



University of Pannonia
Chemical Engineering and Material Sciences Doctoral School

**Analytical Method Development for the
Separation of N-Glycans by Capillary
Electrophoresis**

Ph.D. THESIS

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2025



Introduction

Glycosylation is one of the most influential post-translational modifications, playing a pivotal role in determining the structure, stability, and biological activity of glycoproteins. The structural and quantitative analysis of N-glycans is of strategic importance not only for the biotechnology industry but also for biomarker research and the characterization of therapeutic proteins. The aim of this doctoral dissertation was to develop novel and reliable analytical methods enabling the rapid and efficient examination of N-glycans using capillary electrophoresis (CE), with special emphasis on glycoproteins that are sterically hindered or artificially modified. During the research, four major developmental directions were defined, which, by building upon each other, contributed to the establishment of a versatile and widely applicable analytical method portfolio. First, a tryptic pre-digestion-based protocol was developed, capable of mitigating the steric hindrance effects frequently encountered in artificially glycoengineered proteins. Through this trypsin pretreatment, glycosylation sites became accessible to the enzyme without causing qualitative or quantitative alterations in the glycan profile. The second phase of development involved the elaboration of a temperature gradient-based denaturation protocol, which effectively reduced precipitation effects associated with increasing protein concentration. Given the typically low ionization efficiency of CE, especially in CE-MS coupled systems, highly concentrated yet fully soluble protein samples are essential. By optimizing detergent concentrations and applying gradual temperature elevation, the efficiency of the denaturation process was improved without triggering solubility-related issues. Complementing the temperature gradient denaturation step, a magnetic microparticle-based sample preparation method was developed. This technique selectively captured glycoproteins via specific amino-functional groups. Such targeted immobilization not only further enhanced denaturation efficiency without inducing precipitation but also enabled the selective removal of analytically interfering matrix components – such as glucose – from the sample. This proved especially beneficial for improving compatibility with mass spectrometric detection. To achieve precise qualitative characterization of the digested and fluorescently labelled N-glycan structures, the sequencing process using exoglycosidase enzymes was automated. Through the optimization of individual steps – including the combined application of specific enzymes and the fine-tuning of digestion parameters – the total analysis time, including sample preparation and measurement, was reduced to less than one hour. The



methodological developments presented in this dissertation make a significant contribution to the advancement of glycan-based analytics, particularly in terms of sample handling automation, integration with high-throughput systems, and the analysis of low-volume samples. The developed techniques may also be potentially integrated into pharmaceutical manufacturing environments, supporting in-process control and glycosylation monitoring at various stages of therapeutic protein production.

Experimental

During the doctoral research, various recombinant and therapeutic proteins (e.g., Humira, Enbrel), biological samples (human serum), and reference standards (such as hIgG1) were used for the experiments. These samples exhibit glycosylation patterns that are representative of glycoproteins commonly utilized in the pharmaceutical industry. The sample preparation process consisted of four main steps: denaturation, enzymatic release of N-glycans, fluorescent labeling, and removal of excess labeling reagent. The aim of the denaturation step was to unfold the native protein structure to render the asparagine-linked glycan moieties accessible to the PNGase F enzyme. For this purpose, various detergent combinations (e.g., SDS, NP-40) were used in conjunction with a temperature gradient-based denaturation protocol (30°C → 80°C), which allowed for the efficient yet gentle unfolding of protein structures. This approach minimized precipitation effects, which were particularly prominent at high protein concentrations. To selectively cleave N-glycans, Peptide-N-glycosidase F (PNGase F) was employed. In order to reduce steric hindrance and enhance enzymatic accessibility, proteins were subjected to a tryptic pre-digestion step. This method significantly improved the efficiency of PNGase F, especially for fusion or glycoengineered proteins. Following release, the liberated glycans were labeled using 8-aminopyrene-1,3,6-trisulfonic acid (APTS), enabling high-sensitivity laser-induced fluorescence (LIF) detection during capillary electrophoresis (CE) analysis. After labeling, magnetic microparticles were applied to selectively remove the excess fluorescent dye, resulting in cleaner baseline signals and enhanced analytical reliability. The qualitative determination of glycan structures – i.e., their sequencing – was carried out using exoglycosidase enzymes, which selectively cleave monosaccharide residues from glycan side chains. By optimizing the enzymatic reaction parameters and employing novel configurations of the CE instrumentation, the sequencing process was rendered automatable. Consequently,



the entire analytical workflow, including sample preparation and measurement, could be completed within one hour.

New scientific results: Thesis

1) To enable the N-glycan analysis of fusion and glycoengineered glycoproteins developed during the production of biopharmaceuticals, I developed a sample preparation method based on tryptic pre-digestion. This approach eliminates the steric hindrance that typically arises during deglycosylation with PNGase F, thereby allowing for high-efficiency and representative N-glycan analysis. Despite effective denaturation of glycoproteins, the accessibility of N-glycan structures to PNGase F can be significantly restricted in artificially engineered proteins, which may prolong enzymatic reaction times or render the enzyme entirely ineffective. By applying trypsin digestion, this steric hindrance can be resolved without altering the glycan profile, thereby enabling accurate qualitative and quantitative analyses.

2) To reduce precipitation effects associated with protein denaturation – particularly at higher concentrations depending on the protein's properties – I developed a temperature gradient-based denaturation method. This allowed for significant increases in protein concentration within the sample, enabling the detection of low-abundance proteins and facilitating their analysis using advanced analytical techniques such as mass spectrometry. While increasing protein concentration is typically limited due to precipitation, this limitation can be overcome by optimizing both the type and amount of detergents used and by gradually increasing the temperature to enhance denaturation efficiency. This enables greater protein content in the sample without compromising solubility, thus supporting more targeted analytical applications.

3) To further increase the protein concentration required for targeted analytical measurements, I developed a magnetic microparticle-based sample preparation method that supports the denaturation step. This method selectively binds glycoproteins through amino-functional groups, facilitates downstream enzymatic reactions (e.g., PNGase F digestion), and removes interfering matrix components such as glucose (which can inhibit enzymatic reactions). The application of functionalized magnetic microparticles significantly increased the amount of denaturable protein without inducing precipitation. Moreover, through targeted immobilization, unwanted matrix components could be efficiently removed from the system.



Filep, Csenge; **Szigeti, Marton**; Farsang, Robert; Habberger, Markus; Reusch, Dietmar; Guttman, Andras; Multilevel capillary gel electrophoresis characterization of new antibody modalities, ANALYTICA CHIMICA ACTA 1166 Paper: 338492 (2021)
Közlemény:32018177 Folyóiratcikk (Szakcikk) Tudományos Nyilvános idéző összesen: 17 | Független: 12 | Független: 5 | Nem jelölt: 0 | WoS jelölt: 16 | Scopus jelölt: 17 | WoS/Scopus jelölt: 17 | DOI jelölt: 17 , Folyóirat szakterülete: Scopus - Analytical Chemistry SJR indikátor: Q1, IF: 6.911

Scientometrics

Number of publications in international journals:	41
Number of publications in national journals:	3
Cumulative impact factor (expected):	176,0 (15,2)
Total impact factor:	191,2
First-author impact factor:	27,7
Number of publications in D1 journals:	12
Number of publications in Q1 journals:	15
Number of citations – independent / total:	597 / 860
Total number of citations:	860
Hirsch index; independent (total):	15 (18)