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Research paper

Standardization, evaluation and early-phase method validation of an analytical scheme for batch-consistency *N*-glycosylation analysis of recombinant produced glycoproteins

Stefan Zietze [a,*], Rainer H. Müller [b], René Brecht [a]

[a] *ProBioGen AG, Berlin, Germany*
[b] *Free University of Berlin – Department of Pharmaceutical Technology, Biotechnology and Quality Management, Berlin, Germany*

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Abstract

In order to set up a batch-to-batch-consistency analytical scheme for *N*-glycosylation analysis, several sample preparation steps including enzyme digestions and fluorophore labelling and two HPLC-methods were established. The whole method scheme was standardized, evaluated and validated according to the requirements on analytical testing in early clinical drug development by usage of a recombinant produced reference glycoprotein (RGP). The standardization of the methods was performed by clearly defined standard operation procedures. During evaluation of the methods, the major interest was in the loss determination of oligosaccharides within the analytical scheme. Validation of the methods was performed with respect to specificity, linearity, repeatability, LOD and LOQ. Due to the fact that reference *N*-glycan standards were not available, a statistical approach was chosen to derive accuracy from the linearity data. After finishing the validation procedure, defined limits for method variability could be calculated and differences observed in consistency analysis could be separated into significant and incidental ones.
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1. Introduction

Mammalian expression systems are characterized by their ability to perform post-translational modifications in a human like manner. One important modification is glycosylation, because of its influence on pharmacodynamics, pharmacokinetics, immunogenicity and the overall efficacy of the product [1]. Intracellular glycosylation is the enzymatic addition of sugar residues to the peptide-backbone of a protein. More than 50% of all human proteins are glycoproteins [2] and more of them become interesting therapeutic agents for biopharmaceutical companies today. These recombinant produced glycoproteins for pharmaceutical usage include erythropoeitin, interferon-β and monoclonal antibodies. Due to its sensitivity, glycosylation is subject to variations within the biotechnological production process. To demonstrate a reliable product quality, glycosylation analysis has to be part of the regular biopharmaceutical quality control.

2. Theoretical development

According to a draft by PhRMA on "Method Validation by Phase of Development" from 2003 [3], the validation parameters *accuracy*, *precision* and *linearity* need to be examined for early phase bioanalytical method validation (sigmoid binding assays not included) (Table 1). During practical *linearity*-determination, precision and accuracy can be well determined in combination experiments and therefore *linearity* gets a special importance [4].

* Corresponding author. ProBiogen AG, Quality Control, Goethestrasse 54, 13086 Berlin, Germany. Tel.: +49 3092400643.
E-mail address: stefan.zietze@probiogen.de (S. Zietze).

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Table 1
Necessary validation parameters determined in early phase product development

Validation parameter	Assay	Organic impurities
Accuracy	Inferred from precision, *linearity* & specificity	Inferred from precision, *linearity* & specificity
Precision	From 3 sample preparations at 100% of test concentration	From 3 sample preparations at 100% of test concentration
Intermediate precision	Delay	Delay
Specificity	Show resolution of Drug Substance from likely impurities	Show resolution of Drug Substance from likely impurities
Quantitation limit	N/A	Confirmed to be no greater than the reporting limit
Detection limit	N/A	Delay
Linearity	From impurity *linearity* or 3 levels between 80% and 120% of test concentration	Determine from 3 sample concentrations (e.g. attest concentration, 1% of test concentration, and quantitation limit)
Range	Defined by the *linearity* work	Defined by *linearity* and quantification limit experiments
Robustness	Solution stability and information gathering	Solution stability and information gathering

3. Materials and methods

3.1. Scheme for N-glycosylation analysis (Fig. 1)

The glycoanalytical scheme started with the digestion of the purified glycoprotein with trypsin to obtain peptide fragments. Deglycosylation of the peptides was performed by PNGase F digestion. The isolated glycans were labelled with 2-aminobenzamide for fluorimetric detection, resulting in the same response factors ($\lambda_{\text{max-emission}} = 428$ nm) for each *N*-glycan. One part of the glycans was further digested with sialidase to get uncharged oligosaccharides. These desialylated glycans were separated on an amino-phase column by hydrophilic interaction chromatography (HILIC). The sialylated glycans were separated on a DEAE-column by anion exchange chromatography (AEX). These methods can be performed on conventional HPLC-devices and are quite robust against typical interference factors.

3.2. Reagents and instrumentation

3.2.1. For sample preparation

For desalting of the glycoprotein samples, PD-10 columns (Sephadex G-25 M; Product-No. 17-0851-01) from Amersham Biosciences were used. Three enzymes, all from Roche, were purchased for the glycoanalytical scheme: for glycoprotein cleavage trypsin (Product-No. 109819), for deglycosylation of the glycopeptides PNGaseF (Product-No. 1365193) and for desialylation of *N*-glycans sialidase (Product-No. 296611). All enzyme incubation buffers (50 mM *N*-Methyl-2,2′-iminodiethanol, pH 8.0, adjusted with concentrated TFA and 500 mM sodium acetate solution, pH 5.0, acetic acid adjusted with NaOH-solution) were from VWR Merck (Product-No. 8.05851.0100, 1.08262.0025, 1.00066.0250 and 1.05428.0250). For purification of the *N*-glycans after the digestion steps, ion exchange resins from BioRad (AG 50W X12 Resin, 100–200 mesh, hydrogen form, Product-No. 142-1641 and AG 4 X4, 100–200 mesh, free base, Product-No. 140-4341) were used. Disposable polystyrene columns for the resins were from Pierce (Product-No. 29920). 2-aminobenzamide for labelling was purchased from Sigma (Product-No. A8,980-4). Dimethylsulfoxide was from VWR Merck (Product-No. 41647) and sodium cyanoborohydride from Fluka (Product-No. 71435). For separating 2-AB label excess from the *N*-glycans, 3 mm chromatography paper from Whatman (Product-No. 3030917) was used. Butanol and ethanol for paper chromatography were both from VWR Merck. Water was obtained from a Direct-Q system (Millipore). For detection the spot with the labelled *N*-glycans, a UV-lamp from Benda (Product-No. NU-8 k1) was used. Syringes for elution of the oligosaccharides from the chromatography paper was from BD (Product-No. 309585). Membrane filter (0.45 μm) for the separation of cellulose particles after the paper chromatography were from Millipore (Product-No. SLHV R04 NL). Centrifuge tubes were from Biochrom (Product-No. P91015), 0.5 ml screwing vials from Fisher (Product-No. 9479900) and 0.2 ml PCR-tubes from Cotech (Product-No. AXY-PCR-02Y). For incubation at 37 °C, a drying oven (Integra Biosciences, Cellsafe) and a thermo controller (MJ Research, PTC-100™) were used.

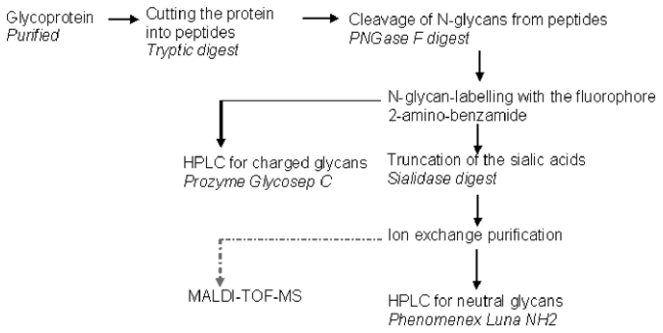


Fig. 1. Established scheme for *N*-glycosylation analysis; black fields were evaluated and validated.

3.2.2. For HPLC-methods

The HPLC-device used for analysis was from Shimadzu (high pressure liquid system: 2xLC-10ATvp, SCL-10AVP, FCV-10ALvp, DGU-14A, SPD-10Avp, FRC-10A, SIL-10ADvp, CTO-10ASvp, RF-10AXL, CLASS-VP-Software

Version 6.12 SP5). For eluents, acetic acid and sodium hydroxide solution both from VWR Merck (Product-No. 1.00066.0250 and 1.05428.0250) and acetonitrile from Rathburn (Product-No. RH1016) were used.

3.3. Sample preparation

At first, 1.6 mg of the reference glycoprotein was desalted with Sephadex PD-10 columns. To assure a complete cleavage of *N*-glycans by PNGase F, the glycoprotein was digested with trypsin before. Therefore, the lyophilized desalted protein sample was dissolved in 860 µl incubation buffer pH 8.0. After that, 90 µl trypsin was added in two portions to the sample and an incubation for 22 h at 37 °C in a drying oven followed. After the incubation, the solution was boiled at 100 °C in a water bath for 10 min to inactivate the trypsin. A cooling down of the solution in crushed ice for 30 min followed. Then 50 µl PNGase F was added in two portions to the sample using desalted PNGase F in a concentration of 1 U/µl. The incubation was performed at 37 °C for another 22 h. After that, 100 µl of 10% (V/V) acetic acid was added to the sample. The pH of the solution changed from 8 to 3. Now 2 ml disposable polystyrene columns were filled with 0.5 ml cation exchange resin and the sample was transferred onto the resin. The flow-through was collected. The column was flushed with 4×0.5 ml of purified water. Then the filtrate was lyophilized to dryness and concentrated to a volume of 10 µl. The lyophilized oligosaccharide sample was mixed with a labelling solution, consisting of 30 mg 2-aminobenzamide, 2100 µl dimethylsulfoxide, 900 µl acetic acid and 36 mg sodium cyanoborohydride. Ten microliters of labelling mixture was added to the sample and an incubation for 16 h at 37 °C in a thermo controller followed. After the incubation, separation of reagent excess was performed by paper chromatography. Each sample was halved in two equal parts of 5 µl and pipetted on paper stripes ($2.5 \text{ cm} \times 8 \text{ cm}$). One part was termed a-sample and the other b-sample. The paper stripes were chromatographed in a glass chamber with a mixture of 4 parts butanol, 1 part ethanol and 1 part purified water as eluent. After the paper stripes were completely dry, they were examined under a UV-lamp at 254 nm for detection of the spot at the start point. To elute the 2-AB-marked oligosaccharides from the paper, the cutted spots were put into a Luer-lock syringe with a Millex®-HV-filter screwed onto it. Now 1 ml purified water was filled into the syringes and a centrifugation at 3000 U/min for 5 min followed. Now the a-samples were transferred into 0.5 ml screwing vials and the b-samples into 0.2 ml PCR-tubes. The a-samples were filled up with water to a volume of 100 µl and frozen at −20 °C. The b-samples were filled up to a volume of 30 µl and then digested with sialidase. Therefore 10 µl sialidase buffer pH 5.0 and 10 µl sialidase (=0.1 U) were added to the solution. This mixture was incubated for 37 °C in a thermo controller for 48 h. The b-samples were then purified over a mixed resin bed consisting of 500 µl anion exchange resin and 500 µl cation exchange resin. The sample was transferred onto the resin and the flow-through was collected. Now the eluate was transferred to a 0.5 ml screwing vial and like the a-samples filled up to a volume of 100 µl with water and frozen at −20 °C.

3.4. HPLC-methods

3.4.1. GlycoSepC-HPLC

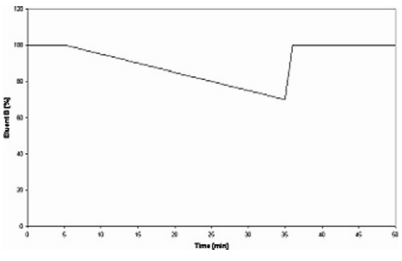
The GlycoSepC-column consisted of a modified weak anion exchanger bound on a polymeric stationary phase with amine functionality (Prozyme, formerly Glyco; 5 µm polymer coated divinyl benzene resin derivatized with DEAE; 4.6 mm*100 mm). It offered optimized resolution of neutral, sialylated, sulfated or phosphorylated glycans into charge classes (AEX). From the a-sample solution a 1% (V/V) dilution was prepared. From this dilution, 20 µl was injected for the HPLC-run. Two eluents were used, A and B. Eluent A was a mixture of 500 ml 500 mM NH_4Ac pH 4.5 and 500 ml 20% acetonitrile (V/V). Eluent B was only 20 % (V/V) acetonitrile solution. The flow rate of the method was 0.5 ml/min. The following gradient was used for separation (Table 2). Column oven temperature was at 25 °C. For fluorimetric detection excitation wavelength was at 330 nm and emission wavelength at 420 nm. Data acquisition was performed for 35 min. The last 15 min were for reequilibration of the column.

3.4.2. Aminophase-HPLC

The aminophase column was an aminopropylsilicate matrix (Phenomenex® Luna NH2; 3 µm spheric particles with a pore size of 100 A; 4.6 mm*150 mm). Desialylated glycans were separated by the amount of their monomers in the way that larger molecules were longer retented than smaller molecules (HILIC). From the b-sample solution a 10% (V/V) dilution was prepared. From this dilution, 20 µl were injected for the HPLC-run. Two eluents were used, A and B. Eluent A was 15 mM NH_4Ac (pH 5.2) solution. Eluent B was a mixture of 25% (V/V) 15 mM NH_4Ac (pH 5.2) solution and 75% (V/V) acetonitrile. The flow rate of the method was 1.5 ml/min. The following gradient was

Table 2
Gradient program for GlycoSepC-HPLC

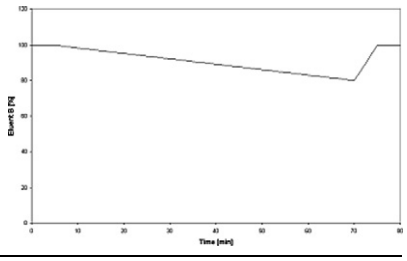
Time (min)	Eluent B (%)
0[a]	100
5	100
35	70
36	100
50	100



[a] Time of injection.

Table 3
Gradient program for aminophase-HPLC

Time (min)	Eluent B (%)
0	100
5[a]	100
70	80
75	100
80	100



[a] Time of injection.

used for separation (Table 3). Column oven temperature was at 25 °C. For fluorimetric detection excitation wavelength was at 330 nm and emission wavelength at 420 nm. Data acquisition was performed up to 70 min. The last 10 min were for reequilibration of the column.

3.5. Standardization

Standardization of the method was performed by creating standard operation procedures (SOPs) for the sample preparation and the HPLC methods. In the SOP for sample preparation each working step had to be documented in working protocols (exemplified in Table 4). One hundred and fifty working steps at all had to be documented during sample preparation.

3.6. Validation approach

Before validation started, a risk assessment in form of an Ishikawa-diagram (cause–effect-diagram) of the analytical methods including all sample preparation steps was performed (Fig. 2). Nearly all sample preparation steps had an influence on the recovery (accuracy) and repeatability (precision) of the methods. An efficient way to validate the whole analytical scheme for the early phase of drug development was to determine the overall validation parameters linearity, precision, specificity and accuracy. These were examined for one reference glycoprotein. The reference protein was therefore analyzed on five different concentration levels in a four time repetition (Table 5). The complete analytical scheme was performed and peak areas of the resulting HPLC-chromatograms were evaluated (Fig. 3).

4. Results

4.1. Evaluation of the method

4.1.1. Determination of response factors

An important criteria for relative quantification of HPLC-chromatograms was the examination of comparable response factors for each glycan fraction and structure. Therefore emission spectra of all HPLC-peaks, exemplified for the GlycoSepC-HPLC, were examined (Table 6). Emission spectra were taken during normal HPLC runs. When a peak occurred, the pumps were stopped and the emission spectrum was recorded at comparable peak intensities. The spectrum scans were performed between 300 and 500 nm. The wavelength with the maximal emission (λ_{max}) was calculated from 380 to 500 nm and is shown in the right column in Table 6. Excitation took place at 330 nm.

As result, it became obvious that all labelled oligosaccharides had the same maximal emission wavelength and therefore the same response factors. Relative quantification of glycan structures was possible. For electrochemical detection systems (e.g., High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection, HPAEC-PAD), this is not possible without using reference standards for each glycan structure because of different responses of the analytes at the electrochemical detector [5].

4.1.2. Determination of recovery

To determine the recovery of the oligosaccharides during the sample preparation procedure, several methods were used including UV-spectroscopy (UV), a colorimetric sialic acid determination (SA) and HPAEC-PAD-monosaccharide analysis (MA). The combination of the different methods was necessary because not all methods were able to quantify the glycans in each status of the preparation procedure. The results of this study are shown in Fig. 4.

The highest loss of oligosaccarides occurred after sialidase digestion, maybe due to the mixed bed resin purification. In the sample for GlycoSepC-HPLC, 73% of all oligosaccharides was found, for the Aminophase-HPLC only 37% were recovered.

4.2. Validation of the method

The glycoanalytical scheme was validated with respect to specificity, linearity, accuracy, repeatability, limit of detection (LOD) and quantification (LOQ).

Table 4
Desalting of samples: small quadrates in the table were marked after performing the specific step

Sample	Adjusting volume of sample to 2.5 ml	Flushing of the PD-10-column with 25 ml purified water	Pipetting of samples on the column	Elution of the samples with 3.5 ml purified water	Lyophilization of the eluate
1	□	□	□	□	□
2	□	□	□	□	□
3	□	□	□	□	□
4	□	□	□	□	□
5	□	□	□	□	□

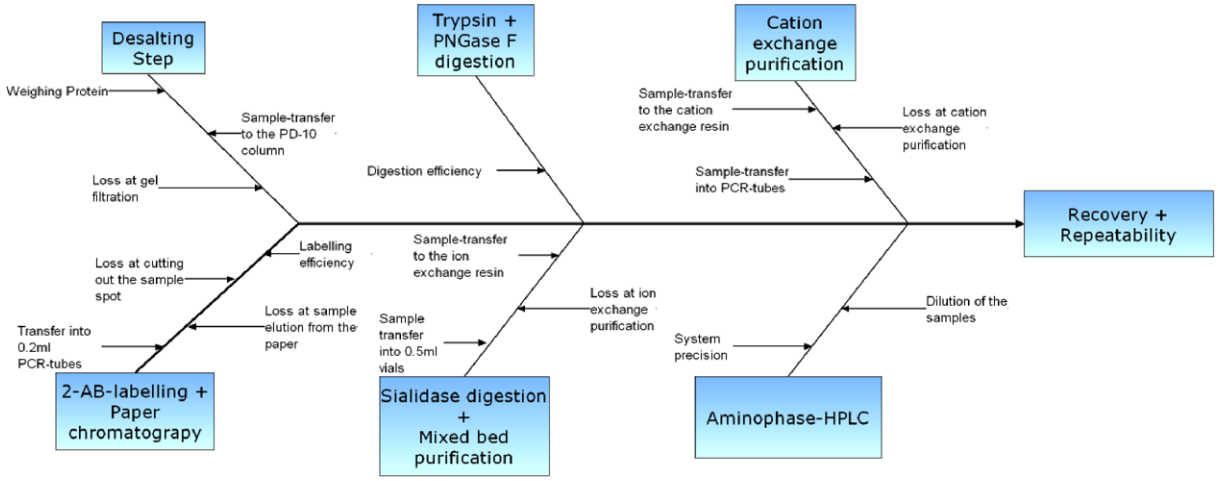


Fig. 2. Risk assessment, exemplified for HILIC-analysis in form of an Ishikawa-diagram.

Table 5
Reference glycoprotein on five different concentration levels

Protein (nmol)	Repetition
3.5	4
14	4
24.5	4
35	4
45.5	4

4.2.1. Repeatability

The variation coefficients of most analytical methods correspond to the Horwitz-equation [6]

$$\mathrm{CV}(\%) = 2^{(1-0.5\log C)},$$

where C is the concentration expressed as powers of 10 (Fig. 5, copied from [6]).

Regarding Fig. 6, no clear trend in the relative standard deviations comparable with the Horwitz-graph was observed. The lowest relative standard deviation (RSD) for GlycoSepC-analysis was at 24.5 nmol reference glycoprotein (RGP), whereas the lowest RSD for Aminophase-analysis was at 45.5 nmol.

4.2.2. Linearity and accuracy

Linear regression analysis was performed from the resulting peak areas for each charged glycan fraction (GlycoSepC-HPLC, Fig. 7) and for each uncharged structure (Aminophase-HPLC, Fig. 8a). In general, accuracy can be inferred from the fact that the prolonged linearity curve at sample amounts of 0 nmol does not have a significant difference from 0 in the y-axis-direction [7]. Therefore linear regression was performed and the estimated 95% confidence interval was laid over $x = 0$. If the confidence interval enclosed $y = 0$, then the method would be accurate by a probability of 95%, implying that precision, linearity and specificity were taken for granted. For GlycoSepC-analysis, accuracy could be inferred with this procedure, whereas for Aminophase-analysis the confidence interval did not overlap $x = 0$. This was due to the first data point (concentration $x = 3.5$ nmol) which did not only narrow the confidence interval extremely, but at which a specific

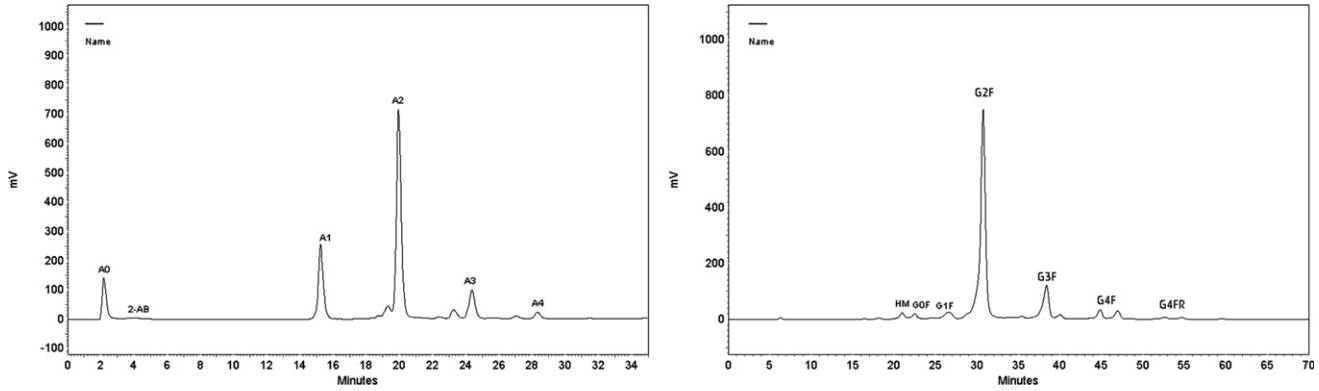
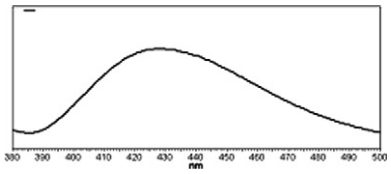
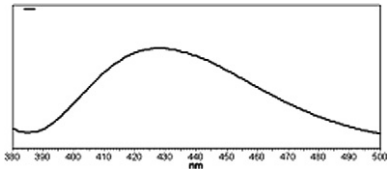
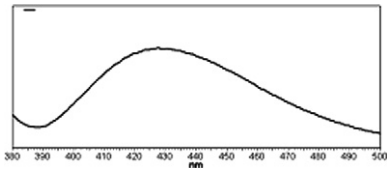
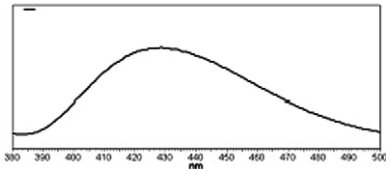
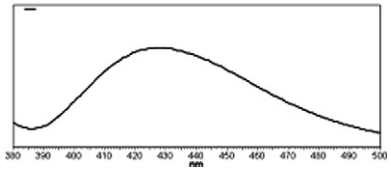


Fig. 3. HPLC-chromatograms: (left) GlycoSepC-chromatography (AEX) of sialylated, charged N-glycans; (right) Aminophase-chromatography (HILIC) of desialylated, uncharged N-glycans A0… 4 = number of negative charges in the anion fraction. HM = High mannose structure, G0… 4 = Number of endstanding galactose residues in the neutral N-glycan structure, F = Core fucose in the neutral N-glycan structure, R = Repeating structure component consisting of N-acetylglucosamine and galactose.

Table 6
Emission spectra of GlycoSepC-peaks (A0–A4)

	A0: $\lambda_{max} = 428$ nm
	A1: $\lambda_{max} = 428$ nm
	A2: $\lambda_{max} = 428$ nm
	A3: $\lambda_{max} = 428$ nm
	A4: $\lambda_{max} = 428$ nm

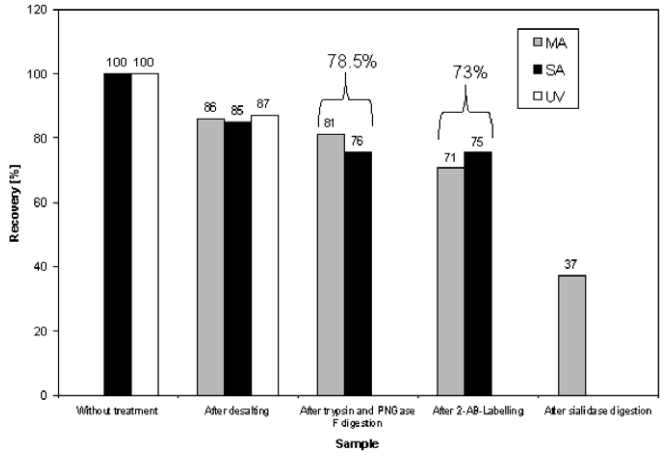


Fig. 4. Loss of oligosaccharides during sample preparation measured by monosaccharide analysis, sialic acid determination and UV.

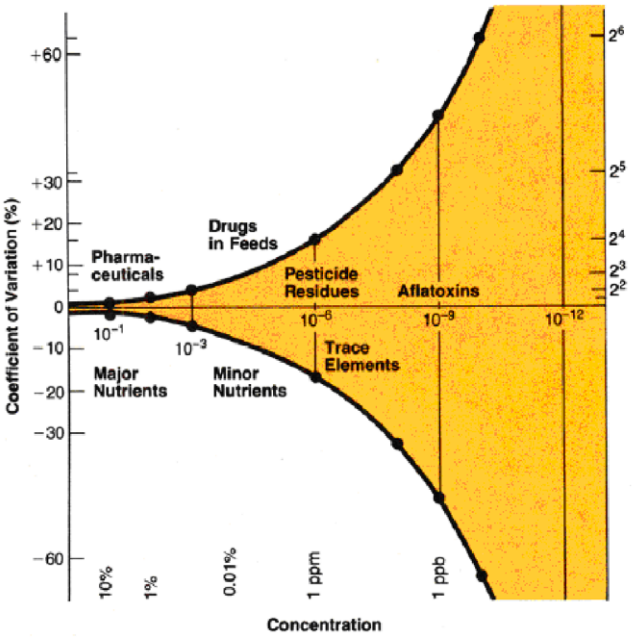


Fig. 5. The general curve for relating interlaboratory coefficients of variation with concentration along the horizontal center axis.

loss in glycan structures seemed to occur. As a consequence of this problem, the analytical range for Aminophase-analysis was shortened so that the first data point was outside this new linear range. Within the new range (14–45.5 nmol) accuracy could be inferred (Fig. 8b).

Before linear regression was performed, an F-test was used to examine if the standard deviations of the different concentration levels were comparable. Therefore the highest and the lowest standard deviations determined at all concentration levels were used to calculate the F-test statistic:

$$F = \left(\frac{\mathrm{sdv}_{\mathrm{high}}}{\mathrm{sdv}_{\mathrm{low}}}\right)^2$$

To test only representative data for both methods instead of the individual fractions (GlycoSepC) and the individual structures (Aminophase), only the average peak area linearity curves of all fractions and structures were used. GlycoSepC:

$$F = \left(\frac{\mathrm{sdv}_{\mathrm{high}}}{\mathrm{sdv}_{\mathrm{low}}}\right)^2 = \left(\frac{8334867}{869527}\right)^2 = \frac{7E13}{7.6E11} = 92$$

Aminophase:

$$F = \left(\frac{\mathrm{sdv}_{\mathrm{high}}}{\mathrm{sdv}_{\mathrm{low}}}\right)^2 = \left(\frac{18460729}{742508}\right)^2 = \frac{3.4E14}{5.5E11} = 618$$

$F^*(f1 = n - 1 = 3; f2 = n - 1 = 3; p = 0.05) = 9.277$

$F > F^*$, standard deviations were not homogenous. As a consequence, normal linear regression could not be performed, but linear regression which considered the different standard deviations. That was by using weighted linear regression, where a weighing factor of $1/\mathrm{sdv}(y)$ was used.

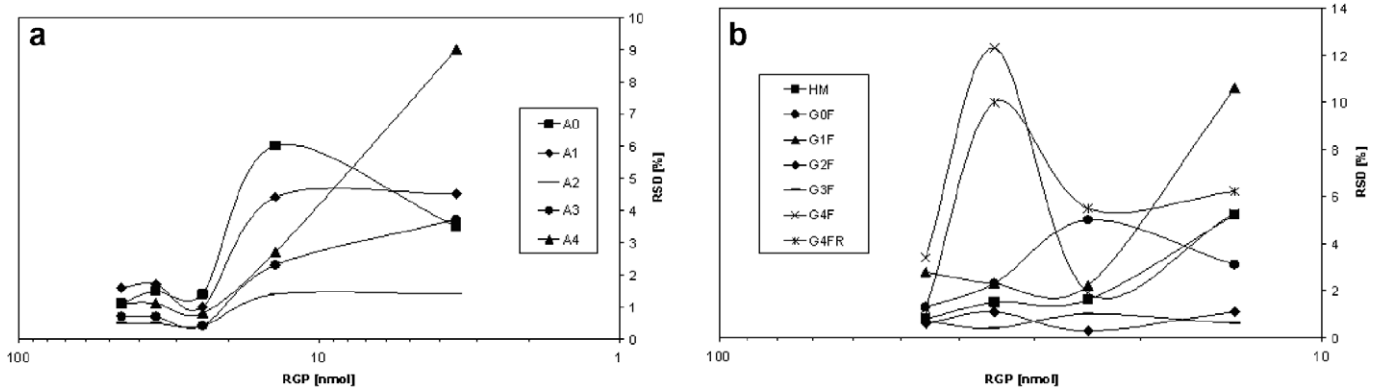


Fig. 6. Relative standard deviations for (a) GlycoSepC-analysis and (b) Aminophase-analysis in dependence on the reference glycoprotein (RGP) concentration.

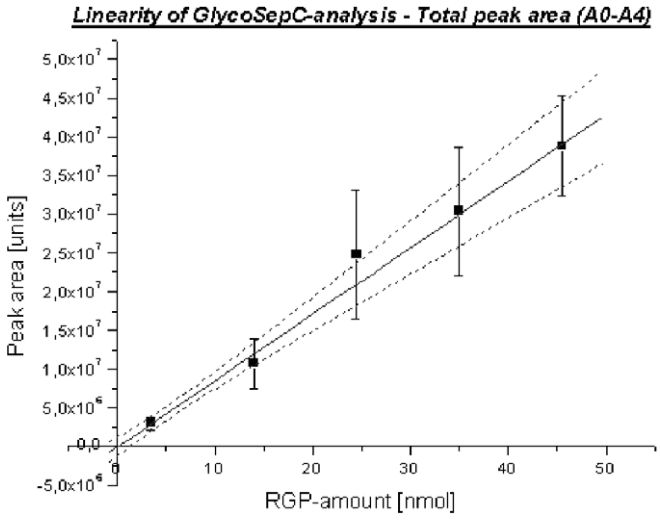


Fig. 7. Linearity determination of GlycoSepC-analysis (solid line = linear regression curve, dashed line = 95% confidence interval).

To test linearity, linear and quadratic regression curves were calculated and the residual standard deviations of both regressions were determined. A statistical F-test was performed to test for significant differences [8].

For Aminophase-analysis, data without $x = 3.5$ nmol were chosen to calculate whether the accurate Aminophase-analysis was also linear. Linear residual standard deviations

$$S_{y(L)} = \sqrt{\frac{\sum (r_\mathrm{i} - r_\mathrm{o})^2}{N-2}}$$

Quadratic residual standard deviations:

$$s_{y(Q)} = \sqrt{\frac{\sum (r_\mathrm{i} - r_\mathrm{o})^2}{N-3}}$$

N = number of concentration levels
r_i = individual residuals
r_o = optimal regression value

Test statistic:

$$\text{test statistic} = \left(\frac{S_{y(L)}}{S_{y(Q)}}\right)^2$$

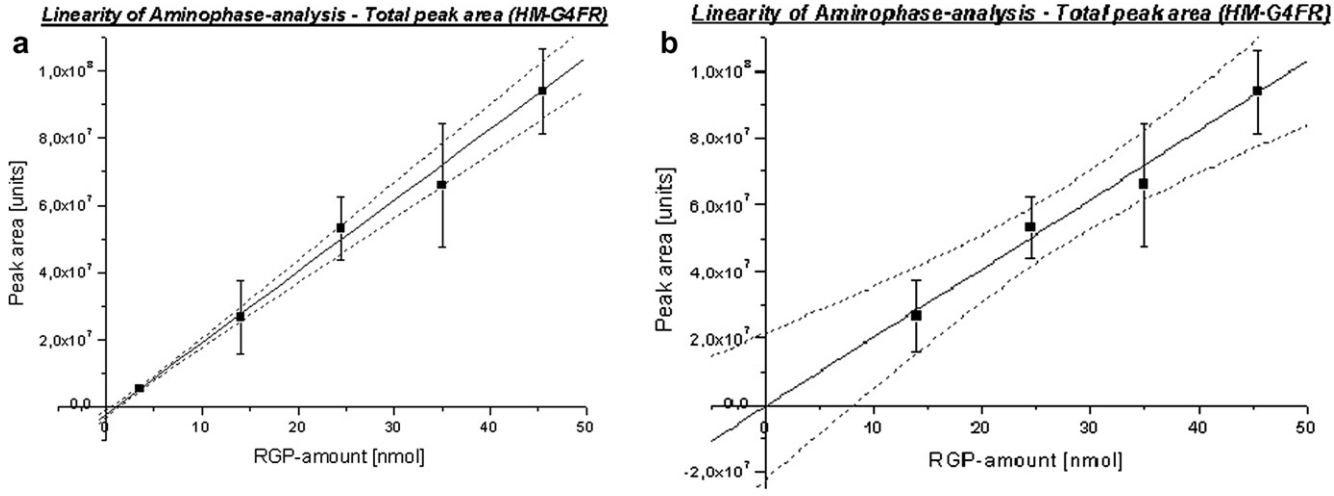


Fig. 8. Solid line = linear regression curve, dashed line = 95% confidence interval: (a) linearity determination for Aminophase-analysis over the whole working range. (b) Linearity determination for Aminophase-analysis without RGP = 3.5 nmol.

This test statistic was compared with the *F*-table ($p = 0.05$, $f1 = N - 2$, $f2 = N - 3$). If the test statistic was higher than the critical value in the *F*-table, a significant difference between both residual standard deviations would exist and the quadratic regression would be preferred. If the test statistic was lower than the critical value in the *F*-table, linear regression would be preferred (Table 10 and Table 11).

As it became obvious from Tables 7 and 8, linearity of both methods could be demonstrated for all structures.

4.2.3. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. The best way to examine the specificity of a chromatographic method is to combine it with another method which is able to identify the analytes, for example mass spectrometry. Peaks were fractionated and analyzed by MALDI-TOF-MS. Due to the fact that the established MALDI-TOF-MS method only worked in the positive ion mode, it was only useful for Aminophase-analysis (Fig. 9 and Table 9). GlycoSepC-chromatograms were examined regarding peak resolution which was calculated to be higher than 1.4 for each charge fraction (not shown).

As it became obvious from the MS-spectra in Table 9, each peak fraction showed only the expected structure, representing high specificity for the Aminophase-HPLC.

4.2.4. LOD and LOQ

The determination of the detection and quantification limit was performed by examining the linearity data of the GlycoSepC- and the Aminophase-analyses. Because

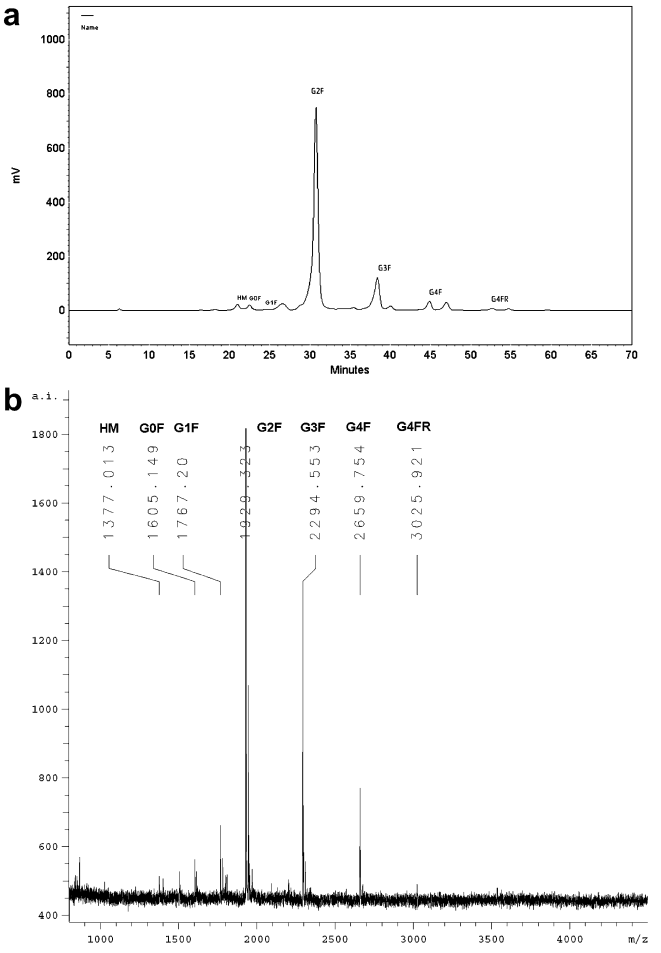


Fig. 9. (a) Aminophase chromatogram of RGP-*N*-glycans which were fractionated for MALDI-TOF-MS analysis. (b) MALDI-TOF-MS spectrum of RGP-*N*-glycan mixture.

Table 7
GlycoSepC-analysis – test of linearity

Fraction	Test statistic	Critical test statistic	Result
All (A0–A4)	0.662	19.16	Linear
A0	0.665	19.16	Linear
A1	0.667	19.16	Linear
A2	0.660	19.16	Linear
A3	0.661	19.16	Linear
A4	0.670	19.16	Linear

Table 8
Arninophase-analysis – test of linearity

Structure	Test statistic	Critical test statistic	Result
AII(HM-G4FR)	0.499	199.5	Linear
HM	0.500	199.5	Linear
G0F	0.493	199.5	Linear
G1F	0.503	199.5	Linear
G2F	0.497	199.5	Linear
G3F	0.499	199.5	Linear
G4F	0.500	199.5	Linear
G4FR	0.497	199.5	Linear

of the weighted linear regression approach, specialized mathematical calculations for LOD and LOQ had to be used [9,10].

In this case, the approach of Zorn and Oppenheimer was chosen [10,11].

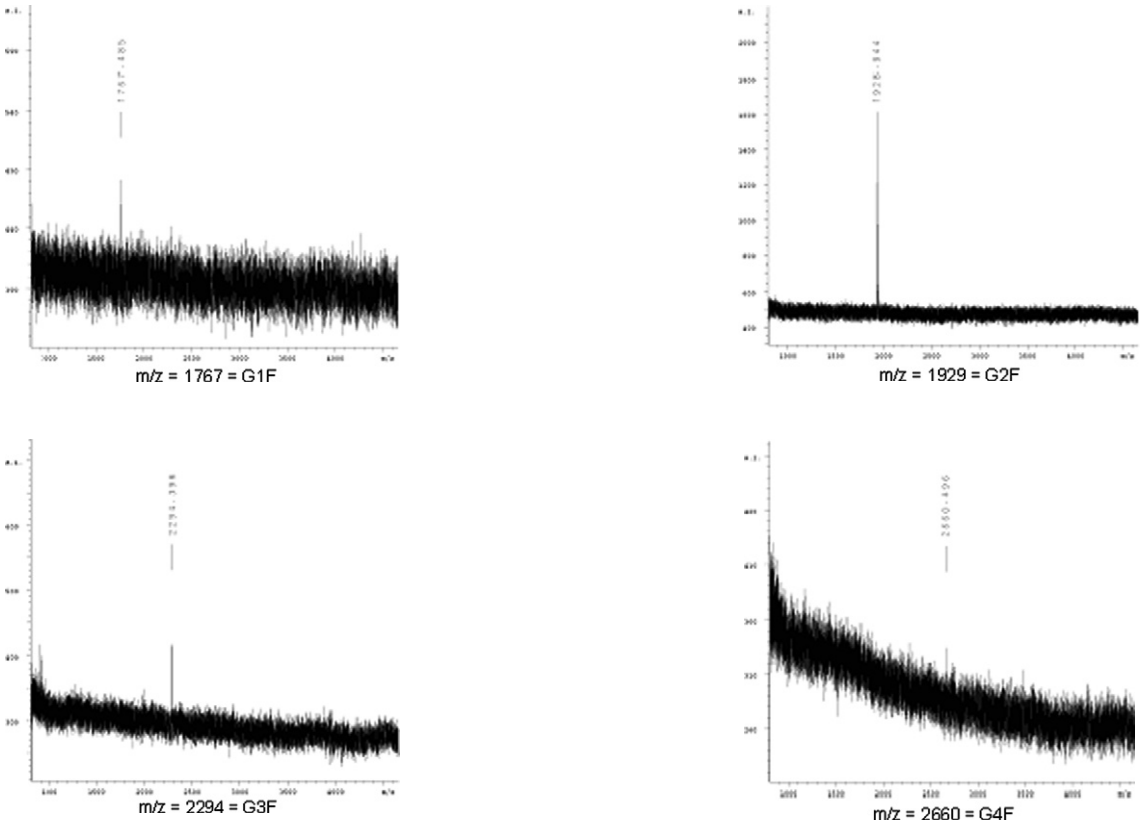
$$\mathrm{LOD} = \mathrm{LC} + \frac{t_{(1-\beta,n-p-2)} s_{\mathrm{w}}}{b_{1\mathrm{w}}} \left[\frac{1}{w_{\mathrm{LOD}}} + \frac{1}{\sum w_{\mathrm{i}}} + \frac{(\mathrm{LOD} - \overline{X}_{\mathrm{w}})^2}{Sxx_{\mathrm{w}}} \right]^{\frac{1}{2}}$$

LC = Critical Level in concentration units
$b_{1\mathrm{w}}$ = weighted slope
s_{w} = weighted residual standard deviation
w_{LOD} = weight at the detection limit
w_i = weight at concentration i
$\overline{X}_{\mathrm{w}}$ = weighted arithmetic average of x
Sxx_{w} = weighted sum of x-deviation-squares
$t_{(1-\beta,n-p-2)} = t$ – factor in dependence of β – failure and p

with

$$\mathrm{LC} = \frac{Y_{\mathrm{C}} - b_{0\mathrm{w}}}{b_{1\mathrm{w}}}$$

Table 9
Exemplified MALDI-TOF-MS spectra of fractionated anninophase peaks from Fig. 9



Y_C = Critical level in response units
b_{0w} = weighted intercept and

$$Y_C = b_{0w} + t_{(1-\alpha,n-p-2)} s_w \left[\frac{1}{w_0} + \frac{1}{\sum w_i} + \frac{\overline{X}_w^2}{Sxx_w}\right]^{\frac{1}{2}}$$

Based on the weighted least-squares model

$$Y = b_{0w} + b_{1w}X + \varepsilon$$

the variables above were defined as follows:

$$b_{1w} = \frac{Sxy_w}{Sxx_w} \qquad Sxy_w = \sum w_i (X_i - \overline{X}_w) Y_i$$

$$Sxx_w = \sum w_i (X_i - \overline{X}_w)^2 \quad \overline{X}_w = \frac{\sum w_i X_i}{\sum w_i} \quad \overline{Y}_w = \frac{\sum w_i Y_i}{\sum w_i}$$

p = number of parameters used to model the weights, in this case $p = 3$, due to a quadratic model to calculate the standard deviation at an unknown concentration x:

$$s_x = a_0 + a_1 X + a_2 X^2$$

To avoid an iterative solution for LOD, Oppenheimer's conservative assumptions were used, resulting in larger estimates of LOD:

$$w_0 \approx 1$$

$$w_{LOD} \approx 1$$

$$(LOD - \overline{X}_w)^2 \approx \overline{X}_w^2$$

LOQ was calculated by the approach of Gibbons et al. [12].

$$LOQ = \frac{Y_Q - b_{0w}}{b_{1w}} \quad \text{with} \quad Y_Q = 10 s_{LOC} + b_{0w}$$

Alpha- and beta-failures were set to 5% [11].

As it was not surprising, LC, LOD and LOQ laid higher for Aminophase-analysis than for GlycoSepC-analysis (Tables 10 and 11).

Table 10
LC, LOD and LOQ for GlycoSepC-analysis in nmol RGP (calculated from regression line in Fig. 7)

LC	LOD	LOQ
0.88	1.75	3.72

Table 11
LC, LOD and LOQ for Aminophase-analysis in nmol RGP (calculated from regression line in Fig. 8(b))

LC	LOD	LOQ
2,02	4,03	11,76

5. Conclusions

Highly sensitive methods for batch-consistency glycosylation analysis were established and validated at ProBioGen. Although the established analytical scheme consisted of multiple steps that were difficult to validate individually, it was possible to evaluate the overall procedure to obtain statistical values that could be used as representative validation parameters. This top-down approach was efficient and characterized the analytical methods quite well for early phase product requirements. Linearity could be confirmed for the examined analytical range of the methods. By examining precision and specificity, accuracy of the methods could be statistically inferred. In later development stages, more detailed characterization of individual working steps could follow (e.g. examination of 2-AB-labelling efficiency by ESI-MS) as well as a determination of an uncertainty budget for each individual sample preparation step. By knowing the most critical preparation steps with the highest uncertainties, one goal would be a simplification of working procedures to shorten analysis time. After validation, the high precise HPLC-methods could be used to even detect minor differences in apparently consistent biotechnological production processes and to distinct observed variations in variabilities that resulted from the analytical method themselves and in variabilities that resulted from product inconsistencies [13].

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