A quantitative MS-based multi-attribute method (MAM) approach for new biological entities and biosimilar candidates presented by a full method qualification for an IgG1 market product

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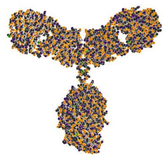
Summary

- Development of an MS-based multi-attribute method (MAM) for monitoring the product quality attributes (PQA) on a biotherapeutic protein IgG1 market product
- Characterization and quantification of PQAs: amino acid sequence, terminal processing, truncation, deamidation, oxidation and glycosylation
- Introduction of a concept for full method qualification of a MAM approach using reversed-phase chromatography in combination with high resolution mass spectrometry
- Evaluation of the qualification parameters: analyte autosampler stability, repeatability, intermediate precision, linearity and specificity of the method using appropriate stress samples
- MAM approach offers the technical potential for validation and usage as GMP release testing

Monitored Product Quality Attributes (PQAs) by MAM

- Amino acid sequence and sequence variants
- Terminal truncations, signal peptide residue
- N-Terminal pyro-Glu
- C-Terminal Lys and Amidation
- Deamidation
- Oxidation
- Glycation
- N-Glycosylation profile
- Disulfide linkages, thioether, trisulfide, free thiols



Methods and Instrumentation

Sample	Monoclonal antibody, IgG1 Qualification sample, innovator Lot Specificity sample, stress induced innovator Lot
Reduction and Alkylation	Dithiothreitol 2-Iodoacetamide
Digestion	Trypsin (Promega) Trypsin/Chymotrypsin Chymotrypsin (Roche)
Chromatography	UltiMate™ 3000 RSLC (Thermo Fisher Scientific) Accucore RP, 2.1x100 mm, 2 µm particle size
Mass Spectrometry	Q Exactive™ Orbitrap MS (Thermo Fisher Scientific) R: 140.000, scan range: 300–1400 *m/z*, MS/MS: TOP 4 HCD, NCE: 25%
Data Analysis	Byonic™ and Byologic® software packages (Protein Metrics Inc.)

Fig. 1

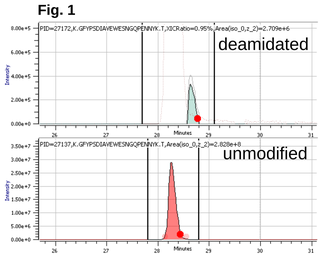
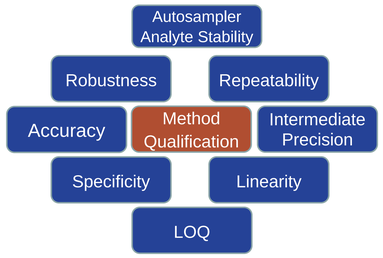


Fig. 1: XIC of deamidated and unmodified PENNY-peptide for quantitative evaluation of modification level. The co-elution and resulting overlap of the integrated mass window of deamidated peptide with first ^{13}C isotope of unmodified peptide is overcome with Byologic deconvolution algorithm.

Analytical Method Qualification

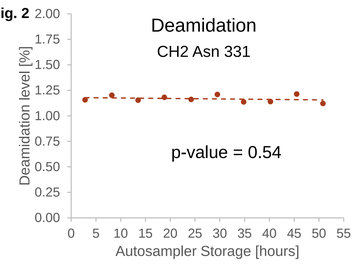


The **qualification** of analytical methods serves to prove that the method is **suitable for the intended application** with method results which are meaningful and reliable. Various **qualification parameters** are addressed. In accordance to quality assurance **qualified equipment** and **Good Documentation Practice** should be mandatory. A qualification study identifies the **method performance** and can still be a part of the **method development process** with the option to optimize the method, if required. In contrast to validation, where results have to meet strictly pre-determined acceptance criteria, the qualification can serve to **define acceptance criteria**.

Autosampler Analyte Stability — Deamidation & Oxidation

Design	Assessment
1 sample preparation analyzed at 10 time points over 50 hours autosampler storage	unweighted linear regression, stability: p-value ≥ 0.05 for slope = 0

Fig. 2



For the most **deamidation** sites the p-value is ≥0.05 confirming no significant increase of modification level (example CH2 Asn331, Fig. 2). Other sites prone for deamidation (example PENNY-peptide Fig. 3) show a **slight, but significant increase at acetic condition** (0.1%FA dilution). The observed significant increase of total deamidation (sum all sites, Fig. 4) shows an **absolute increase of 0.5% deamidation over 50 hours**, which is within the range of method variation. For **oxidation the analyte stability is confirmed** over tested time range.

Fig. 3

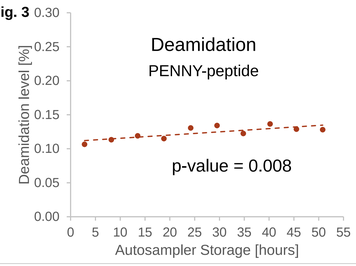
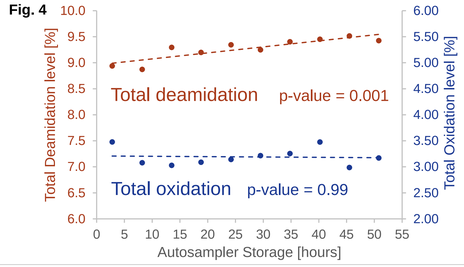


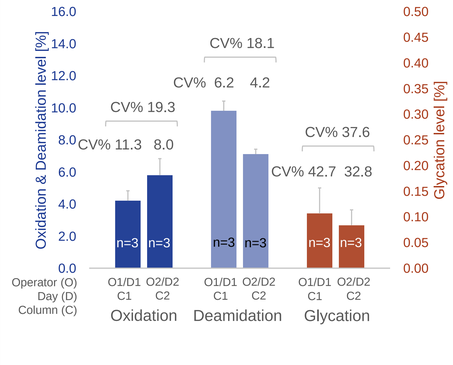
Fig. 4



Precision — Deamidation, Oxidation, Glycation

Design	Assessment
6 independent analysis including sample preparation	Coefficient of variation (CV) determination

Fig. 5 Total modification level



The presented precision study includes the evaluation of:

- **Repeatability** (within-day variability), three independent analyses (including sample preparation)
- **Intermediate precision** (day-to-day variability), six analysis carried out by two different operators, on two different days, using two different RP-column lots

Due to the general low modification level (glycation) the CV is high. The absolute error is still low. However, the intermediate precision with CV up to approx. 20% (oxidation, deamidation) highlights the need for **robust protocols** of sample preparation a **reference standard** in each measurement campaign to judge the variability between different studies.

Linearity & Quantification Limit — N-Glycosylation

Design Linearity	Assessment
Test of 6.25%, 12.5%, 25%, 50%, 75%, 100%, 125% and 150% of nominal test concentration, n = 2 per point	unweighted linear regression, linearity: p-value ≥ 0.05 for slope = 0

Fig. 6 Linearity of N-Glycosylation analysis

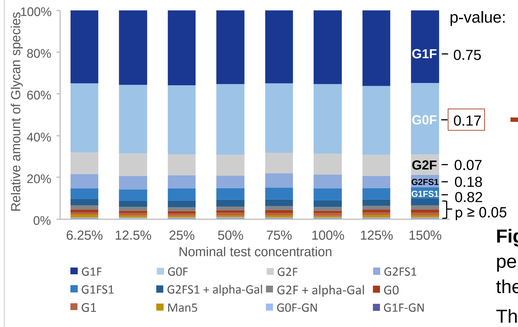


Fig. 6: The N-glycan structures attached to the IgG1 Fc N-glycosylation site peptide were quantified via XICs over the tested range.

Fig. 7

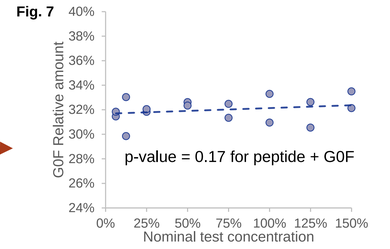
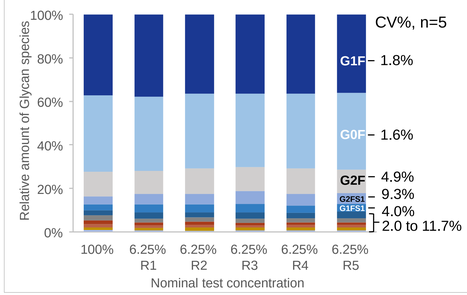


Fig. 7: An unweighted linear regression was performed to test the hypothesis that the slope of the trendline is zero.

The site-specific N-glycan analysis was determined as **linear** for the tested **range 6.25% to 150% for all N-glycan species** (all p-values ≤0.05), even for low abundant N-glycans.

Design Precision LOQ	Assessment
5 analyses at concentration level with confirmed linearity	Coefficient of variation (CV) determination

Fig. 8 Precision of N-Glycosylation analysis at LOQ

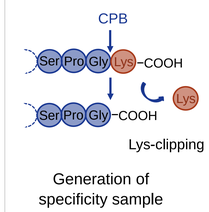


The **LOQ at 6.25% sample concentration** was determined (last point of the tested range, where linear behavior is maintained). The precision at LOQ was verified by five analyses. The obtained **precision at LOQ is between CV 2% and 12**% for the quantified N-glycan species.

As linearity and LOQ were tested in dilution series, the matrix compounds are diluted too. Due to the missing **matrix effects** both parameters are probably overestimated. Although the procedure is compliant to ICH guidelines, in MS the testing with the presence of matrix would be more reliable.

Specificity — C-Terminal Variants

Design	Assessment
Comparative analysis of impurity samples (stressed sample), controls (other IgG)	Detectable and reliable differences in physicochemical properties and structure



Generation of specificity sample

Sample for specificity testing to evaluate the analysis of C-terminal Lysine clipping was generated by Carboxypeptidase B (CPB) treatment.

The **specificity of the method is confirmed** as differences in the Lys-clipping level were detected between untreated IgG1 and specificity sample.

C-Terminus HC	Untreated IgG1	Specificity sample CPB
…SLSLSPGK	**2.72%**	---
…SLSLSPG	**97.23%**	**99.95%**
…SLSLSP^{amidated}	0.05%	0.05%

Outlook: MAM and GMP

LC-MS is an extremely powerful, but several considerations and necessary preconditions should be addressed to use MS in fully compliant routine analyses:

- LC-MS is a highly sophisticated technique with many different instrument types
- Method validation is feasible, if robustness of the complex MAM method is addressed properly by the creation of robust protocols for sample preparation and the analytical workflow
- Implementation of system suitability tests with appropriate acceptance criteria
- GMP-compliant software for data acquisition, automated data analysis validated by manufacturer and in user environment