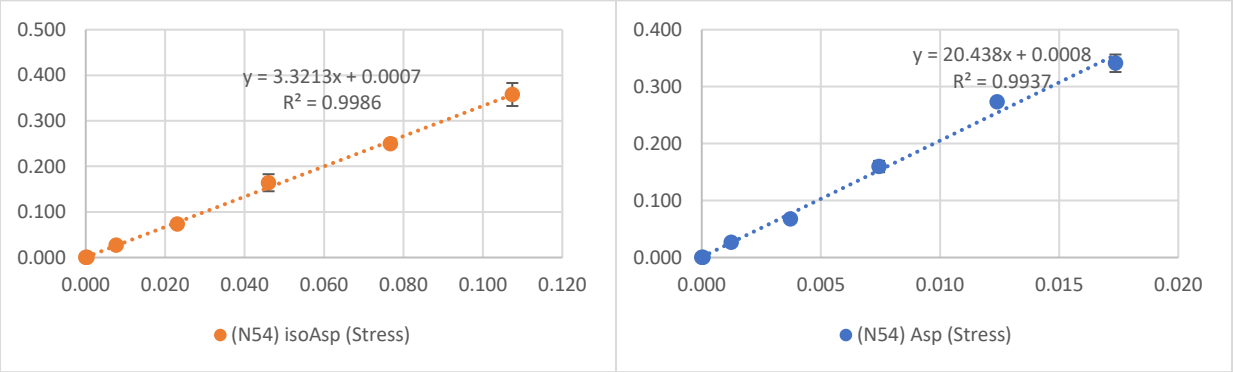
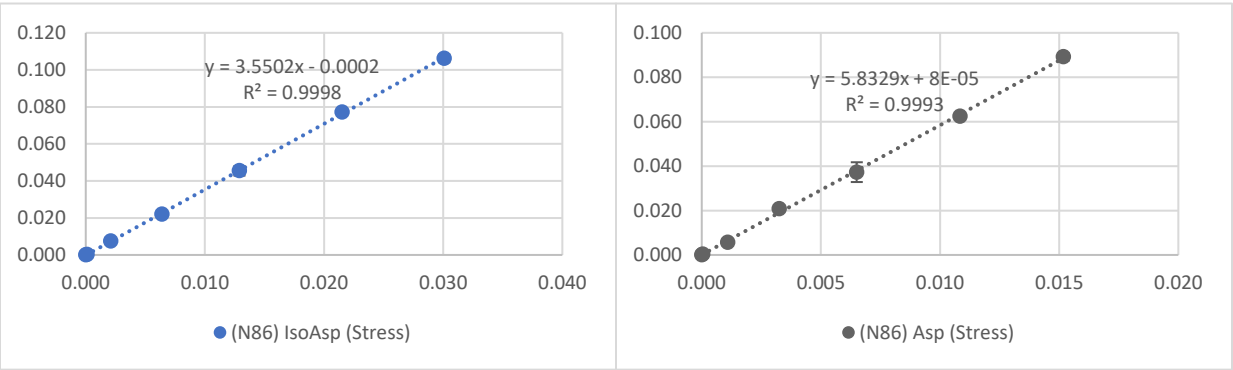


Figure 37. Calibration curves for both stressed (pH: 9-10, temperature: 40 °C, oxidative: H_2O_2, incubation time: 45 days) and non-stressed (stored at -80 °C and digested immediately after thawing) reference standards. A) Stable tryptic peptide DST used for quantification of Pertuzumab present in exploratory animal study samples and PK curve plotting. B) Tryptic NTLYL peptide from the Fab region prone to oxidation. C) Tryptic GLEW peptide from the CDR region surveilled for deamidation occurrence. D) Tryptic PENNY peptide from the Fc region prone to deamidation. Standard deviations between triplicates are indicated by grey whiskers. Full data is available in Supplemental Material (Tables S6, S7). Y axis: Signal intensity corrected by the IS (added at the beginning of the digestion protocol). X axis: Total concentration (in mg/ml) of the non-modified variant of the peptide.


y = 0.6817x + 9E-05
R² = 0.9987
M83 Oxidation
y = 23.803x - 0.0114
R² = 0.9981
(N61) isoAsp (Stress)


y = 155.08x + 0.0205
R² = 0.9904
(N52) isoAsp (Stress)
y = 2.5407x - 0.0003
R² = 0.9994
(N52) Asp (Stress)


y = 3.3213x + 0.0007
R² = 0.9986
(N54) isoAsp (Stress)
y = 20.438x + 0.0008
R² = 0.9937
(N54) Asp (Stress)


y = 3.5502x - 0.0002
R² = 0.9998
(N86) IsoAsp (Stress)
y = 5.8329x + 8E-05
R² = 0.9993
(N86) Asp (Stress)

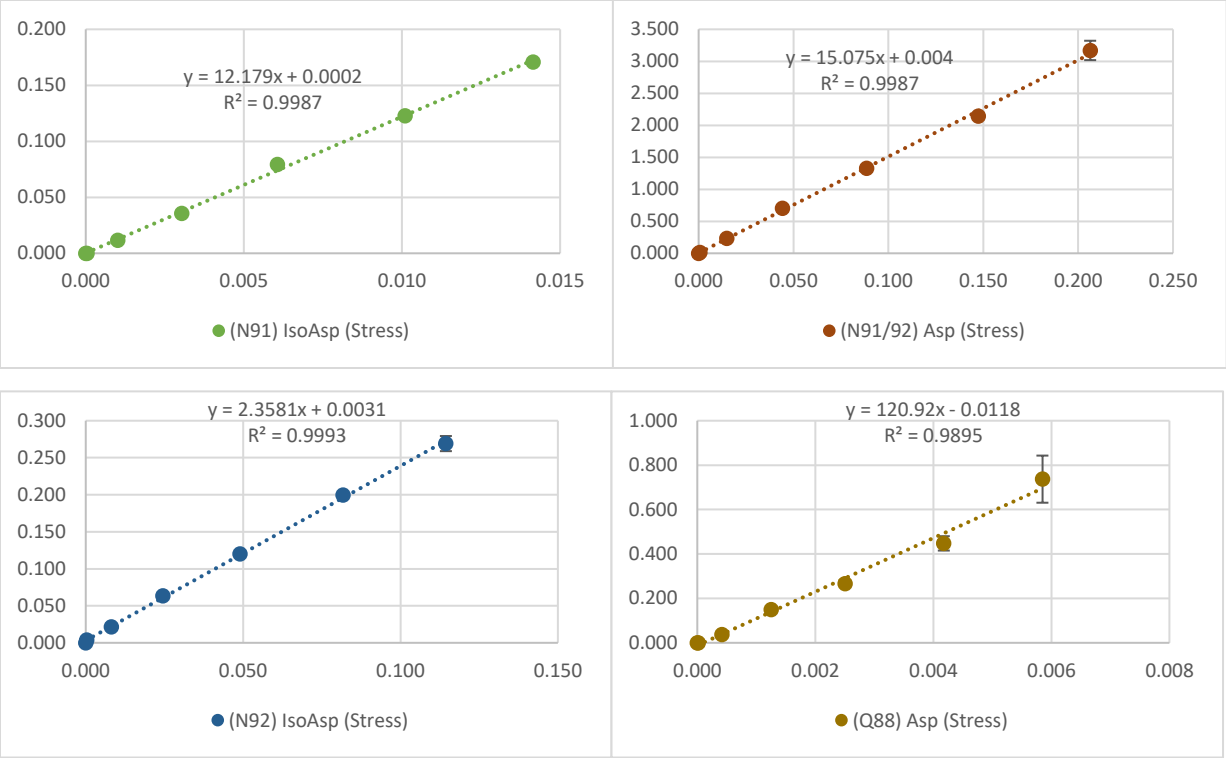


Figure 38. Calibration curves per tryptic modified variant of the studied peptides in Pertuzumab derived from stressed reference standard (pH: 9-10, temperature: 40 °C, oxidative: H_2O_2, incubation time: 45 days). An equation of the calibration curve per peptide modification variant is obtained and depicted above the corresponding graph. Standard deviations between triplicates are indicated by grey whiskers. Full obtained data is available in Supplemental Material (Tables S6, S7). Y axis: Signal intensity corrected by the IS. X axis: Total concentration (in mg/ml).

5) Assessment of the biological matrix effect on the quantification of deamidation and oxidation reactions in Pertuzumab

Besides fulfilling the FDA criteria for method validation presented in Chapter IV, 4, it was important to investigate and manage a matrix effect that may lead to inaccurate measurements of the target peptides. The FDA states in their "Guidance for Industry: Bioanalytical Method Validation"[125]:

"It may be important to consider the variability of the matrix due to the physiological nature of the sample. In the case of LC-MS/MS-based procedures, appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method, especially if the nature of the matrix changes from the matrix used during method validation."

In the case of the method here developed, endogenous substances in the biological samples may be primary sources of matrix effects on peptide quantification. Endogenous substances may include salts, carbohydrates, lipids, peptides, or metabolites present in the animal serum[126].

Two quantification strategies on how to assess and overcome this matrix effect were compared. These two strategies were:

A. Each modification rate was calculated based on the absolute signals of all peak areas for the corresponding peptide. No correction with IS was performed and the rate of a certain modification was given as:

$$\frac{Area\ under\ the\ peak\ of\ the\ modification\ of\ interest\ in\ peptide\ "X"}{Sum\ of\ all\ areas\ under\ the\ peaks\ of\ all\ peptide\ "X"\ variants\ (modified\ and\ non-modified)}$$

This calculation was performed per calibration curve point and an average modification rate (from all calibration curve points) was calculated.

B. The modification rate was calculated through the equation derived from each calibration curve of the corresponding modification of peptide "X". By substituting the signal (AUC) corrected with the IS in each equation, the concentration in mg/ml of each modification per point of the curve was determined. Based on the calculated concentrations of all peptide "X" variants (modified and non-modified species), the modification rate per calibration curve point was determined for every peptide's variant. Afterwards an average modification rate (from all calibration curve points) was calculated. (see Table 12).

The average modification rate (from all points of the curve) obtained through strategy B was compared to the corresponding average from strategy A. These two averages were then compared to the average rates seen in a six-fold analysis in PBS (see Table 11 and Figure 36 for values in PBS) and the observed deviation from PBS values was reported. As additional measure of comparison, standard deviations between points of the curve were assessed for both strategies A and B.

The quantification discrepancies between strategies A and B due to a matrix effect (observed in the deviation from PBS values) were first assessed in calibration curves built with pooled serum (Table 12) and later in samples with individual animal serum (Table 13). It was observed that the discrepancy of both strategies varied from mild to high depending on the peptide. While for some peptides (e. g. stressed NTLYL) the obtained deviation from PBS modification rates differed highly between strategies (Strategy A: 21.80%, Strategy B: 1.42%), other peptides (e. g. oxidized stressed NTLYL) showed low deviations from PBS values in both strategies (Strategy A: 3.87%, Strategy B: 1.20%). However, it could be seen that strategy B yielded significantly smaller deviations from PBS modification rates than strategy A for all peptides.

For strategy B, relative deviations of average modification rates from PBS values were ranging from 0.03% (N92 iso-asp PENNY) to 3.65% (N52 iso-asp GLEW) (See Supplemental Material, Table S7). In contrast, strategy A resulted in relative deviations of up to 21.80% (NTLYL) from PBS.

As additional measure, the relative standard deviation of modification rates between all points of the curve per peptide was assessed. It could be observed that strategy B yielded significantly lower standard deviations than strategy A. For strategy B, deviation values between 1.17% (PENNY)

and 8.25% (N52 iso-asp GLEW) were measured (data for all peptides may be found in the Supplemental Material, Table S6-S7). For strategy A, the corresponding relative standard deviations ranged from 4.56% (PENNY) to 31.95% (NTLYL).

Average of 3 calibration curves from stressed Pertuzumab							
NTLYL (Stressed)							
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
6th	0.4425	0.103048	0.450600	**1.83%**	0.001138	**1.10%**	**64.24%**
5th	0.3161	0.068946	0.301485	**4.62%**	0.000115	**0.17%**	**62.04%**
4th	0.1896	0.042348	0.185187	**2.35%**	0.001082	**2.55%**	**61.79%**
3rd	0.0948	0.021310	0.093198	**1.71%**	0.001136	**5.33%**	**65.37%**
2nd	0.0316	0.006998	0.030615	**3.14%**	0.000298	**4.26%**	**62.46%**
1st	0.0013	0.000322	0.001426	**12.78%**	0.000035	**10.89%**	**68.80%**
Zero	0.0000	0.000000	0.000000	**0**			
					Average Strategy B		64.12%
					Rel. deviation PBS		**1.42%**
					RSD Strategy B		**2.42%**
					Average Strategy A		49.44%
					Rel. deviation PBS		**21.80%**
					RSD Strategy A		**31.95%**

Average of 3 calibration curves from stressed Pertuzumab							
Oxidized NTLYL (Stressed)							
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
6th	0.2542	0.171110	0.250872	**1.32%**	0.003011	**1.76%**	**35.76%**
5th	0.1816	0.125833	0.184456	**1.57%**	0.004213	**3.35%**	**37.96%**
4th	0.1090	0.078165	0.114529	**5.11%**	0.006844	**8.76%**	**38.21%**
3rd	0.0545	0.033749	0.049376	**9.37%**	0.000791	**2.34%**	**34.63%**
2nd	0.0182	0.012632	0.018399	**1.32%**	0.000837	**6.62%**	**37.54%**
1st	0.0007	0.000531	0.000647	**10.99%**	0.000085	**16.05%**	**31.20%**
Zero	0.0000	0.000000	0.000000	**0**			
					Average Strategy B		35.88%
					Rel. deviation PBS		**1.20%**
					RSD Strategy B		**4.33%**
					Average Strategy A		34.91%
					Rel. deviation PBS		**3.87%**
					RSD Strategy A		**12.28%**

Table 12. Data analysis of spiked stressed reference standard in pooled animal serum and comparison of two strategies to assess the matrix effect on quantification of low-abundant modifications. As example, the data analyses of the stressed NTLYL peptide and the oxidized stressed NTLYL peptide are shown. The entire data obtained for all peptides can be found in the Supplemental Material (Tables S6 and S7). Columns are distributed as follows: Sample name/number corresponding to the calibration curve point, theoretical spiked concentration of corresponding modification in the stressed reference standard, signal corrected by IS, concentration calculated through the equation obtained from the calibration curve of the corresponding peptide, relative deviation of the calculated concentration from the theoretical spiked value, absolute and relative standard deviation between triplicates, obtained modification rate (calculated through the concentrations reported in the fourth column for all peptide's variants). In blue (Strategy B), in red (Strategy A): Average modification rate of all points per calibration curve, relative deviation from the modification rate observed in PBS, relative deviation between all points within the calibration curve.

After assessing the matrix effect on quantification accuracy in pooled serum, it was observed that the matrix effect was exacerbated in individual serum (Table 13). The impact of using strategy B (IS correction and equation per modification) varied significantly between individual animals. Compared to the measurements in pooled serum, the difference between strategy B and strategy A in terms of accuracy and precision was even higher in individual animals. However, while strategy A yielded significantly higher deviations from PBS than those observed in pooled serum, strategy B allowed to reach accurate quantification, low deviation from PBS, and acceptable standard deviation in all seven animals.

The following examples illustrated in Table 13 confirm these results:

- Iso-aspartic formation N61 in GLEW peptide:
    - Relative deviations from PBS in animal 1:
        - Strategy A: 14.20%
        - Strategy B: 3.56%
    - Relative deviations from PBS in animal 6:
        - Strategy A: 139.54%
        - Strategy B: 0.14%

- Aspartic formation N54 in GLEW peptide:
    - Relative deviations from PBS in animal 3:
        - Strategy A: 9.96%
        - Strategy B: 1.83%
    - Relative deviations from PBS in animal 7:
        - Strategy A: 20.05%
        - Strategy B: 1.00%

- Oxidized NTLYL peptide:

- Relative deviations from PBS in animal 2:
  - Strategy A: 36.74%
  - Strategy B: 4.11%
- Relative deviations from PBS in animal 4:
  - Strategy A: 4.41%
  - Strategy B: 1.65%

A full overview on the analyzed data for all peptides and animals can be found in the Supplemental Material, Table S11.

Animal 1					**Animal 6**				
(N61) iso-Asp (Stress)					**(N61) iso-Asp (Stress)**				
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*
0.0397	0.802200	0.034181	**13.81%**	**5.04%**	0.0397	0.852114	0.036278	**8.52%**	**5.35%**
0.0283	0.581265	0.024899	**12.10%**	**5.33%**	0.0283	0.631336	0.027002	**4.67%**	**5.78%**
0.0028	0.053255	0.002716	**4.11%**	**6.02%**	0.0028	0.051854	0.002657	**6.19%**	**5.89%**
		Average Strategy B		5.46%			*Average Strategy B*		5.67%
		Rel. deviation PBS		**3.56%**			*Rel. deviation PBS*		**0.14%**
		RSD Strategy B		**9.20%**			RSD Strategy B		**4.99%**
		Average Strategy A		6.47%			*Average Strategy A*		13.57%
		Rel. deviation PBS		**14.20%**			*Rel. deviation PBS*		**139.54%**
		RSD Strategy A		**9.82%**			RSD Strategy A		**47.64%**

Animal 3					**Animal 7**				
(N54) Asp (Stress)					**(N54) Asp (Stress)**				
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*
0.0174	0.324767	0.015851	**8.70%**	**2.34%**	0.0174	0.335855	0.016394	**5.57%**	**2.42%**
0.0124	0.273024	0.013319	**7.41%**	**2.85%**	0.0124	0.246890	0.012041	**2.91%**	**2.58%**
0.0012	0.022823	0.001078	**13.11%**	**2.39%**	0.0012	0.024043	0.001137	**8.30%**	**2.52%**
		Average Strategy B		2.53%			*Average Strategy B*		2.51%
		Rel. deviation PBS		**1.83%**			*Rel. deviation PBS*		**1.00%**
		RSD Strategy B		**11.16%**			RSD Strategy B		**3.19%**
		Average Strategy A		2.73%			*Average Strategy A*		2.98%
		Rel. deviation PBS		**9.96%**			*Rel. deviation PBS*		**20.05%**
		RSD Strategy A		**8.14%**			RSD Strategy A		**20.27%**

Animal 2	**Animal 4**
Ox (Stress)	**Ox (Stress)**

Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*
0.2542	0.177531	0.260292	**2.38%**	**36.15%**	0.2542	0.159719	0.234163	**7.90%**	**36.36%**
0.1816	0.111083	0.162818	**10.34%**	**34.79%**	0.1816	0.127916	0.187510	**3.26%**	**34.89%**
0.0182	0.011255	0.016378	**9.81%**	**33.54%**	0.0182	0.011165	0.016246	**10.54%**	**35.92%**
		Average Strategy B		34.83%			*Average Strategy B*		35.72%
		Rel. deviation PBS		**4.11%**			*Rel. deviation PBS*		**1.65%**
		RSD Strategy B		**3.76%**			RSD Strategy B		**2.11%**
		Average Strategy A		22.98%			*Average Strategy A*		34.72%
		Rel. deviation PBS		**36.74%**			*Rel. deviation PBS*		**4.41%**
		RSD Strategy A		**20.27%**			RSD Strategy A		**5.15%**

Table 13. Data analysis of spiked stressed reference standard in individual animals' serum and comparison of two strategies to assess the matrix effect on quantification of low-abundant modifications. As example, the data analysis of the following peptides is shown: N61 iso-aspartic formation, N54 aspartic formation (both in GLEW peptide), and oxidized NTLYL peptide. The entire data obtained for all peptides and animals can be found in the Supplemental Material (Table S11). Columns are distributed as follows: Theoretical spiked concentration of corresponding modification in the stressed reference standard, signal corrected by IS, concentration calculated through the equation obtained from the calibration curve of the corresponding peptide, relative deviation of the calculated concentration from the theoretical spiked value, obtained modification rate (calculated through the concentrations reported in the third column for all peptide's variants per animal). In blue (Strategy B), in red (Strategy A): Average modification rate of all points per calibration curve, relative deviation from the modification rate observed in PBS, relative deviation between all points within the calibration curve.

The significant difference between strategy A and strategy B both in pooled and individual serum showed that the matrix effect in the biological matrix could be corrected by the proposed quantification strategy B (by means of an equation per modification and correction with an IS per modification). With this step, a proper method validation according to FDA guidelines was finalized. From the results seen in this chapter, it was possible to conclude that the method may be applied to quantify low-abundant deamidation and oxidation modifications in both in vitro and in vivo samples with animal serum as biological matrix. The method further allowed to correct the impact of a matrix effect on the quantification of the modifications.

Discussion

The validation of the developed method provided insights into some of the difficulties of MAM implementation within a GMP environment. Major findings focusing on the quantification of low-

abundant oxidation and deamidation modifications and a matrix effect on the quantification accuracy will be discussed in the following.

The reference standard (stressed Pertuzumab) and the internal standard were both subject to stressed conditions being incubated at pH, temperature, and oxidative stress. It was observed that stability differed highly between mAbs. As previously stated, (Discussion section of Chapter II, 1) lower modification propensity was expected for Pertuzumab due to its increased stability designed in the CDR regions. However, higher stability was also seen in the Fc region when compared to other mAbs of the IgG1 subtype. To reach high deamidation rates in the Fc region (e. g. at the PENNY peptide), the required incubation times differed widely between Pertuzumab and SIL IgG1 or Trastuzumab under the same stress conditions. As possible explanation, it was hypothesized that the 3D and folding structure of these mAbs may influence their overall stability and therefore their deamidation propensity.

After both, the stressed reference standard and IS were obtained and characterized, it was observed (see Results section) that two species of the PENNY peptide (iso-aspartic formation of the Asparagine residue closest to the N-terminal and the non-deamidated peptide) coeluted despite an optimized gradient. This coelution may be explained by the extremely minimal change in hydrophobicity caused by a modification in an Asparagine residue close to the N-terminus. However, as presented in the Results section, the coelution of these two peptides had no effect on their separate quantification. This could be handled through means of narrow mass extraction ranges for the proper product ion chromatograms. In the case of the GLEW peptide in the CDR region, the corresponding product ion chromatograms were also extracted by means of narrow mass extraction ranges. No coelution of species and no overlapping ion chromatograms (same ion chromatogram observed in both different mass extraction ranges) were observed. This allowed to confidently observe that quantification was not biased nor overestimated. This elucidated the difficulty of monitoring all degradation products, the high possibility of coeluting species, and a possible bias during degradation products analysis.

Characterization of all detected peptide variants for both the reference and the internal standard allowed to continue with validation of the method. Stability tests of both (reference standard and IS) were performed aiming to proof that deamidation and oxidation rates (derived from the stress incubation conditions) did not significantly vary during the time of validation. This was achieved by a six-fold analysis comparing the tryptic digest of the IS and of the reference standard (Pertuzumab with high modification rates) in PBS at validation day 1 against a six-fold analysis of their tryptic digest after the validation had been completed. Minimal changes over time were observed and allowed to validate the stability of both IS and stressed reference standard during the time of study. This stability was supported and confirmed by the minimal standard deviations between the six analyses performed.

The two types of reference standards (stressed and non-stressed Pertuzumab) were used for calibration curves and QC samples preparation. In the non-stressed mAb, artificial deamidation and oxidation were observed to be depreciable. It could be concluded that the modifications caused by the optimized sample preparation were minimal or inexistent. On the other side, modification rates (total deamidation and total oxidation) in the stressed reference standard were above the

method's variability. The presence of peptide variants at different rates in the stressed reference standard required an adjustment of the theoretical spiked concentration per point of calibration curve. In contrast to non-stressed reference standard, the spiked concentration of stressed reference standard was divided into the different peptide variants. To plot the actual concentration of each peptide variant present per point of the curve, a correction of the spiked concentration was needed. This correction was performed by multiplying the spiked concentration with the modification rate seen in the stability results (six-fold analysis of digested stressed reference standard in PBS). The concentration correction allowed to plot:

- both reference standards in the same graph (in the case of non-modified peptide variants)
- one graph per modification only with the stressed reference standard (since in non-stressed reference standard these modifications are inexistent/depreciable)

Through this approach, a calibration curve with a corresponding linear equation was obtained per peptide variant.

When comparing the plotted calibration curves for both types of reference standards, an almost identical slope was observed for the peptide DST. This confirmed not only the stability of this peptide but also the correct concentration determination for the stressed reference standard. However, as presented in the Results section of this chapter, divergent slopes were observed for the non-modified peptides prone to both oxidation and deamidation. This difference in slopes was hypothesized to be an indicator for the presence of additional modifications. These modifications may have occurred in those peptides and were failed to be detected and included in the assay. The deviation observed between slopes for the GLEW peptide located in the CDR region was minimal (0.9-fold). By this, the stability of the CDR regions in Pertuzumab was confirmed once more.

In contrast to that, a higher divergence between slopes was observed for both the NTLYL and the PENNY peptide. In the case of NTLYL, it was hypothesized that the divergence between slopes (1.31-fold) could be explained by possible deamidation and iso-aspartic formation of the first Asparagine residue in the non-oxidized and oxidized tryptic peptide variants. Not surprisingly, the PENNY peptide showed the highest divergence between slopes (1.83-fold). This means a remarkable slope deviation by almost double. The PENNY peptide had the longest amino acid sequence of all monitored peptides and showed less stability during the experiments of incubation under stressed conditions (performed in Chapter IV, 1). It was presumed that other unexpected modifications occurring in the PENNY peptide (which were not monitored) caused this slope diversion effect. Such modifications were hypothesized to be oxidation of a tryptophan residue and/or succinimide formation.

These findings were a clear indicator of the complexity of an assay that is focused on monitoring several degradation products at once. Reaching an absolute quantification of PTMs and degradation products may not be always confidently assured without intact heavy labeled standards and modified/characterized reference standards. The use of calibration curves from stressed material allowed to elucidate that some modifications will inevitably remain undetected and unquantified. A complete characterization and quantification of multiple modifications in a mAb would represent an enormous and challenging task.

These observations might be of great impact for MAMs methods development, in which several attributes are aimed to be monitored with one single method. Results obtained from MAMs must be carefully interpreted and validated as information on low-abundant modifications might be missed. The approach shown here presents just some insights into the wide challenges of monitoring the presence of low-abundant modified peptides in complex matrices and validating such methods. The challenge becomes particularly hard, when starting materials such as the controlled and fully characterized mAb and an intact heavy-labeled standard in their non-modified and modified versions are not available. Although through the methodology developed in this study an absolute quantification may not be reported, it provides the possibility of giving an initial assessment of the relative presence of these modifications. The application of this method may give a first insight into the molecule's propensity to suffer modifications of interest (such as deamidation and oxidation) and which may have an undesired impact. Furthermore, the developed method concept may be applied to other mAbs and biosimilars when a targeted analysis and quantification of low abundant modifications is of interest.

Matrix Effect

During validation, two quantification strategies (A and B) were proposed and compared both in pooled and individual animal serum (see Results section of Chapter IV, 5). The purpose of this comparison was to assess the effect of the matrix on the quantification of peptides. In pooled serum, the impact of the serum on quantification through strategy A was dependent on the peptide. On the other hand, a more accurate quantification in pooled serum was obtained through strategy B with modification rates closer to those seen in PBS. It was possible to conclude that a strategy B (making use of an IS and a calibration curve per modification of study) was needed to avoid a serum matrix effect on the quantification of peptides. Additionally, this matrix effect was concluded to be not only dependent on the peptide, but also dependent on the concentration, as through strategy A different deviation values from PBS rates were seen within the different points of the calibration curve. In contrast, a consistency of modification rates within the different points of the calibration curve was seen through strategy B, confirming the accuracy and precision obtained through this strategy.

These first conclusions reached during the study in pooled serum implied the need for a reference standard and an internal standard which contained all modifications to be monitored. The need for an IS may be challenging during MAMs validation as all peptide variants must be synthesized. That increases the cost per assay significantly. In the case, that an intact heavy-labeled mAb (such as Trastuzumab) was available in the market and that absolute quantification should be reached, the intact IS would need to be submitted to degradation to obtain the required modifications.

In addition to the use of an IS containing all sites of modification, it was proven that also a reference standard containing all sites of modification was required to build calibration curves per modification. It was furthermore proven that quantification based on these calibration curves from stressed reference standard and stressed internal standard is more accurate, precise, and reproducible. In contrast, a "conventional" quantification approach to report modification rates

(area under the peak of a peptide variant divided by the sum of all areas under the peaks of variants of a peptide) was proven to be highly inaccurate as well as dependent on the peptide and the concentration.

Furthermore, it was observed that different modifications were affected in a different way by the serum matrix in those peptides yielding a high number of variants (such as the PENNY peptide with several iso-aspartic and aspartic acid formations). The total deviation of the modification rates from the rates seen in PBS and within the calibration curve points was highly dependent on the modification. However, this deviation could be minimized through strategy B (see Results of Chapter IV, 5 and Supplemental Material Table S7). It was hypothesized that the difference in MS response between modified variants of the same peptide was caused by two possible explanations:

1) The potential coelution of peptides both endogenous to the serum and to the mAb of interest generate a matrix effect on the quantification of the targets of interest.
2) A potential different response at MS level (ionization and/or fragmentation) was caused by the punctually modified site.

Moreover, the simultaneous combination of these two possible explanations should not be discarded, as it would probably represent the most real scenario for a matrix effect dependent on the modification.

When studying individual serum, the same effect on quantification accuracy as already observed in pooled serum was seen. However, it was of interest that this effect was highly dependent on the individual animal for the same peptide. This observation combined with the previous one seen in the study of pooled serum allowed to conclude that the matrix effect on quantification of low-abundant modifications was dependent on the

- peptide
- concentration
- individual

Response variability in LC-MS/MS analysis derived from a matrix effect has been studied in other applications. For example, Olson et al. reported highly variable IS responses in the LC–MS/MS analysis mouse plasma samples[127]. Although their method validation using a control matrix had been completed without any problems, 22 of the 158 analyzed individual matrices showed a more than two-fold increase in IS response, while calibration curve samples and QC samples remained unaffected. The root cause for this effect was a co-eluting compound to the analyte of interest (endogenous bile acid taurocholic acid) causing the enhancement of the IS ionization efficiency. Although levels of this compound are endogenously usually low, physiological changes in some samples may occur. These affect the ionization of analytes and IS in the case of insufficient chromatographic separation.

In contrast to the previous example (where an enhancing matrix effect on the analyte ionization was observed), analyte and IS response variability may be also affected by other mechanisms, for instance by matrix-induced degradation. This was the case for the analysis of plasma from malaria patients through an LC-MS/MS method developed for the quantification of an antimalarial drug

and its main metabolite observed by Lindegardh et al.[128]. Although method validation did not present any complications nor did calibration curves or quality control samples show any response variability, an extreme variability of the IS response in the analysis of plasma from malaria patients was observed. As the IS response remained affected in the presence of healthy subjects' plasma (matrix used for calibration curves and quality control samples), this effect was hypothesized to be caused by the IS degradation in the presence of disease-related compounds in plasma. The exact reason for this degradation may be explained by the presence of iron in the blood steam released from the hemoglobin consumption by the malaria-causing parasite. Plasma samples from malaria patients may have varying levels of iron depending on the disease severity, resulting in a different degradation rate of the method's analytes (malaria drug and its metabolite) and their IS.

These examples emphasize the importance of using an appropriate internal standard and testing several lots of the biological matrix during method validation. Within the method developed in this study, this was applied buy testing seven different biological matrices as previously presented. In the case of analyte or IS variability response, the impact on results and the most probable root cause must be assessed. It cannot be assumed that the use of an IS will always compensate for fluctuations. A variability that is too high may be a sign of poor method performance. Similarly, IS responses falling into a predefined acceptable range does not necessarily mean that the data should not be closely looked at for trends that may indicate analytical or sample-related issues and thus, potentially incorrect results[122].

Through this method validation, it was possible to conclude that the proposed quantification strategy allowed to overcome an unpredictable matrix effect from individuals and pooled serum in the quantification and reporting of modification rates. However, depending on the scope of the assay, the lengthy work that this strategy implies (stress of characterized reference standards and IS, creation of calibration curves per modification, and data analysis per modification under study) must be considered. Depending on the question whether a close (to exact) quantification or just an approximation that may indicate a tendency is required, it must be decided in either of the scenarios which scheme fits the best to answer the posed scientific question.

In summary, the LC-MS/MS method developed in Chapter II could be validated according to FDA guidelines. Moreover, an accurate and precise quantification strategy (despite the matrix effect of serum on the quantification of modifications) was proposed. The overall goal of this study was to assess with the developed and validated method, if in vivo samples showed variability in deamidation and oxidation rates over time after dosage and if these rates may be correlated/reproduced to incubated in vitro samples. An in-depth analysis for the application of the method to both in vivo and in vitro samples will be presented in Chapter V.

V. Application of the validated LC-MS/MS method to in vivo and in vitro samples for comparison of deamidation rates over time in exploratory animal study samples and incubated spiked models

Results

In Chapter IV, the validation of an LC-MS/MS method according to FDA guidelines was presented. This method was now used to compare in vivo with in vitro models. In concrete terms, deamidation and oxidation rates were determined both in vivo and in vitro and assessed afterwards on similarities, and differences. The validated LC-MS/MS method was used for the measurements. Quantification of deamidation and oxidation modifications rates was performed over time in all samples with the following goals:

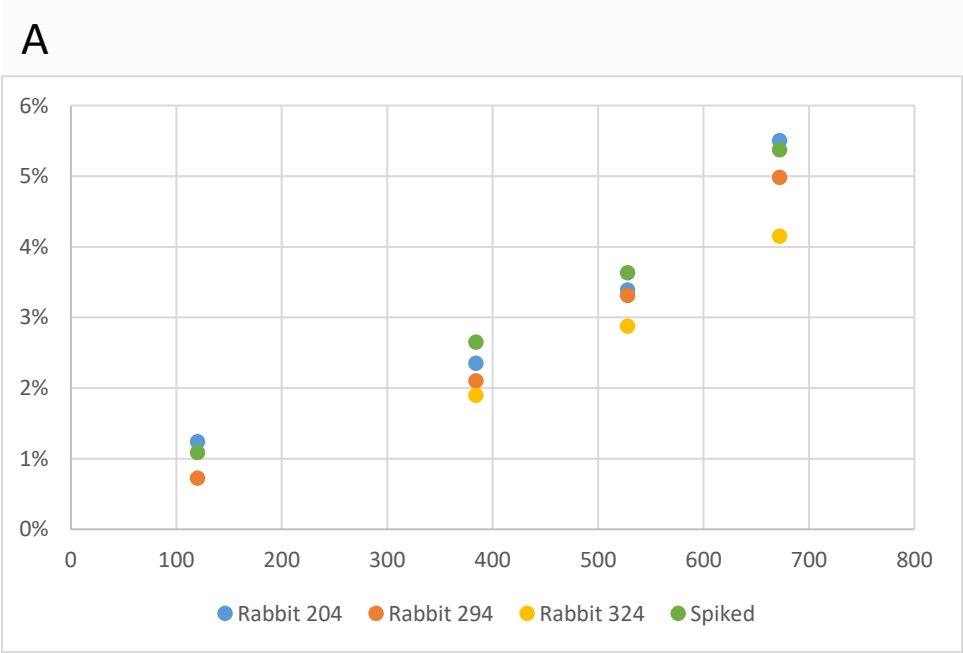
- assess a variability between individuals
- assess a correlation/non-correlation between in vivo and in vitro models

A mini cohort of three animals having received intravenous Pertuzumab treatment (see Material and Methods section V) was analyzed. These three animals were selected for having the highest concentration of Pertuzumab within a 28-day period after dosage. Pertuzumab concentration was determined by the validated LC-MS/MS method and compared to ELISA determinations (see Table 14). The obtained concentrations presented only slight variations from those obtained through ELISA analysis. Moreover, the observed relative standard deviation between triplicates was below 15% for all samples. Hence, the method's accuracy and suitability were confirmed.

Pharmacokinetic profile curve					
Hours	**Animal ID**	**LC-MS/MS (in mg/ml)**	**RSD**	**ELISA (in mg/ml)**	**Deviation (MS vs. ELISA)**
0.83	1	**0.58173**	5.60%	**0.55382**	**4.80%**
	2	**0.51412**	1.44%	**0.47785**	**7.05%**
	3	**0.54961**	9.40%	**0.48334**	**12.06%**
120	1	**0.29048**	8.55%	**0.32394**	**-11.52%**
	2	**0.14819**	13.02%	**0.13407**	**9.52%**
	3	**0.17800**	8.91%	**0.16975**	**4.63%**
384	1	**0.07361**	0.41%	**0.06644**	**9.74%**
	2	**0.05948**	7.70%	**0.05629**	**5.35%**
	3	**0.08736**	2.64%	**0.08046**	**7.90%**
528	1	**0.03287**	13.27%	**0.02904**	**11.63%**
	2	**0.02529**	14.14%	**0.02630**	**-3.99%**
	3	**0.05869**	7.81%	**0.05425**	**7.57%**
672	1	**0.01772**	7.56%	**0.01602**	**9.61%**
	2	**0.01295**	7.66%	**0.01195**	**7.74%**
	3	**0.03109**	6.18%	**0.02726**	**12.32%**

Table 14. Concentration of Pertuzumab in exploratory animal study samples over time through quantification of the stable tryptic DST peptide through the validated LC-MS/MS method. Columns are distributed as follows: Time points after dosage in hours, animal identification number, Pertuzumab concentration obtained through LC-MS/MS method, relative standard deviation between triplicate measurements, Pertuzumab concentration obtained through ELISA analysis, deviation between LC-MS/MS and ELISA.

In Figure 39, it can be observed that the deamidation rates differed between individual animals. Moreover, it was not possible to correlate the deamidation rate for individuals to the rate seen in incubated spiked pooled animal serum over time. A difference in deamidation rate between the four measured samples (animal 3, animal 1, animal 2, spiked pooled animal serum) was only evident after long periods of incubation (21 days). Before day 21 however, this difference between samples may not be reported confidently as only after 21 days the difference between samples is higher than the method's intermediate precision of 2.68% (obtained from inter-reproducibility of QC samples within different days during validation).



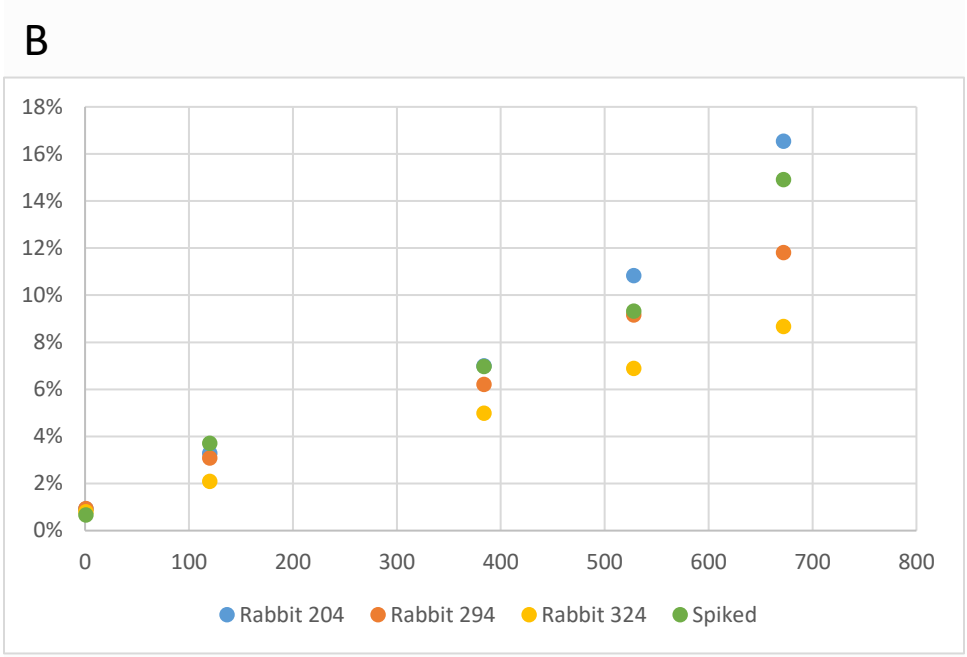


Figure 39. Deamidation rates over time for three animals and spiked pooled animal serum at two different deamidation sites in Pertuzumab. A) N86 (DG) deamidation. B) N91/92 moiety (PEDNY/PENDY) deamidation. Both deamidation variants of the tryptic PENNY peptide located in the Fc region of Pertuzumab. Y axis: Deamidation rate. X axis: Time after Pertuzumab administration (in hours).

Interestingly, the observation that animal 3 presented the highest deamidation propensity in the Fc region of Pertuzumab was correlated to the observation that animal 3 also presented the highest deamidation propensity in the CDR region of Pertuzumab (Table 15). This correlation could be also observed in animal 2 that presented the lowest deamidation rates in both the Fc and CDR region indicating a higher stability (lower propensity to deamidation) in the CDR region.

		(N54) PDSG				PEDNY/PENDY (N91/92)			
		Animal 1	*Animal 2*	*Animal 3*	*Spiked*	*Animal 1*	*Animal 2*	*Animal 3*	*Spiked*
50 min	Deamidation rate	-	-	-	-	**0.94%**	**0.80%**	**0.93%**	**0.67%**
	RSD	-	-	-	-	*12.53%*	*8.52%*	*12.51%*	*14.17%*
5 days	Deamidation rate	-	-	-	-	**3.09%**	**2.09%**	**3.29%**	**3.71%**
	RSD	-	-	-	-	*12.24%*	*11.73%*	*3.46%*	*7.47%*
16 days	Deamidation rate	-	-	-	-	**6.22%**	**5.00%**	**7.01%**	**6.97%**
	RSD	-	-	-	-	*4.61%*	*14.66%*	*1.80%*	*7.45%*
22 days	Deamidation rate	-	-	**1.57%**	**1.03%**	**9.16%**	**6.89%**	**10.84%**	**9.33%**
	RSD	-	-	*2.42%*	*13.19%*	*7.38%*	*2.25%*	*4.10%*	*3.23%*
28 days	Deamidation rate	**1.92%**	-	**3.77%**	**1.20%**	**11.82%**	**8.68%**	**16.55%**	**14.91%**
	RSD	*7.65%*	-	*4.57%*	*5.47%*	*10.78%*	*5.54%*	*3.01%*	*10.59%*

Table 15. Deamidation rate over time detected at two sites of Pertuzumab (N54 in GLEW peptide in the CDR region and N91/N92 moiety in the PENNY peptide in the Fc region) in exploratory animal study samples (animals) and a spiked model (spiked Pertuzumab in pooled animal serum incubated at 37 °C). Relative standard deviations between triplicates are depicted for each sample and each analyzed point in time.

As final step, different deamidation sites of the PENNY peptide were compared per sample in terms of deamidation rate over time. It was observed that all samples (3 individual animals and spiked pooled animal serum) showed a similar deamidation pattern, i.e., a similar linear regression curve (see Figure 40). In specific, the deamidation patterns of the individuals (between all PENNY peptide variants) were observed to be almost identical when normalized (see Table 16), despite forming at different velocities.

Regarding oxidation rates, no significant difference between samples (3 individual animals and spiked pooled animal serum) could be observed. Differences between samples were below the method's intermediation precision of 2.68% for all samples and hence within the method's own variability (see Supplemental Material, Table S16).

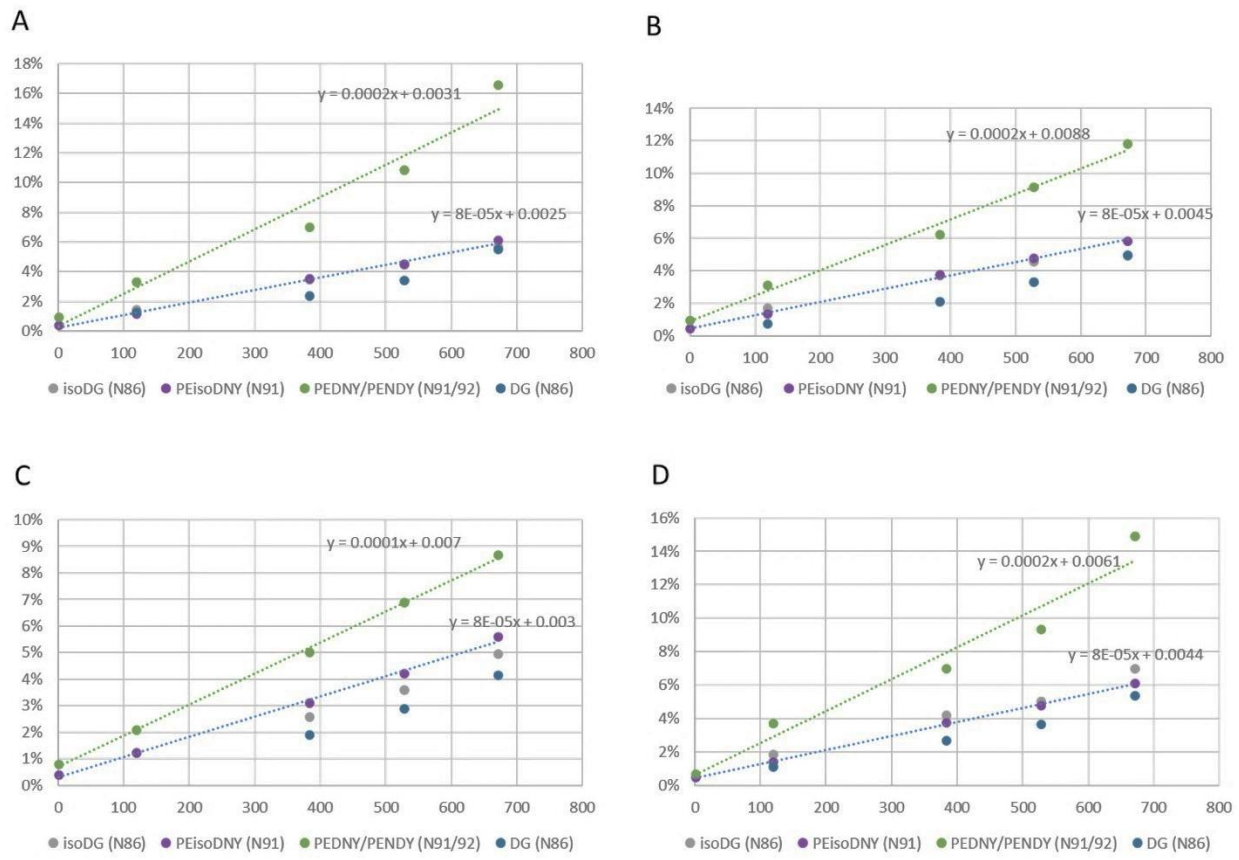


Figure 40. Deamidation pattern of main deamidation variants of the PENNY peptide over time for 3 individual animals and in vitro incubated spiked pooled animal serum. A) Animal 1. B) Animal 2. C) Animal 3. D) Incubated spiked pooled animal serum. Y axis: Deamidation rate. X axis: Time after administration (in hours).

Sample	*isoDG (N86)*	*PENisoDY (N92)*	*PEisoDNY (N91)*	*PEDNY/PENDY (N91/92)*	*DG (N86)*
Animal 1	15.63%	12.70%	18.46%	37.42%	15.79%
Animal 2	17.88%	15.33%	20.27%	31.46%	15.06%
Animal 3	14.38%	14.25%	15.42%	41.98%	13.97%
Spiked	16.12%	22.95%	14.05%	34.46%	12.42%

Table 16. Detected relative deamidation rates at the tryptic PENNY peptide in the Fc region normalized to 100% per sample. The considered time point is 28 days after administration. Deamidation rates for 3 individual animals and in vitro incubated spiked pooled animal serum were considered.

The observations of this chapter allowed to obtain three main results:

- Rates of deamidation over time are dependent on the individual

- Despite the different rate over time, a similar pattern of deamidation between possible peptide variants was observed between models (in vivo and in vitro)
- No correlation between pooled serum (in vitro samples) and in vivo samples was seen

Discussion

The developed and validated LC-MS/MS method for quantification of low-abundant modifications in animal serum (along with the proposed quantification strategy for modifications) was applied to the following samples as proof of concept:

- In vivo: mini cohort of three animals
- In vitro: spiked incubations in pooled animal serum

As presented in Chapter II, 3, only three out of seven available animal models could be included in the study due to the low concentration of Pertuzumab at day 28 after administration present in the other 4 animals (see Table 7 in Chapter II). The animal individuals had received intravenous treatment with Pertuzumab, and samples were taken at certain time points (see Material and Methods section V). The in vitro incubations were performed by spiking Pertuzumab in pooled animal serum and by taking samples at the same time points as the ones when samples were taken from the individual animals.

After analysis of these samples with the validated LC-MS/MS method, it was possible to determine the concentration of Pertuzumab in in vivo samples over time through the stable DST peptide. The concentrations obtained through the LC-MS/MS method correlated well with ELISA results. By this, accuracy and precision of the method were confirmed. Although it was expected to obtain a slight deviation from values determined through the ELISA method, these deviations were observed to be minimal between the two methodologies.

In addition to the determination of Pertuzumab concentration, the analysis of the samples allowed to quantify and compare rates of degradation between animals and models (in vivo and in vitro). Main findings from this analysis (as previously presented in the Results section) were:

1) The rates of deamidation over time differed between individuals and none of the individual models could be correlated with the in vitro model (incubated pooled animal serum).
2) The difference in deamidation formation over time was evident only after long periods of incubation posterior to 3 weeks.
3) A higher propensity to deamidation seen in the Fc region correlated to a higher propensity for deamidation in the CDR region of animal 3. Similarly, in animal 2 a lower propensity to deamidation seen in the Fc region correlated to a lower propensity for deamidation in the CDR region.
4) Despite the different velocities of deamidation formation over time within individuals and the in vitro model, a similar pattern of distribution was found between the possible sites of deamidation in the PENNY peptide.

From these results, it was possible to draw four main hypotheses:

1) The deamidation rate formation in vivo over time is dependent on the individual.
2) In vitro models using pooled serum may not reproduce the in vivo variability for deamidation formation.
3) Deamidation in the Fc region may be used as a "red flag" or an indicator of the overall degradation propensity of the molecule (also in CDR regions) in a particular individual.
4) Despite forming at different velocities, the deamidation pattern between individuals would remain almost identical.

Being able to draw these conclusions may be of major impact for the feasibility and complexity of quantification methods for low-abundant modifications. The correlation between Fc and CDR deamidation propensity within individuals is of particular interest. Monitoring solely the Fc region of a mAb would make it possible to assess if further efforts for degradation studies should be taken to the study of CDR regions. The observed differences in degradation propensity in the Fc region between mAbs correlated with the previous findings observed during incubation of mAbs (SIL IgG1 and Trastuzumab) at high pH, temperature, and oxidative stress conditions (Chapter IV, subsection 1). It was found that very different time frames between SIL IgG1 and Pertuzumab (15 vs. 44 days respectively) were needed to reach ~40% deamidation in one Asparagine of the PENNY peptide. Similarly, a higher deamidation in the Fc region (PENNY peptide) was observed for Trastuzumab compared to Pertuzumab under the same conditions. This can be explained by Trastuzumab showing higher deamidation rates in CDR regions while Pertuzumab had a higher stability. Through the observations on the stress incubations in Chapter IV, 1 and the analysis of in vivo and in vitro samples in this chapter, it was possible to conclude that stability in the Fc region (deamidation propensity) may be affected by the 3D folding structure of the protein. Furthermore, it could be concluded that stability in the Fc region may vary between different mAbs. Its study may serve as an indicator of the overall deamidation propensity of the molecule in different mAbs and biosimilars.

Another main conclusion of impact that could be drawn was that in vivo deamidation rates were dependent on the individual. Unknown in vivo factors in individuals may influence the deamidation rates of Pertuzumab. These results correlated to the results seen in reported studies in the literature with Trastuzumab in clinical samples[129], where deamidation rates were also dependent on the individual. However, it was additionally assessed within this study that although deamidation velocity formation was also dependent on the individual, a similar distribution pattern was observed between the different degradation products of the PENNY peptide (both for individual samples and for pooled serum). A theoretical ~3:1 ratio between iso-aspartic vs. aspartic acid that was reported (ex vivo) in the literature[130,131] could not be observed in this distribution of deamidated products. This was hypothesized to be an indicator of the enzymatic in vivo function of the PIMT enzyme. This function should have a repairing mechanism activity in the conversion of iso-Asp to Asp.

Results observed during this analysis elucidated the challenges and further efforts needed in the study and understanding of individual variability in deamidation pathways. As expected, it was confirmed that incubation using individual serum of each animal at pre-dose must be performed to reproduce the individual deamidation rates in vitro.

During the analysis of in vivo and in vitro samples, individual variability in deamidation formation was concluded. Incubations using individual serum would be of interest to assess if the same deamidation formation over time is reproducible (in vitro vs. in vivo) just by the presence of the individual matrix, or if another underlying biological mechanism plays a role in this different deamidation seen in vivo. Proving the first scenario (reproducibility observed) would be of major interest for the clinic as it would enable to study the treatment response per patient beforehand and assess if a potency decrease or bioactivity of the drug will occur in a particular individual. A dose adjustment and an individualized regime in the clinic could be implemented like that.

Proving the later scenario (no reproducibility in vitro despite the use of individual serum for incubation) would imply that higher efforts are needed to study the biological in vivo mechanisms that are responsible for the observed variability. In here, the use of cell culture and cancerous tissue models would be interesting to explore. Furthermore, 3D protein structural conformation studies could be envisioned to study the impact of the 3D structure on

- the incubation conditions
- the interaction with other proteins
- the presence of other modifications

This would allow to acquire a broader understanding of the underlying mechanisms for individual degradation propensity and provide further knowledge of in vivo variability and response to treatment.

To date, very limited quantitative studies with the aim of characterizing individual variation in post-translation modification levels have been reported. For example, Brett et al. have recently explored the quantification of phosphorylation levels for three genotyped human cell lines through an LC-MS/MS method. Phosphopeptide variability between individuals was found[132]. In their studies they concluded that protein length, connectivity, and/or expression level may serve as a functional buffer against inter-individual phosphorylation variation. However, further studies are required with a broader sample size.

Inter-individual variability in protein modification may underlie phenotypic differences as in any other inter-individual phosphorylation variational molecular regulatory phenotype. However, as in the case of deamidation (being a non-enzymatic reaction), deeper understanding and research of the underlying mechanism for this inter-variability is needed.

Conclusion

The growing development and research behind bio-therapeutics and biosimilars must be paired with the development and optimization of platforms and methodologies for their correct characterization and assessment. It is essential to be able to reliably characterize species and quantify quality attributes in vivo that may have an impact on the safety and bioactivity of biotherapeutics. The approach presented within this study did not only show the challenges when monitoring and validating the quantification of low-abundant modified species in biological matrices or in vivo. It also elucidated the complexity of such assays when starting materials such as controlled, fully modified, and characterized mAbs or intact heavy-labeled standards are not available.

Nevertheless, the developed method shows the possibility of giving an initial assessment of the relative presence of modifications with a potential undesired pharmacokinetic impact. It also allowed to propose a quantification strategy to overcome the theoretical risk of a matrix effect on the quantification of peptide variants. Furthermore, the method may be extrapolated to the analysis of Fc regions of different mAbs and biosimilars, providing a first rapid screening of stability and possible estimation of overall deamidation. This can be taken as a first indicator for the decision whether further efforts to monitor CDR regions should be developed.

Finally, it was possible to observe a correlation between the obtained results and the studies of clinic sample in the literature[129], where deamidation was also observed to be dependent on the individual. However, it was additionally observed that the deamidation pattern distributions between possible peptide variants seemed similar between individuals and in vitro models despite different velocities of formation.

For a holistic picture on the analysis of biotherapeutics and PTMs in vivo and in complex matrices, a deeper examination of the processes ranging from sample preparation to validation and analysis is needed to envision the observed individual variability. Further optimizations and applications of robust and sensitive methodologies that allow the quantification of PTMs in vivo are crucial to provide a better scope of information for the metabolic fate of biotherapeutics in the clinic. Additionally, the question of how to analyze possible PTMs occurring through bottom-up approaches in absence of heavy-labeled intact standards is a field of interest to further explore.

Outlook

Although this study specifically focused on the quantification of low-abundant deamidation and oxidation in mAbs by using NWZ animal serum, the developed method and quantification strategy may be applied to other modifications, antibodies, and biological matrices. The developed method provides an insight not only on the challenges encountered within MAMs validation, but also on further examinations when studying and quantifying low-abundant degradation products of therapeutics mAbs after dosage in both exploratory animal study and clinical samples. Additionally, the observed variability for in vivo deamidation may serve as ground for the design of simulated in vitro incubation systems with the use of individual serum. In this respect, not only individual animal serum may be used in vitro, but also in vivo experiment designs with a bigger cohort of individual animals and a higher initially administered dose (to not encounter sensitivity constraints derived from low concentrations in vivo at day 28 or posterior times) may be explored. Additionally, further conformation studies, cell culture, and tissue models may be investigated. Further scopes of research in this area may focus on the in vivo variability of mAbs degradation, and incubation systems to reproduce this degradation in vitro.

Material and Methods

For full details on the documentation of experiments in lab journals and storage of raw data, please refer to Tables S1 and S2 in the Supplemental Material.

In Table 16 all the chemicals, instruments and software used during all experiments are presented. For each experiment in this section, the specific chemicals used are stated for comprehension purposes.

Chemical/Instrument/Software	Distributor
AccuMAP 10x low pH reaction buffer	Promega
AccuMAP denaturing solution	Promega
AccuMAP low pH resistant rLys-C solution	Promega
AccuMAP modified trypsin solution	Promega
Acetic acid	Merck
Acetonitrile	VWR
Acquity Peptide BEH C18, 300A column	Waters
Acquity UPLC system	Waters
Albumin	Sigma-Aldrich
Amicon® tubes MWCO 30k Da	Merck
Amicon® Ultra MWCO 10K Da	Merck
BCA Protein Assay kit	Thermo Fischer
Chromeleon 7.2 software	Thermo Fischer
DTT	Merck
Dulbecco's PBS	Life Technologies
EDTA	Sigma-Aldrich
Excel sheets	Microsoft Office
Formic acid	Honeywell
GeneData software	GeneData
Guanidine HCL	Sigma-Aldrich
Guanidine HCL Solution 8M	Thermo Fischer
HCL 0.1mol/L	Merck
Histidine monohydrochloride	Sigma-Aldrich
Hydrogen peroxide	Sigma-Aldrich
Iodoacetamide	Merck
Iodoacetamide	Promega
LC system 1290 Infinity II	Agilent
L-histidine	Sigma-Aldrich
L-Methionine	Merck
Lys-C mass spectrometry grade	Promega
Mass Hunter Qualitative Analysis	Agilent
Mass Hunter Workstation Data Acquisition	Agilent
Orbitrap Fusion mass spectrometer	Thermo Fischer
PENNY variant 1:iso-aspartic acid N386 (DG) synthetic non-heavy labeled peptide 100ug/ml in 0.1%FA in water	Bachem
PENNY variant 2: aspartic acid N386 (DG) synthetic non-heavy labeled peptide 100ug/ml in 0.1%FA in water	Bachem

PENNY variant 3: aspartic acid N391 (PENDY) synthetic non-heavy labeled peptide 100ug/ml in 0.1%FA in water	Bachem
PENNY variant 4: iso-aspartic acid N391 (PEisoDNY) synthetic non-heavy labeled peptide 100ug/ml in 0.1%FA in water	Bachem
PENNY variant 5: aspartic acid N392 (PEDNY) synthetic non-heavy labeled peptide 100ug/ml in 0.1%FA in water	Bachem
PENNY variant 6: iso-aspartic acid N392 (PENisoDY) synthetic non-heavy labeled peptide 100ug/ml in 0.1%FA in water	Bachem
PENNY variant 7: non-deamidated synthetic non-heavy labeled peptide 100ug/ml in 0.1%FA in water	Bachem
Perjeta® (Pertuzumab) batch H050B03	Roche
Plates 96 wells	Thermo Fischer
Pooled gender animal serum recovered from whole blood donations, non-filtrated, without anticoagulants	BioIVT
Q-Exactive Plus	Thermo Fischer
Sodium hydroxide	Sigma-Aldrich
SOLAu SPE plate	Thermo Fischer
Stable isotopically labeled IgG1	Promega
Stable isotopically labeled peptide H-GLEWVADVNPNSGGSIYNQR*-OH, 99% purity, MW 2186.4 Da, 1mg aliquots	PepScan
Stable isotopically labeled peptide H-NTLYLQMNSLR*-OH, 93.9% purity, MW 1362.6 Da, 1mg aliquots	PepScan
TCEP	Promega
TFA	Honeywell
Herceptin® (Trastuzumab) batch 872468	Genentech
Triple quadrupole mass spectrometer Agilent 6400 series	Agilent
Tris HCL	VWR
Trizma	Sigma-Aldrich
Trypsin Gold, mass spectrometry grade trypsin	Promega
tween 20(aqueous)	CalbioChem
Urea	Sigma-Aldrich
V bottom 96 well plates	Thermo Fischer
Xcalibur 3.1 Qual Browser	Thermo Fischer
Xcalibur Data Acquisition and Interpretation software	Thermo Fischer

Table 17. List of all chemicals, instruments and software used during this study.

I. Comparison of sample digestion parameters in terms of efficiency and side reactions including deamidation and oxidation

1. Digestion protocols

A. Digestion with trypsin at pH value of 7.0

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml. *Herceptin® (Trastuzumab)* authorized from Genentech (US) batch 872468 kept at -80 °C in aliquots of 100ul at concentration of 21.30 mg/ml.

Dulbecco's PBS was obtained from Life Technologies. Tris HCL (hydrogen chloride) was obtained from VWR. Trizma, EDTA (ethylenediaminetetraacetic acid), guanidine HCL, histidine monohydrochloride, L-histidine and urea were all obtained from Sigma-Aldrich. Acetic acid, DTT (dithiothreitol), iodoacetamide and HCL 0.1mol/L were obtained from Merck. Formic acid was obtained from Honeywell. Trypsin Gold, mass spectrometry grade trypsin from was obtained from Promega. Amicon® Ultra 10K centrifugal filter devices were obtained from Merck.

All buffers were adjusted to a pH of 7.0 by dropwise addition of 1 M HCL and sterile filtered through a 0.22 um cellulose acetate membrane. 0.1 M Tris solution was prepared by diluting Tris HCL in MiliQ water. 10mM EDTA was prepared by dissolving EDTA in 0.1 M Tris already at a pH of 7.0. Denaturizing buffer was 6 M guanidine HCL, 1mM EDTA in 0.1M Tris at pH 7.0. Acetic acid 0.05 M was prepared in MiliQ water. DTT 500 mM solution was prepared by dissolving DTT in MiliQ water. IAM (Iodoacetamide) 500 mM solution was prepared by dissolving IAM in MiliQ water. Trypsin 1ug/ul solution was prepared by dissolving 25 ug of trypsin in 25 ul 0.05 M acetic acid. Digestion buffer was 1M urea dissolved in 0.1M Tris at pH of 7.0.

Sample preparation

All experiments were performed in triplicate. 500 ug of either Pertuzumab or Trastuzumab were added to 445 ul of denaturizing buffer. 50 ul of MiliQ water were added and 5 ul of 500 mM DTT solution. Samples were then reduced by incubation at 4 °C for 60 minutes at the dark. Alkylation of samples was performed by addition of 10 ul of 500 mM IAM solution and incubating at 4 °C for 60 minutes in the dark. A buffer exchange step was performed using Amicon® tubes. Centrifugation was performed at 14,000 rcf for 10 minutes at room temperature after each washing steps (3 in total) with 450 ul of digestion buffer. The remaining volume was recovered via reverse centrifugation of the filter unit and volume was adjusted to 50 ul with digestion buffer. To the total volume, 3 ul of trypsin solution 1 ug/ul were added for a 1:17 enzyme/mAb ratio. 92ul of digestion buffer were added to this solution and the digestion was carried at room temperature (20 °C) for 4 hours. After this incubation time, the reaction was stopped with addition of 100 ul of 0.1% FA (formic acid) in MiliQ water. The final protein concentration was of 0.25 ug/ul.

B. Digestion with trypsin at pH value of 7.8

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml. *Herceptin® (Trastuzumab)* authorized from Genentech (US) batch 872468 kept at -80 °C in aliquots of 100ul at concentration of 21.30 mg/ml. Dulbecco's PBS was obtained from Life Technologies. Tris HCL was obtained from VWR. Trizma, EDTA, guanidine HCL, histidine monohydrochloride, L-histidine and urea were all obtained from Sigma-Aldrich. Acetic acid, DTT, iodoacetamide and HCL 0.1mol/L were obtained from Merck. Formic acid was obtained from Honeywell. Trypsin Gold, mass spectrometry grade

trypsin from was obtained from Promega. Amicon® Ultra 10K centrifugal filter devices were obtained from Merck.

All buffers were adjusted to a pH of 7.8 by dropwise addition of 1 M HCL and sterile filtered through a 0.22 um cellulose acetate membrane. 0.1 M Tris solution was prepared by diluting Tris HCL in MiliQ water. 10mM EDTA was prepared by dissolving EDTA in 0.1 M Tris already at a pH of 7.8. Denaturizing buffer was 6 M guanidine HCL, 1mM EDTA in 0.1M Tris at pH 7.8. Acetic acid 0.05 M was prepared in MiliQ water. DTT 500 mM solution was prepared by dissolving DTT in MiliQ water. IAM 500 mM solution was prepared by dissolving IAM in MiliQ water. Trypsin 1ug/ul solution was prepared by dissolving 25 ug of trypsin in 25 ul 0.05 M acetic acid. Digestion buffer was 1M urea dissolved in 0.1M Tris at pH of 7.8.

Sample preparation

All experiments were performed in triplicate. 500 ug of either Pertuzumab or Trastuzumab were added to 445ul of denaturizing buffer. 50ul of MiliQ water were added and 5 ul of 500mM DTT solution. Samples were then reduced by incubation at 4 °C for 60 minutes at the dark. Alkylation of samples was performed by addition of 10 ul of 500 mM IAM solution and incubating at 4 °C for 60 minutes in the dark. A buffer exchange step was performed using Amicon® tubes. Centrifugation was performed at 14,000 rcf for 10 minutes at room temperature after each washing steps (3 in total) with 450 ul of digestion buffer. The remaining volume was recovered via reverse centrifugation of the filter unit and volume was adjusted to 50 ul with digestion buffer. To the total volume, 3 ul of trypsin solution 1 ug/ul were added for a 1:17 enzyme/mAb ratio. 92ul of digestion buffer were added to this solution and the digestion was carried at room temperature (20 °C) for 4 hours. After this incubation time, the reaction was stopped with addition of 100 ul of 0.1% FA in MiliQ water. The final protein concentration was of 0.25ug/ul.

C. Digestion with recombinant Lys-C + recombinant trypsin at pH value of 5.5

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml. *Herceptin® (Trastuzumab)* authorized from Genentech (US) batch 872468 kept at -80 °C in aliquots of 100ul at concentration of 21.30 mg/ml. Dulbecco's PBS was obtained from Life Technologies. L-Methionine was obtained from Merck. TCEP (tris(2-carboxyethyl) phosphine), IAM, AccuMAP denaturing solution, AccuMAP 10x low pH reaction buffer, AccuMAP low pH resistant rLys-C solution and AccuMAP modified trypsin solution were all obtained from Promega from their AccuMAP low pH protein digestion mini kit.

Sample preparation:

All experiments were performed in triplicate. 50ug of either Pertuzumab or Trastuzumab was added to 20ul of denaturing solution, 6ul of AccuMAP 10x low pH reaction buffer, and 1ul of 100mM TCEP. The sample was denaturized and reduced though incubation at 37°C for 30 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37

°C for 30 minutes in the dark. A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 3 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample. The final estimated concentration was of 0.30ug/ul. The samples were diluted with 2% v/v FA in water to obtain a final protein concentration of 0.25ug/ul.

D. Digestion with recombinant Lys-C at pH value of 5.5

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml. *Herceptin® (Trastuzumab)* authorized from Genentech (US) batch 872468 kept at -80 °C in aliquots of 100ul at concentration of 21.30 mg/ml. Dulbecco's PBS was obtained from Life Technologies. L-Methionine was obtained from Merck. TCEP, IAM, AccuMAP denaturing solution, AccuMAP 10x low pH reaction buffer, AccuMAP low pH resistant rLys-C solution and AccuMAP modified trypsin solution were all obtained from Promega from their AccuMAP low pH protein digestion mini kit.

Sample preparation:

All experiments were performed in triplicate. 50ug of either Pertuzumab or Trastuzumab was added to 20ul of denaturing solution, 6ul of AccuMAP 10x low pH reaction buffer, and 1ul of 100mM TCEP. The sample was denaturized and reduced though incubation at 37°C for 30 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37 °C for 30 minutes in the dark. A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 75ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C at 37 °C for 3 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample. The final estimated concentration was of 0.30ug/ul. The samples were diluted with 2% v/v FA in water to obtain a final protein concentration of 0.25ug/ul.

E. Digestion with Lys-C at pH value of 7.0

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml. *Herceptin® (Trastuzumab)* authorized from

Genentech (US) batch 872468 kept at -80 °C in aliquots of 100ul at concentration of 21.30 mg/ml. Dulbecco's PBS was obtained from Life Technologies. Tris HCL was obtained from VWR. EDTA was obtained from Sigma-Aldrich. DTT, iodoacetamide and HCL 0.1mol/L were all obtained from Merck. Formic acid was obtained from Honeywell and tween 20(aqueous) from CalbioChem. Lys-C mass spectrometry grade obtained from Promega was used for enzymatic digestion.

The denaturing solution was prepared as follows: 6M guanidine hydrocholide, 50mM Tris HCL and 5mM EDTA were dissolved in MiliQ water. Digestion buffer consisted in: 50mM sodium citrate tribasic dehydrate in MiliQ water and the pH was adjusted to 7.0 with 0.1M HCL. 0.5M DTT solution, 1M IAA, 10% w/w Tween 20(aq) and Lys-C solution 0.5ug/ul were all prepared in MiliQ water.

Sample preparation:

All experiments were performed in triplicate. 480 ug of either Pertuzumab or Trastuzumab were added to 5ul of 0.5M DTT solution. The sample was denaturized 37 °C for 15minutes at 350 rpm. 5ul of 1M IAA were added to the sample and alkylation was performed at 25 °C for 1 hour at 350 rpm in the dark. The sample was removed and let to cool down at room temperature for 5 minutes. 740ul of digestion buffer were added to the sample ad 460ul of this solution was pipetted into a new 1.5 ml Eppendorf tube. 10ul of Lys-C solution (0.5ug/ul) was added to the sample and digestion was performed at 37 °C for 1 hour at 350 rpm. After this time, another 10ul of Lys-C solution (0.5ug/ul) were added to the sample. The sample was incubated at 37 °C for 3 hours at 350 rpm. The reaction was terminated by addition of 3ul of FA. Final protein concentration estimated was of 0.48ug/ul. The sample was diluted with FA 2% in MiliQ water to a final protein concentration of 0.25ug/ul and divided in 20ul aliquots.

2. Analysis of all samples

Peptide mapping: Analysis of all samples was conducted using an Agilent 1290 Infinity II LC system coupled to a Q-Exactive Plus mass spectrometer from Thermo Fischer.

Chromatographic separation was performed at 60 °C on a 2.1x 150mm, 1.7um particle size, Acquity Peptide BEH C18, 300A column obtained from Waters. Mobile phase A consisted of 0.1% TFA (trifluoroacetic acid) in water and mobile phase B was 0.08% TFA in acetonitrile. The peptides were separated by gradient elution using the following settings: 0.0 - 2.0min: 2% B; 2.0 - 17.0 min: 2 - 22% B; 17.0 - 47.0 min: 22 - 42% B; 47.0 - 47.5 min: 42 - 95% B; 47.5 - 51.5 min: 95% B; 51.5 - 52.5 min: 95 - 2% B; 52.5 -60 min: 2% B. A total of 2.5ug of protein for samples from each protocol was injected per analysis.

Mass spectrometric settings for peptide mapping were as follows:

- Full MS/ddMS2 (TopN) method. Full MS scan resolution of 70,000, positive mode, microscans: 1, AGC (automatic gain control) target 3e6, maximum IT (injection time): 100 ms, number of scan ranges: 200 to 3000m/z.

- dd-MS2 Scan: resolution of 17,500, AGC target 1e5, maximum IT 100ms, isolation window 3.0m/z, isolation offset 0.0 m/z, scan range: 200 to 2000m/z.
- ddS settings: minimum AGC target: 1.00e3, intensity threshold 1.0e4, isotope exclusion, dynamic exclusion 3.0s.
- Source parameters: sheat gas flow rate: 55, aux. gas flow rate: 15, spray voltage: 3.5, capillary temperature: 300. Valve was diverted to waste at minutes 0-2.0 and 50.0-60.0.

Data evaluation was performed in GeneData software and Excel sheets (Microsoft Office). Optimal parameters for analysis in GeneData workflows were tested and chosen. These included but were not limited to: Intensity thresholding, RT alignment, peak detection settings and isotope clustering settings. All detected peptides were verified and confirmed with MS/MS data.

II. Development of an LC-MS/MS method for the quantification of side reactions including deamidation and oxidation during digestion of Pertuzumab in a biological matrix

1. Incubations at oxidative, pH and temperature stress conditions (Peptides selection)

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml. Dulbecco's PBS was obtained from Life Technologies. L-Methionine was obtained from Merck. TCEP, IAM, AccuMAP denaturing solution, AccuMAP 10x low pH reaction buffer, AccuMAP low pH resistant rLys-C solution and AccuMAP modified trypsin solution were all obtained from Promega from their AccuMAP low pH protein digestion mini kit. Amicon® tubes MWCO (molecular weight cut off) 30k Da were obtained from Merck. Sodium hydroxide 1M and 0.1M as well as hydrogen peroxide were obtained from Sigma-Aldrich.

Sample preparation:

All the following incubations were performed in triplicate.

- *pH stress*: Aliquots of 1ml of Pertuzumab originator at a concentration of 30mg/ml were added dropwise 1M NaOH (sodium hydroxide) and 0.1M NaOH until a pH of 8.5 was reached. The aliquots were incubated at 25 °C for 4 and 7 days. Two controls at the formulation's original pH of 6.15 were incubated for the same period of time and at the same conditions of temperature.
- *Temperature stress*: Aliquots of 100ul of Pertuzumab originator at a concentration of 30mg/ml were incubated at 25 °C for 4 weeks and at 40 °C for 2 weeks.
- *Oxidative (H_2O_2) stress*: Aliquots of 100ul of Pertuzumab originator at a concentration of 30mg/ml were added hydrogen peroxide (H_2O_2) to a final concentration of 0.1% v/v. The final protein concentration was of 25mg/ml.

- *Non-stressed sample*: An aliquot of 1ml Pertuzumab at a concentration of 30mg/ml at the formulation's original pH of 6.15 and kept at all times at -80 °C served as negative control. These samples were thawed until the moment of sample preparation and digested at the same time that the stressed samples.

After stress conditions all aliquots were buffer exchanged through 3 washes with 300ul of PBS using Amicon® tubes MWCO 30k Da, adjusting the final volume of PBS to a theoretical protein concentration of 10mg/ml in the solution. 5ul of this solution in all samples (50ug of protein) were digested with protocol C (digestion with recombinant Lys-C + recombinant trypsin at pH value of 5.5) presented in Chapter I and analyzed by LC-MS/MS. Peptides with highest percentage of deamidation and oxidation were shortlisted.

LC-MS/MS analysis

All samples were analyzed in triplicate using an Agilent 1290 Infinity II LC system coupled to a Q-Exactive Plus mass spectrometer from Thermo Fischer. A peptide mapping was performed for analysis of samples.

Chromatographic separation was performed at 60 °C on a 2.1x 150mm, 1.7um particle size, Acquity Peptide BEH C18, 300A column obtained from Waters. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.08% TFA in acetonitrile. The peptides were separated by gradient elution using the following settings: 0.0 - 2.0min: 2% B; 2.0 - 17.0 min: 2 - 22% B; 17.0 - 47.0 min: 22 - 42% B; 47.0 - 47.5 min: 42 - 95% B; 47.5 - 51.5 min: 95% B; 51.5 - 52.5 min: 95 - 2% B; 52.5 -60 min: 2% B.

Mass spectrometric settings for peptide mapping were as follows:

- Full MS/ddMS2 (TopN) method. Full MS scan resolution of 70,000, positive mode, microscans: 1, AGC target 3e6, maximum IT: 100 ms, number of scan ranges: 200 to 3000m/z.
- dd-MS2 Scan: resolution of 17,500, AGC target 1e5, maximum IT 100ms, isolation window 3.0m/z, isolation offset 0.0 m/z, scan range: 200 to 2000m/z.
- ddS settings: minimum AGC target: 1.00e3, intensity threshold 1.0e4, isotope exclusion, dynamic exclusion 3.0s.
- Source parameters: sheat gas flow rate: 55, aux. gas flow rate: 15, spray voltage: 3.5, capillary temperature: 300. Valve was diverted to waste at minutes 0-2.0 and 50.0-60.0.

Data evaluation was performed in GeneData software and Excel sheets (Microsoft Office). All detected peptides were verified and confirmed with MS/MS data.

2. Tested methods for chromatographic separation optimization

Triplicates of samples of Pertuzumab incubated under pH stress conditions as explained in the previous section (Chapter II.1) were digested with protocol C (digestion with recombinant Lys-C + recombinant trypsin at pH value of 5.5) presented in Chapter I. The samples were then analyzed

in triplicate using an Agilent 1290 Infinity II LC system coupled to a Q-Exactive Plus mass spectrometer from Thermo Fischer. A peptide mapping was performed for analysis of samples.

Chromatographic separation for all tested methods was performed at 60 °C on a 2.1x 150mm, 1.7um particle size, Acquity Peptide BEH C18, 300A column obtained from Waters. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.08% TFA in acetonitrile.

Different chromatographic methods by gradient elution using the following settings were tested:

- Gradient A: 0.0 - 2.0 min: 2% B; 2.0 - 15.0 min: 2 - 19% B; 15.0 - 35.0 min: 19 - 19% B; 35.0 – 55 min: 30% B; 55 – 70 min: 42% B; 70 - 70.5 min: 42 - 95% B; 70.5 - 74.5 min: 95% B; 74.5 - 75.5 min: 2% B; 75.5 – 83 min: 95% B
- Gradient B: 0.0 - 2.0 min: 2% B; 2.0 - 17.0 min: 2 - 20% B; 17.0 - 47.0 min: 20 - 30% B; 47.0 - 87.0 min: 30 - 42% B; 87 - 87.5 min: 42 - 95% B; 87.5 – 91 min: 95% B; 91.0 - 92.5 min: 95 - 2% B; 92.5 – 100 min: 2% B
- Gradient C: 0.0 - 2.0 min: 15% B; 2.0 - 32.0 min: 15 - 25% B; 32.0 - 33.0 min: 25 - 95% B; 33.0 - 36.0 min: 95% B; 36.0 – 37 min: 95 - 15% B; 37.0 - 40.0 min: 15% B
- Gradient D: 0.0 - 5.0 min: 2 - 15% B; 5.0 - 38.0 min: 15 - 22.62% B; 38.0 - 38.5 min: 22.62 - 95% B; 38.5 - 41.5 min: 95% B; 41.5 - 42.0 min: 95 - 2% B; 42.0 - 45.0 min: 2% B.

Mass spectrometric settings for peptide mapping were as follows:

- Full MS/ddMS2 (TopN) method. Full MS scan resolution of 70,000, positive mode, microscans: 1, AGC target 3e6, maximum IT: 100 ms, number of scan ranges: 200 to 3000m/z.
- dd-MS2 Scan: resolution of 17,500, AGC target 1e5, maximum IT 100ms, isolation window 3.0m/z, isolation offset 0.0 m/z, scan range: 200 to 2000m/z.
- ddS settings: minimum AGC target: 1.00e3, intensity threshold 1.0e4, isotope exclusion, dynamic exclusion 3.0s.
- Source parameters: sheat gas flow rate: 55, aux. gas flow rate: 15, spray voltage: 3.5, capillary temperature: 300.

Data evaluation was performed in GeneData software and Excel sheets (Microsoft Office). All detected peptides were verified and confirmed with MS/MS data.

3. Protein digestion efficiency optimization

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml. Dulbecco's PBS was obtained from Life Technologies. 8M guanidine HCL Solution was obtained from Thermo Fischer, L-Methionine was obtained from Merck. TCEP, IAM, AccuMAP denaturing solution, AccuMAP 10x low pH reaction buffer, AccuMAP low pH resistant rLys-C solution and AccuMAP modified trypsin solution were all obtained from Promega from their AccuMAP low pH protein digestion mini kit. SOLAu SPE plate and V bottom 96 well plates were both obtained from Thermo Fischer. Stable

isotopically labeled IgG1 from Promega 100ug aliquots. Stable isotopically labeled peptides from PepScan 1) H-GLEWVADVNPNSGGSIYNQR*-OH, 99% purity, MW (molecular weight) 2186.4 Da, 1mg aliquots. 2) H-NTLYLQMNSLR*-OH, 93.9% purity, MW 1362.6 Da, 1mg aliquots. Pooled gender animal serum recovered from whole blood donations, non-filtrated, without anticoagulants, obtained from BioIVT.
IS mix: stable isotopically labeled IgG1 and stable isotopically labeled peptides from PepScan were mixed in a solution with a final concentration of 0.2ug/ul per peptide in 0.1% FA in MiliQ water.

Sample preparation:

All experiments were performed in triplicate. Samples of Pertuzumab at a concentration of 0.01ug/ul in animal serum were prepared through a stepwise dilution as following:

10ug of Pertuzumab at 30mg/ml was diluted in 140ul of animal serum. (2mg/ml)

10ul of the 2mg/ml solution was diluted with 10ul of animal serum. (1mg/ml)

10ul of the 1mg/ml solution was diluted with 90ul of animal serum. (0.1ug/ul)

10ul of the 0.1mg/ml solution was diluted with 90ul of animal serum. (0.01ug/ul)

All samples were subjected to a clean-up step (SPE solid phase extraction) described at the end of this subsection.

- ***Test 1: no denaturation***

10ul of the IS mix was added to 10ul of Pertuzumab at a concentration of 0.01ug/ul in animal serum. 6ul of AccuMAP 10x low pH reaction buffer, and 1ul of 100mM TCEP were added. Samples were reduced though incubation at 37°C for 30 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37 °C for 30 minutes in the dark. A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 3 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample.

- ***Test 2: denaturation solution from vendor (Promega) for 30min***

10ul of the IS mix was added to 10ul of Pertuzumab at a concentration of 0.01ug/ul in animal serum. 20ul of denaturing solution, 6ul of AccuMAP 10x low pH reaction buffer, and 1ul of 100mM TCEP were added. Samples were denaturized and reduced though incubation at 37°C for 30 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37 °C for 30 minutes in the dark. A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and

4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 3 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample.

- ***Test 3: conditions of test 2 + guanidine HCL addition***

10ul of the IS mix was added to 10ul of Pertuzumab at a concentration of 0.01ug/ul in animal serum. 5ul of 6M guanidine HCL, 20ul of denaturing solution, 6ul of AccuMAP 10x low pH reaction buffer, and 1ul of 100mM TCEP were added. Samples were denaturized and reduced though incubation at 37°C for 30 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37 °C for 30 minutes in the dark. A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 3 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample.

- ***Test 4: conditions of test 3 + increase of time (30 min to 45 min)***

10ul of the IS mix was added to 10ul of Pertuzumab at a concentration of 0.01ug/ul in animal serum. 5ul of 6M guanidine HCL, 20ul of denaturing solution, 6ul of AccuMAP 10x low pH reaction buffer, and 1ul of 100mM TCEP were added. Samples were denaturized and reduced though incubation at 37°C for 45 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37 °C for 30 minutes in the dark. A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 3 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample.

- ***Test 5: conditions of test 4 + increase of time (45 min to 90 min)***

10ul of the IS mix was added to 10ul of Pertuzumab at a concentration of 0.01ug/ul in animal serum. 5ul of 6M guanidine HCL, 20ul of denaturing solution, 6ul of AccuMAP 10x low pH reaction buffer, and 1ul of 100mM TCEP were added. Samples were denaturized and reduced though incubation at 37°C for 90 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37 °C for 30 minutes in the dark. A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final

concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 3 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample.

- ***Different Reduction conditions (Test 1-3)***

10ul of the IS mix was added to 10ul of Pertuzumab at a concentration of 0.01ug/ul in animal serum. 5ul of 6M guanidine HCL, 20ul of denaturing solution, 6ul of AccuMAP 10x low pH reaction buffer, and 1ul of 100mM, 200mM or 300mM TCEP (test 1-3 respectively) were added. Samples were denaturized and reduced though incubation at 37°C for 90 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37 °C for 30 minutes in the dark. A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 3 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample.

- ***Pre-digestion with Lys-C tests (Test 1 and 2)***

10ul of the IS mix was added to 10ul of Pertuzumab at a concentration of 0.01ug/ul in animal serum. 5ul of 6M guanidine HCL, 20ul of denaturing solution, 6ul of AccuMAP 10x low pH reaction buffer, and 1ul of 100mM TCEP were added. Samples were denaturized and reduced though incubation at 37°C for 90 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37 °C for 30 minutes in the dark.

Test 1: Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 50ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 3 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample.

Test 2: A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 25ul (0.2ug/ul) of AccuMAP Low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 3 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample.

- ***Digestion times tests (Test 1-3)***

10ul of the IS mix was added to 10ul of Pertuzumab at a concentration of 0.01ug/ul in animal serum. 5ul of 6M guanidine HCL, 20ul of denaturing solution, 6ul of AccuMAP 10x low pH

reaction buffer, and 1ul of 100mM was added. Samples were denaturized and reduced though incubation at 37°C for 90 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37 °C for 30 minutes in the dark. A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 3, 4 or 5 hours (test 1-3 respectively) in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2% in the sample.

Solid phase extraction for all sample preparations

After digestion of the samples, a solid phase extraction step was performed in a SOLAu SPE plates. The SPE plate was equilibrated and conditioned with 200ul acetonitrile at centrifugation at 1000g for 1 min, and 200ul 0.1%TFA and centrifugation. The digested sample was prepared 1:1 v/v with 0.1%TFA in water and loaded into the plate wells and centrifuged. Sample was cleaned by addition of 500ul 0.1%TFA and centrifugation. Finally, the sample was eluted in a collection plate by 2 steps of 25ul 70% acetonitrile, 30% water addition into the well and centrifugation, Final volume of 50ul was collected and transferred into low protein binding 1.5ml Eppendorf tubes and evaporated until dryness in a Speedvac for 45 minutes at 22 °C. Samples were reconstituted in 10ul 0.1% FA in water.

LC-MS/MS analysis

Samples were analyzed in triplicate using an Agilent 1290 Infinity II LC system coupled to a Q-Exactive Plus mass spectrometer from Thermo Fischer. A PRM method was used for analysis of samples (See Table S2 in Supplemental Material for the inclusion list with specified precursors, scan time segments and CE applied per peptide).

Chromatographic separation was performed at 60 °C on a 2.1x 150mm, 1.7um particle size, Acquity Peptide BEH C18, 300A column obtained from Waters. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.08% TFA in acetonitrile. The peptides were separated by gradient elution using the following settings: 0.0 - 5.0 min: 2 - 15% B; 5.0 - 38.0 min: 15 - 22.62% B; 38.0 - 38.5 min: 22.62 - 95% B; 38.5 - 41.5 min: 95% B; 41.5 - 42.0 min: 95 - 2% B; 42.0 - 45.0 min: 2% B.

Mass spectrometric settings for PRM analysis were as follows:

- PRM method: positive mode, MS2 resolution of 17,500, AGC target 2e5, maximum injection time: 50 ms, isolation window: 1.6m/z, isolation offset: 0.0 m/z.
- Source parameters: sheat gas flow rate: 55, aux. gas flow rate: 15, spray voltage: 3.5, capillary temperature: 300. Valve was diverted to waste at minutes 0-2.0 and 42.0-45.0.

Data evaluation was performed in Chromeleon 7.2 software from Thermo Fisher and Excel sheets (Microsoft Office).

III. Comparison between SRM and PRM approaches in terms of sensitivity and specificity achieved in complex biological matrices

1. SRM method development

Analysis was performed in an Acquity UPLC (ultra-performance liquid chromatography) system coupled to an Agilent 6400 series triple quadrupole mass spectrometer from Agilent. A sample of digested Pertuzumab in PBS was analyzed in a full scan mode. Precursors with the highest intensity signal were selected for every peptide. Next an MS2 analysis varying collision energies was performed. Product ions with the highest intensity signal were selected per peptide. Next a "Dynamic SRM" method was created with the specified precursors, product ions, scan time segments and CE per peptide. (See Table S3 in Supplemental Material).

Chromatographic separation was performed at 60 °C on a 2.1x 150mm, 1.7um particle size, Acquity Peptide BEH C18, 300A column obtained from Waters. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.08% TFA in acetonitrile. The peptides were separated by gradient elution using the following settings: 0.0 - 5.0 min: 2 - 15% B; 5.0 - 38.0 min: 15 - 22.62% B; 38.0 - 38.5 min: 22.62 - 95% B; 38.5 - 41.5 min: 95% B; 41.5 - 42.0 min: 95 - 2% B; 42.0 - 45.0 min: 2% B.

Mass spectrometric settings for SRM analysis were as follows:

- Source parameters: gas temperature: 300 C, gas flow: 7 L/min, nebulizer: 25 psi, capillary voltage: 4000 V, cycle time 500 ms.

Data acquisition was performed in Agilent Mass Hunter Workstation Data Acquisition, while data analysis was performed in Mass Hunter Qualitative Analysis and Excel sheets (Microsoft Office).

2. PRM method development

Analysis was performed in an Agilent 1290 Infinity II LC system coupled to a Q-Exactive Plus mass spectrometer from Thermo Fischer. A sample of digested Pertuzumab in PBS was analyzed in a full scan mode. Precursors with the highest intensity signal were selected for every peptide. Next an MS2 analysis varying collision energies was performed. Product ions with the highest intensity signal were selected per peptide. A PRM method was created with the specified precursors, product ions, scan time segments and CE per peptide (See Table S2 in Supplemental Material)

Chromatographic separation was performed at 60 °C on a 2.1x 150mm, 1.7um particle size, Acquity Peptide BEH C18, 300A column obtained from Waters. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.08% TFA in acetonitrile. The peptides were

separated by gradient elution using the following settings: 0.0 - 5.0 min: 2 - 15% B; 5.0 - 38.0 min: 15 - 22.62% B; 38.0 - 38.5 min: 22.62 - 95% B; 38.5 - 41.5 min: 95% B; 41.5 - 42.0 min: 95 - 2% B; 42.0 - 45.0 min: 2% B.

Mass spectrometric settings for PRM analysis were as follows:

- PRM method: positive mode, MS2 resolution of 17,500, AGC target 2e5, maximum injection time: 50 ms, isolation window: 1.6m/z, isolation offset: 0.0 m/z.
- Source parameters: sheat gas flow rate: 55, aux. gas flow rate: 15, spray voltage: 3.5, capillary temperature: 300. Valve was diverted to waste at minutes 0-2.0 and 42.0-45.0.

Acquisition was performed in Xcalibur Data Acquisition and Interpretation software from Thermo Fischer. Data evaluation was performed in Xcalibur 3.1 Qual Browser from Thermo Fischer and Excel sheets (Microsoft Office). Monitored precursors and product ions used for quantification were: GLEW (m/z>1088.5245, y11>1192.57057), PENNY (m/z>1272.5693, y6>764.35739), NTLYL (m/z> 676.85323, y8>1024.52447), DST (m/z>751.8829, y8>836.47242).

3. Targeted SIM/dd-MS2 method development

Analysis was performed in an Agilent 1290 Infinity II LC system coupled to a Q-Exactive Plus mass spectrometer from Thermo Fischer. A "Targeted SIM/dd-MS2" method was used for analysis of the sample of digested Pertuzumab in PBS as analyzed in the PRM method. The same inclusion list as in PRM was used excluding the scan time segments per peptide.

Chromatographic separation was performed at 60 °C on a 2.1x 150mm, 1.7um particle size, Acquity Peptide BEH C18, 300A column obtained from Waters. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.08% TFA in acetonitrile. The peptides were separated by gradient elution using the following settings: 0.0 - 5.0 min: 2 - 15% B; 5.0 - 38.0 min: 15 - 22.62% B; 38.0 - 38.5 min: 22.62 - 95% B; 38.5 - 41.5 min: 95% B; 41.5 - 42.0 min: 95 - 2% B; 42.0 - 45.0 min: 2% B.

Mass spectrometric settings for targeted-SIM/ddMS2 analysis were as follows:

- t-SIM/ddMS2 method: positive mode.
- SIM: resolution of 70,000, AGC target 3e6, maximum IT: 100 ms, isolation window 4.0m/z
- dd-MS2: resolution of 17,500, AGC target 2e5, maximum IT: 50 ms, isolation window 1.6m/z.
- dd settings: minimum AGC: 1.00e3, dynamic exclusion: 3.0s.
- Source parameters: sheat gas flow rate: 55, aux. gas flow rate: 15, spray voltage: 3.5, capillary temperature: 300. Valve was diverted to waste at minutes 0-2.0 and 42.0-45.0.

Acquisition was performed in Xcalibur Data Acquisition and Interpretation software from Thermo Fischer. Data evaluation was performed in Xcalibur 3.1 Qual Browser from Thermo Fischer and Excel sheets (Microsoft Office).

IV. LC-MS/MS (PRM) method validation according to FDA guidelines

1. Generation of a stressed reference standard and an internal standard through incubation conditions

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml. *Herceptin® (Trastuzumab)* authorized from Genentech (US) batch 872468 kept at -80 °C in aliquots of 100ul at concentration of 21.30 mg/ml. Stable isotopically labeled IgG1 from Promega 100ug aliquot, reconstituted in 0.1% FA in MiliQ water. Stable isotopically labeled peptides from PepScan 1) H-GLEWVADVNPNSGGSIYNQR*-OH, 99% purity, MW 2186.4 Da, 1mg aliquots. 2) H-NTLYLQMNSLR*-OH, 93.9% purity, MW 1362.6 Da, 1mg aliquots. All synthetic peptides were reconstituted in 0.1% FA in MiliQ water. Amicon® tubes MWCO 30k Da were obtained from Merck. Sodium hydroxide 1M and 0.1M as well as hydrogen peroxide were obtained from Sigma-Aldrich.

Sample preparation

Pertuzumab, Trastuzumab and IS mix (Stable isotopically labeled IgG1 from Promega and stable isotopically labeled peptides from PepScan) aliquots (all at a 5ug/ul concentration in MiliQ water in a total solution volume of 1 ml) were incubated under the following conditions:

- *pH and temperature stress*: Aliquots of Pertuzumab 5ug/ul were added with dropwise 1M NaOH and 0.1M NaOH until a basic pH value between 9 and 10 was reached. The pH value was assessed through a reactive paper. The same volumes of NaOH were spiked into Trastuzumab and IS mix aliquots. The samples were incubated at 40 °C for 44 days. Aliquots of 100ul were taken at days: 7, 15, 21, 28 and 44 for each mAb.
- *Oxidative (H_2O_2) stress*: Aliquots of 100ul taken at days 7, 15, 21, 28 and 44 from pH and temperature stress conditions, were added hydrogen peroxide (H_2O_2) to a final concentration of 0.1% v/v. Samples were incubated at 40 °C for 24 hours. Reaction was quenched with addition of 250mM L-Methionine solution. A buffer exchange step was performed with Amicon® tubes by three washes with 300ul of PBS and posterior centrifugation at 14 krcf and room temperature for 3 minutes. The final volume was adjusted with PBS to 100ul.

2. Characterization of the stressed reference standard and the internal standard for validation

Chemical and reagents

Dulbecco's PBS was obtained from Life Technologies. 8M guanidine HCL Solution was obtained from Thermo Fischer, L-Methionine was obtained from Merck. TCEP, IAM, AccuMAP denaturing solution, AccuMAP 10x low pH reaction buffer, AccuMAP low pH resistant rLys-C solution and AccuMAP modified trypsin solution were all obtained from Promega from their AccuMAP low pH protein digestion mini kit. SOLAu SPE plate and V-bottom 96 well plates were both obtained from Thermo Fischer. Synthetic non-heavy labeled peptides corresponding to the PENNY peptide were obtained from a donation and reconstituted in 0.1%FA in water depending on purity to a concentration of 100ug/ml (Peptides originally synthesized by Bachem). peptide 1: iso-aspartic acid N386 (DG), peptide 2: aspartic acid N386 (DG), peptide 3: aspartic acid N391 (PENDY), peptide 4: iso-aspartic acid N391 (PEisoDNY), peptide 5: aspartic acid N392 (PEDNY), peptide 6: iso-aspartic acid N392 (PENisoDY), peptide 7: non-deamidated.

MS3 method analysis

The obtained stressed reference standard after 45 days of incubation was digested following the optimized digestion protocol (See Chapter II.3 - Digestion times, Test 3)

Analysis was performed in an Agilent 1290 Infinity II LC system coupled to an Orbitrap Fusion mass spectrometer from Thermo Fischer. A MS3 method was created to analyze the samples. Selected product ions (y1 to y8) of the PENNY and GLEW peptide were included in an inclusion list for MS3 fragmentation. (See Table S5 in Supplemental Material).

Chromatographic separation was performed at 60 °C on a 2.1x 150mm, 1.7um particle size, Acquity Peptide BEH C18, 300A column obtained from Waters. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.08% TFA in acetonitrile. The peptides were separated by gradient elution using the following settings: 0.0 - 5.0 min: 2 - 15% B; 5.0 - 38.0 min: 15 - 22.62% B; 38.0 - 38.5 min: 22.62 - 95% B; 38.5 - 41.5 min: 95% B; 41.5 - 42.0 min: 95 - 2% B; 42.0 - 45.0 min: 2% B.

Mass spectrometric settings for MS3 method analysis were as follows:

- ddMS2: Orbitrap HCD and ddMS2 orbitrap ETD. Orbitrap resolution: 6000, scan range: 200-2000, maximum IT: 50 ms, Positive polarity.
- ddMS3: Orbitrap HCD followed by ddMS3 ion trap HCD. 6 scans, orbitrap resolution: 6000, scan range: 200-2000, maximum IT: 50ms, positive polarity, quadrupole isolation.
- Source parameters: ESI ion source, spray voltage: static, positive ion V: 3500, sheat gas: 45, sweep gas: 3, ion transfer temperature: 300 °C, vaporizer temperature: 50 °C

Acquisition was performed in Xcalibur Data Acquisition and Interpretation software from Thermo Fischer. Data evaluation was performed in Xcalibur 3.1 Qual Browser from Thermo Fischer and Excel sheets (Microsoft Office).

Synthetic peptides analysis

The characterization assigned through MS3 methods for the PENNY peptide was confirmed through analysis of non-heavy labeled synthetic peptides. Analysis was performed in an Agilent 1290 Infinity II LC system coupled to a Q-Exactive Plus mass spectrometer from Thermo Fischer.

A mixture comprising all seven synthetic peptides at equal concentrations, and a second one with peptides 1 to 6 (excluding non-deamidated peptide 7) at equal concentrations were prepared. These two preparations were analyzed, injecting 1ug of the total mix of peptides. Additionally, 0.5ug of each peptide was analyzed individually.

Chromatographic separation was performed at 60 °C on a 2.1x 150mm, 1.7um particle size, Acquity Peptide BEH C18, 300A column obtained from Waters. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.08% TFA in acetonitrile. The peptides were separated by gradient elution using the following settings: 0.0 - 5.0 min: 2 - 15% B; 5.0 - 38.0 min: 15 - 22.62% B; 38.0 - 38.5 min: 22.62 - 95% B; 38.5 - 41.5 min: 95% B; 41.5 - 42.0 min: 95 - 2% B; 42.0 - 45.0 min: 2% B.

Mass spectrometric settings for PRM analysis were as follows:

- PRM method: positive mode, MS2 resolution of 17,500, AGC target 2e5, maximum IT: 50 ms, isolation window: 1.6m/z, isolation offset: 0.0 m/z.
- Source parameters: sheat gas flow rate: 55, aux. gas flow rate: 15, spray voltage: 3.5, capillary temperature: 300. Valve was diverted to waste at minutes 0-2.0 and 42.0-45.0.

Acquisition was performed in Xcalibur Data Acquisition and Interpretation software from Thermo Fischer. Data evaluation was performed in Xcalibur 3.1 Qual Browser from Thermo Fischer and Excel sheets (Microsoft Office).

3. Stressed standard concentration's determination through a BCA test

Chemical and reagents

BCA Protein Assay kit was obtained from Thermo Fisher. Albumin was obtained from Sigma-Aldrich and 96 well plates were obtained from Thermo Fischer. Dulbecco's PBS was obtained from Life Technologies.

Sample preparation

Calibration curve: Samples of 20ul with albumin ranging from 1mg/ml to 0.1mg/ml and a blank of 20ul PBS were prepared in triplicate in dwells of a 96 well plate.

Samples of stressed standard after buffer exchanged were diluted 1:100 in PBS in triplicate next to the albumin calibration curve sample in the 96 well plate.

The reaction was performed according to the vendor's kit manual and the plate was incubated for 30 minutes at 37 °C. After incubation the plate was placed in a plate reader and absorbance was measured at a wavelength 562nm. The readout data was exported to an Excel sheet and the average of the triplicates of the calibration curve was calculated. The calibration curve was used to determine the concentration of each triplicate of sample and an average was calculated.

4. Stressed standard concentration's determination through stable DST tryptic peptide

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml. Dulbecco's PBS was obtained from Life Technologies. 8M guanidine HCL Solution was obtained from Thermo Fischer, L-Methionine was obtained from Merck. TCEP, IAM, AccuMAP denaturing solution, AccuMAP 10x low pH reaction buffer, AccuMAP low pH resistant rLys-C solution and AccuMAP modified trypsin solution were all obtained from Promega from their AccuMAP low pH protein digestion mini kit.

Sample preparation

Based on the concentration pre-determination of the stressed standard performed through a BCA test, triplicate stressed reference samples at estimated concentration of 2mg/ml in PBS were prepared. A calibration curve with non-stressed mAb in PBS (kept at -80 °C) was prepared with the following concentrations: 3mg/ml, 2.5mg/ml, 2mg/ml, 1.5mg/ml, 0.5mg/ml, 0mg/ml. These samples were prepared as following:

1. 4 mg/ml: 12ul (30mg/ml Pertuzumab) in 78ul PBS
2. 3 mg/ml: 9ul (4mg/ml Pertuzumab) in 3ul PBS
3. 2.5 mg/ml: 10ul (4mg/ml Pertuzumab) in 6ul PBS
4. 2 mg/ml: 10ul (4mg/ml Pertuzumab) in 10ul PBS
5. 1.5 mg/ml: 12ul (4mg/ml Pertuzumab) in 20ul PBS
6. 1 mg/ml: 10ul (4mg/ml Pertuzumab) in 30ul PBS
7. 0.5 mg/ml: 10ul (4mg/ml Pertuzumab) in 70ul PBS
8. 0 mg/ml: 10ul PBS

10ul of the samples (from calibration curve and from stressed reference standard at 2mg/ml in triplicates) were digested following the optimized digestion protocol. (See Chapter II.3 - Digestion times, Test 3)

LC-MS/MS analysis

Samples were analyzed in triplicate using an Agilent 1290 Infinity II LC system coupled to a Q-Exactive Plus mass spectrometer from Thermo Fischer through the PRM method described in Chapter III.2 (Same parameters for chromatographic separation and MS setting as previously described were used)

Acquisition was performed in Xcalibur Data Acquisition and Interpretation software from Thermo Fischer. Data evaluation was performed in Xcalibur 3.1 Qual Browser from Thermo Fischer and Excel sheets (Microsoft Office).

Only the DST stable peptide (m/z>751.8829, y8>836.47242) was monitored and quantified through product ion y8. A calibration curve was built and used to determine the concentration of each triplicate of stressed reference standard and an average was calculated. This concentration was compared to the theoretical of 2mg/ml and the exact initial concentration on stressed standards after buffer exchange was calculated.

5. LC-MS/MS method validation

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml.

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 stressed as described in section IV.1 for 45 days and kept afterwards at -80 °C in aliquots of 100ul at concentration of 10.48mg/ml.

Stable isotopically labeled IgG1 from Promega 100ug aliquot, reconstituted in 0.1% FA in MiliQ water. Stable isotopically labeled peptides from PepScan 1) H-GLEWVADVNPNSGGSIYNQR*-OH, 99% purity, MW 2186.4 Da, 1mg aliquots. 2) H-NTLYLQMNSLR*-OH, 93.9% purity, MW 1362.6 Da, 1mg aliquots. All synthetic peptides were reconstituted in 0.1% FA in MiliQ water. All stressed as described in section IV.1 and kept afterwards at -80 °C in aliquots of 500ul at concentration of 0.2mg/ml.

Pooled gender animal serum recovered from whole blood donations, non-filtrated, without anticoagulants, obtained from BioIVT.

Dulbecco's PBS was obtained from Life Technologies. 8M guanidine HCL Solution was obtained from Thermo Fischer, L-Methionine was obtained from Merck. TCEP, IAM, AccuMAP denaturing solution, AccuMAP 10x low pH reaction buffer, AccuMAP low pH resistant rLys-C solution and AccuMAP modified trypsin solution were all obtained from Promega from their AccuMAP low pH protein digestion mini kit. SOLAu SPE plate and V-bottom 96 well plates were both obtained from Thermo Fischer.

Stability samples

- *Internal standard:* 25ul of the stressed internal standard with a concentration of 0.2mg/ml were digested at the beginning and at the end of the study. No SPE or reconstitution was performed as the digestion was performed in PBS and not in serum. The final volume of the samples was 190ul with a final concentration of 0.026ug/ul. 20ul injection was analyzed.
- *Stressed reference standard:* 2ul of the stressed reference standard with a concentration of 10.48mg/ml was diluted in 8ul of PBS. The volume of 10ul was divided in 2 aliquots of 5ul at 2mg/ml and digested at the beginning and at the end of the study. No SPE or reconstitution was performed as no serum was added. The final volume of the samples was 170ul with a final concentration of 0.06ug/ul. 10ul injection was analyzed.

Calibration curves and QCs samples preparation for both stressed and non-stressed reference standards (Pertuzumab)

- 2 stocks with stressed reference standard at 2mg/ml were prepared. One for calibration curves (60ul) and one for QCs and matrix effect samples(165ul). 13ul of stressed reference

standard at 10.48ug/ul was diluted in 55.1ul of pooled serum for calibration curves. For QC samples and matrix effect samples, 34ul of stressed reference standard at 10.48ug/ul was diluted in 144.1ul of pooled serum.

- 2 stocks with non-stressed reference standard at 2mg/ml prepared. One for calibration curves and for QCs. 10ul of non-stressed reference standard Pertuzumab at 30mg/ml was diluted in 140ul of pooled serum.

Calibration curve samples for both types of reference standard (stressed and non-stressed) were prepared by consequent dilutions as following:

- *6^{th} sample:* 11ul of 2mg/ml stock + 20ul of pooled serum (0.7mg/ml)
- *5^{th} sample:* 10ul of 2mg/ml stock + 30ul of pooled serum (0.5mg/ml)
- *4^{th} sample:* 10ul of 2mg/ml stock + 55ul of pooled serum (0.3mg/ml)
- *3^{rd} sample:* 10ul of 2mg/ml stock + 123ul of pooled serum (0.15mg/ml)
- *2^{nd} sample*: 20ul of 0.15mg/ml sample + 40ul of pooled serum (0.05mg/ml)
- *1^{st} sample:* 10ul of 0.05mg/ml sample + 240ul of pooled serum (0.002mg/ml)
- *Zero sample:* 50ul of pooled serum (to be spiked with IS)
- *Blank sample:* 50ul of pooled serum (to be spiked with PBS instead of IS)

QC samples for both types of reference standard (stressed and non-stressed) were prepared by consequent dilutions as following:

- *High QC sample*: 20ul of the stock at 2mg/ml + 46ul of pooled serum (0.6mg/ml)
- *Middle QC sample*: 10ul of the stock at 2mg/ml + 70ul of pooled serum (0.25mg/ml)
- *Low QC sample*: 10ul of the middle QC Sample + 90ul of pooled serum (0.025mg/ml)

QC samples combining for both types of reference standard (stressed and non-stressed) in three different ratios were prepared by consequent dilutions as following:

- *High QC sample (600ug/ul)*
    - 1:1: 10ul of 2mg/ml of stressed reference standard + 10ul of 2mg/ml of non-stressed reference standard + 46ul of pooled serum
    - 3:1: 15ul of 2mg/ml of stressed reference standard + 5ul of 2mg/ml of non-stressed reference standard + 46ul of pooled serum
    - 1:3: 5ul of 2mg/ml of stressed reference standard + 15ul of 2mg/ml of non-stressed reference standard + 46ul of pooled serum

- *Middle QC sample (250ug/ul)*
    - 1:1: 10ul of 2mg/ml of stressed reference standard + 10ul of 2mg/ml of non-stressed reference standard + 140ul of pooled serum
    - 3:1: 15ul of 2mg/ml of stressed reference standard + 5ul of 2mg/ml of non-stressed reference standard + 140ul of pooled serum
    - 1:3: 5ul of 2mg/ml of stressed reference standard + 15ul of 2mg/ml of non-stressed reference standard + 140ul of pooled serum

- *Low QC sample (25ug/ul)*
    - 1:1: 10ul of the 1:1 middle QC Sample + 90ul of pooled serum
    - 3:1: 10ul of the 3:1 middle QC Sample + 90ul of pooled serum
    - 1:3: 10ul of the 1:3 middle QC Sample + 90ul of pooled serum

Low concentration samples were prepared in order to confirm detection of deamidation at levels close to the LLOQ. These samples were prepared combining for both types of reference standard (stressed and non-stressed) in three different ratios (for final low % of deamidation) by consequent dilutions as following:

10ul of the stressed reference standard at 2mg/ml with high % deamidation was diluted with 45ul of non-stressed reference standard at 2mg/ml (both from the stocks at 2mg/ml prepared already in serum) in order to lower the percentage of deamidation prior to preparing the low concentration samples.

- *First Low sample (100ug/ml)*
    - 1:1: 5ul of 2mg/ml of stressed reference standard + 5ul of 2mg/ml of non-stressed reference standard + 180ul of pooled serum (2.7% deamidation expected in N391/392 moiety at PENNY peptide)
    - 3:1: 15ul of 2mg/ml of stressed reference standard + 5ul of 2mg/ml of non-stressed reference standard + 380ul of pooled serum (4% deamidation expected in N391/392 moiety at PENNY peptide)
    - 1:3: 5ul of 2mg/ml of stressed reference standard + 15ul of 2mg/ml of non-stressed reference standard + 380ul of pooled serum (1.3% deamidation expected in N391/392 moiety at PENNY peptide)

- *Second Low sample (25ug/ml)*
    - 1:1: 5ul of 2mg/ml of stressed reference standard + 5ul of 2mg/ml of non-stressed reference standard + 790ul of pooled serum (2.7% deamidation expected in N391/392 moiety at PENNY peptide)
    - 3:1: 15ul of 2mg/ml of stressed reference standard + 5ul of 2mg/ml of non-stressed reference standard + 1580ul of pooled serum (4% deamidation expected in N391/392 moiety at PENNY peptide)
    - 1:3: 5ul of 2mg/ml of stressed reference standard + 15ul of 2mg/ml of non-stressed reference standard + 1580ul of pooled serum (1.3% deamidation expected in N391/392 moiety at PENNY peptide)

Selectivity samples

Serum samples of 7 different animals taken at baseline (before dosage) were digested and analyzed. No reference standard nor internal standard was added. 10ul of each animal sample was digested.

Samples for assessment of matrix effect on quantification of deamidation and oxidation reactions

3ul of stressed reference standard at 10.48ug/ul (in PBS) was diluted in 12.7ul of PBS to obtain a concentration of 2mg/ml. And 2.5ul of this 2mg/ml solution was diluted in 22.5ul of PBS (for the lowest concentration sample). These solutions were used to spike samples of serum from 7 different animals at three different concentrations. The serum samples were taken at baseline (before dosage).

- *Sample corresponding to 6th point of calibration curve* (0.7mg/ml)
  - 5ul of a stressed reference standard at 2ug/ul in PBS spiked into 9ul of each animal (7 different animals, 7 different samples)
- *Sample corresponding to 5th point of calibration curve* (0.5mg/ml)
  - 3ul of a stressed reference standard at 2ug/ul in PBS spiked into 9ul of each animal (7 different animals, 7 different samples)
- *Sample corresponding to 2nd point of calibration curve* (0.05mg/ml)
  - 3ul of a stressed reference standard at 0.2ug/ul in PBS spiked into 9ul of each animal (7 different animals, 7 different samples)

Analytical method validation

Method was validated according to FDA guidelines[124] including demonstrations of 1) Selectivity, 2) Accuracy and precision, 3) Linearity, 4) Sensitivity, 5) Reproducibility, 6) Quality control samples, 7) Stability on autosampler, and 8) Stability of analyte.

Stability of the used standards during the validation process was confirmed by comparison of six measurements at beginning and end of the study.

An equation per curve was obtained and used for the proposed quantification strategy B (see below). Proper linearity was assessed in calibration curves, as well as accuracy, precision as well as in the QC samples. No signal for monitored peptides was confirmed in blank samples. Sensitivity was proved at LLOQ with a deviation lower than 20% of the theoretical concentration. 3 points of the calibration curve were reanalyzed at the end of each sequence to assess reproducibility. Stability in the autosampler was proved with re injections of the first analyzed samples. The inter-reproducibility of the method was calculated with the variability of QCs determinations between the three days.

Concentration correction: In the calibration curves derived from stressed reference standard, a correction of the actual spiked concentration from each modification was performed. This was performed for all points of the calibration curve in order to plot both reference standard (stressed and non-stressed) within one graph. The correction was based on the rate seen of the modification in PBS in a 6-fold analysis. This average of this six-fold analysis was assessed to be the rate the present of that variant. This rate was used to multiply the nominal spiked Pertuzumab's concentration. The correction was only necessary for the stressed reference standard, as in the non-stressed reference standard modifications were depreciable or inexistent. The corrected concentration was then plotted against the corrected signals (corrected with the internal standard per modification). Plots with both stressed and non-stressed reference standards were obtained for the non-modified variants of peptides PENNY, NTLYL and GLEW. In the case of the rest of modified variants (as they were not present in the non-stressed reference standard_ the obtained

plots were solely with the stressed reference standard. No correction was needed for the stable DST peptide as this peptide had no modification variants.

Quantification strategies

Strategy A: each modification's rate was based on the absolute signals of all areas under the peak for that EIC of the product ions used for quantification per peptide. No correction with IS was performed. The rate of a certain modification is given as the area under the peak of the modification over the sum of all areas under the peaks of all variants of that peptide.

Strategy B: the modification's concentration was calculated through the equation derived from each modification's calibration curve. For which a correction with IS was performed and the reported rate is based on a concentration calculation. The concentration present per point of the curve was determined in mg/ml. By using all calculated concentrations of all variants of a peptide, the rate of the modification was reported in terms of percentage. Average between the curve points was reported.

For both strategies standard deviation between triplicates and average of all the rates per point of the curve was calculated. This average was then compared in terms of relative deviation from the modification's rate seen in PBS.

Sample preparation

10ul of the internal standard was added to 10ul of each sample (reference standards) or 10ul of PBS (blanks and selectivity samples). 5ul of 6M guanidine HCL, 20ul of denaturing solution, 6ul of AccuMAP 10x low pH reaction buffer, and 1ul of 100mM were added to the samples. Samples were denaturized and reduced though incubation at 37°C for 90 minutes. 2ul of 300mM IAM were added and alkylation was carried out through incubation at 37 °C for 30 minutes in the dark. A pre-digestion with Lys-C was performed by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and incubating the sample for 1 hour at 37 °C in the dark. Next, it was added to the sample: 6ul low pH reaction buffer, 44ul of MiliQ water and 4ul of 268.125mM L-Methionine solution (to obtain a final concentration of 15mM L-Methionine in the solution). The samples were digested by addition of 25ul (0.2ug/ul) of AccuMAP low pH resistant rLys-C and 20ul (0.5ug/ul) of AccuMAP modified trypsin solution at 37 °C for 5 hours in the dark. The reaction was terminated with addition of FA to reach a final concentration of 2%v/v in the sample.

Solid phase extraction for all sample preparations

After digestion of the samples, a solid phase extraction step was performed in a SOLAu SPE plates. The SPE plate was equilibrated and conditioned with 200ul acetonitrile at centrifugation at 1000g for 1 min, and 200ul 0.1%TFA and centrifugation. The digested sample was prepared 1:1 v/v with 0.1%TFA in water and loaded into the plate wells and centrifuged. Sample was cleaned by addition of 500ul 0.1%TFA and centrifugation. Finally, the sample was eluted in a collection plate by 2 steps of 25ul 70% acetonitrile, 30% water addition into the well and centrifugation, Final volume of 50ul was collected and transferred into low protein binding 1.5ml Eppendorf tubes and evaporated until dryness in a Speedvac for 45 minutes at 22 °C. Samples were reconstituted in 10ul 0.1% FA in water.

LC-MS/MS analysis

Analysis was performed in an Agilent 1290 Infinity II LC system coupled to a Q-Exactive Plus mass spectrometer from Thermo Fischer. Chromatographic separation was performed at 60 °C on a 2.1x 150mm, 1.7um particle size, Acquity Peptide BEH C18, 300A column obtained from Waters. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.08% TFA in acetonitrile. The peptides were separated by gradient elution using the following settings: 0.0 - 5.0 min: 2 - 15% B; 5.0 - 38.0 min: 15 - 22.62% B; 38.0 - 38.5 min: 22.62 - 95% B; 38.5 - 41.5 min: 95% B; 41.5 - 42.0 min: 95 - 2% B; 42.0 - 45.0 min: 2% B.

Mass spectrometric settings for PRM analysis (See Table S2 in Supplemental Material for inclusion list) were as follows:

- PRM method: positive mode, MS2 resolution of 17,500, AGC target 2e5, maximum IT: 50 ms, isolation window: 1.6m/z, isolation offset: 0.0 m/z.
- Source parameters: sheat gas flow rate: 55, aux. gas flow rate: 15, spray voltage: 3.5, capillary temperature: 300. Valve was diverted to waste at minutes 0-2.0 and 42.0-45.0.

Acquisition was performed in Xcalibur Data Acquisition and Interpretation software from Thermo Fischer. Data evaluation was performed in Xcalibur 3.1 Qual Browser from Thermo Fischer and Excel sheets (Microsoft Office). Monitored precursors and product ions used for quantification and analysis were: GLEW (m/z>1088.5245, y11>1192.57057), PENNY (m/z>1272.5693, y6>764.35739), NTLYL (m/z> 676.85323, y8>1024.52447), DST (m/z>751.8829, y8>836.47242).

V. Application of the validated LC-MS/MS method to in vivo and in vitro samples for comparison of deamidation rates over time in exploratory animal study samples and incubated spiked models

Chemical and reagents

Perjeta® (Pertuzumab) EU authorized from Roche (United Kingdom) batch H050B03 kept at -80 °C in aliquots of 50ul at concentration of 30mg/ml.

Pooled gender animal serum recovered from whole blood donations, non-filtrated, without anticoagulants, obtained from BioIVT.

Dulbecco's PBS was obtained from Life Technologies. 8M guanidine HCL Solution was obtained from Thermo Fischer, L-Methionine was obtained from Merck. TCEP, IAM, AccuMAP denaturing solution, AccuMAP 10x low pH reaction buffer, AccuMAP low pH resistant rLys-C solution and AccuMAP modified trypsin solution were all obtained from Promega from their AccuMAP low pH protein digestion mini kit. SOLAu SPE plate and V-bottom 96 well plates were both obtained from Thermo Fischer.

In vivo and in vitro samples

In vivo samples: Perjeta® administered to 3 animal animals as a single IV (intravenous) injection (15 mg/kg dosage) on test day 0; serum samples obtained at time points (pre-dose, 50 min, 24 hours, day 5, day 16, day 22, day 25, day 28). Aliquots of 50ul were stored at -80 °C until analysis.

In vitro samples: Perjeta® (30mg/ml) 15ul spiked into 2.25ml pooled animal serum to obtain a final concentration of 200ug/ml. This was performed in triplicate. The samples were incubated at 37 °C for 28 days. Aliquots of 100ul were collected at the same time points as the in vivo samples (pre-dose, 50 min, 24 hours, day 5, day 16, day 22, day 25, day 28) and stored at -80°C until analysis.

LC-MS/MS analysis of in vivo and in vitro samples

Three different animals (animals 3, 1, and 2) were selected for analysis with the validated LC-MS/MS method (see Chapter IV.5). These animals were selected for presenting the highest concentration of Pertuzumab 28 days after administration. The same time points in in vivo and in vitro samples were analyzed (50 min, 5 days, 16 days, 22 days, 28 days) in triplicate.

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Supplemental Material

Table/Figure	ID computer of MS data storage	ID sequence raw data
Table 2	ESI-7	181024_LC36_01
Table 3	ESI-7	181024_LC36_01
Table 4	ESI-7	181024_LC36_01
Table 5	ESI-7	191129_LC36_01
Table 6	ESI-7	191129_LC36_01
Table 7	NA	Taken from: Report XXX (data cannot be disclosed)
Table 9	ESI-11	200819_LC36_01, 200820_LC36_01, 200820_LC36_02
Table 10	ESI-11	201022_LC36_02
Table 11	ESI-11	201022_LC36_02, 201030_LC36_01, 201106_LC36_01
Table 12	ESI-11	201022_LC36_02, 201030_LC36_01, 201106_LC36_01
Table 13	ESI-11	201022_LC36_02, 201030_LC36_01, 201106_LC36_01
Table 14	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Table 15	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Table 16	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Figure 5	ESI-7	181024_LC36_01
Figure 6	ESI-7	181024_LC36_01
Figure 7	ESI-7	191129_LC36_01
Figure 8	ESI-7	191129_LC36_01
Figure 12	ESI-7	190225_LC36_01
Figure 13	ESI-7	190225_LC36_01
Figure 14	ESI-7	190225_LC36_01
Figure 16	ESI-7	190523_LC36_01
Figure 17	ESI-7	190523_LC36_01
Figure 18	ESI-7	190523_LC36_01
Figure 19	ESI-7	190523_LC36_01
Figure 21	QQQ	N/A (UKE)
Figure 22	QQQ	N/A (UKE)
Figure 23	QQQ	N/A (UKE)
Figure 24	QQQ	N/A (UKE)
Figure 25	ESI-11	200814_LC36_01
Figure 26	ESI -6, ESI-5	ESI6_200619, ESI5_200911
Figure 27	ESI-9	201008_ESI9

Figure 28	ESI-11	201016_LC36_01
Figure 29	ESI-11	201014_LC36_01, 201109_LC36_01
Figure 30	ESI-11	201014_LC36_01
Figure 31	ESI-11	201014_LC36_01
Figure 32	ESI-11	200821_LC36_01
Figure 33	ESI-11	201014_LC36_01
Figure 34	ESI-11	200819_LC36_01, 200820_LC36_01, 200820_LC36_02
Figure 35	ESI-11	200821_LC36_01, 201022_LC36_02
Figure 36	ESI-11	201022_LC36_02, 201030_LC36_01, 201106_LC36_01
Figure 37	ESI-11	201022_LC36_02, 201030_LC36_01, 201106_LC36_01
Figure 38	ESI-11	201022_LC36_02, 201030_LC36_01, 201106_LC36_01
Figure 39	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Figure 40	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Table S2 (supplemental material)	ESI-11	N/A (Methods file)
Table S4 (supplemental material)	ESI-9	N/A (Methods file)
Table S5 (supplemental material)	ESI-11	201022_LC36_02, 201022_LC36_02, 201030_LC36_01
Table S6 (supplemental material)	ESI-11	201022_LC36_02, 201022_LC36_02, 201030_LC36_01
Table S7 (supplemental material)	ESI-11	201022_LC36_02, 201022_LC36_02, 201030_LC36_01
Table S8 (supplemental material)	ESI-11	201022_LC36_02, 201022_LC36_02, 201030_LC36_01
Table S9 (supplemental material)	ESI-11	201022_LC36_02, 201022_LC36_02, 201030_LC36_01
Table S10 (supplemental material)	ESI-11	201022_LC36_02, 201022_LC36_02, 201030_LC36_01
Table S11 (supplemental material)	ESI-11	201022_LC36_02, 201022_LC36_02, 201030_LC36_01
Table S12 (supplemental material)	ESI-11	201022_LC36_02, 201022_LC36_02, 201030_LC36_01
Table S11 (supplemental material)	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Table S13 (supplemental material)	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Table S14 (supplemental material)	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01

Table S15 (supplemental material)	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Table S16 (supplemental material)	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Table S17 (supplemental material)	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Figure S2 (supplemental material)	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Figure S3 (supplemental material)	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Figure S4 (supplemental material)	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01
Figure S5 (supplemental material)	ESI-11	210127_ESI11_LC36_01, 210129_ESI11_LC36_01

Table S1A. Documentation of raw data storage from figures/tables (lab books and raw data stored at University of Hamburg).

Chapter in thesis	Experiment	Lab journal	Pages	ID sequence data storage
Chapter 1	Analysis of five distinct sample preparations (protein digestion protocols) and analysis through LC-MS/MS	DEOB-495	1-17, 24-29, 33	181024_LC36_01 (ESI-7)
Chapter 5	In vitro samples (incubation of pooled NWZ animal sera)	DEOB-495	18-23, 30-33, 45-47	NA
Chapter 2, Subsection 1	Conditions for pH stress of mAbs	DEOB-495	34-35	NA
Chapter 2, Subsection 1	LC-MS/MS analysis of stressed Trastuzumab and Pertuzumab (oxidative, pH, and temperature stress) for peptides of interest selection	DEOB-495	36-41	181129_LC36_01 (ESI-7), 181130_LC36_01 (ESI-7), 190114_LC36_01 (ESI-7)
Chapter 2, Subsection 4	Sample preparation (Protein digestion) optimization - SPE step assessment	DEOB-495	64-69	190327_LC36_01 (ESI-7)
Chapter 2, Subsection 4	Sample preparation (Protein digestion) optimization - enzyme ratios	DEOB-495	71-75	190403_LC36_01 (ESI-7), 190402_LC36_01 (ESI-7), 190404_LC36_01 (ESI-7), 190408_LC36_01 (ESI-7)
Chapter 2, Subsection 4	Sample preparation (Protein digestion) optimization - different denaturation/reduction conditions	DEOB-495	90-94	190502_LC36_02 (ESI-7), 190502_LC36_03 (ESI-7), 190523_LC36_01 (ESI-7)
Chapter 2, Subsection 4	Sample preparation (Protein digestion) optimization	DEOB-495	100-102	190523_LC36_01 (ESI-7)
Chapter 2, Subsection 4	Sample preparation (Protein digestion) optimization - predigestion step, times of digestion, ratios of trypsin assessment	DEOB-495	130-136	191021_LC36_01 (ESI7), 191021_LC36_02 (ESI7), 191021_LC36_03 (ESI7), 190923_LC36_01 (ESI7), 190923_LC36_02 (ESI7)
Chapter 2, Subsection 4	Sample preparation (Protein digestion) optimization - samples at 150ng/ml, different TCEP concentrations and times of reduction, times of alkylation and digestion	DEOB-522	15-21	200122_LC36_01 (ESI-7)

Chapter 4, Subsection 1	Pertuzumab and SilumAb oxidative (H_2O_2), temperature and pH stress conditions.	DEOB-522	29-30, 33-41, 47-49, 60-65, 67-72, 83	200821_LC36_01 (ESI-7), 201014_LC36_01 (ESI-7), 201109_LC36_01 (ESI-7), ESI6_200619, ESI5_200911, UKE(Hamburg), 200805_LC36_01 (ESI-11), 200805_LC36_02 (ESI-11), 200914_LC36_01 (ESI-7)
Chapter 3	SRM analysis at LC MS/MS (triple quadrupole instrument)	DEOB-522	31	UKE (Hamburg)
Chapter 4, Subsection 1,2	Highly stressed Pertuzumab concentration determination through BCA test	DEOB-522	32, 50-52	UKE (Hamburg)
Chapter 4, Subsection 1,2	Analysis of stressed internal standard through LC-MS/MS	DEOB-522	53-59, 68-69	ESI6_200619, ESI5_200911, 200820_LC36_01 (ESI-11), 200820_LC36_01 (ESI-11)
Chapter 4, Subsection 3	Assessment of signal to noise ratio at LLOQ concentration of Pertuzumab in serum	DEOB-522	68	200819_LC36_01 (ESI-11), 200820_LC36_01 (ESI-11), 200820_LC36_02 (ESI-11)
Chapter 4, Subsection 2	MS3 (LC-MS/MS/MS) analysis in Orbitrap-Ion trap hybrid instrument (Fusion Lumos)	DEOB-522	92	201008_ESI9
Chapter 4, Subsection 1,2	Analysis of stressed reference standard (Pertuzumab) used in validation	DEOB-522	97	201013_LC36_02 (ESI-11)
Chapter 4, Subsection 1,2	Calibration curve with non-stressed Pertuzumab and analysis of stressed reference standard for concentration determination through LC-MS/MS	DEOB-522	98-99	201014_LC36_01 (ESI-11)
Chapter 4, Subsection 1,2	Analysis of highly stressed (45 days, high pH, temperature and oxidative stress) mAbs Trastuzumab and Pertuzumab	DEOB-522	101	ESI6_200619, ESI5_200911, 201016_LC36_02 (ESI-11)
Chapter 4, Subsection 2	PENNY peptide deamidation sites characterization through synthetic peptides LC-MS/MS analysis	DEOB-522	102	201016_LC36_01 (ESI-11)
Chapter 4, Subsection 4,5	LC-MS/MS (PRM) method validation-Seq1	DEOB-522	106,107	201022_LC36_02 (ESI-11)
Chapter 4, Subsection 4,5	LC-MS/MS (PRM) method validation-Seq2	DEOB-522	112	201030_LC36_01 (ESI-11)
Chapter 4, Subsection 4,5	LC-MS/MS (PRM) method validation-Seq3	DEOB-522	114	201106_LC36_01 (ESI-11)
Chapter 5	In vitro (incubated spiked pooled sera) and in vivo (NWZ animal exploratory animal study study) samples LC-MS/MS analysis -Seq1	DEOB-544	2,3	210127_ESI11_LC36_01
Chapter 5	In vitro (incubated spiked pooled sera) and in vivo (NWZ animal exploratory animal study study) samples LC-MS/MS analysis -Seq2	DEOB-544	4	210129_ESI11_LC36_01

Table S1B. Documentation of raw data (lab books and raw data stored at University of Hamburg).

Sequence of Pertuzumab

>sp|Pertuzumab _LC|Pertuzumab light chain

DIQMTQSPSS LSASVGDRVT ITCKASQDVS IGVAWYQQKP GKAPKLLIYS ASYRYTGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YYIYPYTFGQ

GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC

>sp|Pertuzumab _HC|Pertuzumab heavy chain

EVQLVESGGG LVQPGGSLRL SCAASGFTFT DYTMDWVRQA PGKGLEWVAD VNPNSGGSIY NQRFKGRFTL SVDRSKNTLY LQMNSLRAED TAVYYCARNL GPSFYFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY ICNVNHKPSN TKVDKKVEPK SCDKTHTCPP CPAPELLGGP SVFLFPPKPK DTLMISRTPE VTCVVVDVSH EDPEVKFNWY VDGVEVHNAK TKPREEQYNS TYRVVSVLTV LHQDWLNGKE YKCKVSNKAL PAPIEKTISK AKGQPREPQV YTLPPSREEM TKNQVSLTCL VKPENNYSDIA VEWESNGQPE NNYKTTPPVL DSDGSFFLYS KLTVDKSRWQ QGNVFSCSVM HEALHNHYTQ KSLSLSPG

Sequence of Trastuzumab

>sp|Trastuzumab _LC|Trastuzumab light chain

DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYS ASFLYSGVPS RFSGSRSGTD FTLTISSLQP EDFATYYCQQ HYTTPPTFGQ GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC

>sp|Trastuzumab _HC|Trastuzumab heavy chain

EVQLVESGGG LVQPGGSLRL SCAASGFNIK DTYIHWVRQA PGKGLEWVAR IYPTNGYTRY ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCSRWG GDGFYAMDYW GQGTLVTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKPENNYSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPG

Figure S1. Amino acid sequence of mAbs Pertuzumab and Trastuzumab

Mass [m/z]	[z]	Polarity	Start [min]	End [min]	CE	ID Comment
676.85323	2	Positive	16	22	20	NTLYL
681.85737	2	Positive	16	22	20	NTLYL*
684.85323	2	Positive	13	19	20	NTLYL_Ox

689.85737	2	Positive	13	19	20	NTLYL*_Ox
692.84084	2	Positive	13	19	20	NTLYL_2Ox
697.84498	2	Positive	13	19	20	NTLYL*_2Ox
1088.5245	2	Positive	19	25	23	GLEW
1093.5286	2	Positive	19	25	23	GLEW*
1089.02457	2	Positive	19	25	23	GLEW_Deam
1094.0287	2	Positive	19	25	23	GLEW*_Deam
1272.5693	2	Positive	21	27	20	PENNY
1276.5764	2	Positive	21	27	20	PENNY*
1273.0621	2	Positive	22	28	20	PENNY_Deam
1277.0612	2	Positive	22	28	20	PENNY*_Deam
751.8829	2	Positive	15	21	18	DST
755.89	2	Positive	15	21	18	DST*

Table S2. Inclusion list for peptides of interest in PRM method on a quadrupole orbitrap system. In columns is presented: m/z of precursor ion in Daltons, charge state, polarity, range of scan in minutes (start and end), collision energy and identification per peptide.

Precursor ion [m/z]	[z]	Product ion	Polarity	Start [min]	End [min]	CE	Compound name
676.8	2	748.2	Positive	18	22	30	NTLYL
681.8	2	758.2083	Positive	18	22	30	NTLYL*
684.8	2	1040.5	Positive	14	18	25	NTLYL_Ox
689.8	2	1050.508	Positive	14	18	25	NTLYL*_Ox
726	3	894.4429	Positive	19.5	23.5	27	GLEW
729.3	3	904.4511	Positive	19.5	23.5	27	GLEW*
726.3	3	894.4429	Positive	19	24	27	GLEW_Deam
729.7	3	904.4511	Positive	19	24	27	GLEW*_Deam
848.7	3	764.3574	Positive	22.5	26.5	25	PENNY
851.4	3	772.3716	Positive	22.5	26.5	25	PENNY*
849	3	1466.6	Positive	22	27	25	PENNY_Deam
851.7	3	1474.614	Positive	22	27	25	PENNY*_Deam
751.9	2	836.47	Positive	17	21	25	DST
755.9	2	844.4842	Positive	17	21	25	DST*

Table S3: Inclusion list for peptides of interest in SRM method on a triple quadrupole system. In columns is presented: m/z of precursor ion in Daltons, charge state, polarity, range of scan in minutes (start and end), collision energy and identification per peptide.

Compound	m/z	z	t start (min)	t stop (min)
PENNY y3 (NYK)	424.2191	1	25	38

PENNY y3 1*Deam	425.2031	1	25	38
PENNY y4 (NNYK)	538.262	1	25	38
PENNY y4 1*deam	539.2461	1	25	38
PENNY y4 2*deam	540.2301	1	25	38
PENNY y8 (GQPENNYK)	949.4374	1	25	38
PENNY y8 1*deam	950.4214	1	25	38
PENNY y8 2*deam	951.4055	1	25	38
PENNY y8 3*deam	952.3895	1	25	38
PENNY y13 (EWESNGQPENNYK)	1595.6609	1	25	38
PENNY y13 1*deam	1596.6449	1	25	38
PENNY y13 2*deam	1597.6289	1	25	38
PENNY y13 3*deam	1598.6129	1	25	38
PENNY y6 (PENNYK)	764.3574	1	25	38
PENNY y6 1*deam	765.3414	1	25	38
PENNY y6 2*deam	766.3254	1	25	38
GLEW y3 (NQR)	417.2205	1	22	34
GLEW y3 1*deam	418.2045	1	22	34
GLEW y9 (SGGSIYNQR)	981.4749	1	22	34
GLEW y9 1*deam	982.4589	1	22	34
GLEW y10 (NSGGSIYNQR)	1095.5178	1	22	34
GLEW y10 1*deam	1096.5018	1	22	34
GLEW y10 2*deam	1097.4858	1	22	34
GLEW y12 (NPNSGGSIYNQR)	1306.6135	1	22	34
GLEW y12 1*deam	1307.5975	1	22	34
GLEW y12 2*deam	1308.5818	1	22	34
GLEW y12 3*deam	1309.5655	1	22	34
GLEW y12 4*deam	1310.5496	1	22	34
NTLYL y8	1024.4	1	15	26
NTLYL ox y8	1040.5	1	8	15
DST y8	836.47	1	12	25

Table S4: Inclusion list for peptides of interest in MS3 method on a fusion orbitrap/ion trap system. In columns is presented: identification per peptide, m/z of precursor ion in Daltons, charge state, range of scan in minutes (start and end).

Method validation data analysis

Stability

Stability internal standard

		*NTLYL ox**	*NTLYL 2ox**	*NTLYL**	*(N52) isoAsp**	*(N54) isoAsp**	*(N61) isoAsp**	*GLEW**	*(N54) Asp**	*(N52) Asp**
Beginning of study	**Rate Modification**	24.48%	2.63%	72.79%	0.15%	7.28%	1.10%	80.58%	1.13%	9.75%
	STD	0.0033	0.0029	0.0043	0.0002	0.0027	0.0008	0.0030	0.0003	0.0028
	RSD	1.34%	11.19%	0.59%	12.03%	3.68%	7.50%	0.38%	2.40%	2.89%
End of study	**Rate Modification**	24.58%	2.67%	72.75%	0.14%	7.40%	1.12%	80.56%	1.14%	9.63%
	STD	0.0079	0.0004	0.0078	0.0001	0.0021	0.0005	0.0039	0.0002	0.0022
	RSD	3.22%	1.36%	1.07%	4.31%	2.89%	4.63%	0.48%	2.15%	2.24%
Rel. deviation beginning vs. end		0.42%	1.26%	0.05%	4.97%	1.64%	1.71%	0.03%	0.87%	1.21%

		*(Q88) isoGlu**	*(N86) isoAsp**	*(N91) isoAsp**	*(N92) isoAsp**	*PENNY**	*(N91/92) Asp**	*(N86) Asp**	*(Q88) Glu**
Beginning of study	**Rate Modification**	0.15%	13.48%	3.63%	17.10%	54.43%	2.88%	7.91%	0.41%
	STD	0.0001	0.0034	0.0029	0.0036	0.0072	0.0009	0.0020	0.0004
	RSD	8.72%	2.56%	8.07%	2.12%	1.33%	3.17%	2.58%	10.01%
End of study	**Rate Modification**	0.15%	13.48%	3.70%	17.06%	54.54%	2.88%	7.76%	0.43%
	STD	0.0001	0.0040	0.0028	0.0033	0.0051	0.0008	0.0033	0.0002
	RSD	8.07%	2.94%	7.46%	1.94%	0.93%	2.87%	4.29%	5.31%
Rel. deviation beginning vs. end		0.01%	0.00%	1.99%	0.28%	0.19%	0.20%	1.90%	3.74%

Stability stressed reference standard Pertuzumab

		NTLYL ox	*NTLYL 2ox*	*NTLYL*	*(N52) isoAsp*	*(N54) isoAsp*	*(N61) isoAsp*	*GLEW*	*(N54) Asp*	*(N52) Asp*
Beginning of study	**Rate Modification**	35.37%	0.45%	64.36%	3.24%	15.47%	5.72%	69.51%	2.75%	3.32%
	STD	0.0038	0.0008	0.0064	0.0015	0.0025	0.0032	0.0021	0.0015	0.0024
	RSD	1.07%	16.67%	1.00%	4.60%	1.62%	5.68%	0.31%	5.60%	7.16%
End of study	**Rate Modification**	36.12%	0.45%	63.43%	4.49%	15.30%	5.74%	68.24%	2.49%	3.74%
	STD	0.0074	0.0007	0.0075	0.0045	0.0032	0.0035	0.0050	0.0016	0.0015
	RSD	2.06%	16.33%	1.18%	10.02%	2.07%	6.01%	0.73%	6.41%	3.89%
Rel. deviation beginning vs. end		0.55%	0.53%	0.33%	5.23%	0.30%	1.34%	0.39%	0.37%	0.12%

		(Q88) isoGlu	*(N86) IsoAsp*	*(N91) IsoAsp*	*(N92) IsoAsp*	*PENNY*	*(N91/92) Asp*	*(N86) Asp*	*(Q88) Glu*
Beginning of study	**Rate Modification**	0.05%	4.28%	1.25%	16.32%	45.22%	29.86%	2.18%	0.84%
	STD	0.0002	0.0026	0.0017	0.0033	0.0044	0.0042	0.0004	0.0009
	RSD	37.18%	6.02%	13.24%	2.02%	0.98%	1.41%	1.68%	10.22%
End of study	**Rate Modification**	0.05%	4.08%	2.03%	16.27%	45.03%	29.51%	2.17%	0.85%
	STD	0.0002	0.0042	0.0014	0.0027	0.0064	0.0072	0.0008	0.0010
	RSD	48.52%	10.19%	6.78%	1.68%	1.41%	2.45%	3.68%	11.78%
Rel. deviation beginning vs. end		3.13%	5.05%	0.54%	0.48%	0.50%	0.13%	0.19%	2.20%

Table S5. Chemical stability of both the internal standard and the stressed reference standard (stressed Pertuzumab) before and after the method's validation.

Calibration curves for all peptides

Cal curve 1		DST peptide (Non-Stress)			NTLYL (Non-Stress)			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.7000	3.529498	0.703816	**0.55%**	0.7000	0.211278	0.696786	**0.46%**
5th sample	0.5000	2.460099	0.490453	**1.91%**	0.5000	0.152376	0.502068	**0.41%**
4th sample	0.3000	1.557734	0.310416	**3.47%**	0.3000	0.087741	0.288399	**3.87%**
3rd sample	0.1500	0.711891	0.141655	**5.56%**	0.1500	0.044651	0.145952	**2.70%**
2nd sample	0.0500	0.274393	0.054367	**8.73%**	0.0500	0.015033	0.048044	**3.91%**
1st sample	0.0020	0.010045	0.001625	**18.75%**	0.0020	0.000992	0.001627	**18.63%**
blank	0.0000	0.000000	0.000000	**NA**	0.0000	0.000000	0.000000	**0.00%**

	DST peptide (Stress)				NTLYL (Stress)				Ox (Stress)			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.7000	3.646326	0.702931	**0.42%**	0.4425	0.102925	0.444429	**0.43%**	0.2542	0.174235	0.255448	**0.48%**
5th sample	0.5000	2.576763	0.496647	**0.67%**	0.3161	0.062100	0.312339	**1.18%**	0.1816	0.125835	0.184460	**1.58%**
4th sample	0.3000	1.545729	0.297793	**0.74%**	0.1896	0.038160	0.191864	**1.17%**	0.1090	0.076448	0.112023	**2.81%**
3rd sample	0.1500	0.785433	0.151157	**0.77%**	0.0948	0.019094	0.095913	**1.15%**	0.0545	0.033758	0.049410	**9.30%**
2nd sample	0.0500	0.272170	0.052165	**4.33%**	0.0316	0.006300	0.031532	**0.24%**	0.0182	0.012502	0.018235	**0.41%**
1st sample	0.0020	0.010026	0.001606	**19.71%**	0.0013	0.000241	0.001041	**17.64%**	0.0007	0.000537	0.000685	**5.64%**
blank	0.0000	0.000000	0.000000	**NA**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**

	(N52) isoAsp (Stress)				(N54) isoAsp (Stress)				(N61) isoAsp (Stress)			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*

6th sample	0.0299	4.437869	0.028661	**4.11%**	0.1074	0.359143	0.107721	**0.28%**	0.0397	0.936404	0.039750	**0.24%**
5th sample	0.0214	3.579763	0.023145	**8.41%**	0.0767	0.249906	0.074920	**2.36%**	0.0283	0.678868	0.028940	**2.17%**
4th sample	0.0128	2.222852	0.014423	**12.59%**	0.0460	0.163062	0.048843	**6.10%**	0.0170	0.376645	0.016254	**4.36%**
3rd sample	0.0064	0.916367	0.006025	**5.94%**	0.0230	0.073491	0.021947	**4.65%**	0.0085	0.164301	0.007341	**13.61%**
2nd sample	0.0029	0.406254	0.002746	**3.87%**	0.0077	0.027083	0.008012	**4.42%**	0.0028	0.066463	0.003235	**14.19%**
1st sample	0.0001	0.000000	NA	**NA**	0.0003	0.000000	NA	**NA**	0.0001	0.000000	NA	**NA**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**
GLEW (Stress)					**(N54) Asp (Stress)**				**(N52) Asp (Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.4796	0.148871	0.480578	**0.21%**	0.0174	0.341933	0.016717	**3.71%**	0.0261	0.066203	0.025885	**0.88%**
5th sample	0.3425	0.104643	0.338090	**1.30%**	0.0124	0.273486	0.013371	**7.82%**	0.0187	0.047547	0.018557	**0.52%**
4th sample	0.2055	0.065360	0.211534	**2.92%**	0.0074	0.153933	0.007526	**1.14%**	0.0112	0.028219	0.010966	**2.02%**
3rd sample	0.1028	0.030462	0.099104	**3.56%**	0.0037	0.067733	0.003311	**10.99%**	0.0056	0.012827	0.004920	**12.08%**
2nd sample	0.0343	0.010052	0.033350	**2.64%**	0.0012	0.026374	0.001289	**3.97%**	0.0019	0.004984	0.001840	**1.38%**
1st sample	0.0014	0.000086	0.001244	**9.18%**	0.0000	0.000000	NA	**NA**	0.0001	0.000000	NA	**NA**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**
GLEW (Non-Stress)					**(N86) IsoAsp (Stress)**				**(N91) IsoAsp (Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.7000	0.244407	0.704043	**0.58%**	0.0301	0.107357	0.030055	**0.11%**	0.0141	0.170793	0.009150	**11.49%**
5th sample	0.5000	0.170712	0.492276	**1.54%**	0.0215	0.077237	0.021639	**0.65%**	0.0101	0.122731	0.003629	**8.52%**
4th sample	0.3000	0.104946	0.303292	**1.10%**	0.0129	0.045464	0.012760	**1.08%**	0.0061	0.079233	0.071108	**5.06%**
3rd sample	0.1500	0.051624	0.150070	**0.05%**	0.0064	0.022078	0.006225	**2.73%**	0.0030	0.035622	0.039878	**2.62%**
2nd sample	0.0500	0.016782	0.049949	**0.10%**	0.0021	0.007407	0.002126	**1.23%**	0.0010	0.011534	0.078340	**0.79%**
1st sample	0.0020	0.000159	0.002180	**9.02%**	0.0001	0.000094	0.000110	**9.94%**	0.0000	0.000000	NA	**NA**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**

	(N92) IsoAsp (Stress)				PENNY (Stress)				(N91/92) Asp (Stress)			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.1144	0.268071	0.114863	**0.39%**	0.3137	0.250076	0.316226	**0.81%**	0.2063	3.167947	0.209998	**1.77%**
5th sample	0.0817	0.199630	0.085172	**4.22%**	0.2241	0.176002	0.222519	**0.68%**	0.1474	2.141613	0.141880	**3.73%**
4th sample	0.0490	0.120014	0.050633	**3.26%**	0.1344	0.102317	0.129307	**3.81%**	0.0884	1.328128	0.087889	**0.61%**
3rd sample	0.0245	0.063584	0.026153	**6.67%**	0.0672	0.056165	0.070924	**5.52%**	0.0442	0.703679	0.046444	**5.04%**
2nd sample	0.0082	0.021594	0.007936	**2.89%**	0.0224	0.017557	0.022083	**1.44%**	0.0147	0.231973	0.015137	**2.71%**
1st sample	0.0003	0.004180	0.000382	**16.77%**	0.0009	0.000869	0.000764	**14.73%**	0.0006	0.013097	0.000610	**3.54%**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**

	(N86) Asp (Stress)				(Q88) Asp (Stress)				PENNY (Non-Stress)			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.0152	0.089115	0.015269	**0.58%**	0.0058	0.724715	0.006158	**5.30%**	0.7000	1.027611	0.706181	**0.88%**
5th sample	0.0108	0.062507	0.010705	**1.27%**	0.0042	0.447966	0.003836	**8.16%**	0.5000	0.714318	0.490800	**1.84%**
4th sample	0.0065	0.037546	0.006423	**1.27%**	0.0025	0.265993	0.002310	**7.85%**	0.3000	0.434600	0.298502	**0.50%**
3rd sample	0.0033	0.020891	0.003566	**9.64%**	0.0013	0.150204	0.001338	**6.78%**	0.1500	0.226518	0.155450	**3.63%**
2nd sample	0.0011	0.005719	0.000964	**11.10%**	0.0004	0.041142	0.000423	**1.31%**	0.0500	0.072342	0.049458	**1.08%**
1st sample	0.0000	0.000319	0.000038	**13.31%**	0.0000	0.000000	NA	**NA**	0.0020	0.003470	0.002110	**5.51%**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**

Cal curve 2		**DST peptide (Non-Stress)**				**NTLYL (Non-Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	
6th sample	0.7000	3.529414	0.703999	**0.57%**	0.7000	0.194469	0.642186	**8.26%**	
5th sample	0.5000	2.459600	0.490365	**1.93%**	0.5000	0.140337	0.463062	**7.39%**	

4th sample	0.3000	1.557634	0.310249	**3.42%**	0.3000	0.080806	0.266070	**11.31%**				
3rd sample	0.1500	0.712106	0.141403	**5.73%**	0.1500	0.040977	0.134273	**10.48%**				
2nd sample	0.0500	0.276278	0.054372	**8.74%**	0.0500	0.013843	0.044482	**11.04%**				
1st sample	0.0020	0.015802	0.002357	**17.83%**	0.0020	0.000948	0.001814	**9.30%**				
blank	0.0000	0.000000	0.000000	**NA**	0.0000	0.000000	0.000000	**0.00%**				
		DST peptide (Stress)				**NTLYL (Stress)**					**Ox (Stress)**	
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.7000	3.645860	0.702932	**0.42%**	0.4425	0.102922	0.444287	**0.40%**	0.2542	0.170868	0.250510	**1.47%**
5th sample	0.5000	2.576620	0.496706	**0.66%**	0.3161	0.072349	0.312218	**1.22%**	0.1816	0.125954	0.184635	**1.67%**
4th sample	0.3000	1.545036	0.297743	**0.75%**	0.1896	0.044452	0.191715	**1.09%**	0.1090	0.076599	0.112245	**3.02%**
3rd sample	0.1500	0.785445	0.151239	**0.83%**	0.0948	0.022231	0.095728	**0.95%**	0.0545	0.033748	0.049395	**9.33%**
2nd sample	0.0500	0.271403	0.052095	**4.19%**	0.0316	0.007349	0.031442	**0.52%**	0.0182	0.012620	0.018407	**1.36%**
1st sample	0.0020	0.009596	0.001600	**19.99%**	0.0013	0.000310	0.001037	**18.01%**	0.0007	0.000499	0.000630	**13.32%**
blank	0.0000	0.000000	0.000000	**NA**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**
		(N52) isoAsp (Stress)				**(N54) isoAsp (Stress)**					**(N61) isoAsp (Stress)**	
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.0299	4.435766	0.028628	**4.22%**	0.1074	0.359219	0.107731	**0.29%**	0.0397	0.930610	0.039646	**0.03%**
5th sample	0.0214	3.583103	0.023147	**8.41%**	0.0767	0.249896	0.074908	**2.37%**	0.0283	0.678903	0.029054	**2.57%**
4th sample	0.0128	2.220344	0.014386	**12.30%**	0.0460	0.162961	0.048807	**6.02%**	0.0170	0.376290	0.016319	**3.98%**
3rd sample	0.0064	0.911895	0.005975	**6.71%**	0.0230	0.073486	0.021943	**4.67%**	0.0085	0.164431	0.007404	**12.88%**
2nd sample	0.0029	0.396489	0.002662	**6.81%**	0.0077	0.027085	0.008012	**4.42%**	0.0028	0.060585	0.003033	**7.09%**
1st sample	0.0001	0.000000	NA	**NA**	0.0003	0.000000	NA	**NA**	0.0001	0.000000	NA	**NA**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**
		GLEW (Stress)				**(N54) Asp (Stress)**					**(N52) Asp (Stress)**	

Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.4796	0.148871	0.480576	**0.21%**	0.0174	0.341860	0.016716	**3.72%**	0.0261	0.066135	0.025920	**0.75%**
5th sample	0.3425	0.104625	0.338033	**1.32%**	0.0124	0.273492	0.013373	**7.84%**	0.0187	0.047548	0.018613	**0.22%**
4th sample	0.2055	0.065350	0.211503	**2.91%**	0.0074	0.153991	0.007530	**1.19%**	0.0112	0.028235	0.011021	**1.53%**
3rd sample	0.1028	0.030461	0.099102	**3.56%**	0.0037	0.067769	0.003314	**10.93%**	0.0056	0.012886	0.004987	**10.88%**
2nd sample	0.0343	0.010052	0.033349	**2.64%**	0.0012	0.026379	0.001290	**4.00%**	0.0019	0.004985	0.001881	**0.85%**
1st sample	0.0014	0.000086	0.001244	**9.21%**	0.0000	0.000000	NA	**NA**	0.0001	0.000000	NA	**NA**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**
GLEW (Non-Stress)					**(N86) IsoAsp (Stress)**				**(N91) IsoAsp (Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.7000	0.233746	0.692239	**1.11%**	0.0301	0.107387	0.030074	**0.05%**	0.0141	0.170698	0.014009	**0.95%**
5th sample	0.5000	0.170832	0.505438	**1.09%**	0.0215	0.077140	0.021627	**0.59%**	0.0101	0.122734	0.010068	**0.34%**
4th sample	0.3000	0.104887	0.309639	**3.21%**	0.0129	0.045468	0.012782	**0.92%**	0.0061	0.079362	0.006504	**7.31%**
3rd sample	0.1500	0.051625	0.151499	**1.00%**	0.0064	0.022079	0.006250	**2.35%**	0.0030	0.035595	0.002908	**4.04%**
2nd sample	0.0500	0.015007	0.042776	**14.45%**	0.0021	0.007407	0.002152	**2.50%**	0.0010	0.011535	0.000931	**7.80%**
1st sample	0.0020	0.001314	0.002118	**5.92%**	0.0001	0.000102	0.000112	**12.16%**	0.0000	0.000000	NA	**NA**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**
(N92) IsoAsp (Stress)					**PENNY (Stress)**				**(N91/92) Asp (Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.1144	0.270111	0.112972	**1.26%**	0.3137	0.251435	0.316839	**1.01%**	0.2063	3.168516	0.210002	**1.78%**
5th sample	0.0817	0.199717	0.083200	**1.80%**	0.2241	0.176006	0.221805	**1.00%**	0.1474	2.141928	0.141876	**3.74%**
4th sample	0.0490	0.119984	0.049477	**0.90%**	0.1344	0.102324	0.128971	**4.06%**	0.0884	1.327537	0.087832	**0.68%**

3rd sample	0.0245	0.063538	0.025604	**4.43%**	0.0672	0.056165	0.070814	**5.35%**	0.0442	0.704980	0.046518	**5.21%**
2nd sample	0.0082	0.021586	0.007861	**3.81%**	0.0224	0.017556	0.022170	**1.05%**	0.0147	0.231942	0.015127	**2.63%**
1st sample	0.0003	0.003886	0.000375	**14.65%**	0.0009	0.000527	0.001041	**16.16%**	0.0006	0.013106	0.000604	**2.50%**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**
	(N86) Asp (Stress)				**(Q88) Asp (Stress)**				**PENNY (Non-Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.0152	0.089073	0.015265	**0.56%**	0.0058	0.741918	0.006137	**4.94%**	0.7000	1.031322	0.706268	**0.90%**
5th sample	0.0108	0.062523	0.010710	**1.22%**	0.0042	0.447362	0.003799	**9.06%**	0.5000	0.718473	0.491753	**1.65%**
4th sample	0.0065	0.037537	0.006423	**1.27%**	0.0025	0.266457	0.002363	**5.73%**	0.3000	0.434501	0.297039	**0.99%**
3rd sample	0.0033	0.020863	0.003562	**9.51%**	0.0013	0.149431	0.001434	**14.42%**	0.1500	0.226528	0.154435	**2.96%**
2nd sample	0.0011	0.005721	0.000964	**11.05%**	0.0004	0.027916	0.000469	**12.34%**	0.0500	0.072392	0.048747	**2.51%**
1st sample	0.0000	0.000319	0.000038	**13.22%**	0.0000	0.000000	NA	**NA**	0.0020	0.003954	0.001820	**8.99%**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.003954	0.000000	**0.00%**
Cal curve 3		**DST peptide (Non-Stress)**				**NTLYL (Non-Stress)**						
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*				
6th sample	0.7000	3.481478	0.699486	**0.07%**	0.7000	0.190846	0.640235	**8.54%**				
5th sample	0.5000	2.475199	0.496685	**0.66%**	0.5000	0.140838	0.472647	**5.47%**				
4th sample	0.3000	1.548817	0.309986	**3.33%**	0.3000	0.079037	0.265539	**11.49%**				
3rd sample	0.1500	0.716084	0.142160	**5.23%**	0.1500	0.041448	0.139573	**6.95%**				
2nd sample	0.0500	0.278632	0.053998	**8.00%**	0.0500	0.014069	0.047817	**4.37%**				
1st sample	0.0020	0.019884	0.001851	**7.45%**	0.0020	0.000333	0.001787	**10.65%**				
blank	0.0000	0.000000	0.000000	**NA**	0.0000	0.000000	0.000000	**0.00%**				
	DST peptide (Stress)				**NTLYL (Stress)**				**Ox (Stress)**			
Sample (point of	*Theoretical spiked*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from*	*Theoretical spiked*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml]*	*Rel. deviation from*	*Theoretical spiked concentra*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml]*	*Rel. deviation from*

calibration curve)	*concentration [in mg/ml]*			*theoretical spiked value*	*concentration [in mg/ml]*		*from equation*	*theoretical spiked value*	*tion [in mg/ml]*		*from equation*	*theoretical spiked value*
6th sample	0.7000	3.722775	0.708745	**1.25%**	0.4425	0.103297	0.445008	**0.56%**	0.2542	0.168227	0.246637	**2.99%**
5th sample	0.5000	2.575492	0.490903	**1.82%**	0.3161	0.072388	0.311722	**1.38%**	0.1816	0.125711	0.184279	**1.48%**
4th sample	0.3000	1.537693	0.293850	**2.05%**	0.1896	0.044432	0.191171	**0.80%**	0.1090	0.081447	0.119357	**9.54%**
3rd sample	0.1500	0.782933	0.150540	**0.36%**	0.0948	0.022607	0.097053	**2.35%**	0.0545	0.033742	0.049388	**9.35%**
2nd sample	0.0500	0.273313	0.053775	**7.55%**	0.0316	0.007344	0.031237	**1.17%**	0.0182	0.012775	0.018635	**2.61%**
1st sample	0.0020	0.002130	0.002284	**14.21%**	0.0013	0.000415	0.001359	**7.46%**	0.0007	0.000556	0.000712	**1.92%**
blank	0.0000	0.000000	0.000000	**NA**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**

	(N52) isoAsp (Stress)				**(N54) isoAsp (Stress)**				**(N61) isoAsp (Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.0299	4.364518	0.028501	**4.65%**	0.1074	0.354801	0.107058	**0.34%**	0.0397	0.932299	0.039647	**0.03%**
5th sample	0.0214	3.587858	0.023458	**9.87%**	0.0767	0.249505	0.075178	**2.02%**	0.0283	0.680551	0.029079	**2.66%**
4th sample	0.0128	2.217357	0.014558	**13.64%**	0.0460	0.166249	0.049971	**8.55%**	0.0170	0.376716	0.016325	**3.94%**
3rd sample	0.0064	0.907049	0.006049	**5.56%**	0.0230	0.073408	0.021862	**5.02%**	0.0085	0.161993	0.007312	**13.95%**
2nd sample	0.0028	0.390125	0.002692	**4.38%**	0.0077	0.026850	0.007766	**1.21%**	0.0028	0.060257	0.003041	**7.37%**
1st sample	0.0001	0.000000	NA	**NA**	0.0003	0.000000	NA	**NA**	0.0001	0.000000	NA	**NA**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**

	GLEW (Stress)				**(N54) Asp (Stress)**				**(N52) Asp (Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.4796	0.148762	0.479763	**0.04%**	0.0174	0.339232	0.016464	**5.17%**	0.0261	0.066189	0.025942	**0.67%**
5th sample	0.3425	0.105296	0.339866	**0.78%**	0.0124	0.273190	0.013236	**6.73%**	0.0187	0.047635	0.018614	**0.21%**
4th sample	0.2055	0.065280	0.211073	**2.70%**	0.0074	0.171176	0.008249	**10.87%**	0.0112	0.025073	0.009704	**13.30%**
3rd sample	0.1028	0.030473	0.099044	**3.62%**	0.0037	0.067668	0.003190	**14.25%**	0.0056	0.013223	0.005024	**10.22%**
2nd sample	0.0343	0.010051	0.033316	**2.74%**	0.0012	0.027217	0.001213	**2.18%**	0.0019	0.005127	0.001827	**2.04%**

1st sample	0.0014	0.000088	0.001250	**8.76%**	0.0000	0.000000	NA	**NA**	0.0001	0.000000	NA	**NA**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**
	GLEW (Non-Stress)				**(N86) IsoAsp (Stress)**				**(N91) IsoAsp (Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.7000	0.232821	0.690517	**1.35%**	0.0301	0.103695	0.029685	**1.34%**	0.0141	0.171117	0.014018	**0.88%**
5th sample	0.5000	0.172005	0.509679	**1.94%**	0.0215	0.077081	0.022052	**2.57%**	0.0101	0.122745	0.010050	**0.51%**
4th sample	0.3000	0.103599	0.306271	**2.09%**	0.0129	0.045421	0.012971	**0.55%**	0.0061	0.079459	0.006500	**7.25%**
3rd sample	0.1500	0.051704	0.151958	**1.31%**	0.0064	0.021957	0.006240	**2.49%**	0.0030	0.035600	0.002903	**4.20%**
2nd sample	0.0500	0.015375	0.043934	**12.13%**	0.0021	0.007427	0.002073	**1.29%**	0.0010	0.011529	0.000929	**8.02%**
1st sample	0.0020	0.001176	0.001712	**14.38%**	0.0001	0.000490	0.000083	**16.84%**	0.0000	0.000000	NA	**0.03%**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**
	(N92) IsoAsp (Stress)				**PENNY (Stress)**				**(N91/92) Asp (Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.1144	0.269165	0.112796	**1.42%**	0.3137	0.250800	0.315690	**0.64%**	0.2063	3.173456	0.210058	**1.80%**
5th sample	0.0817	0.199876	0.083432	**2.09%**	0.2241	0.175990	0.221637	**1.08%**	0.1474	2.142375	0.141720	**3.84%**
4th sample	0.0490	0.119973	0.049571	**1.09%**	0.1344	0.102482	0.129220	**3.88%**	0.0884	1.332423	0.088038	**0.44%**
3rd sample	0.0245	0.063265	0.025539	**4.17%**	0.0672	0.056125	0.070939	**5.54%**	0.0442	0.704822	0.046442	**5.04%**
2nd sample	0.0082	0.021434	0.007812	**4.41%**	0.0224	0.017616	0.022524	**0.53%**	0.0147	0.232415	0.015132	**2.67%**
1st sample	0.0003	0.003909	0.000385	**17.89%**	0.0009	0.000540	0.001056	**17.78%**	0.0006	0.013114	0.000597	**1.34%**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**
	(N86) Asp (Stress)				**(Q88) Asp (Stress)**				**PENNY (Non-Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretic*

												al spiked value
6th sample	0.0152	0.089396	0.015312	**0.87%**	0.0058	0.744072	0.006216	**6.29%**	0.7000	1.029136	0.706227	**0.89%**
5th sample	0.0108	0.062477	0.010703	**1.29%**	0.0042	0.447865	0.003780	**9.52%**	0.5000	0.714753	0.490067	**1.99%**
4th sample	0.0065	0.036770	0.006301	**3.15%**	0.0025	0.264474	0.002271	**9.39%**	0.3000	0.438795	0.300327	**0.11%**
3rd sample	0.0033	0.020685	0.003547	**9.04%**	0.0013	0.149022	0.001321	**5.43%**	0.1500	0.226731	0.154518	**3.01%**
2nd sample	0.0011	0.005757	0.000991	**8.61%**	0.0004	0.041802	0.000439	**5.16%**	0.0500	0.071100	0.047511	**4.98%**
1st sample	0.0000	0.000268	0.000051	**17.61%**	0.0000	0.000000	NA	**NA**	0.0020	0.004558	0.001759	**12.05%**
blank	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.000000	0.000000	**0.00%**	0.0000	0.004558	0.000000	**0.00%**

Table S6. Full data obtained for all peptides in three calibration curves in pooled animal serum for both stressed and non-stressed reference standard. Columns are distributed as follows: Sample name/number corresponding to the calibration curve point, theoretical spiked concentration of corresponding modification in the stressed reference standard, signal corrected by IS, concentration calculated through the equation obtained from the calibration curve of the corresponding peptide and relative deviation of the calculated concentration from the theoretical spiked value. Concentrations are given in mg/ml.

3 Cal Curves: Non-Stress												
DST peptide (Non-Stress)							**NTLYL (Non-Stress)**					
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*
6thsample	0.7000	3.513464	0.702450	**0.35%**	0.027700	**3.94%**	0.7000	0.198864	0.660233	**5.68%**	0.002092	**1.05%**
5thsample	0.5000	2.464966	0.492494	**1.50%**	0.008866	**1.80%**	0.5000	0.144517	0.479617	**4.08%**	0.007713	**5.34%**
4thsample	0.3000	1.554728	0.310224	**3.41%**	0.005120	**1.65%**	0.3000	0.082528	0.273606	**8.80%**	0.001811	**2.19%**
3rdsample	0.1500	0.713360	0.141745	**5.50%**	0.002361	**1.67%**	0.1500	0.042359	0.140109	**6.59%**	0.004623	**10.91%**
2ndsample	0.0500	0.276434	0.054253	**8.51%**	0.002124	**3.91%**	0.0500	0.014315	0.046909	**6.18%**	0.001412	**9.86%**
1stsample	0.0020	0.015244	0.001951	**2.45%**	0.000294	**15.09%**	0.0020	0.000758	0.001854	**7.29%**	NA	**NA**
blank	0.0000	0.000000	0.000000	**0**			0.0000	0.000040	0.000000	**0**		

		GLEW (Non-Stress)							**PENNY (Non-Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*
6thsample	0.7000	0.236991	0.695537	**0.64%**	0.006439	**2.72%**	0.7000	1.029356	0.706079	**0.87%**	0.026237	**2.55%**
5thsample	0.5000	0.171183	0.502154	**0.43%**	0.007144	**4.17%**	0.5000	0.715848	0.490904	**1.82%**	0.029377	**4.10%**
4thsample	0.3000	0.104477	0.306133	**2.04%**	0.007611	**7.29%**	0.3000	0.435965	0.298810	**0.40%**	0.007033	**1.61%**
3rdsample	0.1500	0.051651	0.150899	**0.60%**	0.000456	**0.88%**	0.1500	0.226592	0.155108	**3.41%**	0.006769	**2.99%**
2ndsample	0.0500	0.015721	0.045317	**9.37%**	0.000937	**5.96%**	0.0500	0.071945	0.048967	**2.07%**	0.003569	**4.96%**
1st sample	0.0020	0.000883	0.001712	**14.38%**	0.000631	**NA**	0.0020	0.003994	0.002330	**16.48%**	0.000343	**8.58%**
blank	0.0000	0.000000	0.000000	**0**			0.0000	0.000000	0.000000	**0.00%**		
		DST peptide (Stress)							**Ox (Stress)**			
Sample (point of calibration curve)	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
6thsample	0.7000	3.671653	0.704889	**0.70%**	0.044273	**6.28%**	0.2542	0.171110	0.250872	**1.32%**	0.003011	**1.76%**	35.76%
5thsample	0.5000	2.576292	0.494732	**1.05%**	0.000696	**0.14%**	0.1816	0.125833	0.184456	**1.57%**	0.004213	**3.35%**	37.96%
4thsample	0.3000	1.542819	0.296449	**1.18%**	0.004453	**1.50%**	0.1090	0.078165	0.114529	**5.11%**	0.006844	**8.76%**	38.21%
3rdsample	0.1500	0.784604	0.150976	**0.65%**	0.001447	**0.96%**	0.0545	0.033749	0.049376	**9.37%**	0.000791	**2.34%**	34.63%
2ndsample	0.0500	0.272295	0.052684	**5.37%**	0.000961	**1.82%**	0.0182	0.012632	0.018399	**1.32%**	0.000837	**6.62%**	37.54%
1stsample	0.0020	0.007251	0.001832	**8.38%**	0.000240	**13.09%**	0.0007	0.000531	0.000647	**10.99%**	0.000085	**16.05%**	31.20%
blank	0.0000	0.000000	0.000000	**0**			0.0000	0.000000	0.000000	**0**		***Average Strategy B***	**35.88%**
												Rel. deviation PBS	**1.20%**
												RSD Strategy B	**4.33%**
												Average Strategy A	**34.91%**
												Rel. deviation PBS	**3.87%**
												RSD Strategy A	**12.28%**

NTLYL (Stress)							(N52) isoAsp (Stress)						
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
0.4425	0.103048	0.450600	**1.83%**	0.001138	**1.10%**	64.24%	0.0299	4.412718	0.028322	**5.25%**	0.041756	**0.95%**	4.05%
0.3161	0.068946	0.301485	**4.62%**	0.000115	**0.17%**	62.04%	0.0214	3.583575	0.022976	**7.61%**	0.040680	**1.14%**	4.62%
0.1896	0.042348	0.185187	**2.35%**	0.001082	**2.55%**	61.79%	0.0128	2.220184	0.014184	**10.73%**	0.027510	**1.24%**	4.58%
0.0948	0.021310	0.093198	**1.71%**	0.001136	**5.33%**	65.37%	0.0064	0.911770	0.005747	**10.27%**	0.046599	**5.11%**	4.03%
0.0316	0.006998	0.030615	**3.14%**	0.000298	**4.26%**	62.46%	0.0029	0.397623	0.002432	**14.87%**	0.008124	**2.04%**	4.85%
0.0013	0.000322	0.001426	**12.78%**	0.000035	**10.89%**	68.80%	0.0001	0.000000	NA	**NA**	0.000000	**NA**	NA
0.0000	0.000000	0.000000	**0**	***Average Strategy B***		**64.12%**	0.0000	0.000000	0.000000	**0.00%**		***Average Strategy B***	**4.43%**
				Rel. deviation PBS		**1.42%**						***Rel. deviation PBS***	**3.65%**
				RSD Strategy B		**2.42%**						**RSD Strategy B**	**8.25%**
				Average Strategy A		**49.44%**						***Average Strategy A***	**3.45%**
				Rel. deviation PBS		**21.80%**						***Rel. deviation PBS***	**19.30%**
				RSD Strategy A		**31.95%**						**RSD Strategy A**	**25.39%**

(N54) isoAsp (Stress)							(N61) isoAsp (Stress)						
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
0.1074	0.357721	0.107494	**0.07%**	0.025291	**7.07%**	15.39%	0.0397	0.933104	0.039680	**0.06%**	0.029801	**3.19%**	5.68%
0.0767	0.249769	0.074991	**2.26%**	0.002286	**0.92%**	15.06%	0.0283	0.679441	0.029023	**2.46%**	0.009617	**1.42%**	5.83%
0.0460	0.164091	0.049195	**6.86%**	0.018698	**11.40%**	15.89%	0.0170	0.376550	0.016298	**4.10%**	0.002286	**0.61%**	5.26%
0.0230	0.073461	0.021908	**4.83%**	0.000463	**0.63%**	15.36%	0.0085	0.163575	0.007351	**13.50%**	0.013712	**8.38%**	5.16%
0.0077	0.027006	0.007920	**3.23%**	0.001354	**5.01%**	15.79%	0.0028	0.062435	0.003102	**9.51%**	0.003492	**5.59%**	6.18%
0.0003	0.000000	NA	**NA**	0.000000	**NA**	NA	0.0001	0.000000	NA	**NA**	0.000000	**NA**	NA
0.0000	0.000000	0.000000	**0.00%**	***Average Strategy B***		**15.50%**	0.0000	0.000000	0.000000	**0.00%**		***Average Strategy B***	**5.62%**
				Rel. deviation PBS		**1.00%**						***Rel. deviation PBS***	**0.75%**
				RSD Strategy B		**2.18%**						**RSD Strategy B**	**7.49%**
				Average Strategy A		**14.41%**						***Average Strategy A***	**5.11%**

				Rel. deviation PBS		**6.13%**						***Rel. deviation PBS***	**9.78%**
				RSD Strategy A		**22.18%**						**RSD Strategy A**	**24.70%**
GLEW (Stress)							**(N54) Asp (Stress)**						
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
0.4796	0.148835	0.480306	**0.16%**	0.006280	**4.22%**	68.75%	0.0174	0.341008	0.016646	**4.12%**	0.015388	**4.51%**	2.38%
0.3425	0.104855	0.338664	**1.13%**	0.003824	**3.65%**	68.03%	0.0124	0.273389	0.013337	**7.55%**	0.001726	**0.63%**	2.68%
0.2055	0.065330	0.211370	**2.84%**	0.000435	**0.67%**	68.26%	0.0074	0.159700	0.007775	**4.49%**	0.009939	**6.22%**	2.51%
0.1028	0.030465	0.099083	**3.58%**	0.000649	**2.13%**	69.49%	0.0037	0.067723	0.003274	**11.99%**	0.000514	**0.76%**	2.30%
0.0343	0.010052	0.033338	**2.67%**	0.000275	**2.73%**	66.47%	0.0012	0.026657	0.001265	**2.02%**	0.000486	**1.82%**	2.52%
0.0014	0.000087	0.001246	**9.05%**	0.000001	**1.48%**	NA	0.0000	0.000000	NA	**NA**	0.000000	**NA**	NA
0.0000	0.000000	0.000000	**0.00%**	***Average Strategy B***		**68.20%**	0.0000	0.000000	0.000000	**0.00%**		***Average Strategy B***	**2.48%**
				Rel. deviation PBS		**0.45%**						***Rel. deviation PBS***	**0.08%**
				RSD Strategy B		**1.64%**						**RSD Strategy B**	**5.90%**
				Average Strategy A		**70.93%**						***Average Strategy A***	**2.45%**
				Rel. deviation PBS		**3.54%**						***Rel. deviation PBS***	**1.15%**
				RSD Strategy A		**9.07%**						**RSD Strategy A**	**23.40%**

(N52) Asp (Stress)						
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
0.0261	0.066176	0.026164	**0.19%**	0.003593	**5.43%**	3.75%
0.0187	0.047577	0.018844	**1.02%**	0.000507	**1.06%**	3.79%
0.0112	0.027176	0.010814	**3.38%**	0.001821	**6.70%**	3.49%
0.0056	0.012979	0.005226	**6.61%**	0.000213	**1.64%**	3.67%
0.0019	0.005032	0.002099	**12.51%**	0.000083	**1.64%**	4.18%

0.0001	0.000000	NA	**NA**	0.000000	**NA**	NA
0.0000	0.000000	0.000000	**0.00%**	***Average Strategy B***		**3.77%**
				Rel. deviation PBS		**1.17%**
				RSD Strategy B		**6.76%**
				Average Strategy A		**3.65%**
				Rel. deviation PBS		**2.05%**
				RSD Strategy A		**24.30%**

PENNY (Stress)							**(N86) IsoAsp (Stress)**						
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
0.3137	0.250770	0.316402	**0.87%**	0.006798	**2.71%**	44.90%	0.0301	0.106146	0.029955	**0.44%**	0.002123	**2.00%**	4.25%
0.2241	0.175999	0.222137	**0.85%**	0.008337	**4.74%**	44.99%	0.0215	0.077153	0.021788	**1.34%**	0.000787	**1.02%**	4.41%
0.1344	0.102374	0.129317	**3.80%**	0.000930	**0.91%**	43.86%	0.0129	0.045451	0.012859	**0.32%**	0.002583	**5.68%**	4.36%
0.0672	0.056152	0.071043	**5.70%**	0.002313	**4.12%**	45.20%	0.0064	0.022038	0.006264	**2.13%**	0.000703	**3.19%**	3.99%
0.0224	0.017576	0.022411	**0.03%**	0.000341	**1.94%**	44.99%	0.0021	0.007414	0.002145	**2.12%**	0.000112	**1.51%**	4.31%
0.0009	0.000645	0.001066	**18.90%**	0.000086	**13.26%**	48.65%	0.0001	0.000171	0.000105	**4.59%**	0.000006	**3.25%**	4.77%
0.0000	0.000000	0.000000	**0**	***Average Strategy B***		**45.43%**	0.0000	0.000000	0.000000	**0.00%**	***Average Strategy B***		**4.35%**
				Rel. deviation PBS		**1.39%**					***Rel. deviation PBS***		**1.17%**
				RSD Strategy B		**1.17%**					**RSD Strategy B**		**3.84%**
				Average Strategy A		**44.12%**					***Average Strategy A***		**4.36%**
				Rel. deviation PBS		**1.55%**					***Rel. deviation PBS***		**1.54%**
				RSD Strategy A		**4.56%**					**RSD Strategy A**		**12.80%**
(N91) IsoAsp (Stress)							**(N92) IsoAsp (Stress)**						
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
0.0141	0.170869	0.014013	**0.91%**	0.002197	**1.29%**	1.99%	0.1144	0.269116	0.112809	**1.40%**	0.010211	**3.79%**	16.01%

0.0101	0.122737	0.010061	**0.40%**	0.000737	**0.60%**	2.04%	0.0817	0.199741	0.083390	**2.04%**	0.001246	**0.62%**	16.89%
0.0061	0.079352	0.006499	**7.23%**	0.001132	**1.43%**	2.20%	0.0490	0.119990	0.049570	**1.09%**	0.000211	**0.18%**	16.81%
0.0030	0.035606	0.002907	**4.07%**	0.001441	**4.05%**	1.85%	0.0245	0.063462	0.025598	**4.41%**	0.001728	**2.72%**	16.29%
0.0010	0.011533	0.000931	**7.88%**	0.000322	**2.79%**	1.87%	0.0082	0.021538	0.007819	**4.33%**	0.000903	**4.19%**	15.70%
0.0000	0.000000	NA	**NA**	0.000000	**NA**	NA	0.0003	0.003992	0.000378	**15.69%**	0.000163	**4.09%**	NA
0.0000	0.000000	0.000000	**0.00%**	***Average Strategy B***		**1.99%**	0.0000	0.000000	0.000000	**0.00%**	***Average Strategy B***		**16.34%**
				Rel. deviation PBS		**1.51%**					***Rel. deviation PBS***		**0.03%**
				RSD Strategy B		**7.23%**					**RSD Strategy B**		**3.14%**
				Average Strategy A		**1.76%**					***Average Strategy A***		**16.86%**
				Rel. deviation PBS		**12.84%**					***Rel. deviation PBS***		**3.17%**
				RSD Strategy A		**24.54%**					**RSD Strategy A**		**22.19%**
	(N91/92) Asp (Stress)							**(N86) Asp (Stress)**					
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
0.2063	3.169973	0.210015	**1.78%**	0.151480	**4.78%**	29.80%	0.0152	0.089195	0.015278	**0.64%**	0.001756	**1.97%**	2.17%
0.1474	2.141972	0.141822	**3.77%**	0.038319	**1.79%**	28.73%	0.0108	0.062503	0.010702	**1.30%**	0.000235	**0.38%**	2.17%
0.0884	1.329363	0.087918	**0.58%**	0.026664	**2.01%**	29.82%	0.0065	0.037284	0.006378	**1.96%**	0.004454	**11.95%**	2.16%
0.0442	0.704494	0.046467	**5.09%**	0.007102	**1.01%**	29.57%	0.0033	0.020813	0.003554	**9.27%**	0.001120	**5.38%**	2.26%
0.0147	0.232110	0.015132	**2.67%**	0.002645	**1.14%**	30.38%	0.0011	0.005733	0.000969	**10.62%**	0.000214	**3.73%**	1.95%
0.0006	0.013106	0.000604	**2.46%**	0.000860	**6.56%**	NA	0.0000	0.000302	0.000038	**12.18%**	0.000030	**9.82%**	NA
0.0000	0.000000	0.000000	**0.00%**	***Average Strategy B***		**29.66%**	0.0000	0.000000	0.000000	**0.00%**	***Average Strategy B***		**2.14%**
				Rel. deviation PBS		**0.62%**					***Rel. deviation PBS***		**1.26%**
				RSD Strategy B		**2.03%**					**RSD Strategy B**		**5.46%**
				Average Strategy A		**29.83%**					***Average Strategy A***		**2.19%**
				Rel. deviation PBS		**1.19%**					***Rel. deviation PBS***		**0.96%**
				RSD Strategy A		**6.45%**					**RSD Strategy A**		**13.60%**

(Q88) Asp (Stress)

Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Absolute standard deviation (triplicates)*	*Relative standard deviation (triplicates)*	*Modification rate (Strategy B)*
0.0058	0.736902	0.006192	**5.87%**	0.106085	**14.40%**	0.88%
0.0042	0.447731	0.003800	**9.03%**	0.032326	**7.22%**	0.77%
0.0025	0.265641	0.002294	**8.46%**	0.010370	**3.90%**	0.78%
0.0013	0.149552	0.001334	**6.47%**	0.006006	**4.02%**	0.85%
0.0004	0.036953	0.000403	**3.48%**	0.007833	**21.20%**	0.81%
0.0000	0.000000	NA	**NA**	0.000000	**NA**	NA
0.0000	0.000000	0.000000	**0.00%**		***Average Strategy B***	**0.82%**
					Rel. deviation PBS	**2.21%**
					RSD Strategy B	**5.68%**
					Average Strategy A	**0.88%**
					Rel. deviation PBS	**5.13%**
					RSD Strategy A	**30.22%**

Table S7. Full data obtained for all peptides in three calibration curves in pooled animal serum for both stressed and non-stressed reference standard and comparison of two strategies to assess the matrix effect on quantification of low-abundant modifications. Columns are distributed as follows: Sample name/number corresponding to the calibration curve point, theoretical spiked concentration of corresponding modification in the stressed reference standard, signal corrected by IS, concentration calculated through the equation obtained from the calibration curve of the corresponding peptide, relative deviation of the calculated concentration from the theoretical spiked value, absolute and relative standard deviation between triplicates, obtained modification rate (calculated through the concentrations reported in the fourth column for all peptide's variants). In blue (Strategy B), in red (Strategy A): Average modification rate of all points per calibration curve, relative deviation from the modification rate observed in PBS, relative deviation between all points within the calibration curve.

Quality control samples

QC samples												
	QC DST				QC Ox (Stress)				QC NTLYL (Stress)			
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
Low QC	0.0250	0.115871	0.022101	**11.60%**	0.0091	0.003379	0.007740	**14.76%**	0.0158	0.002255	0.015256	**3.46%**
Middle QC	0.2500	1.131981	0.225571	**9.77%**	0.0908	0.038137	0.086449	**4.79%**	0.1580	0.024832	0.169752	**7.41%**
High	0.6000	3.009422	0.601518	**0.25%**	0.2179	0.109812	0.248758	**14.15%**	0.3793	0.057537	0.393553	**3.76%**
Low QC	0.0250	0.140912	0.027116	**8.46%**	0.0091	0.004263	0.009740	**7.27%**	0.0158	0.002540	0.017207	**8.88%**
Middle QC	0.2500	1.132372	0.225650	**9.74%**	0.0908	0.039774	0.090156	**0.71%**	0.1580	0.025104	0.171613	**8.59%**
High	0.6000	2.675368	0.534626	**10.90%**	0.2179	0.109170	0.247304	**13.49%**	0.3793	0.059493	0.406940	**7.29%**
Low QC	0.0250	0.138069	0.026546	**6.18%**	0.0091	0.004023	0.009198	**1.30%**	0.0158	0.002142	0.014483	**8.36%**
Middle QC	0.2500	1.108277	0.220825	**11.67%**	0.0908	0.036785	0.083386	**8.16%**	0.1580	0.022976	0.157049	**0.63%**
High QC	0.6000	2.883562	0.576316	**3.95%**	0.2179	0.108746	0.246342	**13.04%**	0.3793	0.058737	0.401765	**5.92%**

	QC NTLYL (Non-Stress)				QC (N52) isoAsp (Stress)				QC (N54) isoAsp (Stress)			
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
0.0250	0.008140	0.02638775	**5.55%**	0.0011	0.123534	0.001108	**3.83%**	0.0038	0.007514	0.003411	**11.08%**
0.2500	0.065450	0.21684869	**13.26%**	0.0107	1.019641	0.010123	**5.17%**	0.0384	0.089639	0.042004	**9.49%**
0.6000	0.162613	0.53975859	**10.04%**	0.0256	3.849337	0.024609	**3.95%**	0.0921	0.181793	0.085310	**7.35%**
0.0250	0.007245	0.02341192	**6.35%**	0.0011	0.121820	0.001091	**2.21%**	0.0038	0.007255	0.003289	**14.26%**
0.2500	0.068132	0.22576302	**9.69%**	0.0107	1.005783	0.009984	**6.48%**	0.0384	0.074368	0.034828	**9.22%**
0.6000	0.198074	0.65760678	**9.60%**	0.0256	2.911446	0.029154	**13.79%**	0.0921	0.176078	0.082624	**10.26%**
0.0250	0.007658	0.02478687	**0.85%**	0.0011	0.121423	0.001087	**1.84%**	0.0038	0.007213	0.003269	**14.78%**
0.2500	0.067451	0.22349885	**10.60%**	0.0107	0.989333	0.009818	**8.03%**	0.0384	0.080848	0.037872	**1.28%**
0.6000	0.156727	0.5201948	**13.30%**	0.0256	2.907905	0.029119	**13.65%**	0.0921	0.197363	0.092626	**0.60%**

	QC (N61) isoAsp (Stress)				QC GLEW (Stress)				QC (N54) Asp (Stress)			
Theoretical spiked concentrati	*AUC (peptide) / AUC (IS)*	*Concentration [in*	*Rel. deviation from*	*Theoretical spiked concentrati*	*AUC (peptide) / AUC (IS)*	*Concentration [in*	*Rel. deviation from*	*Theoretical spiked concentrati*	*AUC (peptide) / AUC (IS)*	*Concentration [in*	*Rel. deviation from*

on [in mg/ml]		*mg/ml] from equation*	*theoretical spiked value*	*on [in mg/ml]*		*mg/ml] from equation*	*theoretical spiked value*	*on [in mg/ml]*		*mg/ml] from equation*	*theoretical spiked value*
0.0014	0.016012	0.001492	**5.37%**	0.0171	0.002738	0.014773	**13.74%**	0.0006	0.007007	0.000536	**13.56%**
0.0142	0.198050	0.013415	**5.28%**	0.1713	0.030961	0.157069	**8.29%**	0.0062	0.085264	0.006524	**5.21%**
0.0340	0.582131	0.038572	**13.48%**	0.4110	0.080861	0.408659	**0.58%**	0.0149	0.167070	0.012783	**14.10%**
0.0014	0.015593	0.001465	**3.43%**	0.0171	0.002796	0.015065	**12.04%**	0.0006	0.007136	0.000546	**11.96%**
0.0142	0.182567	0.012401	**12.44%**	0.1713	0.032667	0.165667	**3.27%**	0.0062	0.080258	0.006141	**0.97%**
0.0340	0.528707	0.035073	**3.18%**	0.4110	0.076471	0.386523	**5.97%**	0.0149	0.174552	0.013355	**10.25%**
0.0014	0.015864	0.001483	**4.69%**	0.0171	0.002862	0.015397	**10.10%**	0.0006	0.006959	0.000532	**14.15%**
0.0142	0.219906	0.014847	**4.83%**	0.1713	0.035398	0.179440	**4.77%**	0.0062	0.073192	0.005600	**9.69%**
0.0340	0.500257	0.033209	**2.30%**	0.4110	0.079106	0.399812	**2.73%**	0.0149	0.196634	0.015045	**1.10%**

QC (N52) Asp (Stress)				**QC GLEW (Non-Stress)**				**QC (N86) IsoAsp (Stress)**			
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
0.0009	0.001587	0.000858	**8.04%**	0.0250	0.007640	0.021568	**13.73%**	0.0011	0.002633	0.001235	**14.90%**
0.0093	0.014686	0.008910	**4.47%**	0.2500	0.085479	0.250305	**0.12%**	0.0107	0.024445	0.010767	**0.20%**
0.0224	0.035114	0.021467	**4.10%**	0.6000	0.193500	0.567735	**5.38%**	0.0258	0.065291	0.028618	**10.96%**
0.0009	0.001608	0.000871	**6.64%**	0.0250	0.008495	0.024082	**3.67%**	0.0011	0.002403	0.001134	**5.51%**
0.0093	0.013747	0.008332	**10.66%**	0.2500	0.083124	0.243386	**2.65%**	0.0107	0.023220	0.010232	**4.79%**
0.0224	0.035105	0.021461	**4.13%**	0.6000	0.197294	0.578884	**3.52%**	0.0258	0.065423	0.028676	**11.19%**
0.0009	0.001926	0.001066	**14.32%**	0.0250	0.008583	0.024340	**2.64%**	0.0011	0.002571	0.001208	**12.37%**
0.0093	0.014380	0.008722	**6.49%**	0.2500	0.085936	0.251648	**0.66%**	0.0107	0.022430	0.009886	**8.00%**
0.0224	0.035179	0.021506	**3.92%**	0.6000	0.202737	0.594877	**0.85%**	0.0258	0.065866	0.028869	**11.94%**

QC (N91) IsoAsp (Stress)				**QC (N92) IsoAsp (Stress)**				**QC PENNY (Stress)**			
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
0.0005	0.004113	0.000512	**1.42%**	0.0041	0.007886	0.003922	**4.02%**	0.0112	0.006430	0.012603	**12.50%**
0.0051	0.038204	0.004895	**3.09%**	0.0409	0.064476	0.042343	**3.62%**	0.1120	0.053075	0.104948	**6.32%**
0.0121	0.106914	0.013728	**13.25%**	0.0981	0.138291	0.092458	**5.72%**	0.2689	0.142997	0.282974	**5.25%**

0.0005	0.004052	0.000505	**0.11%**	0.0041	0.007349	0.003558	**12.92%**	0.0112	0.005623	0.011006	**1.75%**
0.0051	0.041342	0.005298	**4.90%**	0.0409	0.060086	0.039362	**3.67%**	0.1120	0.054743	0.108252	**3.37%**
0.0121	0.094223	0.012096	**0.21%**	0.0981	0.152555	0.102143	**4.15%**	0.2689	0.144486	0.285922	**6.35%**
0.0005	0.003958	0.000492	**2.50%**	0.0041	0.007576	0.003712	**9.16%**	0.0112	0.005393	0.010549	**5.83%**
0.0051	0.036781	0.004712	**6.71%**	0.0409	0.062179	0.040783	**0.19%**	0.1120	0.059440	0.117550	**4.93%**
0.0121	0.096415	0.012378	**2.11%**	0.0981	0.150731	0.100904	**2.89%**	0.2689	0.148030	0.292938	**8.96%**
	QC (N91/92) Asp (Stress)				**QC (N86) Asp (Stress)**				**QC (Q88) Asp (Stress)**		
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
0.0074	0.077984	0.007841	**6.41%**	0.0005	0.002154	0.000561	**3.47%**	0.0002	0.009085	0.000197	**5.54%**
0.0737	0.671211	0.069459	**5.74%**	0.0054	0.020898	0.005593	**3.16%**	0.0021	0.160369	0.002184	**4.55%**
0.1769	1.862619	0.193210	**9.24%**	0.0130	0.048013	0.012872	**1.07%**	0.0050	0.378092	0.005042	**0.59%**
0.0074	0.069952	0.007007	**4.91%**	0.0005	0.001945	0.000505	**6.87%**	0.0002	0.008474	0.000189	**9.38%**
0.0737	0.700222	0.072473	**1.65%**	0.0054	0.019996	0.005351	**1.30%**	0.0021	0.157244	0.002143	**2.58%**
0.1769	1.914701	0.198620	**12.30%**	0.0130	0.048136	0.012905	**0.82%**	0.0050	0.346189	0.004624	**7.77%**
0.0074	0.077993	0.007842	**6.42%**	0.0005	0.002352	0.000614	**13.30%**	0.0002	0.008807	0.000194	**7.28%**
0.0737	0.632960	0.065486	**11.14%**	0.0054	0.019255	0.005152	**4.98%**	0.0021	0.170445	0.002316	**10.88%**
0.1769	1.783508	0.184993	**4.60%**	0.0130	0.047459	0.012723	**2.22%**	0.0050	0.357193	0.004768	**4.88%**
	QC PENNY (Non-Stress)										
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*								
0.0250	0.040607	0.027459	**9.83%**								
0.2500	0.348348	0.238674	**4.53%**								
0.6000	0.852974	0.585020	**2.50%**								
0.0250	0.040328	0.027267	**9.07%**								
0.2500	0.343521	0.235361	**5.86%**								
0.6000	0.846158	0.580342	**3.28%**								
0.0250	0.040540	0.027412	**9.65%**								
0.2500	0.369845	0.253428	**1.37%**								

0.6000	0.821814	0.563634	**6.06%**

Table S8. Full data obtained for all peptides in quality control samples. Columns are distributed as follows: Theoretical spiked concentration of corresponding modification in the stressed reference standard, signal corrected by IS, concentration calculated through the equation obtained from the calibration curve of the corresponding peptide and relative deviation of the calculated concentration from the theoretical spiked value.

QC samples at 3 ratios										
	DST peptide (Stress)					**Ox (Stress)**				
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*
High QC 1:1	0.6000	3.192460	0.612951	2.16%	0.1090	0.077273	0.113222	3.91%	20.00%	20.12%
High QC 3:1	0.6000	2.836932	0.544739	9.21%	0.1634	0.095388	0.139794	14.47%	27.67%	27.07%
High QC 1:3	0.6000	3.421378	0.656871	9.48%	0.0545	0.042539	0.062269	14.30%	8.11%	8.10%
Mid QC 1:1	0.2500	1.269614	0.244031	2.39%	0.0454	0.027163	0.039714	12.52%	18.10%	17.57%
Mid QC 3:1	0.2500	1.368770	0.263055	5.22%	0.0681	0.040932	0.059912	12.02%	31.71%	31.69%
Mid QC 1:3	0.2500	1.368345	0.262974	5.19%	0.0227	0.014894	0.021717	4.33%	7.82%	8.07%
Low QC 1:1	0.0250	0.124203	0.024271	2.92%	0.0045	0.003110	0.004431	2.41%	17.28%	16.36%
Low QC 3:1	0.0250	0.116478	0.022789	8.84%	0.0068	0.004288	0.006158	9.58%	37.75%	32.66%
Low QC 1:3	0.0250	0.119523	0.023373	6.51%	0.0023	0.001865	0.002603	14.68%	7.83%	8.04%
							QCs 1:1	*Average*	18.46%	18.02%
					Theoretical modification rate	NTLYLox		*Rel. deviation PBS*	1.65%	0.79%
					QC 1:1	18.16%		RSD	7.55%	10.64%
					QC 3:1	27.24%	QCs 3:1	*Average*	32.38%	30.47%
					QC 1:3	9.08%		*Rel. deviation PBS*	18.86%	11.87%
								RSD	15.66%	9.79%
							QCs 1:3	*Average*	7.92%	8.07%
								Rel. deviation PBS	12.78%	11.13%

								RSD	2.09%	0.35%		
			NTLYL (Stress)							**(N52) isoAsp (Stress)**		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*
High QC 1:1	0.4896	0.102811	0.449561	8.19%	79.82%	79.88%	0.0128	2.139066	0.013661	6.64%	1.96%	2.09%
High QC 3:1	0.3396	0.086112	0.376545	10.86%	72.02%	72.93%	0.0192	3.118605	0.019977	3.97%	3.04%	3.27%
High QC 1:3	0.6396	0.161631	0.706755	10.49%	91.77%	91.90%	0.0064	1.102402	0.006976	8.92%	1.07%	1.07%
Mid QC 1:1	0.2040	0.042611	0.186338	8.67%	81.81%	82.43%	0.0053	0.752618	0.004721	11.55%	2.01%	2.13%
Mid QC 3:1	0.1415	0.029536	0.129163	8.73%	68.11%	68.31%	0.0080	1.214185	0.007697	3.86%	3.41%	3.39%
Mid QC 1:3	0.2665	0.056565	0.247352	7.19%	92.18%	91.93%	0.0027	0.405196	0.002481	7.05%	0.88%	0.90%
Low QC 1:1	0.0204	0.005175	0.022646	11.00%	82.72%	83.64%	0.0005	0.096508	0.000490	8.18%	2.86%	2.06%
Low QC 3:1	0.0142	0.002900	0.012697	10.28%	62.25%	67.34%	0.0008	0.135782	0.000743	7.15%	3.42%	3.11%
Low QC 1:3	0.0267	0.006805	0.029772	11.71%	92.17%	91.96%	0.0003	0.059881	0.000254	4.85%	1.74%	1.13%
			QCs 1:1	*Average*	81.45%	81.98%			QCs 1:1	*Average*	2.28%	2.10%
	Theoretical modification rate	NTLYL		*Rel. deviation PBS*	0.20%	0.46%	Theoretical modification rate	(N52) DVisoDP		*Rel. deviation PBS*	6.62%	1.84%
	QC 1:1	81.61%		RSD	1.82%	2.34%	QC 1:1	2.14%		RSD	22.33%	1.65%
	QC 3:1	72.41%	QCs 3:1	*Average*	67.46%	69.53%	QC 3:1	3.20%	QCs 3:1	*Average*	3.29%	3.26%
	QC 1:3	90.80%		*Rel. deviation PBS*	6.84%	3.98%	QC 1:3	1.07%		*Rel. deviation PBS*	2.76%	1.73%
				RSD	7.29%	4.29%				RSD	6.49%	4.29%
			QCs 1:3	*Average*	92.04%	91.93%			QCs 1:3	*Average*	1.23%	1.03%
				Rel. deviation PBS	1.36%	1.24%				*Rel. deviation PBS*	15.02%	3.16%
				RSD	0.25%	0.03%				RSD	37.05%	11.74%
			(N54) isoAsp (Stress)							**(N61) isoAsp (Stress)**		

Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*
High QC 1:1	0.0460	0.169563	0.050842	10.44%	7.53%	7.79%	0.0170	0.442214	0.019057	12.13%	2.94%	2.92%
High QC 3:1	0.0691	0.242990	0.072950	5.64%	11.62%	11.92%	0.0255	0.641442	0.027427	7.58%	4.63%	4.48%
High QC 1:3	0.0230	0.080030	0.023885	3.77%	3.57%	3.66%	0.0085	0.187929	0.008374	1.46%	1.30%	1.28%
Mid QC 1:1	0.0192	0.066947	0.019946	3.98%	8.86%	9.01%	0.0071	0.160209	0.007210	1.81%	3.24%	3.26%
Mid QC 3:1	0.0288	0.089112	0.026620	7.48%	11.56%	11.74%	0.0106	0.208497	0.009238	13.03%	4.15%	4.07%
Mid QC 1:3	0.0096	0.032491	0.009572	0.20%	3.32%	3.47%	0.0035	0.074952	0.003628	2.46%	1.25%	1.31%
Low QC 1:1	0.0019	0.007244	0.001970	2.71%	9.87%	8.29%	0.0007	0.006622	0.000757	6.92%	1.30%	3.19%
Low QC 3:1	0.0029	0.009461	0.002638	8.32%	11.76%	11.05%	0.0011	0.016924	0.001190	12.02%	3.50%	4.99%
Low QC 1:3	0.0010	0.004028	0.001002	4.47%	5.42%	4.47%	0.0004	0.000000	NA	NA	NA	NA
			QCs 1:1	*Average*	8.75%	8.36%			QCs 1:1	*Average*	2.49%	3.12%
	Theoretical modification rate	(N54) PisoDSG		*Rel. deviation PBS*	14.04%	8.98%	Theoretical modification rate	(N61) YisoDQR		*Rel. deviation PBS*	11.94%	10.16%
	QC 1:1	7.67%		RSD	13.42%	7.31%	QC 1:1	2.83%		RSD	42.05%	5.69%
	QC 3:1	11.51%	QCs 3:1	*Average*	11.65%	11.57%	QC 3:1	4.25%	QCs 3:1	*Average*	4.09%	4.51%
	QC 1:3	3.84%		*Rel. deviation PBS*	1.19%	0.54%	QC 1:3	1.42%		*Rel. deviation PBS*	3.68%	6.24%
				RSD	0.90%	3.97%				RSD	13.83%	10.12%
			QCs 1:3	*Average*	4.10%	3.87%			QCs 1:3	*Average*	1.28%	1.30%
				Rel. deviation PBS	6.94%	0.79%				*Rel. deviation PBS*	9.96%	8.27%
				RSD	27.96%	13.74%				RSD	2.97%	1.58%
			GLEW (Stress)						**(N54) Asp (Stress)**			
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*

High QC 1:1	0.5055	0.170696	0.550712	8.94%	84.83%	84.36%	0.0074	0.159359	0.007758	4.26%	1.14%	1.19%
High QC 3:1	0.4583	0.143060	0.461707	0.75%	76.00%	75.47%	0.0112	0.244025	0.011901	6.63%	1.83%	1.95%
High QC 1:3	0.5528	0.186690	0.602223	8.95%	92.51%	92.39%	0.0037	0.087462	0.004240	13.97%	0.63%	0.65%
Mid QC 1:1	0.2106	0.056363	0.182491	13.36%	82.77%	82.40%	0.0031	0.055661	0.002684	13.42%	1.18%	1.21%
Mid QC 3:1	0.1910	0.052860	0.171207	10.34%	75.70%	75.48%	0.0047	0.099335	0.004821	3.67%	2.02%	2.13%
Mid QC 1:3	0.2303	0.079310	0.256393	11.32%	93.12%	92.85%	0.0016	0.035142	0.001680	8.40%	0.60%	0.61%
Low QC 1:1	0.0211	0.005833	0.019753	6.22%	82.60%	83.13%	0.0003	0.007699	0.000338	8.88%	1.87%	1.42%
Low QC 3:1	0.0191	0.005320	0.018098	5.22%	76.43%	75.82%	0.0005	0.009943	0.000447	3.80%	2.11%	1.87%
Low QC 1:3	0.0230	0.006144	0.020755	9.88%	91.34%	92.58%	0.0002	0.003781	0.000146	5.92%	0.83%	0.65%
			QCs 1:1	*Average*	83.40%	83.30%			QCs 1:1	*Average*	1.40%	1.27%
	Theoretical modification rate	GLEW		*Rel. deviation PBS*	1.02%	1.14%	Theoretical modification rate	(N54) PDSG		*Rel. deviation PBS*	12.54%	2.71%
	QC 1:1	84.25%		RSD	1.49%	1.19%	QC 1:1	1.24%		RSD	29.53%	10.04%
	QC 3:1	76.38%	QCs 3:1	*Average*	76.04%	75.59%	QC 3:1	1.86%	QCs 3:1	*Average*	1.99%	1.98%
	QC 1:3	92.13%		*Rel. deviation PBS*	0.44%	1.03%	QC 1:3	0.62%		*Rel. deviation PBS*	6.89%	6.53%
				RSD	0.48%	0.26%				RSD	7.05%	6.54%
			QCs 1:3	*Average*	92.33%	92.61%			QCs 1:3	*Average*	0.69%	0.64%
				Rel. deviation PBS	0.22%	0.52%				*Rel. deviation PBS*	10.80%	2.65%
				RSD	0.98%	0.25%				RSD	18.12%	3.81%
			(N52) Asp (Stress)						**(N86) IsoAsp (Stress)**			
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*
High QC 1:1	0.0112	0.027016	0.010751	3.94%	1.61%	1.65%	0.0129	0.050376	0.014246	10.48%	2.82%	2.54%
High QC 3:1	0.0168	0.044944	0.017808	6.07%	2.87%	2.91%	0.0193	0.072448	0.020463	5.79%	4.00%	3.54%
High QC 1:3	0.0056	0.015297	0.006139	9.69%	0.92%	0.94%	0.0064	0.023454	0.006663	3.34%	1.35%	1.20%
Mid QC 1:1	0.0047	0.010952	0.004429	5.03%	1.94%	2.00%	0.0054	0.018465	0.005257	2.15%	2.48%	2.19%
Mid QC 3:1	0.0070	0.018069	0.007230	3.36%	3.16%	3.19%	0.0081	0.026257	0.007452	7.53%	3.43%	3.06%

Mid QC 1:3	0.0023	0.005773	0.002390	2.51%	0.84%	0.87%	0.0027	0.010494	0.003012	12.12%	1.30%	1.19%
Low QC 1:1	0.0005	0.000853	0.000454	2.73%	1.51%	1.91%	0.0005	0.001509	0.000481	10.42%	1.80%	1.87%
Low QC 3:1	0.0007	0.001611	0.000752	7.55%	2.78%	3.15%	0.0008	0.002986	0.000897	11.36%	3.78%	3.71%
Low QC 1:3	0.0002	0.000365	0.000262	12.18%	0.67%	1.17%	0.0003	0.000654	0.000241	10.46%	0.76%	0.93%
			QCs 1:1	*Average*	1.69%	1.85%			QCs 1:1	*Average*	2.36%	2.20%
	Theoretical modification rate	(N52) DVDP		*Rel. deviation PBS*	9.60%	0.72%	Theoretical modification rate	isoDG (N86)		*Rel. deviation PBS*	10.02%	2.29%
	QC 1:1	1.87%		RSD	13.45%	9.89%	QC 1:1	2.15%		RSD	21.91%	15.08%
	QC 3:1	2.80%	QCs 3:1	*Average*	2.94%	3.08%	QC 3:1	3.22%	QCs 3:1	*Average*	3.74%	3.44%
	QC 1:3	0.93%		*Rel. deviation PBS*	4.97%	10.20%	QC 1:3	1.07%		*Rel. deviation PBS*	15.91%	6.66%
				RSD	6.76%	4.88%				RSD	7.75%	9.80%
			QCs 1:3	*Average*	0.81%	0.99%			QCs 1:3	*Average*	1.14%	1.11%
				Rel. deviation PBS	13.51%	6.29%				*Rel. deviation PBS*	5.69%	3.00%
				RSD	15.58%	15.80%				RSD	28.78%	13.65%
			(N91) IsoAsp (Stress)						**(N92) IsoAsp (Stress)**			
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*
High QC 1:1	0.0061	0.083371	0.006829	12.67%	1.25%	1.22%	0.0490	0.110807	0.045675	6.85%	7.93%	8.13%
High QC 3:1	0.0091	0.099806	0.008179	10.04%	1.40%	1.42%	0.0736	0.195711	0.081681	11.05%	13.38%	14.13%
High QC 1:3	0.0030	0.037968	0.003101	2.33%	0.56%	0.56%	0.0245	0.064011	0.025831	5.36%	4.59%	4.63%
Mid QC 1:1	0.0025	0.034813	0.002842	12.54%	1.21%	1.18%	0.0204	0.052072	0.020768	1.65%	8.76%	8.64%
Mid QC 3:1	0.0038	0.048244	0.003945	4.14%	1.58%	1.62%	0.0306	0.079220	0.032280	5.33%	12.98%	13.26%
Mid QC 1:3	0.0013	0.017740	0.001440	14.06%	0.57%	0.57%	0.0102	0.024899	0.009244	9.51%	3.97%	3.66%
Low QC 1:1	0.0003	0.003442	0.000266	5.40%	1.03%	1.04%	0.0020	0.007759	0.001976	3.30%	11.70%	7.69%
Low QC 3:1	0.0004	0.005317	0.000420	10.91%	1.60%	1.74%	0.0031	0.010727	0.003234	5.54%	16.87%	13.38%
Low QC 1:3	0.0001	0.001785	0.000130	3.06%	0.52%	0.50%	0.0010	0.005602	0.001061	3.86%	8.48%	4.11%
			QCs 1:1	*Average*	1.17%	1.14%			QCs 1:1	*Average*	9.46%	8.15%

	Theoretical modification rate	PEisoDNY (N91)		*Rel. deviation PBS*	15.49%	13.30%	Theoretical modification rate	PENisoDy (N92)		*Rel. deviation PBS*	15.76%	0.25%
	QC 1:1	1.01%		RSD	10.12%	8.35%	QC 1:1	8.17%		RSD	20.93%	5.82%
	QC 3:1	1.52%	QCs 3:1	*Average*	1.53%	1.59%	QC 3:1	12.26%	QCs 3:1	*Average*	14.41%	13.59%
	QC 1:3	0.51%		*Rel. deviation PBS*	0.84%	5.02%	QC 1:3	4.09%		*Rel. deviation PBS*	17.55%	10.88%
				RSD	7.09%	10.26%				RSD	14.82%	3.48%
			QCs 1:3	*Average*	0.55%	0.54%			QCs 1:3	*Average*	5.68%	4.14%
				Rel. deviation PBS	8.39%	7.64%				*Rel. deviation PBS*	38.96%	1.21%
				RSD	4.24%	6.39%				RSD	43.10%	11.75%
				PENNY (Stress)						**(N91/92) Asp (Stress)**		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*
High QC 1:1	0.4344	0.316787	0.399630	8.01%	70.47%	71.14%	0.0884	1.307526	0.086469	2.22%	15.81%	15.39%
High QC 3:1	0.3516	0.258815	0.326544	7.14%	55.67%	56.51%	0.1326	1.932032	0.127896	3.58%	22.87%	22.13%
High QC 1:3	0.5172	0.372903	0.470377	9.06%	83.88%	84.37%	0.0442	0.711720	0.046947	6.18%	8.72%	8.42%
Mid QC 1:1	0.1810	0.133088	0.168038	7.17%	68.83%	69.88%	0.0368	0.604074	0.039806	8.03%	17.04%	16.55%
Mid QC 3:1	0.1465	0.113865	0.143803	1.85%	58.36%	59.09%	0.0553	0.759963	0.050147	9.27%	21.12%	20.61%
Mid QC 1:3	0.2155	0.173332	0.218775	1.52%	86.14%	86.65%	0.0184	0.276041	0.018046	2.05%	7.22%	7.15%
Low QC 1:1	0.0181	0.014897	0.019033	5.15%	69.52%	74.07%	0.0037	0.057182	0.003528	4.25%	14.64%	13.73%
Low QC 3:1	0.0147	0.011061	0.014196	3.11%	54.80%	58.72%	0.0055	0.077392	0.004868	11.91%	20.89%	20.14%
Low QC 1:3	0.0216	0.017440	0.022239	3.20%	81.33%	86.20%	0.0018	0.034288	0.002009	9.06%	8.38%	7.79%
			QCs 1:1	*Average*	69.61%	71.70%			QCs 1:1	*Average*	15.83%	15.22%
	Theoretical modification rate	PENNY		*Rel. deviation PBS*	3.87%	0.98%	Theoretical modification rate	PEDNY/PENDY (N91/92)		*Rel. deviation PBS*	7.42%	3.30%
	QC 1:1	72.41%		RSD	1.19%	3.00%	QC 1:1	14.74%		RSD	7.59%	9.32%

	QC 3:1	58.61%	QCs 3:1	*Average*	56.28%	58.11%	QC 3:1	22.11%	QCs 3:1	*Average*	21.63%	20.96%
	QC 1:3	86.20%		*Rel. deviation PBS*	3.98%	0.86%	QC 1:3	7.37%		*Rel. deviation PBS*	2.18%	5.20%
				RSD	3.29%	2.40%				RSD	5.01%	4.98%
			QCs 1:3	*Average*	83.78%	85.74%			QCs 1:3	*Average*	8.11%	7.79%
				Rel. deviation PBS	2.81%	0.54%				*Rel. deviation PBS*	10.04%	5.64%
				RSD	2.88%	1.41%				RSD	9.71%	8.17%
				(N86) Asp (Stress)						**(Q88) Asp (Stress)**		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*
High QC 1:1	0.0065	0.037132	0.006352	2.36%	1.19%	1.13%	0.0025	0.298710	0.002568	2.45%	0.53%	0.46%
High QC 3:1	0.0098	0.052499	0.008987	7.91%	1.84%	1.56%	0.0038	0.485608	0.004114	9.41%	0.83%	0.71%
High QC 1:3	0.0033	0.019280	0.003292	1.19%	0.64%	0.59%	0.0013	0.150002	0.001338	6.77%	0.26%	0.24%
Mid QC 1:1	0.0027	0.015231	0.002598	4.17%	1.16%	1.08%	0.0010	0.128794	0.001163	11.33%	0.53%	0.48%
Mid QC 3:1	0.0041	0.025653	0.004384	7.83%	1.92%	1.80%	0.0016	0.151921	0.001354	13.57%	0.61%	0.56%
Mid QC 1:3	0.0014	0.008454	0.001436	5.92%	0.60%	0.57%	0.0005	0.050581	0.000516	1.20%	0.20%	0.20%
Low QC 1:1	0.0003	0.001801	0.000295	8.87%	1.23%	1.15%	0.0001	0.002318	0.000117	11.80%	0.09%	0.45%
Low QC 3:1	0.0004	0.002313	0.000383	5.87%	1.70%	1.58%	0.0002	0.009554	0.000177	12.73%	0.37%	0.73%
Low QC 1:3	0.0001	0.000777	0.000119	11.88%	0.53%	0.46%	0.0001	0.000000	NA	NA	NA	NA
			QCs 1:1	*Average*	1.19%	1.12%			QCs 1:1	*Average*	0.38%	0.46%
	Theoretical modification rate	DG (N86)		*Rel. deviation PBS*	9.73%	3.27%	Theoretical modification rate	GEP (Q88)		*Rel. deviation PBS*	8.60%	11.31%
	QC 1:1	1.08%		RSD	3.00%	3.16%	QC 1:1	0.42%		RSD	66.66%	3.46%
	QC 3:1	1.63%	QCs 3:1	*Average*	1.82%	1.65%	QC 3:1	0.63%	QCs 3:1	*Average*	0.60%	0.67%
	QC 1:3	0.54%		*Rel. deviation PBS*	11.70%	1.24%	QC 1:3	0.21%		*Rel. deviation PBS*	3.48%	6.32%
				RSD	6.18%	8.19%				RSD	38.83%	14.35%
			QCs 1:3	*Average*	0.59%	0.54%			QCs 1:3	*Average*	0.23%	0.22%

				Rel. deviation PBS	9.04%	0.27%					*Rel. deviation PBS*	11.28%	6.37%
				RSD	9.62%	12.61%					RSD	19.49%	11.35%
Low concentration samples with final % deamidation lower than 5%						LS=Low sample							
DST peptide (Stress)									**Ox (Stress)**				
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Sample*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*		
1st LS 1:1	0.1000	0.475290	0.091631	8.37%	1st LS 1:1	0.0033	0.002580	0.003653	10.64%	4.06%	3.95%		
1st LS 3:1	0.1000	0.546968	0.105383	5.38%	1st LS 3:1	0.0050	0.003803	0.005447	9.97%	5.57%	5.25%		
1st LS 1:3	0.1000	0.523914	0.100960	0.96%	1st LS 1:3	0.0017	0.001303	0.001779	7.76%	2.14%	1.91%		
2nd LS 1:1	0.0250	0.114595	0.022428	10.29%	2nd LS 1:1	0.0008	0.000637	0.000803	2.75%	3.58%	3.16%		
2nd LS 3:1	0.0250	0.120597	0.023579	5.68%	2nd LS 3:1	0.0012	0.001040	0.001393	12.52%	5.57%	5.20%		
2nd LS 1:3	0.0250	0.128661	0.025126	0.51%	2nd LS 1:3	0.0004	0.000334	0.000357	13.39%	1.87%	1.39%		
								Low S 1:1	*Average*	3.82%	3.56%		
						Theoretical modification rate	NTLYLox		*Rel. deviation PBS*	15.63%	7.69%		
						QC 1:1	3.30%	Low S 3:1	*Average*	5.57%	5.23%		
						QC 3:1	4.95%		*Rel. deviation PBS*	12.47%	5.51%		
						QC 1:3	1.65%	Low S 1:3	*Average*	2.00%	1.65%		
									Rel. deviation PBS	21.44%	0.02%		
NTLYL (Stress)									**(N52) isoAsp (Stress)**				
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	
1st LS 1:1	0.0967	0.020324	0.088886	8.04%	95.94%	96.05%	0.0004	0.086467	0.000425	9.58%	0.62%	0.47%	

1st LS 3:1	0.0950	0.022471	0.098274	3.46%	94.43%	94.75%	0.0006	0.118750	0.000634	8.80%	0.80%	0.72%
1st LS 1:3	0.0983	0.020886	0.091343	7.10%	97.86%	98.09%	0.0002	0.046227	0.000166	14.53%	0.24%	0.16%
2nd LS 1:1	0.0242	0.005616	0.024573	1.69%	96.42%	96.84%	0.0001	0.000000		NA	NA	NA
2nd LS 3:1	0.0237	0.005805	0.025402	6.97%	94.43%	94.80%	0.0001	0.046014	0.000165	13.02%	1.10%	0.70%
2nd LS 1:3	0.0246	0.005787	0.025322	3.01%	98.13%	98.61%	0.0000	0.000000	NA	NA	NA	NA
			Low S 1:1	*Average*	96.18%	96.44%			Low S 1:1	*Average*	0.62%	0.47%
	Theoretical modification rate	NTLYL		*Rel. deviation PBS*	0.49%	0.22%	Theoretical modification rate	(N52) DVisoDP		*Rel. deviation PBS*	59.54%	21.82%
	QC 1:1	96.66%	Low S 3:1	*Average*	94.43%	94.77%	QC 1:1	0.39%	Low S 3:1	*Average*	0.95%	0.71%
	QC 3:1	94.98%		*Rel. deviation PBS*	0.58%	0.22%	QC 3:1	0.58%		*Rel. deviation PBS*	63.13%	21.46%
	QC 1:3	98.33%	Low S 1:3	*Average*	98.00%	98.35%	QC 1:3	0.19%	Low S 1:3	*Average*	0.24%	0.16%
				Rel. deviation PBS	0.34%	0.02%				*Rel. deviation PBS*	23.17%	19.41%
			(N54) isoAsp (Stress)							**(N61) isoAsp (Stress)**		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*
1st LS 1:1	0.0014	0.005725	0.001513	8.46%	1.88%	1.68%	0.0005	0.002329	0.000577	11.99%	0.12%	0.64%
1st LS 3:1	0.0021	0.006804	0.001838	12.17%	2.27%	2.08%	0.0008	0.004795	0.000680	11.93%	0.24%	0.77%
1st LS 1:3	0.0007	0.003164	0.000742	6.37%	0.88%	0.70%	0.0003	0.000000	NA	NA	NA	NA
2nd LS 1:1	0.0003	0.002024	0.000399	14.33%	2.74%	1.80%	0.0001	0.000000	NA	NA	NA	NA
2nd LS 3:1	0.0005	0.002291	0.000479	8.45%	2.93%	2.03%	0.0002	0.000000	NA	NA	NA	NA
2nd LS 1:3	0.0002	0.001340	0.000193	10.43%	1.78%	0.85%	0.0001	0.000000	NA	NA	NA	NA
			Low S 1:1	*Average*	2.31%	1.74%			Low S 1:1	*Average*	0.12%	0.64%
	Theoretical modification rate	(N54) PisoDSG		*Rel. deviation PBS*	65.58%	24.78%	Theoretical modification rate	(N61) YisoDQR		*Rel. deviation PBS*	77.37%	24.50%
	QC 1:1	1.40%	Low S 3:1	*Average*	2.60%	2.06%	QC 1:1	0.52%	Low S 3:1	*Average*	0.24%	0.77%

	QC 3:1	2.09%		*Rel. deviation PBS*	24.20%	1.79%	QC 3:1	0.77%		*Rel. deviation PBS*	69.18%	0.17%
	QC 1:3	0.70%	Low S 1:3	*Average*	1.33%	0.77%	QC 1:3	0.26%	Low S 1:3	*Average*	NA	NA
				Rel. deviation PBS	90.54%	10.82%				*Rel. deviation PBS*	NA	NA
				GLEW (Stress)						**(N54) Asp (Stress)**		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*
1st LS 1:1	0.0971	0.026673	0.086868	10.57%	96.85%	96.57%	0.0002	0.004843	0.000198	12.26%	0.25%	0.22%
1st LS 3:1	0.0957	0.025836	0.084175	12.05%	95.81%	95.41%	0.0003	0.007823	0.000344	1.59%	0.40%	0.39%
1st LS 1:3	0.0986	0.032249	0.104826	6.35%	98.66%	98.84%	0.0001	0.003424	0.000128	13.89%	0.15%	0.12%
2nd LS 1:1	0.0243	0.006456	0.021758	10.40%	97.26%	98.20%	0.0001	0.000000	NA	NA	NA	NA
2nd LS 3:1	0.0239	0.006837	0.022984	3.94%	95.97%	97.28%	0.0001	0.000000	NA	NA	NA	NA
2nd LS 1:3	0.0246	0.006704	0.022557	8.46%	98.22%	99.15%	0.0000	0.000000	NA	NA	NA	NA
			Low S 1:1	*Average*	97.06%	97.39%			Low S 1:1	*Average*	0.25%	0.22%
	Theoretical modification rate	GLEW		*Rel. deviation PBS*	0.08%	0.26%	Theoretical modification rate	(N54) PDSG		*Rel. deviation PBS*	12.24%	2.46%
	QC 1:1	97.14%	Low S 3:1	*Average*	95.89%	96.34%	QC 1:1	0.23%	Low S 3:1	*Average*	0.40%	0.39%
	QC 3:1	95.71%		*Rel. deviation PBS*	0.19%	0.67%	QC 3:1	0.34%		*Rel. deviation PBS*	17.06%	15.16%
	QC 1:3	98.57%	Low S 1:3	*Average*	98.44%	99.00%	QC 1:3	0.11%	Low S 1:3	*Average*	0.15%	0.12%
				Rel. deviation PBS	0.13%	0.43%				*Rel. deviation PBS*	30.73%	7.38%
				(N52) Asp (Stress)								
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical*	*Modification rate (Strategy A)*	*Modification rate (Strategy B)*						

				spiked value		
1st LS 1:1	0.0003	0.000641	0.000370	9.23%	0.28%	0.41%
1st LS 3:1	0.0005	0.001104	0.000553	8.62%	0.49%	0.63%
1st LS 1:3	0.0002	0.000194	0.000194	14.55%	0.07%	0.18%
2nd LS 1:1	0.0001	0.000000	NA	NA	NA	NA
2nd LS 3:1	0.0001	0.000000	NA	NA	NA	NA
2nd LS 1:3	0.0000	0.000000	NA	NA	NA	NA
			Low S 1:1	*Average*	0.28%	0.41%
	Theoretical modification rate	(N54) PDSG		*Rel. deviation PBS*	17.14%	21.43%
	QC 1:1	0.34%	Low S 3:1	*Average*	0.49%	0.63%
	QC 3:1	0.51%		*Rel. deviation PBS*	4.33%	23.12%
	QC 1:3	0.17%	Low S 1:3	*Average*	0.07%	0.18%
				Rel. deviation PBS	56.75%	8.01%

Table S9. Full data obtained for all peptides for the quality control samples at different ratios of stressed and non-stressed reference standard as well as at final low concentration of stressed reference standard. Columns are distributed as follows: theoretical spiked amount of each specie in pooled sera, signal corrected by IS, concentration calculated through the equation obtained from the respective calibration curve, deviation from the spiked value. Additional columns for modified peptides: Strategy A and relative standard deviation between replicates, calculated percentage of the modified and non-modified peptides per point of the calibration curve (calculated through the concentrations reported in the third column). In blue (Strategy B), in red (Strategy A): percentages of all points of the calibration curve, its deviation from the modification percentage observed in PBS, relative deviation between all points of the calibration curve. Concentrations are given in mg/ml. LS stands for low sample (low concentration of stressed reference standard mixed with non-stressed reference standard)

Selectivity

Selectivity										
Animal 1	**NTLYLox**	**NTLYLox***	**NTLYL2ox**	**NTLYL2ox***	**NTLYL**	**NTLYL***	**DST**	**DST***	**(N52) isoAsp**	**(N52) isoAsp***
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 2										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 3										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 4										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 5										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 6										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 7										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 1	**(N54) isoAsp**	**(N54) isoAsp***	**(N61) isoAsp**	**(N61) isoAsp***	**GLEW**	**GLEW***	**(N54) Asp**	**(N54) Asp***		
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 2										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 3										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 4										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 5										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 6										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 7										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		

Animal 1	(N52) Asp	(N52) Asp*	(Q88) isoGlu	(Q88) isoGlu*	(N86) IsoAsp	(N86) IsoAsp*	(N91) IsoAsp	(N91) IsoAsp*	(N92) IsoAsp	(N92) IsoAsp*
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 2										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 3										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 4										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 5										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 6										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 7										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Animal 1	**PENNY**	**PENNY***	**(N91/92) Asp**	**(N91/92) Asp***	**(N86) Asp**	**(N86) Asp***	**(Q88) Glu**	**(Q88) Glu***		
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 2										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 3										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 4										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 5										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 6										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
Animal 7										
Area in blank	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		

Table S10. Full data obtained for all peptides in selectivity tests. Area detected in 7 different blank animals in the same retention times as the peptides of interest are reported.

Matrix effect

Matrix Effect										
Animal 1		DST peptide (Stress)					Ox (Stress)			
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value		Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.7000	3.770089	0.723775	3.40%		0.2542	0.157549	0.230979	9.15%	37.94%
Middle	0.5000	2.398041	0.460532	7.89%		0.1816	0.113093	0.165766	8.72%	37.85%
Low	0.0500	0.233475	0.045236	9.53%		0.0182	0.011243	0.016360	9.91%	37.80%
								Average Strategy B		37.86%
								Rel. deviation PBS		4.25%
								RSD Strategy B		0.19%
								Average Strategy A		29.22%
								Rel. deviation PBS		19.54%
								RSD Strategy A		12.04%
		NTLYL (Stress)					(N52) isoAsp (Stress)			
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4425	0.086401	0.377808	14.62%	62.06%	0.0299	4.350780	0.027923	6.58%	4.12%
Middle	0.3161	0.062252	0.272219	13.88%	62.15%	0.0214	3.246948	0.020805	2.55%	4.45%
Low	0.0316	0.006153	0.026922	14.83%	62.20%	0.0029	0.400428	0.002450	14.23%	5.43%
			Average Strategy B		62.14%			Average Strategy B		4.67%
			Rel. deviation PBS		1.71%			Rel. deviation PBS		9.29%
			RSD Strategy B		0.12%			RSD Strategy B		14.59%
			Average Strategy A		70.47%			Average Strategy A		3.94%
			Rel. deviation PBS		11.47%			Rel. deviation PBS		7.68%
			RSD Strategy A		5.23%			RSD Strategy A		17.52%

			(N54) isoAsp (Stress)					**(N61) isoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.1074	0.341116	0.102495	4.58%	15.12%	0.0397	0.802200	0.034181	13.81%	5.04%
Middle	0.0767	0.264446	0.079410	3.50%	16.99%	0.0283	0.581265	0.024899	12.10%	5.33%
Low	0.0077	0.022365	0.006523	14.98%	14.46%	0.0028	0.053255	0.002716	4.11%	6.02%
			Average Strategy B		15.52%			Average Strategy B		5.46%
			Rel. deviation PBS		1.16%			Rel. deviation PBS		3.56%
			RSD Strategy B		8.46%			RSD Strategy B		9.20%
			Average Strategy A		16.93%			Average Strategy A		6.47%
			Rel. deviation PBS		10.35%			Rel. deviation PBS		14.20%
			RSD Strategy A		7.04%			RSD Strategy A		9.82%
			GLEW (Stress)					**(N54) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4796	0.145136	0.468394	2.33%	69.11%	0.0174	0.359998	0.017575	1.23%	2.59%
Middle	0.3425	0.095717	0.309233	9.72%	66.16%	0.0124	0.252622	0.012321	0.64%	2.64%
Low	0.0343	0.009249	0.030754	10.22%	68.16%	0.0012	0.022639	0.001069	13.84%	2.37%
			Average Strategy B		67.81%			Average Strategy B		2.53%
			Rel. deviation PBS		1.02%			Rel. deviation PBS		2.11%
			RSD Strategy B		2.22%			RSD Strategy B		5.68%
			Average Strategy A		66.22%			Average Strategy A		2.38%
			Rel. deviation PBS		3.33%			Rel. deviation PBS		3.89%
			RSD Strategy A		2.06%			RSD Strategy A		16.28%
			(N52) Asp (Stress)					**(N86) IsoAsp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0261	0.068861	0.027221	4.23%	4.02%	0.0301	0.097848	0.027618	8.21%	4.03%
Middle	0.0187	0.052353	0.020724	11.10%	4.43%	0.0215	0.080213	0.022650	5.35%	4.80%
Low	0.0019	0.003788	0.001609	13.75%	3.57%	0.0021	0.006750	0.001958	6.78%	3.91%
			Average Strategy B		4.01%			Average Strategy B		4.25%
			Rel. deviation PBS		7.36%			Rel. deviation PBS		1.20%
			RSD Strategy B		10.84%			RSD Strategy B		11.38%
			Average Strategy A		4.05%			Average Strategy A		4.23%
			Rel. deviation PBS		8.46%			Rel. deviation PBS		1.53%
			RSD Strategy A		13.36%			RSD Strategy A		8.87%
			(N91) IsoAsp (Stress)					**(N92) IsoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0141	0.149450	0.012255	13.35%	1.79%	0.1144	0.253004	0.105977	7.38%	15.47%
Middle	0.0101	0.116090	0.009516	5.80%	2.02%	0.0817	0.179531	0.074819	8.45%	15.86%
Low	0.0010	0.010764	0.000867	14.13%	1.73%	0.0082	0.023955	0.008844	8.22%	17.66%
			Average Strategy B		1.85%			Average Strategy B		16.33%
			Rel. deviation PBS		8.64%			Rel. deviation PBS		0.11%
			RSD Strategy B		8.17%			RSD Strategy B		7.16%
			Average Strategy A		1.27%			Average Strategy A		16.28%
			Rel. deviation PBS		36.95%			Rel. deviation PBS		0.38%
			RSD Strategy A		14.51%			RSD Strategy A		24.68%
			PENNY (Stress)					**(N91/92) Asp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.3137	0.248258	0.313235	0.14%	45.72%	0.2063	3.118609	0.206608	0.13%	30.16%
Middle	0.2241	0.170586	0.215312	3.90%	45.63%	0.1474	2.025175	0.134075	9.03%	28.42%
Low	0.0224	0.017268	0.022022	1.71%	43.97%	0.0147	0.226413	0.014754	0.10%	29.46%
			Average Strategy B		45.11%			Average Strategy B		29.34%
			Rel. deviation PBS		0.66%			Rel. deviation PBS		0.45%
			RSD Strategy B		2.18%			RSD Strategy B		2.98%
			Average Strategy A		45.56%			Average Strategy A		29.09%
			Rel. deviation PBS		1.68%			Rel. deviation PBS		1.31%
			RSD Strategy A		7.31%			RSD Strategy A		5.78%
			(N86) Asp (Stress)					**(Q88) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0152	0.084496	0.014472	4.66%	2.11%	0.0058	0.591318	0.004988	14.72%	0.73%
Middle	0.0108	0.068896	0.011798	8.81%	2.50%	0.0042	0.431757	0.003668	12.19%	0.78%
Low	0.0011	0.007146	0.001211	11.72%	2.42%	0.0004	0.039673	0.000426	1.90%	0.85%
			Average Strategy B		2.34%			Average Strategy B		0.79%
			Rel. deviation PBS		8.08%			Rel. deviation PBS		6.03%
			RSD Strategy B		8.73%			RSD Strategy B		7.82%
			Average Strategy A		2.58%			Average Strategy A		0.98%
			Rel. deviation PBS		18.79%			Rel. deviation PBS		17.31%
			RSD Strategy A		16.07%			RSD Strategy A		9.23%
Animal 2			**DST peptide (Stress)**					**Ox (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)

High	*0.7000*	*3.367896*	*0.646610*	*7.63%*		*0.2542*	*0.177531*	*0.260292*	*2.38%*	*36.15%*
Middle	*0.5000*	*2.479183*	*0.476100*	*4.78%*		*0.1816*	*0.111083*	*0.162818*	*10.34%*	*34.79%*
Low	*0.0500*	*0.273730*	*0.052959*	*5.92%*		*0.0182*	*0.011255*	*0.016378*	*9.81%*	*33.54%*
								Average Strategy B		*34.83%*
								Rel. deviation PBS		*4.11%*
								RSD Strategy B		*3.76%*
								Average Strategy A		*22.98%*
								Rel. deviation PBS		*36.74%*
								RSD Strategy A		*20.27%*
			NTLYL (Stress)					***(N52) isoAsp (Stress)***		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*
High	*0.4425*	*0.105117*	*0.459647*	*3.87%*	*63.85%*	*0.0299*	*4.700915*	*0.030181*	*0.97%*	*4.45%*
Middle	*0.3161*	*0.069787*	*0.305162*	*3.45%*	*65.21%*	*0.0214*	*2.835402*	*0.018151*	*14.98%*	*3.88%*
Low	*0.0316*	*0.007419*	*0.032456*	*2.68%*	*66.46%*	*0.0029*	*0.407555*	*0.002496*	*12.62%*	*5.53%*
			Average Strategy B		*65.17%*			*Average Strategy B*		*4.62%*
			Rel. deviation PBS		*3.09%*			*Rel. deviation PBS*		*8.26%*
			RSD Strategy B		*2.01%*			*RSD Strategy B*		*18.11%*
			Average Strategy A		*76.76%*			*Average Strategy A*		*3.78%*
			Rel. deviation PBS		*21.42%*			*Rel. deviation PBS*		*11.46%*
			RSD Strategy A		*5.96%*			*RSD Strategy A*		*23.79%*
			(N54) isoAsp (Stress)					***(N61) isoAsp (Stress)***		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*
High	*0.1074*	*0.362952*	*0.109069*	*1.54%*	*16.09%*	*0.0397*	*0.811434*	*0.034569*	*12.83%*	*5.10%*
Middle	*0.0767*	*0.242084*	*0.072678*	*5.28%*	*15.55%*	*0.0283*	*0.571668*	*0.024496*	*13.52%*	*5.24%*
Low	*0.0077*	*0.023697*	*0.006924*	*9.76%*	*15.35%*	*0.0028*	*0.046810*	*0.002445*	*13.67%*	*5.42%*
			Average Strategy B		*15.66%*			*Average Strategy B*		*5.25%*

			Rel. deviation PBS	2.06%				Rel. deviation PBS	7.26%	
			RSD Strategy B	2.46%				RSD Strategy B	3.05%	
			Average Strategy A	15.17%				Average Strategy A	10.31%	
			Rel. deviation PBS	1.12%				Rel. deviation PBS	81.96%	
			RSD Strategy A	5.17%				RSD Strategy A	10.97%	
			GLEW (Stress)					**(N54) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4796	0.150206	0.484723	1.08%	71.52%	0.0174	0.321603	0.015696	9.59%	2.32%
Middle	0.3425	0.096452	0.311600	9.03%	66.67%	0.0124	0.241625	0.011783	4.98%	2.52%
Low	0.0343	0.009024	0.030030	12.33%	66.56%	0.0012	0.022610	0.001067	13.95%	2.37%
			Average Strategy B		68.25%			Average Strategy B		2.40%
			Rel. deviation PBS		0.38%			Rel. deviation PBS		3.21%
			RSD Strategy B		4.15%			RSD Strategy B		4.46%
			Average Strategy A		64.58%			Average Strategy A		2.78%
			Rel. deviation PBS		5.73%			Rel. deviation PBS		12.25%
			RSD Strategy A		2.23%			RSD Strategy A		4.01%
			(N52) Asp (Stress)					**(N86) IsoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0261	0.064227	0.025397	2.75%	3.75%	0.0301	0.105027	0.029640	1.49%	4.33%
Middle	0.0187	0.045078	0.017861	4.25%	3.82%	0.0215	0.070482	0.019909	7.40%	4.22%
Low	0.0019	0.004755	0.001989	6.65%	4.41%	0.0021	0.007146	0.002069	1.46%	4.13%
			Average Strategy B		3.99%			Average Strategy B		4.23%
			Rel. deviation PBS		7.02%			Rel. deviation PBS		1.69%
			RSD Strategy B		9.09%			RSD Strategy B		2.30%
			Average Strategy A		3.37%			Average Strategy A		4.75%
			Rel. deviation PBS		9.59%			Rel. deviation PBS		10.57%

			RSD Strategy A		6.98%			RSD Strategy A		8.84%
			(N91) IsoAsp (Stress)					**(N92) IsoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0141	0.155896	0.012784	9.60%	1.87%	0.1144	0.253433	0.106159	7.22%	15.49%
Middle	0.0101	0.113509	0.009304	7.90%	1.97%	0.0817	0.185671	0.077423	5.26%	16.41%
Low	0.0010	0.012100	0.000977	3.27%	1.95%	0.0082	0.022587	0.008264	1.12%	16.50%
			Average Strategy B		1.93%			Average Strategy B		16.13%
			Rel. deviation PBS		4.49%			Rel. deviation PBS		1.29%
			RSD Strategy B		2.91%			RSD Strategy B		3.45%
			Average Strategy A		1.58%			Average Strategy A		14.98%
			Rel. deviation PBS		21.62%			Rel. deviation PBS		8.35%
			RSD Strategy A		3.33%			RSD Strategy A		6.98%
			PENNY (Stress)					**(N91/92) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.3137	0.227866	0.287526	8.34%	41.97%	0.2063	3.021955	0.200196	2.98%	29.22%
Middle	0.2241	0.168694	0.212927	4.96%	45.13%	0.1474	2.136586	0.141465	4.02%	29.98%
Low	0.0224	0.017807	0.022702	1.32%	45.33%	0.0147	0.232750	0.015174	2.96%	30.30%
			Average Strategy B		44.14%			Average Strategy B		29.83%
			Rel. deviation PBS		1.49%			Rel. deviation PBS		1.21%
			RSD Strategy B		4.27%			RSD Strategy B		1.86%
			Average Strategy A		42.86%			Average Strategy A		32.52%
			Rel. deviation PBS		4.34%			Rel. deviation PBS		10.31%
			RSD Strategy A		2.58%			RSD Strategy A		3.29%
			(N86) Asp (Stress)					**(Q88) Asp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0152	0.085651	0.014670	3.36%	2.14%	0.0058	0.663483	0.005585	4.51%	0.82%
Middle	0.0108	0.056582	0.009687	10.66%	2.05%	0.0042	0.508236	0.004301	2.95%	0.91%
Low	0.0011	0.006104	0.001033	4.75%	2.06%	0.0004	0.039937	0.000428	2.42%	0.85%
			Average Strategy B		2.09%			Average Strategy B		0.86%
			Rel. deviation PBS		3.83%			Rel. deviation PBS		2.97%
			RSD Strategy B		2.32%			RSD Strategy B		5.63%
			Average Strategy A		2.36%			Average Strategy A		0.94%
			Rel. deviation PBS		8.84%			Rel. deviation PBS		12.80%
			RSD Strategy A		7.68%			RSD Strategy A		11.99%
Animal 3			**DST peptide (Stress)**					**Ox (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value		Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.7000	3.327851	0.638927	8.72%		0.2542	0.165245	0.242269	4.71%	33.59%
Middle	0.5000	2.492399	0.478636	4.27%		0.1816	0.115009	0.168577	7.17%	36.98%
Low	0.0500	0.227811	0.044149	11.70%		0.0182	0.011834	0.017227	5.14%	35.49%
								Average Strategy B		35.35%
								Rel. deviation PBS		2.66%
								RSD Strategy B		4.82%
								Average Strategy A		27.63%
								Rel. deviation PBS		23.93%
								RSD Strategy A		8.53%
			NTLYL (Stress)					**(N52) isoAsp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4425	0.109560	0.479072	8.26%	66.41%	0.0299	4.622987	0.029678	0.71%	4.38%
Middle	0.3161	0.065688	0.287243	9.12%	63.02%	0.0214	2.930595	0.018765	12.11%	4.01%
Low	0.0316	0.007157	0.031311	0.94%	64.51%	0.0029	0.415453	0.002547	10.84%	5.64%
			Average Strategy B		64.65%			Average Strategy B		4.68%
			Rel. deviation PBS		2.26%			Rel. deviation PBS		9.58%
			RSD Strategy B		2.63%			RSD Strategy B		18.28%
			Average Strategy A		71.96%			Average Strategy A		4.16%
			Rel. deviation PBS		13.83%			Rel. deviation PBS		2.61%
			RSD Strategy A		3.25%			RSD Strategy A		32.79%
			(N54) isoAsp (Stress)					**(N61) isoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.1074	0.338319	0.101653	5.37%	15.00%	0.0397	1.061441	0.045072	13.66%	6.65%
Middle	0.0767	0.222620	0.066817	12.92%	14.30%	0.0283	0.585267	0.025067	11.51%	5.36%
Low	0.0077	0.025698	0.007526	1.91%	16.68%	0.0028	0.059457	0.002977	5.09%	6.60%
			Average Strategy B		15.32%			Average Strategy B		6.20%
			Rel. deviation PBS		0.14%			Rel. deviation PBS		9.50%
			RSD Strategy B		8.00%			RSD Strategy B		11.74%
			Average Strategy A		14.63%			Average Strategy A		14.63%
			Rel. deviation PBS		4.69%			Rel. deviation PBS		158.31%
			RSD Strategy A		2.00%			RSD Strategy A		23.65%
			GLEW (Stress)					**(N54) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)

High	*0.4796*	*0.150568*	*0.485889*	*1.32%*	*71.69%*	*0.0174*	*0.324767*	*0.015851*	*8.70%*	*2.34%*
Middle	*0.3425*	*0.101377*	*0.327461*	*4.40%*	*70.06%*	*0.0124*	*0.273024*	*0.013319*	*7.41%*	*2.85%*
Low	*0.0343*	*0.009271*	*0.030825*	*10.01%*	*68.32%*	*0.0012*	*0.022823*	*0.001078*	*13.11%*	*2.39%*
			Average Strategy B		*70.02%*			*Average Strategy B*		*2.53%*
			Rel. deviation PBS		*2.21%*			*Rel. deviation PBS*		*1.83%*
			RSD Strategy B		*2.41%*			*RSD Strategy B*		*11.16%*
			Average Strategy A		*60.22%*			*Average Strategy A*		*2.73%*
			Rel. deviation PBS		*12.09%*			*Rel. deviation PBS*		*9.96%*
			RSD Strategy A		*4.81%*			*RSD Strategy A*		*8.14%*
			(N52) Asp (Stress)					***(N86) IsoAsp (Stress)***		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*
High	*0.0261*	*0.059490*	*0.023533*	*9.89%*	*3.47%*	*0.0301*	*0.110486*	*0.031177*	*3.62%*	*4.55%*
Middle	*0.0187*	*0.045911*	*0.018188*	*2.50%*	*3.89%*	*0.0215*	*0.065184*	*0.018417*	*14.34%*	*3.90%*
Low	*0.0019*	*0.004220*	*0.001779*	*4.64%*	*3.94%*	*0.0021*	*0.007396*	*0.002140*	*1.89%*	*4.27%*
			Average Strategy B		*3.77%*			*Average Strategy B*		*4.24%*
			Rel. deviation PBS		*1.01%*			*Rel. deviation PBS*		*1.31%*
			RSD Strategy B		*6.85%*			*RSD Strategy B*		*7.65%*
			Average Strategy A		*3.63%*			*Average Strategy A*		*5.11%*
			Rel. deviation PBS		*2.69%*			*Rel. deviation PBS*		*18.77%*
			RSD Strategy A		*12.09%*			*RSD Strategy A*		*9.04%*
			(N91) IsoAsp (Stress)					***(N92) IsoAsp (Stress)***		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*
High	*0.0141*	*0.163080*	*0.013374*	*5.43%*	*1.95%*	*0.1144*	*0.249393*	*0.104446*	*8.71%*	*15.24%*
Middle	*0.0101*	*0.116121*	*0.009518*	*5.78%*	*2.02%*	*0.0817*	*0.176613*	*0.073582*	*9.97%*	*15.59%*
Low	*0.0010*	*0.013172*	*0.001065*	*5.44%*	*2.13%*	*0.0082*	*0.023732*	*0.008749*	*7.06%*	*17.47%*
			Average Strategy B		*2.03%*			*Average Strategy B*		*16.10%*

			Rel. deviation PBS		*0.58%*			*Rel. deviation PBS*		*1.48%*
			RSD Strategy B		*4.35%*			*RSD Strategy B*		*7.43%*
			Average Strategy A		*1.87%*			*Average Strategy A*		*16.76%*
			Rel. deviation PBS		*7.52%*			*Rel. deviation PBS*		*2.53%*
			RSD Strategy A		*5.07%*			*RSD Strategy A*		*8.06%*
			PENNY (Stress)					***(N91/92) Asp (Stress)***		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*
High	*0.3137*	*0.241797*	*0.305090*	*2.74%*	*44.53%*	*0.2063*	*2.985700*	*0.197791*	*4.14%*	*28.87%*
Middle	*0.2241*	*0.166050*	*0.209594*	*6.45%*	*44.42%*	*0.1474*	*2.065215*	*0.136731*	*7.23%*	*28.98%*
Low	*0.0224*	*0.017277*	*0.022033*	*1.66%*	*43.99%*	*0.0147*	*0.228588*	*0.014898*	*1.08%*	*29.75%*
			Average Strategy B		*44.31%*			*Average Strategy B*		*29.20%*
			Rel. deviation PBS		*1.11%*			*Rel. deviation PBS*		*0.95%*
			RSD Strategy B		*0.64%*			*RSD Strategy B*		*1.64%*
			Average Strategy A		*46.04%*			*Average Strategy A*		*26.36%*
			Rel. deviation PBS		*2.74%*			*Rel. deviation PBS*		*10.58%*
			RSD Strategy A		*5.64%*			*RSD Strategy A*		*13.56%*
			(N86) Asp (Stress)					***(Q88) Asp (Stress)***		
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Modification rate (Strategy B)*
High	*0.0152*	*0.098052*	*0.016796*	*10.65%*	*2.45%*	*0.0058*	*0.653108*	*0.005499*	*5.98%*	*0.80%*
Middle	*0.0108*	*0.064373*	*0.011023*	*1.66%*	*2.34%*	*0.0042*	*0.449596*	*0.003816*	*8.66%*	*0.81%*
Low	*0.0011*	*0.005904*	*0.000998*	*7.91%*	*1.99%*	*0.0004*	*0.044842*	*0.000468*	*12.13%*	*0.94%*
			Average Strategy B		*2.26%*			*Average Strategy B*		*0.85%*
			Rel. deviation PBS		*4.24%*			*Rel. deviation PBS*		*1.60%*
			RSD Strategy B		*10.53%*			*RSD Strategy B*		*8.83%*
			Average Strategy A		*2.51%*			*Average Strategy A*		*1.36%*
			Rel. deviation PBS		*15.70%*			*Rel. deviation PBS*		*63.10%*

			RSD Strategy A		11.28%			RSD Strategy A		39.35%
Animal 4		**DST peptide (Stress)**						**Ox (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.7000	3.362832	0.645639	7.77%		0.2542	0.159719	0.234163	7.90%	36.36%
Middle	0.5000	2.336342	0.448695	10.26%		0.1816	0.127916	0.187510	3.26%	34.89%
Low	0.0500	0.231791	0.044913	10.17%		0.0182	0.011165	0.016246	10.54%	35.92%
								Average Strategy B		35.72%
								Rel. deviation PBS		1.65%
								RSD Strategy B		2.11%
								Average Strategy A		34.72%
								Rel. deviation PBS		4.41%
								RSD Strategy A		5.15%
			NTLYL (Stress)					**(N52) isoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4425	0.093747	0.409930	7.36%	63.64%	0.0299	4.759157	0.030556	2.23%	4.51%
Middle	0.3161	0.080040	0.349995	10.73%	65.11%	0.0214	3.478978	0.022301	4.45%	4.77%
Low	0.0316	0.006624	0.028980	8.31%	64.08%	0.0029	0.422486	0.002592	9.25%	5.74%
			Average Strategy B		64.28%			Average Strategy B		5.01%
			Rel. deviation PBS		1.68%			Rel. deviation PBS		17.29%
			RSD Strategy B		1.18%			RSD Strategy B		13.01%
			Average Strategy A		65.00%			Average Strategy A		6.01%
			Rel. deviation PBS		2.82%			Rel. deviation PBS		40.74%
			RSD Strategy A		2.91%			RSD Strategy A		24.51%
			(N54) isoAsp (Stress)					**(N61) isoAsp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.1074	0.350542	0.105333	1.94%	15.54%	0.0397	0.925518	0.039361	0.74%	5.81%
Middle	0.0767	0.238831	0.071698	6.56%	15.34%	0.0283	0.653509	0.027934	1.38%	5.98%
Low	0.0077	0.023109	0.006747	12.07%	14.95%	0.0028	0.053867	0.002742	3.20%	6.08%
			Average Strategy B		15.28%			Average Strategy B		5.95%
			Rel. deviation PBS		0.44%			Rel. deviation PBS		5.09%
			RSD Strategy B		1.95%			RSD Strategy B		2.29%
			Average Strategy A		14.31%			Average Strategy A		8.29%
			Rel. deviation PBS		6.73%			Rel. deviation PBS		46.36%
			RSD Strategy A		1.53%			RSD Strategy A		11.14%
			GLEW (Stress)					**(N54) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4796	0.152063	0.490701	2.32%	72.40%	0.0174	0.321437	0.015688	9.64%	2.31%
Middle	0.3425	0.099206	0.320470	6.44%	68.57%	0.0124	0.230979	0.011262	9.18%	2.41%
Low	0.0343	0.009536	0.031678	7.52%	70.21%	0.0012	0.024660	0.001167	5.86%	2.59%
			Average Strategy B		70.39%			Average Strategy B		2.44%
			Rel. deviation PBS		2.75%			Rel. deviation PBS		1.74%
			RSD Strategy B		2.73%			RSD Strategy B		5.68%
			Average Strategy A		65.54%			Average Strategy A		2.42%
			Rel. deviation PBS		4.33%			Rel. deviation PBS		2.24%
			RSD Strategy A		1.91%			RSD Strategy A		12.39%
			(N52) Asp (Stress)					**(N86) IsoAsp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0261	0.058578	0.023174	11.26%	3.42%	0.0301	0.101698	0.028702	4.61%	4.19%
Middle	0.0187	0.050013	0.019803	6.16%	4.24%	0.0215	0.070202	0.019830	7.77%	4.20%
Low	0.0019	0.003909	0.001657	11.19%	3.67%	0.0021	0.007083	0.002051	2.32%	4.10%
			Average Strategy B		3.78%			Average Strategy B		4.16%
			Rel. deviation PBS		1.21%			Rel. deviation PBS		3.16%
			RSD Strategy B		11.09%			RSD Strategy B		1.40%
			Average Strategy A		3.42%			Average Strategy A		4.66%
			Rel. deviation PBS		8.29%			Rel. deviation PBS		8.42%
			RSD Strategy A		14.54%			RSD Strategy A		1.77%
			(N91) IsoAsp (Stress)					**(N92) IsoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0141	0.155227	0.012729	9.99%	1.86%	0.1144	0.261772	0.109695	4.13%	16.01%
Middle	0.0101	0.111060	0.009103	9.89%	1.93%	0.0817	0.177874	0.074116	9.31%	15.71%
Low	0.0010	0.011276	0.000909	9.97%	1.82%	0.0082	0.021933	0.007986	2.28%	15.95%
			Average Strategy B		1.87%			Average Strategy B		15.89%
			Rel. deviation PBS		7.56%			Rel. deviation PBS		2.79%
			RSD Strategy B		3.07%			RSD Strategy B		1.00%
			Average Strategy A		1.40%			Average Strategy A		15.44%
			Rel. deviation PBS		30.71%			Rel. deviation PBS		5.55%
			RSD Strategy A		10.75%			RSD Strategy A		7.44%
			PENNY (Stress)					**(N91/92) Asp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.3137	0.234991	0.296510	5.47%	43.28%	0.2063	2.989743	0.198059	4.01%	28.91%
Middle	0.2241	0.167174	0.211011	5.82%	44.72%	0.1474	2.262514	0.149819	1.65%	31.75%
Low	0.0224	0.016834	0.021476	4.15%	42.88%	0.0147	0.240672	0.015700	6.52%	31.35%
			Average Strategy B		43.63%			Average Strategy B		30.67%
			Rel. deviation PBS		2.64%			Rel. deviation PBS		4.05%
			RSD Strategy B		2.22%			RSD Strategy B		5.02%
			Average Strategy A		41.26%			Average Strategy A		33.63%
			Rel. deviation PBS		7.92%			Rel. deviation PBS		14.10%
			RSD Strategy A		4.54%			RSD Strategy A		4.53%
			(N86) Asp (Stress)					**(Q88) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
	0.0152	0.081767	0.014004	7.74%	2.04%	0.0058	0.611334	0.005153	11.89%	0.75%
	0.0108	0.058701	0.010050	7.31%	2.13%	0.0042	0.420759	0.003577	14.37%	0.76%
	0.0011	0.006633	0.001123	3.61%	2.24%	0.0004	0.032807	0.000369	11.69%	0.74%
			Average Strategy B		2.14%			Average Strategy B		0.75%
			Rel. deviation PBS		1.36%			Rel. deviation PBS		10.36%
			RSD Strategy B		4.67%			RSD Strategy B		1.49%
			Average Strategy A		2.61%			Average Strategy A		1.00%
			Rel. deviation PBS		20.32%			Rel. deviation PBS		19.42%
			RSD Strategy A		10.02%			RSD Strategy A		23.14%
Animal 5			**DST peptide (Stress)**					**Ox (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.7000	3.738469	0.717709	2.53%		0.2542	0.182274	0.267250	5.12%	36.75%
Middle	0.5000	2.485528	0.477318	4.54%		0.1816	0.132551	0.194310	7.00%	37.53%
Low	0.0500	0.250240	0.048453	3.09%		0.0182	0.011462	0.016682	8.14%	35.21%
								Average Strategy B		36.49%
								Rel. deviation PBS		0.48%
								RSD Strategy B		3.22%
								Average Strategy A		25.27%
								Rel. deviation PBS		30.42%
								RSD Strategy A		11.54%
			NTLYL (Stress)					**(N52) isoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4425	0.105210	0.460052	3.96%	63.25%	0.0299	4.968422	0.031906	6.74%	4.71%
Middle	0.3161	0.073981	0.323500	2.35%	62.47%	0.0214	3.189937	0.020437	4.28%	4.37%
Low	0.0316	0.007015	0.030693	2.90%	64.79%	0.0029	0.412599	0.002528	11.48%	5.60%
			Average Strategy B		63.51%			Average Strategy B		4.89%
			Rel. deviation PBS		0.46%			Rel. deviation PBS		14.62%
			RSD Strategy B		1.85%			RSD Strategy B		13.00%
			Average Strategy A		74.46%			Average Strategy A		4.92%
			Rel. deviation PBS		17.79%			Rel. deviation PBS		15.22%
			RSD Strategy A		3.98%			RSD Strategy A		21.62%
			(N54) isoAsp (Stress)					**(N61) isoAsp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.1074	0.339339	0.101960	5.08%	15.04%	0.0397	0.891542	0.037934	4.34%	5.60%
Middle	0.0767	0.236904	0.071118	7.31%	15.22%	0.0283	0.597960	0.025600	9.62%	5.48%
Low	0.0077	0.023568	0.006885	10.27%	15.26%	0.0028	0.053062	0.002708	4.39%	6.00%
			Average Strategy B		15.17%			Average Strategy B		5.69%
			Rel. deviation PBS		1.13%			Rel. deviation PBS		0.47%
			RSD Strategy B		0.75%			RSD Strategy B		4.83%
			Average Strategy A		15.97%			Average Strategy A		11.92%
			Rel. deviation PBS		4.04%			Rel. deviation PBS		110.43%
			RSD Strategy A		3.21%			RSD Strategy A		23.60%
			GLEW (Stress)					**(N54) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4796	0.139652	0.450731	6.01%	66.50%	0.0174	0.367406	0.017937	3.32%	2.65%
Middle	0.3425	0.094330	0.304766	11.03%	65.21%	0.0124	0.222860	0.010865	12.39%	2.32%
Low	0.0343	0.009195	0.030579	10.73%	67.77%	0.0012	0.026638	0.001264	1.94%	2.80%
			Average Strategy B		66.49%			Average Strategy B		2.59%
			Rel. deviation PBS		2.94%			Rel. deviation PBS		4.47%
			RSD Strategy B		1.93%			RSD Strategy B		9.40%
			Average Strategy A		60.41%			Average Strategy A		2.98%
			Rel. deviation PBS		11.82%			Rel. deviation PBS		20.04%
			RSD Strategy A		3.10%			RSD Strategy A		21.42%
			(N52) Asp (Stress)					**(N86) IsoAsp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0261	0.062500	0.024718	5.35%	3.65%	0.0301	0.098716	0.027862	7.40%	4.07%
Middle	0.0187	0.042867	0.016990	8.92%	3.64%	0.0215	0.067479	0.019063	11.33%	4.04%
Low	0.0019	0.003988	0.001688	9.52%	3.74%	0.0021	0.007125	0.002063	1.76%	4.12%
			Average Strategy B		3.67%			Average Strategy B		4.08%
			Rel. deviation PBS		1.52%			Rel. deviation PBS		5.19%
			RSD Strategy B		1.58%			RSD Strategy B		0.99%
			Average Strategy A		3.80%			Average Strategy A		4.15%
			Rel. deviation PBS		1.96%			Rel. deviation PBS		3.48%
			RSD Strategy A		3.21%			RSD Strategy A		8.11%
			(N91) IsoAsp (Stress)					**(N92) IsoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0141	0.168196	0.013794	2.46%	2.01%	0.1144	0.251977	0.105541	7.76%	15.40%
Middle	0.0101	0.119058	0.009759	3.39%	2.07%	0.0817	0.182238	0.075967	7.05%	16.10%
Low	0.0010	0.012780	0.001033	2.25%	2.06%	0.0082	0.023455	0.008632	5.62%	17.24%
			Average Strategy B		2.05%			Average Strategy B		16.25%
			Rel. deviation PBS		1.37%			Rel. deviation PBS		0.60%
			RSD Strategy B		1.48%			RSD Strategy B		5.69%
			Average Strategy A		1.60%			Average Strategy A		15.33%
			Rel. deviation PBS		20.94%			Rel. deviation PBS		6.21%
			RSD Strategy A		5.65%			RSD Strategy A		10.19%
			PENNY (Stress)					**(N91/92) Asp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.3137	0.240075	0.302918	3.43%	44.21%	0.2063	2.887886	0.191303	7.29%	27.92%
Middle	0.2241	0.170582	0.215307	3.90%	45.63%	0.1474	2.071454	0.137145	6.95%	29.07%
Low	0.0224	0.017205	0.021942	2.06%	43.81%	0.0147	0.213967	0.013928	5.50%	27.81%
			Average Strategy B		44.55%			Average Strategy B		28.27%
			Rel. deviation PBS		0.58%			Rel. deviation PBS		4.11%
			RSD Strategy B		2.14%			RSD Strategy B		2.46%
			Average Strategy A		42.43%			Average Strategy A		32.76%
			Rel. deviation PBS		5.32%			Rel. deviation PBS		11.13%
			RSD Strategy A		4.81%			RSD Strategy A		2.97%
			(N86) Asp (Stress)					**(Q88) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0152	0.092058	0.015769	3.88%	2.30%	0.0058	0.676757	0.005694	2.63%	0.83%
Middle	0.0108	0.064671	0.011074	2.13%	2.35%	0.0042	0.487995	0.004133	1.06%	0.88%
Low	0.0011	0.007159	0.001214	11.93%	2.42%	0.0004	0.040819	0.000435	4.17%	0.87%
			Average Strategy B		2.36%			Average Strategy B		0.86%
			Rel. deviation PBS		8.70%			Rel. deviation PBS		2.77%
			RSD Strategy B		2.61%			RSD Strategy B		2.81%
			Average Strategy A		2.68%			Average Strategy A		1.06%
			Rel. deviation PBS		23.51%			Rel. deviation PBS		27.20%
			RSD Strategy A		5.46%			RSD Strategy A		18.46%
Animal 6			**DST peptide (Stress)**					**Ox (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value		Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.7000	3.504436	0.672807	3.88%		0.2542	0.166429	0.244006	4.02%	34.12%
Middle	0.5000	2.865388	0.550198	10.04%		0.1816	0.113107	0.165787	8.71%	34.69%
Low	0.0500	0.240488	0.046582	6.84%		0.0182	0.010636	0.015471	14.81%	34.75%
								Average Strategy B		34.52%
								Rel. deviation PBS		4.96%
								RSD Strategy B		1.00%
								Average Strategy A		30.57%
								Rel. deviation PBS		15.84%
								RSD Strategy A		1.93%
			NTLYL (Stress)					**(N52) isoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4425	0.107754	0.471177	6.48%	65.88%	0.0299	4.799352	0.030815	3.09%	4.55%
Middle	0.3161	0.071390	0.312173	1.24%	65.31%	0.0214	3.073009	0.019683	7.81%	4.21%
Low	0.0316	0.006641	0.029054	8.08%	65.25%	0.0029	0.398689	0.002439	14.63%	5.40%
			Average Strategy B		65.48%			Average Strategy B		4.72%
			Rel. deviation PBS		3.59%			Rel. deviation PBS		10.56%
			RSD Strategy B		0.53%			RSD Strategy B		13.04%
			Average Strategy A		69.16%			Average Strategy A		3.65%
			Rel. deviation PBS		9.40%			Rel. deviation PBS		14.63%
			RSD Strategy A		0.76%			RSD Strategy A		25.40%
			(N54) isoAsp (Stress)					**(N61) isoAsp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.1074	0.344432	0.103493	3.65%	15.27%	0.0397	0.852114	0.036278	8.52%	5.35%
Middle	0.0767	0.260028	0.078080	1.76%	16.71%	0.0283	0.631336	0.027002	4.67%	5.78%
Low	0.0077	0.023662	0.006914	9.89%	15.32%	0.0028	0.051854	0.002657	6.19%	5.89%
			Average Strategy B		15.77%			Average Strategy B		5.67%
			Rel. deviation PBS		2.74%			Rel. deviation PBS		0.14%
			RSD Strategy B		5.17%			RSD Strategy B		4.99%
			Average Strategy A		16.64%			Average Strategy A		13.57%
			Rel. deviation PBS		8.45%			Rel. deviation PBS		139.54%
			RSD Strategy A		8.49%			RSD Strategy A		47.64%
			GLEW (Stress)					**(N54) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4796	0.132164	0.426614	11.04%	62.94%	0.0174	0.319585	0.015598	10.16%	2.30%
Middle	0.3425	0.098707	0.318862	6.91%	68.22%	0.0124	0.236615	0.011538	6.96%	2.47%
Low	0.0343	0.009923	0.032924	3.88%	72.97%	0.0012	0.026223	0.001244	0.31%	2.76%
			Average Strategy B		68.04%			Average Strategy B		2.51%
			Rel. deviation PBS		0.68%			Rel. deviation PBS		1.16%
			RSD Strategy B		7.37%			RSD Strategy B		9.19%
			Average Strategy A		60.04%			Average Strategy A		2.62%
			Rel. deviation PBS		12.36%			Rel. deviation PBS		5.75%
			RSD Strategy A		10.30%			RSD Strategy A		16.33%
			(N52) Asp (Stress)					**(N86) IsoAsp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0261	0.059159	0.023403	10.39%	3.45%	0.0301	0.101074	0.028526	5.19%	4.16%
Middle	0.0187	0.040974	0.016245	12.91%	3.48%	0.0215	0.068440	0.019334	10.07%	4.10%
Low	0.0019	0.003885	0.001647	11.70%	3.65%	0.0021	0.007076	0.002049	2.41%	4.09%
			Average Strategy B		3.53%			Average Strategy B		4.12%
			Rel. deviation PBS		5.48%			Rel. deviation PBS		4.20%
			RSD Strategy B		3.07%			RSD Strategy B		0.96%
			Average Strategy A		3.48%			Average Strategy A		4.77%
			Rel. deviation PBS		6.68%			Rel. deviation PBS		11.05%
			RSD Strategy A		9.24%			RSD Strategy A		4.63%
			(N91) IsoAsp (Stress)					**(N92) IsoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0141	0.168322	0.013804	2.39%	2.01%	0.1144	0.284225	0.119217	4.20%	17.40%
Middle	0.0101	0.120881	0.009909	1.91%	2.10%	0.0817	0.169915	0.070741	13.44%	14.99%
Low	0.0010	0.013943	0.001128	11.71%	2.25%	0.0082	0.021526	0.007814	4.39%	15.60%
			Average Strategy B		2.12%			Average Strategy B		16.00%
			Rel. deviation PBS		5.07%			Rel. deviation PBS		2.12%
			RSD Strategy B		5.69%			RSD Strategy B		7.82%
			Average Strategy A		1.66%			Average Strategy A		15.00%
			Rel. deviation PBS		17.75%			Rel. deviation PBS		8.26%
			RSD Strategy A		18.49%			RSD Strategy A		9.02%
			PENNY (Stress)					**(N91/92) Asp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.3137	0.241909	0.305231	2.69%	44.55%	0.2063	2.991732	0.198191	3.95%	28.93%
Middle	0.2241	0.167002	0.210794	5.92%	44.68%	0.1474	2.020570	0.133769	9.24%	28.35%
Low	0.0224	0.017576	0.022411	0.02%	44.75%	0.0147	0.214138	0.013939	5.42%	27.83%
			Average Strategy B		44.66%			Average Strategy B		28.37%
			Rel. deviation PBS		0.34%			Rel. deviation PBS		3.75%
			RSD Strategy B		0.23%			RSD Strategy B		1.93%
			Average Strategy A		43.83%			Average Strategy A		30.80%
			Rel. deviation PBS		2.19%			Rel. deviation PBS		4.49%
			RSD Strategy A		3.32%			RSD Strategy A		1.67%
			(N86) Asp (Stress)					**(Q88) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0152	0.085222	0.014597	3.84%	2.13%	0.0058	0.642124	0.005408	7.53%	0.79%
Middle	0.0108	0.059739	0.010228	5.67%	2.17%	0.0042	0.449249	0.003813	8.73%	0.81%
Low	0.0011	0.007192	0.001219	12.45%	2.43%	0.0004	0.037540	0.000408	2.32%	0.81%
			Average Strategy B		2.24%			Average Strategy B		0.80%
			Rel. deviation PBS		3.49%			Rel. deviation PBS		3.76%
			RSD Strategy B		7.39%			RSD Strategy B		1.64%
			Average Strategy A		2.90%			Average Strategy A		1.04%
			Rel. deviation PBS		33.86%			Rel. deviation PBS		24.07%
			RSD Strategy A		9.28%			RSD Strategy A		9.07%
Animal 7		***DST peptide (Stress)***						***Ox (Stress)***		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value		Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.7000	3.766538	0.723094	3.30%		0.2542	0.181782	0.266527	4.83%	37.22%
Middle	0.5000	2.373955	0.455911	8.82%		0.1816	0.118860	0.174226	4.06%	38.40%
Low	0.0500	0.226376	0.043874	12.25%		0.0182	0.011590	0.016870	7.10%	35.80%
								Average Strategy B		37.14%
								Rel. deviation PBS		2.26%
								RSD Strategy B		3.52%
								Average Strategy A		31.85%
								Rel. deviation PBS		12.32%
								RSD Strategy A		4.35%

			(N54) isoAsp (Stress)					**(N61) isoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.1074	0.369236	0.110961	3.30%	16.37%	0.0397	0.875044	0.037241	6.09%	5.49%
Middle	0.0767	0.236968	0.071137	7.29%	15.22%	0.0283	0.581821	0.024922	12.02%	5.33%
Low	0.0077	0.022407	0.006536	14.82%	14.49%	0.0028	0.050093	0.002583	8.80%	5.73%
			Average Strategy B		15.36%			Average Strategy B		5.52%
			Rel. deviation PBS		0.09%			Rel. deviation PBS		2.61%
			RSD Strategy B		6.19%			RSD Strategy B		3.58%
			Average Strategy A		15.93%			Average Strategy A		9.68%
			Rel. deviation PBS		3.80%			Rel. deviation PBS		70.95%
			RSD Strategy A		3.65%			RSD Strategy A		42.36%

			GLEW (Stress)					**(N54) Asp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.4796	0.138224	0.446132	6.97%	65.82%	0.0174	0.335855	0.016394	5.57%	2.42%
Middle	0.3425	0.098510	0.318228	7.10%	68.09%	0.0124	0.246890	0.012041	2.91%	2.58%
Low	0.0343	0.009037	0.030069	12.22%	66.64%	0.0012	0.024043	0.001137	8.30%	2.52%
			Average Strategy B		66.85%			Average Strategy B		2.51%
			Rel. deviation PBS		2.42%			Rel. deviation PBS		1.00%
			RSD Strategy B		1.71%			RSD Strategy B		3.19%
			Average Strategy A		63.20%			Average Strategy A		2.98%
			Rel. deviation PBS		7.75%			Rel. deviation PBS		20.05%
			RSD Strategy A		3.55%			RSD Strategy A		20.27%
			(N52) Asp (Stress)					**(N86) IsoAsp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0261	0.064163	0.025372	2.85%	3.74%	0.0301	0.104213	0.029410	2.25%	4.29%
Middle	0.0187	0.040844	0.016194	13.19%	3.46%	0.0215	0.073341	0.020715	3.65%	4.39%
Low	0.0019	0.004113	0.001737	6.89%	3.85%	0.0021	0.008182	0.002361	12.43%	4.71%
			Average Strategy B		3.69%			Average Strategy B		4.47%
			Rel. deviation PBS		1.20%			Rel. deviation PBS		3.89%
			RSD Strategy B		5.39%			RSD Strategy B		4.94%
			Average Strategy A		3.57%			Average Strategy A		5.19%
			Rel. deviation PBS		4.32%			Rel. deviation PBS		20.68%
			RSD Strategy A		13.23%			RSD Strategy A		4.49%
			(N91) IsoAsp (Stress)					**(N92) IsoAsp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0141	0.166818	0.013681	3.26%	2.00%	0.1144	0.267339	0.112056	2.06%	16.35%
Middle	0.0101	0.113589	0.009310	7.83%	1.97%	0.0817	0.184429	0.076896	5.91%	16.30%
Low	0.0010	0.012642	0.001022	1.13%	2.04%	0.0082	0.023826	0.008789	7.55%	17.55%
			Average Strategy B		2.00%			Average Strategy B		16.73%
			Rel. deviation PBS		0.84%			Rel. deviation PBS		2.38%
			RSD Strategy B		1.69%			RSD Strategy B		4.23%
			Average Strategy A		2.03%			Average Strategy A		15.63%
			Rel. deviation PBS		0.58%			Rel. deviation PBS		4.36%
			RSD Strategy A		6.56%			RSD Strategy A		15.92%
			PENNY (Stress)					**(N91/92) Asp (Stress)**		
Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.3137	0.244444	0.308427	1.67%	45.02%	0.2063	3.014321	0.199690	3.22%	29.15%
Middle	0.2241	0.168930	0.213224	4.83%	45.19%	0.1474	2.094851	0.138697	5.89%	29.39%
Low	0.0224	0.016722	0.021333	4.78%	42.60%	0.0147	0.222472	0.014492	1.67%	28.94%
			Average Strategy B		44.27%			Average Strategy B		29.16%
			Rel. deviation PBS		1.21%			Rel. deviation PBS		1.08%
			RSD Strategy B		3.27%			RSD Strategy B		0.79%
			Average Strategy A		41.51%			Average Strategy A		31.83%
			Rel. deviation PBS		7.37%			Rel. deviation PBS		7.99%
			RSD Strategy A		3.34%			RSD Strategy A		5.91%
			(N86) Asp (Stress)					**(Q88) Asp (Stress)**		

Sample	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)	Theoretical spiked concentration [in mg/ml]	AUC (peptide) / AUC (IS)	Concentration [in mg/ml] from equation	Rel. deviation from theoretical spiked value	Modification rate (Strategy B)
High	0.0152	0.086643	0.014840	2.24%	2.17%	0.0058	0.688224	0.005789	1.01%	0.84%
Middle	0.0108	0.058230	0.009969	8.06%	2.11%	0.0042	0.506125	0.004283	2.53%	0.91%
Low	0.0011	0.006859	0.001162	7.18%	2.32%	0.0004	0.035372	0.000390	6.62%	0.78%
			Average Strategy B		2.20%			Average Strategy B		0.84%
			Rel. deviation PBS		1.44%			Rel. deviation PBS		1.01%
			RSD Strategy B		4.91%			RSD Strategy B		7.63%
			Average Strategy A		2.69%			Average Strategy A		1.12%
			Rel. deviation PBS		23.90%			Rel. deviation PBS		34.25%
			RSD Strategy A		11.87%			RSD Strategy A		17.86%

Table S11. Full data analysis (all peptides and animals) of spiked stressed reference standard in individual animals' serum and comparison of two strategies to assess the matrix effect on quantification of low-abundant modifications. Columns are distributed as follows: Theoretical spiked concentration of corresponding modification in the stressed reference standard, signal corrected by IS, concentration calculated through the equation obtained from the calibration curve of the corresponding peptide, relative deviation of the calculated concentration from the theoretical spiked value, obtained modification rate (calculated through the concentrations reported in the third column for all peptide's variants per animal). In blue (Strategy B), in red (Strategy A): Average modification rate of all points per calibration curve, relative deviation from the modification rate observed in PBS, relative deviation between all points within the calibration curve.

DST				
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.7	3.47621124	0.692159842	**1.12%**
5th sample	0.5	2.57204929	0.51214472	**2.43%**
4th sample	0.3	1.50920172	0.300535911	**0.18%**
3rd sample	0.15	0.72871168	0.145143385	**3.24%**
2nd sample	0.05	0.24894936	0.049624577	**0.75%**
1st sample	0.002	0.01130539	0.002310588	**15.53%**
Blank	0	0	5.97288E-05	**NA**
High QC-1	0.6	2.83946472	0.565386092	**5.77%**
Middle QC-1	0.25	1.23092814	0.245132725	**1.95%**
Low QC-1	0.025	0.13153664	0.026248162	**4.99%**
High QC-2	0.6	2.81072747	0.559664616	**6.72%**
Middle QC-2	0.25	1.25992809	0.250906502	**0.36%**
Low QC-2	0.025	0.13834538	0.027603756	**10.42%**

Table S12. Data obtained from calibration curve and quality control samples. Samples were analyzed in same sequence as in vitro and in vivo samples. Stable peptide DST is shown. Columns are distributed as follows: sample name, theoretical spiked concentration of corresponding modification in the stressed reference standard, signal corrected by IS, concentration calculated through the equation obtained from the calibration curve of the corresponding peptide, relative deviation of the calculated concentration from the theoretical spiked value.

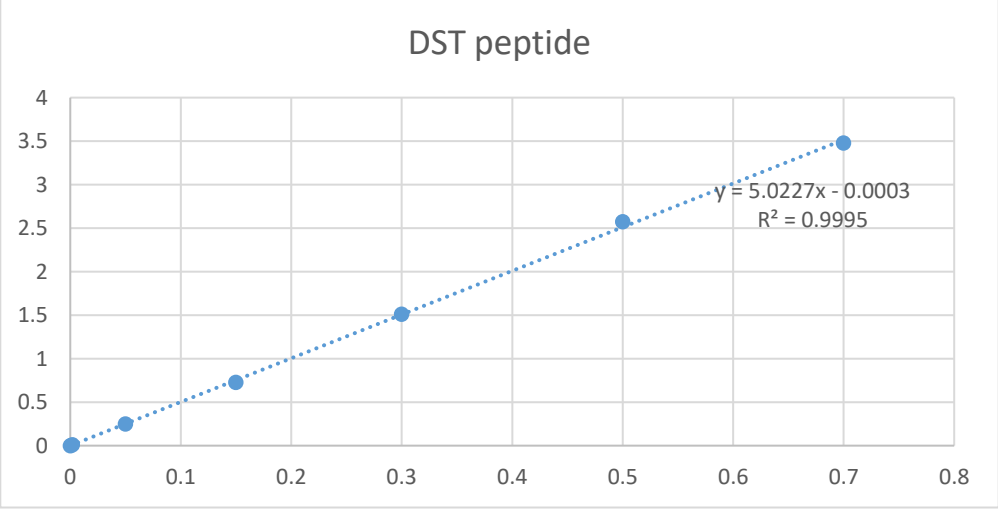

Figure S2. Calibration curve for samples analyzed in same sequence as in vitro and in vivo samples. Y axis: Signal intensity corrected by the IS (added at the beginning of the digestion protocol). X axis: Total concentration (in mg/ml) of the stable DST peptide

	NTLYLox				NTLYL			
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.2542	0.17439646	0.254588011	**0.14%**	0.4425	0.10187118	0.44890675	**1.45%**
5th sample	0.1816	0.12278169	0.179325881	**1.25%**	0.3161	0.06956909	0.30698194	**2.88%**
4th sample	0.1090	0.07734281	0.113069136	**3.77%**	0.1896	0.04341836	0.19208417	**1.28%**
3rd sample	0.0545	0.0362236	0.053111106	**2.51%**	0.0948	0.02050678	0.09141821	**3.59%**
2nd sample	0.0182	0.01187636	0.017609157	**3.03%**	0.0316	0.00763403	0.03485954	**10.29%**
1st sample	0.0007	0.0002047	0.000590118	**18.76%**	0.0013	3.8911E-05	0.00148906	**17.78%**
Blank	0.0000	0	NA	**NA**	0.0000	0	0.0013181	**NA**
High QC-1	0.2179	0.14818255	0.21636418	**0.71%**	0.3793	0.07663142	0.33801151	**10.88%**
Middle QC-1	0.0908	0.07031101	0.102815699	**13.23%**	0.1580	0.03113616	0.1381202	**12.60%**
Low QC-1	0.0091	0.00546305	0.008257586	**9.06%**	0.0158	0.00323208	0.01551881	**1.80%**
High QC-2	0.2179	0.14235457	0.207866095	**4.61%**	0.3793	0.08193153	0.36129846	**4.74%**
Middle QC-2	0.0908	0.06053605	0.08856233	**2.46%**	0.1580	0.03240629	0.14370075	**9.07%**
Low QC-2	0.0091	0.00567534	0.008567131	**5.65%**	0.0158	0.00321784	0.01545624	**2.20%**

Table S13. Data obtained from calibration curve and quality control samples for NTLYL and oxidized NTLYL peptides. Samples were analyzed in same sequence as in vitro and in vivo samples. Columns are distributed as follows: Sample name, theoretical spiked concentration of corresponding modification in the stressed reference standard, signal corrected by IS, concentration calculated through the equation obtained from the calibration curve of the corresponding peptide, relative deviation of the calculated concentration from the theoretical spiked value.

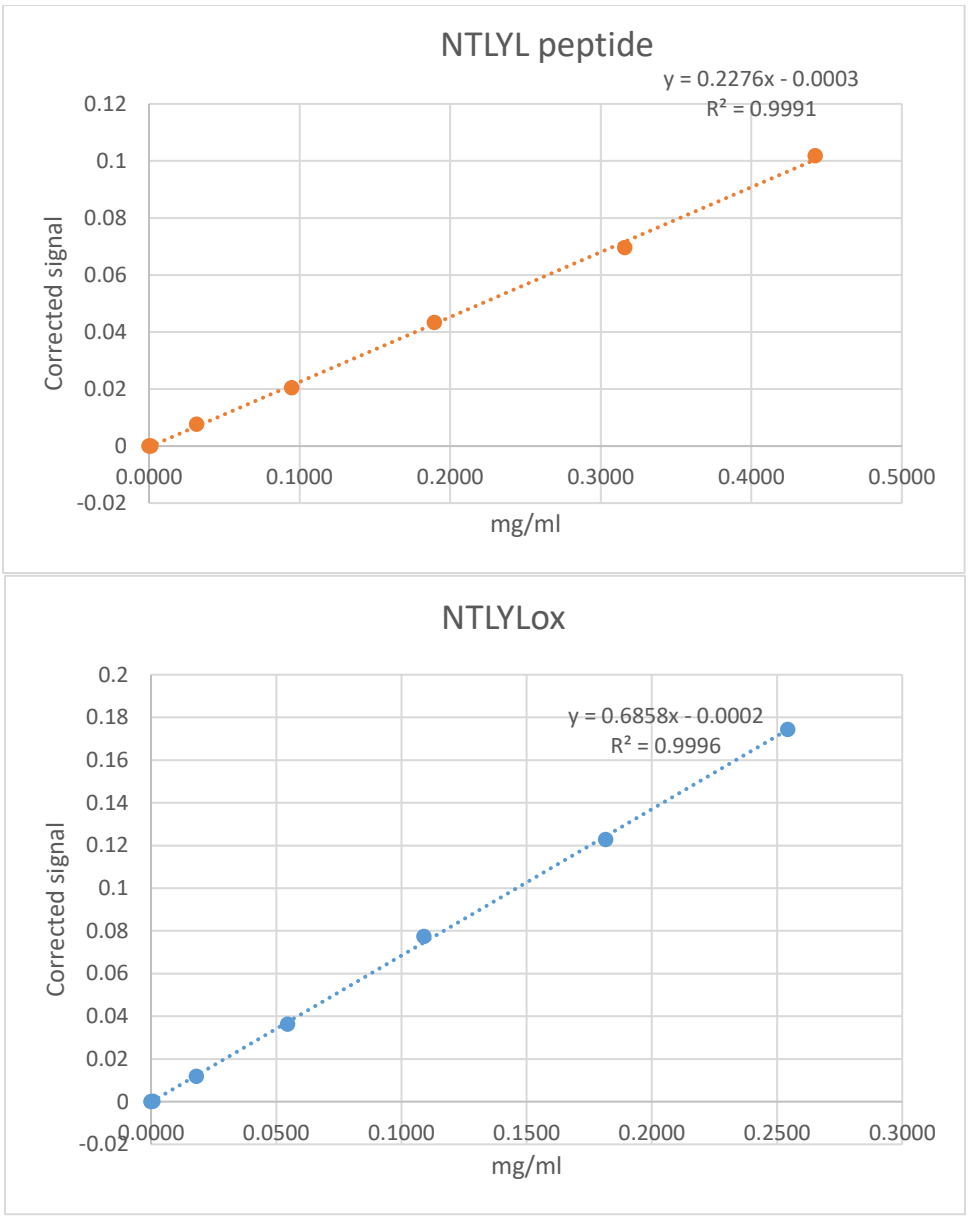

Figure S3. Calibration curve for NTLYL and oxidized NTLYL peptides. Calibration curve samples were analyzed in same sequence as in vitro and in vivo samples. Y axis: Signal intensity corrected by the IS (added at the beginning of the digestion protocol). X axis: Total concentration (in mg/ml).

	(N52) DVisoDP				(N54) PisoDSG			
	Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.0299	4.60402696	0.029907599	**0.06%**	0.1074	0.3278222	0.10735336	**0.06%**
5th sample	0.0214	3.30930218	0.021584071	**1.10%**	0.0767	0.24654874	0.08134918	**6.02%**
4th sample	0.0128	1.90251372	0.012540107	**2.11%**	0.0460	0.11595684	0.03956513	**14.06%**
3rd sample	0.0064	0.91121417	0.00616724	**3.71%**	0.0230	0.05796319	0.02100953	**8.73%**
2nd sample	0.0021	0.24055998	0.001855738	**13.08%**	0.0077	0.01397126	0.00693391	**9.63%**
1st sample	0.0001	0	NA	**NA**	0.0003	0	NA	**NA**
Blank	0.0000	0	NA	**NA**	0.0000	0	NA	**NA**
High QC-1	0.0256	3.43329676	0.022381207	**12.64%**	0.0921	0.24251277	0.08005784	**13.05%**
Middle QC-1	0.0107	1.52434236	0.010108919	**5.30%**	0.0384	0.1170821	0.03992516	**4.07%**
Low QC-1	0.0011	0.13473665	0.00117542	**10.11%**	0.0038	0.00551612	0.00422862	**10.22%**
High QC-2	0.0256	3.53524204	0.023036593	**10.08%**	0.0921	0.25574547	0.08429176	**8.45%**
Middle QC-2	0.0107	1.66809015	0.011033045	**3.35%**	0.0384	0.12598086	0.0427724	**11.49%**
Low QC-2	0.0011	0.13792259	0.001195902	**12.03%**	0.0038	0.00548695	0.00421928	**9.98%**

	(N61) YisoDQR				GLEW			
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
0.0397	0.895169	0.03940561	**0.63%**	0.4796	0.14684801	0.4882072	**1.80%**
0.0283	0.65887232	0.02904081	**2.52%**	0.3425	0.09924486	0.33016221	**3.61%**
0.0170	0.36915971	0.016333	**3.90%**	0.2055	0.06024034	0.20066513	**2.36%**
0.0085	0.19387639	0.00864446	**1.73%**	0.1028	0.03426808	0.11443587	**11.36%**
0.0028	0.05860154	0.00271083	**4.30%**	0.0343	0.00901866	0.03060646	**10.65%**
0.0001	0	NA	**NA**	0.0014	0.00015843	0.00119	**13.15%**
0.0000	0	NA	**NA**	0.0000	0	NA	**NA**
0.0340	0.69504977	0.03062768	**9.90%**	0.4110	0.11218984	0.37314024	**9.22%**
0.0142	0.2775853	0.01231622	**13.04%**	0.1713	0.04724426	0.15751747	**8.03%**
0.0014	0.02429491	0.00120602	**14.85%**	0.0171	0.00465006	0.01610246	**5.98%**
0.0340	0.78883795	0.03474155	**2.21%**	0.4110	0.12056812	0.40095656	**2.46%**
0.0142	0.34216304	0.01514883	**6.96%**	0.1713	0.05410321	0.18028954	**5.27%**
0.0014	0.02803012	0.00136986	**3.28%**	0.0171	0.00436661	0.01516139	**11.48%**

	(N54) PDSG				(N52) DVDP			
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
0.0174	0.39382916	0.01758119	**1.26%**	0.0261	0.0608171	0.02544874	**2.55%**
0.0124	0.27668251	0.01245824	**0.46%**	0.0187	0.04641045	0.019390434	**3.95%**
0.0074	0.15438452	0.00711001	**4.44%**	0.0112	0.02743221	0.011409675	**1.94%**
0.0037	0.06464914	0.00318578	**14.37%**	0.0056	0.01419247	0.005842082	**4.39%**
0.0012	0.01832012	0.00115976	**6.48%**	0.0019	0.00415496	0.001621095	**13.10%**
0.0000	0	NA	**NA**	0.0001	0	NA	**NA**

0.0000	0	NA	**NA**	0.0000	0	NA	**NA**
0.0149	0.30694011	0.01378144	**7.39%**	0.0224	0.04962558	0.020742465	**7.34%**
0.0062	0.12793742	0.00595344	**3.99%**	0.0093	0.0207606	0.00860412	**7.75%**
0.0006	0.00764174	0.00069278	**11.73%**	0.0009	0.00249936	0.000924877	**0.84%**
0.0149	0.31260861	0.01402933	**5.73%**	0.0224	0.04690413	0.019598038	**12.45%**
0.0062	0.14211993	0.00657366	**6.02%**	0.0093	0.02144324	0.008891188	**4.67%**
0.0006	0.00735929	0.00068043	**9.74%**	0.0009	0.00268473	0.001002829	**7.52%**

Table S14. Data obtained from calibration curve and quality control samples for GLEW peptide and iso-aspartic and aspartic acid formations. Samples were analyzed in same sequence as in vitro and in vivo samples. Columns are distributed as follows: Sample name, theoretical spiked concentration of corresponding modification in the stressed reference standard, signal corrected by IS, concentration calculated through the equation obtained from the calibration curve of the corresponding peptide, relative deviation of the calculated concentration from the theoretical spiked value.

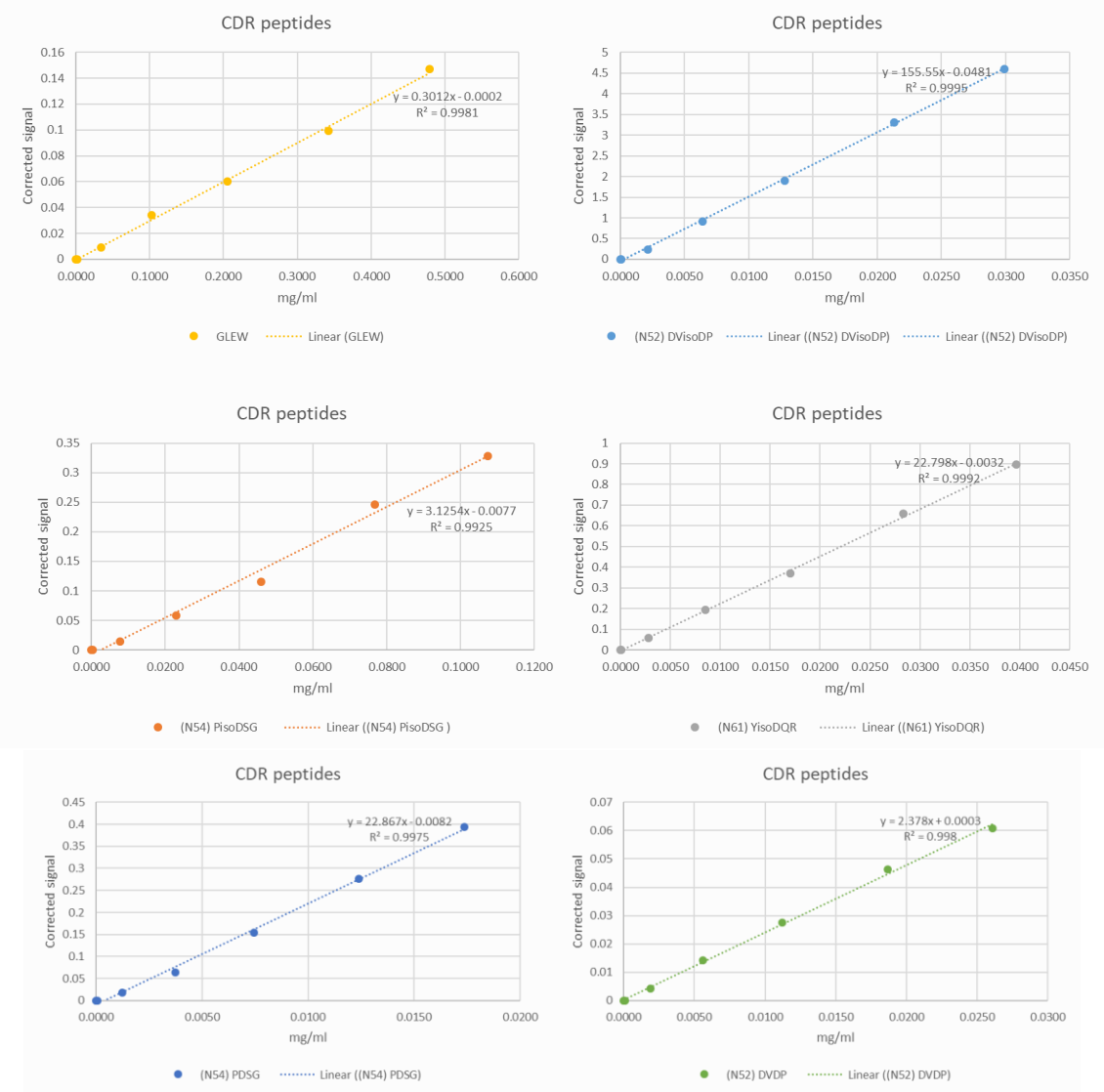

Figure S4. Calibration curves for GLEW and iso-aspartic and aspartic acid formation peptides. Calibration curve samples were analyzed in same sequence as in vitro and in vivo samples. Y axis: Signal intensity corrected by the IS (added at the beginning of the digestion protocol). X axis: Total concentration (in mg/ml).

	isoDG (N86)				**PEisoDNY (N91)**			
Sample	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
6th sample	0.0301	0.10970997	0.030052939	**0.12%**	0.01414	0.17618586	0.01357497	**4.01%**
5th sample	0.0215	0.07939172	0.021648248	**0.73%**	0.01010	0.14184461	0.01088006	**7.71%**
4th sample	0.0129	0.0464447	0.012514816	**2.95%**	0.00606	0.07802933	0.00587219	**3.11%**
3rd sample	0.0064	0.02618624	0.006898855	**7.00%**	0.00303	0.04681813	0.00342291	**12.95%**
2nd sample	0.0021	0.01000456	0.002413042	**12.28%**	0.00101	0.01769626	0.00113759	**12.61%**

1st sample	0.0001	0.0015666	7.39057E-05	**14.03%**	0.00004	0	NA	**NA**
Blank	0.0000	0	NA	**NA**	0.00000	0	NA	**NA**
High QC-1	0.0258	0.09914892	0.027125251	**5.18%**	0.0121	0.14762397	0.01133359	**6.50%**
Middle QC-1	0.0107	0.04483462	0.012068477	**12.31%**	0.0051	0.07555536	0.00567805	**12.42%**
Low QC-1	0.0011	0.00514378	0.001065557	**0.84%**	0.0005	0.00878285	0.00043811	**13.26%**
High QC-2	0.0258	0.09462733	0.025871796	**0.32%**	0.0121	0.1404568	0.01077115	**11.14%**
Middle QC-2	0.0107	0.04459103	0.012000951	**11.68%**	0.0051	0.06566257	0.00490172	**2.95%**
Low QC-2	0.0011	0.00555411	0.001179305	**9.74%**	0.0005	0.00916948	0.00046845	**7.25%**

PENisoDy (N92)				**PENNY**			
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
0.1144	0.2713201	0.11513631	**0.63%**	0.3137	0.23403991	0.31527428	**0.51%**
0.0817	0.18937196	0.07957471	**2.63%**	0.2241	0.1674194	0.22522222	**0.52%**
0.0490	0.12011389	0.04952	**0.99%**	0.1344	0.09512144	0.12749586	**5.16%**
0.0245	0.06774569	0.02679469	**9.29%**	0.0672	0.05200097	0.06920921	**2.97%**
0.0082	0.02737223	0.00927453	**13.48%**	0.0224	0.01979989	0.02568247	**14.63%**
0.0003	0.00687648	0.00038035	**16.35%**	0.0009	0.00144779	0.00087562	**2.30%**
0.0000	0	NA	**NA**	0.0000	0	NA	**NA**
0.0981	0.23367339	0.09879942	**0.74%**	0.2689	0.21922062	0.2952428	**9.81%**
0.0409	0.1124621	0.04619949	**13.06%**	0.1120	0.09147096	0.12256145	**9.40%**
0.0041	0.01522641	0.00400382	**2.02%**	0.0112	0.0099773	0.01240511	**10.73%**
0.0981	0.24557636	0.10396474	**6.01%**	0.2689	0.2165077	0.2915757	**8.45%**
0.0409	0.10680743	0.04374563	**7.06%**	0.1120	0.09600431	0.12868925	**14.87%**
0.0041	0.01605419	0.00436304	**6.77%**	0.0112	0.00816054	0.00994936	**11.19%**

PEDNY/PENDY (N91/92)				**DG (N86)**			
Theoretical spiked concentration [in mg/ml]	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*	*Theoretical spiked concentration [in mg/ml]*	*AUC (peptide) / AUC (IS)*	*Concentration [in mg/ml] from equation*	*Rel. deviation from theoretical spiked value*
0.2063	3.16546178	0.20733482	**0.48%**	0.01518	0.07981478	0.014932239	**1.63%**
0.1474	2.28459497	0.15078925	**2.31%**	0.01084	0.05934986	0.011103872	**2.41%**
0.0884	1.23808509	0.08361055	**5.45%**	0.00651	0.03609632	0.006753839	**3.81%**
0.0442	0.5317069	0.03826595	**13.46%**	0.00325	0.0164178	0.003072583	**5.54%**
0.0147	0.14489779	0.01343547	**8.84%**	0.00108	0.00532092	0.000996693	**8.08%**
0.0006	0	NA	**NA**	0.00004	0.00025289	4.86182E-05	**12.10%**
0.0000	0	NA	**NA**	0.00000	0	NA	**NA**
0.1769	2.31125122	0.1525004	**13.77%**	0.0130	0.06461785	0.012089353	**7.09%**
0.0737	0.99557674	0.06804318	**7.67%**	0.0054	0.02905952	0.005437467	**0.30%**
0.0074	0.06371729	0.00822425	**11.60%**	0.0005	0.00276226	0.000518044	**4.45%**
0.1769	2.43031028	0.16014317	**9.45%**	0.0130	0.06570903	0.012293481	**5.52%**
0.0737	1.24565937	0.08409676	**14.12%**	0.0054	0.0296812	0.005553764	**2.44%**
0.0074	0.06377778	0.00822813	**11.66%**	0.0005	0.0031015	0.000581507	**7.26%**

Table S15. Data obtained from calibration curve and quality control samples for PENNY peptide and iso-aspartic and aspartic acid formations. Samples were analyzed in same sequence as in vitro and in vivo samples. Columns are distributed as follows: Sample name, theoretical spiked concentration of corresponding modification in the stressed reference standard, signal corrected

by IS, concentration calculated through the equation obtained from the calibration curve of the corresponding peptide, relative deviation of the calculated concentration from the theoretical spiked value.

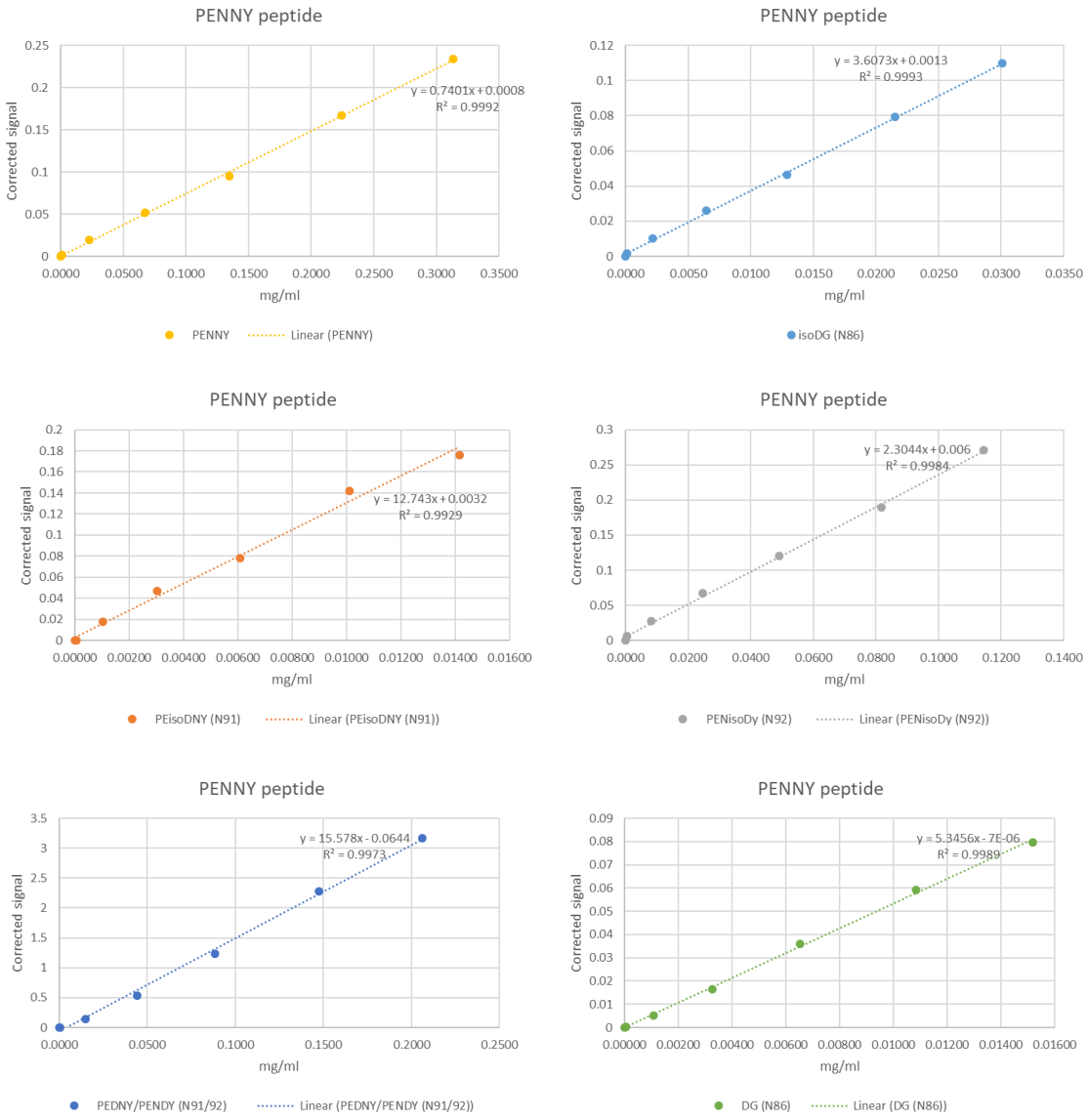

Figure S5. Calibration curves for PENNY and iso-aspartic and aspartic acid formation peptides. Calibration curve samples were analyzed in same sequence as in vitro and in vivo samples. Y axis: Signal intensity corrected by the IS (added at the beginning of the digestion protocol). X axis: Total concentration (in mg/ml).

NTLYLox

		Animal 294	Animal 324	Animal 204	Spiked pooled serum
50 min	Oxidation rate	**3.75%**	**5.26%**	**5.91%**	**2.55%**
	RSD triplicates	*3.70%*	*10.37%*	*10.44%*	*14.79%*
5 days	Oxidation rate	**5.43%**	**5.84%**	**6.74%**	**5.82%**
	RSD triplicates	*12.14%*	*5.93%*	*6.31%*	*9.24%*
16 days	Oxidation rate	**5.95%**	**6.53%**	**6.44%**	**5.58%**
	RSD triplicates	*2.84%*	*3.30%*	*0.18%*	*9.80%*
22 days	Oxidation rate	**7.70%**	**7.90%**	**8.07%**	**9.75%**
	RSD triplicates	*5.95%*	*9.26%*	*13.21%*	*14.11%*
28 days	Oxidation rate	**9.85%**	**8.99%**	**9.46%**	**9.74%**
	RSD triplicates	*11.34%*	*6.69%*	*4.23%*	*6.83%*

Table S16. Oxidation rate over time detected at Pertuzumab (NTLYL peptide in the Fab region) in exploratory animal study samples (animals) and a spiked model (spiked Pertuzumab in pooled animal serum incubated at 37 °C). Relative standard deviations between triplicates are depicted for each sample and each analyzed point in time.

		(N54) PisoDSG				**(N61) YisoDQR**			
		Animal 294	Animal 324	Animal 204	Spiked pooled serum	Animal 294	Animal 324	Animal 204	Spiked pooled serum
50 min	Deamidation rate	-	-	-	-	-	-	-	-
	RSD triplicates	-	-	-	-	-	-	-	-
5 days	Deamidation rate	-	-	-	-	-	-	-	-
	RSD triplicates	-	-	-	-	-	-	-	-
16 days	Deamidation rate	-	-	-	-	-	-	-	-
	RSD triplicates	-	-	-	-	-	-	-	-
22 days	Deamidation rate	**1.15%**	**1.11%**	**1.17%**	**2.03%**	-	-	-	**0.52%**
	RSD triplicates	*13.35%*	*5.66%*	*6.50%*	*6.60%*	-	-	-	*1.20%*
28 days	Deamidation rate	**1.82%**	**1.89%**	**1.68%**	**5.48%**	-	-	-	**1.91%**
	RSD triplicates	*9.38%*	*7.80%*	*12.29%*	*7.69%*	-	-	-	*9.54%*
		(N54) PDSG				**(N52) DVDP**			
		Animal 294	Animal 324	Animal 204	Spiked pooled serum	Animal 294	Animal 324	Animal 204	Spiked pooled serum
50 min	Deamidation rate	-	-	-	-	-	-	-	-
	RSD triplicates	-	-	-	-	-	-	-	-
5 days	Deamidation rate	-	-	-	-	-	-	-	-
	RSD triplicates	-	-	-	-	-	-	-	-
16 days	Deamidation rate	-	-	-	-	-	-	-	-
	RSD triplicates	-	-	-	-	-	-	-	-
22 days	Deamidation rate	-	-	**1.57%**	**1.03%**	-	-	-	-
	RSD triplicates	-	-	*2.42%*	*13.19%*	-	-	-	-
28 days	Deamidation rate	**1.92%**	-	**3.77%**	**1.20%**	-	-	-	**0.96%**
	RSD triplicates	*7.65%*	-	*4.57%*	*5.47%*	-	-	-	*3.52%*

Table S17. Deamidation rate over time detected one sites of Pertuzumab (GLEW peptide in the CDR region) in exploratory animal study samples (animals) and a spiked model (spiked Pertuzumab in pooled animal serum incubated at 37 °C). Relative standard deviations between triplicates are depicted for each sample and each analyzed point in time.

		isoDG (N86)				**PEisoDNY (N91)**			
		Animal 294	Animal 324	Animal 204	Spiked pooled serum	Animal 294	Animal 324	Animal 204	Spiked pooled serum
50 min	Deamidation rate	-	-	-	-	**0.43%**	**0.40%**	**0.39%**	**0.45%**
	RSD triplicates	-	-	-	-	*12.29%*	*12.55%*	*11.49%*	*13.37%*
5 days	Deamidation rate	**1.71%**	**1.21%**	**1.44%**	**1.83%**	**1.37%**	**1.21%**	**1.13%**	**1.41%**
	RSD triplicates	*9.76%*	*10.35%*	*8.80%*	*12.01%*	*7.75%*	*3.40%*	*4.65%*	*9.64%*
16 days	Deamidation rate	**3.72%**	**2.56%**	**3.45%**	**4.20%**	**3.77%**	**3.10%**	**3.53%**	**3.77%**
	RSD triplicates	*3.32%*	*3.90%*	*6.35%*	*4.83%*	*14.31%*	*2.14%*	*13.69%*	*13.23%*
22 days	Deamidation rate	**4.57%**	**3.59%**	**4.46%**	**5.03%**	**4.77%**	**4.21%**	**4.49%**	**4.77%**
	RSD triplicates	*6.34%*	*4.62%*	*1.34%*	*4.95%*	*11.57%*	*1.21%*	*12.36%*	*7.12%*
28 days	Deamidation rate	**4.94%**	**4.93%**	**5.67%**	**6.97%**	**5.83%**	**5.59%**	**6.08%**	**6.08%**
	RSD triplicates	*4.67%*	*12.76%*	*4.84%*	*8.48%*	*5.20%*	*3.76%*	*2.43%*	*12.30%*
		PENisoDy (N92)				**PEDNY/PENDY (N91/92)**			
		Animal 294	Animal 324	Animal 204	Spiked pooled serum	Animal 294	Animal 324	Animal 204	Spiked pooled serum
50 min	Deamidation rate	-	-	-	-	**0.94%**	**0.80%**	**0.93%**	**0.67%**
	RSD triplicates	-	-	-	-	*12.53%*	*8.52%*	*12.51%*	*14.17%*
5 days	Deamidation rate	-	-	-	-	**3.09%**	**2.09%**	**3.29%**	**3.71%**
	RSD triplicates	-	-	-	-	*12.24%*	*11.73%*	*3.46%*	*7.47%*
16 days	Deamidation rate	-	-	-	-	**6.22%**	**5.00%**	**7.01%**	**6.97%**
	RSD triplicates	-	-	-	-	*4.61%*	*14.66%*	*1.80%*	*7.45%*
22 days	Deamidation rate	**1.96%**	**3.56%**	**2.58%**	**1.16%**	**9.16%**	**6.89%**	**10.84%**	**9.33%**
	RSD triplicates	*9.10%*	*12.89%*	*5.04%*	*8.93%*	*7.38%*	*2.25%*	*4.10%*	*3.23%*
28 days	Deamidation rate	**4.01%**	**4.23%**	**5.62%**	**9.93%**	**11.82%**	**8.68%**	**16.55%**	**14.91%**
	RSD triplicates	*4.27%*	*6.88%*	*8.76%*	*9.13%*	*10.78%*	*5.54%*	*3.01%*	*10.59%*
		DG (N86)							
		Animal 294	Animal 324	Animal 204	Spiked				
50 min	Deamidation rate	-	-	-	-				
	RSD triplicates	-	-	-	-				
5 days	Deamidation rate	**0.73%**	-	**1.25%**	**1.09%**				
	RSD triplicates	*9.20%*	-	*6.27%*	*8.74%*				
16 days	Deamidation rate	**2.11%**	**1.90%**	**2.35%**	**2.65%**				
	RSD triplicates	*10.51%*	*10.52%*	*8.83%*	*2.05%*				
22 days	Deamidation rate	**3.31%**	**2.88%**	**3.39%**	**3.63%**				
	RSD triplicates	*12.83%*	*9.17%*	*4.54%*	*14.45%*				
28 days	Deamidation rate	**4.99%**	**4.15%**	**5.50%**	**5.37%**				
	RSD triplicates	*12.06%*	*4.68%*	*5.60%*	*10.97%*				

Table S18. Deamidation rate over time detected one sites of Pertuzumab (PENNY peptide in the Fc region) in exploratory animal study samples (animals) and a spiked model (spiked Pertuzumab in pooled animal serum incubated at 37 °C). Relative standard deviations between triplicates are depicted for each sample and each analyzed point in time.

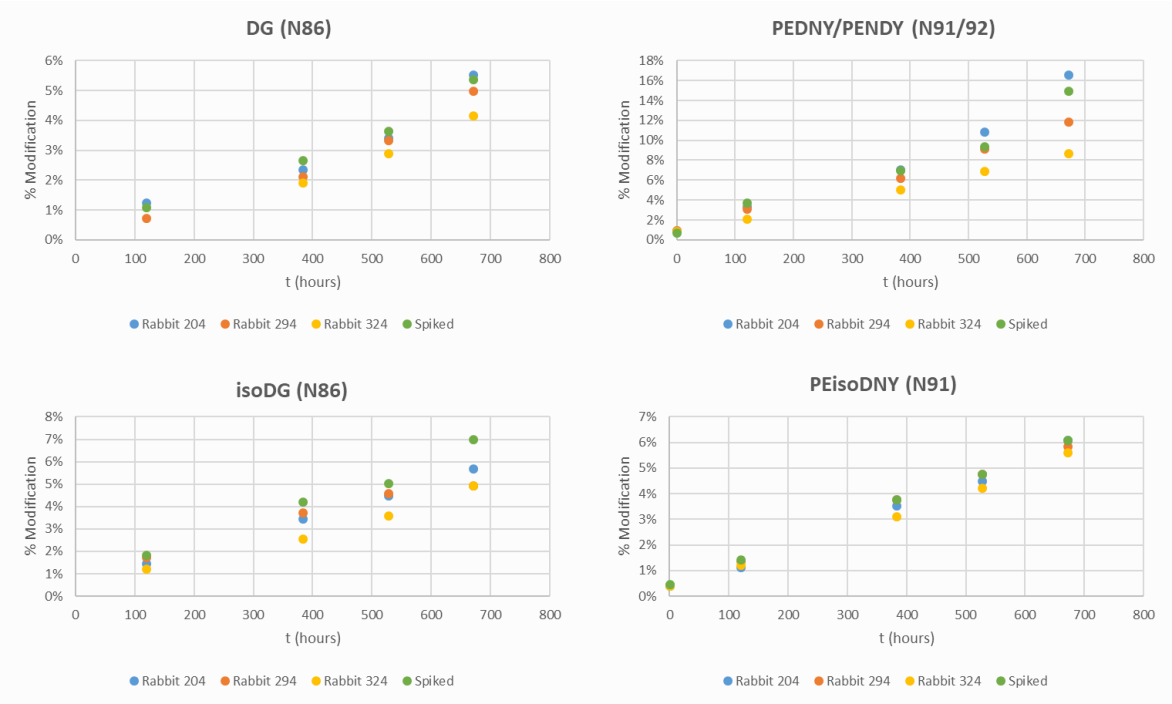

Figure S6. Deamidation rates over time for three animals and spiked pooled animal serum at different deamidation sites of Pertuzumab (PENNY peptide in the Fc region). Y axis: Deamidation rate. X axis: Time after Pertuzumab administration (in hours).

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List of Hazard Substances

Risk and safety pictograms of potentially hazard chemicals used throughout this study based on the Globally Harmonized State of Classification and Labelling of Chemicals (GHS) GHS hazard and precautionary statements.

Chemical	GHS Symbol	GHS hazard statement	GHS precautionary statements
AccuMAP denaturing solution		302, 315, 319	264, 270, 280, 30+312, 302+350, 305+350, 333+313, 337+360
Acetic acid (C2H4O2)		226, 314	210, 280, 301+330+331, 303+361+353, 305+351+338+310
Acetonitrile (C2H3N)		225, 332, 302, 312, 319	210, 240, 302+352+338, 403+233
BCA Protein Assay kit		314	260, 264, 280, 301+330+331, 303+361+353, 304+340, 305+351+338, 310, 321, 363, 405, 501
Dithiothreitol (DTT) (C4H10O2S2)		302, 315, 319, 335	261, 302+352, 305+351+338
Ethylenediaminetetraacetic acid (EDTA) (C10H16N2O8)		319	305 + 351 + 338
Formic acid (CH2O2)		226, 290, 302, 314, 331	P210, P280, P303+P361+P353 P304+P340+P310 P305+P351+P338 P403+P233
Guanidine HCL (CH5N3·HCl)		302+332, 315, 319	261, 264, 301+312, 302+352, 304+340+312, 305+351+338

Hydrochloric acid (HCL)		290, 314, 335	234 261, 271, 280, 303+361+353, 305, 351, 338
Histidine monohydrochloride (C6H9N3O2·HCl·H2O)		319	264, 280, 305+351+338, 337+313
Iodoacetamide (C2H4INO)		301, 317, 334, 413	261, 280, 301+310, 342+311
Sodium hydroxide (NaOH)		290, 314	234, 260, 280, 301+330+331, 303+361+353, 305+351+338
Tris(2-carboxyethyl)phosphine (TCEP)		314	260, 264, 280, 301+330+331, 303+361+353, 304+340, 305+351+338, 310, 363
Trifluoroacetic acid (TFA) (C2HF3O2)		314, 332, 412	273, 380, 305+351+338, 310
Tris HCL (C4H11NO3.HCl)		319, 335, 315	233, 280, 302, 352, 304, 340, 305+351+338, 312, 403+233
Urea (C8H14N4O7)		319	264, 280, 305+351+338, 337+313

Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, die vorliegende Dissertation selbst verfasst und keine anderen als die angegebenen Hilfsmittel benutzt zu haben. Die eingereichte schriftliche Fassung entspricht der auf dem elektronischen Speichermedium. Ich versichere, dass diese Dissertation nicht in einem früheren Promotionsverfahren eingereicht wurde.

Sandra Carolina Mena Pérez

München, Juni. 2021

Declaration on Oath

I hereby declare on oath that this doctoral dissertation is written independently and solely by my own based on the original work of my PhD and has not been used other than the acknowledged resources and aids. The submitted written version corresponds to the version on the electronic storage medium. I declare that the present dissertation was prepared maintaining the Rules of Good Scientific Practice of the German Research Foundation and it has never been submitted in the present form or similar to any other University or board of examiners.

Sandra Carolina Mena Pérez

Munich, June. 2021