# Updates of the current strategies of labeling for N-glycan analysis

---Manuscript Draft---

### Abstract:
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Brussels, 20 February 2024

Dear Professor Wilson,

We thank you for considering our review on released N-glycan analysis for publication in J. Chrom B. We have carefully reviewed the recommendations of the reviewers. We are so pleased to send a revised manuscript. We attached a file with a point to point answer to the comments.

We hope that we have properly fulfilled the requirements.

Sincerely Yours,

Cédric Delporte
Professor
Dear reviewers,

Thank you for your valuable comments and the time you dedicated to our work. We have carefully revised the manuscript and have incorporated changes to include your suggestions. We have highlighted the changes in the manuscript. Please find below a point-by-point response to your comments and concerns.

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- Labeling strategies are discussed based on both liquid chromatography and capillary electrophoresis separation methods.
- The choice of labelings is based on both fluorescence and mass spectrometry detection methods.
- Advantages and disadvantages of labeling agents are discussed.
- Advanced labelings based on multiplex agents for a quantitative approach are discussed.
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Figure 1

A

Mannose (Man)  
N-acetylglucosamine (GlcNAc)  
Galactose (Gal)  
Fucose (Fuc)  
N-acetylneuraminic acid (Neu5A)

B

High mannose

Complex

M6

FA2G2S2

M5A1G1

FA2BG2
Figure 2

(A) Schematic representation of the glycosylation process with key sites indicated for Endo H, PNGase F, and PNGase A.

(B) Chemical reaction showing the cleavage of N-Acetyl glucosamine (GlcNAc) linked to Asn by PNase F and the subsequent hydrolysis with H2O.
Relative Intensities

MS

FLD

Figure 3 new

Click here to access/download; Figure; Fig_3_corrected.pptx
Figure 5

Reductant agent

Sodium cyanoborohydride

2-picolineborane

N-term labeling agent
2-aminobenzamide (2-AB) 2-aminobenzoic acid (2-AA) 2-aminopyridine (PA)

Procaine (Proc)  Procainamide (ProcA)

8-aminopyrene-1,3,6-trisulfonic acid
Conflict of interest:

The authors have no conflict of interest to declare.
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Yosra Helali & Cédric Delporte

RD3-Pharmacognosis, Bioanalysis and Drug Discovery unit & Analytical Platform of the Faculty of Pharmacy (APFP), Faculty of Pharmacy, Université libre de Bruxelles (ULB), Brussels, Belgium

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Contents

1. Introduction ........................................................................................................................................... 4

2. N-glycan release: a comparison between classical and rapid methods ............................................ 6

3. Analytical separation methods for N-glycans ..................................................................................... 8
   3.1. LC separation: HILIC coupled to FLD and MS detection ......................................................... 9
   3.2. CE-FLD analysis ............................................................................................................................ 11

4. Labeling agents and chemistry .......................................................................................................... 11
   4.1. Reductive amination for FLD and/or MS detection ..................................................................... 12
   4.2. Agents for rapid labeling ............................................................................................................. 14
   4.3. Comparison of labeling strategy for sensitivity .......................................................................... 15
   4.4. MS fragmentation and labeling agents ....................................................................................... 17
   4.5. Multiplex labeling agents ........................................................................................................... 18

5. Perspectives ........................................................................................................................................... 19
1. Introduction

Glycosylation of proteins has emerged as a topic of great significance in recent decades. Glycosylation is widely recognized as a crucial post-translational modification of proteins. It is widely acknowledged that glycosylation plays a pivotal role in the functionality of glycoproteins. Glycan variations have been implicated in the pathogenesis of numerous diseases and are being investigated as potential biomarkers [1]. Glycans are oligomers composed of monosaccharides linked together through glycosidic bonds. Among the various types of glycosylation processes, two prominent forms are N-glycosylation and O-glycosylation. N-glycosylation is a widely studied phenomenon, particularly in the field of biopharmaceuticals, where it is recognized as the predominant form of glycosylation [1]. N-glycosylation is an enzymatic process of attaching glycans to asparagine residues located within an Asp-X-Ser/Thr consensus sequence. In the given sequence, X represents any amino acid residue with the exception of proline. This process initiates in the endoplasmic reticulum and is completed in the Golgi apparatus. In mammalian cells, the major monosaccharides that make up N-glycans include mannose (Man), N-acetylglucosamine (GlcNac), galactose (Gal), N-acetyleneuraminic acid (Neu5A), which is a sialic acid, and fucose (Fuc) (Figure 1 A). In the present context, the glycosylation pattern may exhibit variations based on numerous parameters encountered throughout the protein synthesis process. Among the countless potential N-glycan compositions, only a limited number are predominantly found in proteins, particularly in therapeutic glycoproteins such as monoclonal antibodies (mAbs) [2]. N-glycans can be classified into three categories: high-mannose glycans, complex glycans, and hybrid glycans (Figure 1 B). The polysaccharide found in all of them consists of five monosaccharides, the core [3, 4]. Few N-glycans are depicted in Figure 1 B. There exist various symbol nomenclatures employed for the representation of glycans. The Symbol Nomenclature for Glycans (SNFG) (https://www.ncbi.nlm.nih.gov/glycans/snfg.html) is currently the most widely used symbol nomenclature [5]. In this current review, we utilize the Colour Oxford nomenclature as depicted in Figure 1 based on the SNFG. Additionally, various nomenclatures are employed to designate the glycans [5, 6].
In the biopharmaceutical industry, glycosylation has garnered significant attention. Therapeutic proteins can undergo glycosylation, with approximately 60% of the currently approved proteins exhibiting this post-translational modification (PTM). The most important group of glycosylated therapeutic proteins are mAbs. In 2022, a report indicated that within previous four-year period mAbs continued to account for over 53% of all approvals over the past four years [7]. MAbs possess all a conserved N-glycosylated site on the Fc region, which enables the modification of their functional activity, stability, half-life, or toxicity. In this context, the influence of glycosylation on therapeutic proteins is regarded as a crucial quality attribute that necessitates comprehensive characterization and stringent control measures. It is so a regulatory requirement to analyze glycosylation and provide a detailed analyses either during development phase of a new glycosylated biotherapeutics or batch release when authorized on the market [8]. For example, sialylation modification might change half-life of sialylated therapeutic glycoproteins or fucose variation is known as modifying antibody-dependent cell cytotoxicity effect [9].

Analysis of N-glycans has benefited from significant technological advancements in the past few decades, particularly in the fields of liquid chromatography (LC) and mass spectrometry (MS). These advancements include the development of hydrophilic interaction liquid chromatography (HILIC) chemistry and the improvement of MS [10, 11]. N-glycosylation can be analyzed at various levels, including the entire protein-, glycopeptide- or released N-glycan levels. More recently, multi-attribute method (MAM) workflows have also shown their ability to analyze glycosylation among other critical attributes in biotherapeutics [12, 13]. The latter approach Released N-glycan analysis remains the most employed, particularly in the biopharmaceutical field where it is still considered as a gold standard and for biomarker discovery. Usually, N-glycans are enzymatically released from the glycoprotein prior to its purification and subsequent analysis. At first, the analysis of released N-glycans has been conducted using MALDI-TOF MS with or without prior permethylation derivatization [14]. Nowadays LC coupled with fluorescence (FLD) and/or MS detection is the most used approach [1, 15]. Recently, there has been growing interest in the use of unconventional methods, such as infrared spectroscopy, for N-glycosylation analysis. However, these methods are not commonly employed in routine practice.
detailed N-glycan analysis as the sensitivity to light glycosylation changes is lower than the MS-based methods [16, 17].

The major enzyme employed for N-glycan release is peptide-N-Glycanase F (PNGase F) from Flavobacterium meningosepticum [18]. This particular enzyme is capable of hydrolyzing nearly all N-glycans attached to the protein backbone, with the exception of those that contain specific fucosylated N-glycans, which are commonly found in plants. It is more specifically an amidase that necessitates the presence of an Asn-oligosaccharide within a peptide [19]. As N-glycans lack chromophores or fluorophores, they present challenges in their analysis using conventional LC techniques coupled to spectrophotometer. Free glycans are challenging to detect using LC-MS methods due to their high hydrophilicity and the multiple possibilities of ionization in the ion source leading to multi ions and consequently low signals intensities [20]. In this context, released N-glycans are typically subjected to labeling techniques in order to enhance their detectability, either through spectrophotometry and/or MS detectors.

The present mini-review provides an overview of the current strategies commonly employed for the release and labeling of N-glycans, taking into consideration the analytical methods used, along with their respective advantages and disadvantages. Additionally, multiplex analysis using MS is also approached for which specific labeling agents are utilized.

2. N-glycan release: a comparison between classical and rapid methods

As mentioned earlier, one commonly employed method for investigating N-glycosylation involves releasing N-glycans from the protein backbone. There are two primary methods for N-glycan release: enzymatic and chemical ones. The technique employed for release aims to achieve the complete release of intact N-glycans. Among the various methods for chemical releases, β-elimination under alkaline conditions is the most commonly employed technique [14]. Hydrazinolysis, which utilizes anhydrous hydrazine, is another chemical method that is commonly employed [21]. The limitation associated with both chemical methods is the increased potential for undesired alterations of released N-glycans. More
recently, a study has been conducted on the use of sodium hypochlorite. If this method allows for the preservation of N-glycans in a free reducing terminus form, the yield of N-glycans is significantly lower compared to the widely employed enzymatic release method using PNGase F (approximately 20 times lower) [22]. The enzymatic release method continues to be the most widely used and advantageous approach for the complete and intact release of N-glycans.

There are multiple enzymes that are employed for N-glycan release. The most commonly used enzymes for glycan analysis are endoglycosidase H (Endo H), PNGase F, and PNGase A. Endo H specifically cleaves the bond between the GlcNAc attached to the Asn residue and the second GlcNAc in high-mannose and some hybrid N-glycans, but it does not act on complex N-glycans (Figure 2) [19]. This constraint limits its applicability. PNGase F is capable of liberating almost all N-glycans from the peptide backbone, including those with an α1,6 core fucose. However, α1,3 core fucosylated N-glycans found in plants and invertebrate glycoproteins are not cleaved by PNGase F. Conversely, PNGase A is capable of cleaving N-glycans that contain α1,3 core fucose (Figure 2). Due to the higher expense associated with PNGase A and the fact that the majority of studied N-glycans do not possess an α1,3 core fucose, PNGase F is the more commonly used enzyme in research.

The glycans released by PNGase F are in the amine terminal form. However, this form is not highly stable and undergoes rapid deamination (Figure 2). Depending on the nature of the labeling reaction, this may be mandatory or optional (refer to reductive amination labeling below).

If PNGase F is able to cleave a large number of N-glycans, the kinetics may vary depending on the complexity of the glycan and the folding of the protein. It is generally recommended to denature the protein before PNGase F digestion by using reducing and alkylating agents in the presence of a surfactant such as sodium dodecyl sulfate SDS or proteomics specific ones [23]. After that, N-glycan release can be addressed if the denaturation conditions are not excessively harsh. For instance, a high concentration of SDS might be a source of issues, as well as a high concentration of reducing agent. PNGase F has an optimal pH range of around 8, with an acceptable range from 6 to 10 [24]. The digestion process can be lengthy under "classical" conditions, typically taking a minimum of 30 minutes. However, most protocols involve several hours of digestion, ranging from 4 hours up to overnight (15h).
digestion [25, 26]. More recently, a method for rapid digestion using PNGase F has been developed [26]. This rapid digestion, combined with carbamate chemistry labeling, enables quick sample preparation for deglycosylation, labeling, and solid phase extraction (SPE) purification in less than 30 min. The carbamate chemistry requires that the glycosylamine form of the N-glycans (see Figure 2) remains stable until the labeling step. Under the developed conditions using rapid PNGase F, it was demonstrated that the glycosylamine form remains stable for at least 2h [26].

If PNGase F release should be consistent regardless of the enzyme source, it has been demonstrated that this assumption is not entirely true. Several recombinant PNGase F and Rapid PNGase F from different manufacturers were tested, revealing that the profiles of N-glycosylation were slightly different depending on the source of PNGase F. This emphasizes the importance of carefully selecting PNGase F, especially when using protocols for rapid deglycosylation [27, 28].

Altogether, it is now possible to prepare released N-glycan samples within 30 minutes, which includes the release, labeling, and purification steps. If the procedure is coupled with a multi-well system or even a robotic system, this can lead to the preparation of several dozen samples, i.e. 96 samples with a 96-well plate [26]. One limitation of rapid preparation protocols is that they typically require larger quantities of glycoproteins. The sensitivity of these methods is relatively low, making them more suitable for quality control of therapeutic proteins when a large quantity is usually present [29, 30]. However, they may not be as effective for biomarker discovery, where sensitivity is more important.

3. Analytical Separation and detection methods for N-glycans

When analyzing released N-glycans, the choice of the analytical separation method determines the choice of the labeling agent. Currently, HILIC coupled to fluorescence (FLD) or mass spectrometry detectors remains the method of choice and is widely used. They are also coupled in series when possible, with both techniques offering their own advantages. FLD intensities of the N-glycans is less subject to variations of signal with either the LC mobile phase composition or the glycan composition, unlike MS, where ionization may vary with these two parameters. Furthermore, FLD instruments are easier to use and more cost-effective compared to MS instruments. However, a limitation of FLD is that
it does not enable the selective quantification of co-eluting peaks as MS. The latter indeed brings specificity thanks to the m/z values, which is a major advantage in the case of co-elution of N-glycans. Apart from LC separation of N-glycans, capillary electrophoresis (CE) is also a commonly used technique [31-33]. The most important advantage of CE compared to HILIC is its higher resolution compared to LC (HILIC). If CE can be coupled both to fluorescence detectors, such as laser-induced fluorescence (LIF), and to MS detectors, even in series [34], LIF detection is more commonly used due to the complexity of CE-MS instruments. Even though other high-throughput methods have been described for monitoring N-glycans, the CE-based method remains the most commonly used high-throughput one among them [35]. If combined with a robotic platform for sample preparation, the results using CE-based N-glycan analysis can be obtained within a few hours for dozens of samples [15, 36, 37].

In this chapter, we provide a description of HILIC and CE separations for N-glycans. In the following chapter, we will discuss the use of labeling agents that are compatible with these techniques.

### 3.1 LC separation: HILIC coupled to FLD and MS detection

The separation of N-glycans, even if labeled, is poor in reverse-phase (RP) chromatography. This is due to the polar characteristics of N-glycans, which make RP-LC not the best option, even though a few studies have used it [38]. Nano-RPLC of N-glycans has shown improvement and potential for separating isomers [39, 40], but it still has limitations. Using similar mobile phases as those used for RP-LC, porous graphitized carbon (PGC) LC methods coupled to MS are employed for the separation of reduced N-glycans without the need for a labeling step. PGC separation has shown particular interest in distinguishing between α2-3 and α2-6 sialic acids or β1-3 and β1-4 galactose [41]. However, the sensitivity of these methods is lower than when labeling of the N-glycans is applied. In this context, hydrophilic interaction liquid chromatography (HILIC) separation has generated significant interest [1]. The polar characteristics of N-glycans make HILIC columns ideal and enable, with ultra-high performance liquid chromatography (UHPLC) system, to achieve even separation of isomers of some N-glycans [42, 43].
There have been many chemistries developed for HILIC [44, 45]. The amide-based chemistry has shown the best separation of N-glycans. Recently, advancements in HILIC materials for glycans and glycopeptides have been reviewed [46]. HILIC is considered as a variant of normal phase LC, but it is slightly different. In HILIC, the thin aqueous layer (1-2 nm) on the surface of the stationary phase plays a crucial role [47]. The mobile phase is always composed of water and an aprotic solvent that is miscible with water. Acetonitrile (ACN) is the most suitable solvent in this case. The percentage of water varies during the run, starting at a low percentage (5-35%) and increasing accordingly for analyte elution. The retention of analyte in HILIC is a mixture of hydrogen bonding, dipole interactions, and ion interactions [48, 49]. Furthermore, the polarity as well as the size of the analytes determine the elution order. Smaller and neutral N-glycans elute first (M5, A2, FA2), while bigger and charged ones elute later (such as FA2G4S4). Figure 3 illustrates a typical HILIC separation with FLD (A) and MS (B) detection of N-glycans released using PNGase F from immunoglobulins G where the N-glycans have been labeled with procainamide (ProcA) through reductive amination.

Mixed-mode anion-exchange/hydrophilic interaction chromatography (AEX/HILIC) has also been developed. This mixed mode columns have shown to provide better separation of highly charged, i.e. sialylated N-glycans where “classical” HILIC columns have not been able to achieve as efficiently. This has been exploited for the analysis of erythropoietin, which contains many charged variants of N-glycans [50, 51].

If released N-glycans can be directly injected and separated on HILIC columns without labeling, they are usually labeled. The labeling of N-glycans has an impact on their retention in HILIC. So, for example, 2-aminobenzamide (2-AB), procainamide (ProcA), and RapiFluor-MS (RFMS) derivatized N-glycans do not separate with exactly the same retention on an HILIC column under identical ACN/Water mobile phase conditions [29]. In this context, when a new labeling agent is used to modify the N-glycans, it is necessary to adjust the HILIC conditions, specifically the gradient of ACN/Water, in order to achieve the highest resolution of the glycans.
3.2, CE-FLD analysis

High-resolution CE is commonly used for the separation of carbohydrates. This technique has the advantage of being fast and efficient for the separation of N-glycans [52]. CE separates molecules based on their electrophoretic mobilities in a capillary, where a high-voltage electric field is applied. The electrophoretic migration of a molecule is dependent on its own charge in the buffered solution, its attraction or repulsion to the electrodes, as well as its friction forces in the flow generated by the high voltage. CE is usually coupled to LIF or MS detectors, as discussed above. It does not require a high concentration of samples, making it a sensitive method. Furthermore, another advantage of CE is that it enables high-throughput possibilities when using a multichannel system. Callewaert’s group et al has indeed developed a CE-LIF multichannel system based on a DNA sequencer, which can now be used routinely [35, 53, 54]. This technique enables the separation of N-glycan mixtures with high resolution in just a few minutes (less than 5 min) where even UHPLC-based HILIC separation will not resolve the glycans as well and will require longer runs, typically 15-45 minutes. When focusing on N-glycan separation, sialylated N-glycans are better separated with CE than with HILIC separation as CE separates molecules based on their charge.

However, N-glycans have to be labeled with a reactive labeling reagent that is compatible with CE-LIF. 8-amino-pyrene-1,3,6-trisulfonic acid (APTS) is commonly used as it contains three ionizable groups making possible to triple charged the molecules. The charges are due to the three sulfonic acid groups. APTS is also fluorescent thanks to the naphthalene [33]. This agent is described below.

4. Labeling agents and chemistry

If released N-glycans can be detected by MS without labeling, especially when coupled to PGC LC separation [55], the majority of methods still use labeling. The use of a labeling step is a common method to improve signal and sensitivity. The majority of N-glycan labeling reagents are fluorescent, making them detectable by FLD, and several of them also enhance the MS signal. This is because labeling N-glycans increases the signal and, consequently, enhances the sensitivity of the method [56]. The agent is commonly added to the reducing terminal part of N-glycans [57]. Figure 4 represents the four main
steps leading to labeled glycans. There are various strategies for \(N\)-glycan derivatization. The most commonly used labeling technique is reductive amination, where the dye is added to the free \(N\)-glycan through a Schiff base chemistry. Rudd and collaborators et al. have developed a significant method for analyzing \(N\)-glycans. They employ reductive amination using 2-aminobenzamide (2-AB) as a labeling reaction and combine it with HILIC-FLD \(N\)-glycan assignment based on retention. This approach is made possible by the use of a database and a glucose unit ladder [36, 58]. This technique was primarily used for FLD detection. As 2-AB has a low ionization capacity, this strategy has evolved to use labeling agents that are more compatible with mass spectrometry through reductive amination. This is further described below.

Other labeling chemistries, such as Michael addition or hydrazide labeling, also exist [59, 60]. More recently, rapid chemistry has emerged with labeling agents known as “instant” agents. The latter are based on the \(N\)-hydroxysuccinimide (NHS) carbamate chemistry, which involves a reaction on the glycosylamine released glycans.

### 4.1 Reductive amination for FLD and/or MS detection

The reductive amination, utilizing Schiff base chemistry, of released \(N\)-glycans is illustrated in Figure 5. The most commonly used agents for reductive amination are cyanoborohydride and 2-picoline borane (2-PB). The latter is considered to be less toxic than cyanoborohydride [61, 62] (Figure 5). D. Harvey has extensively described the major labeling agents used for reductive amination labeling [57]. Among the most commonly used labeling agents, 2-AB has been the most popular. Focusing on 2-AB, although it is relatively highly fluorescent, its ionization power is low, making it not the optimal choice for MS detection. Other agents such as 2-aminobenzoic acid (2-AA), 2-aminopyridine (PA), 8-aminopyrene-3,6,8-trisulfonic acid (APTS), 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) procaine (Proc), and procainamide (ProcA) (Figure 6) are also used and exhibit higher MS ionization power compared to 2-AB. Concerning the sensitivity they bring in FLD for \(N\)-glycan detection, they have quite similar intensities, even though Proc and ProcA seem to be more intense and thus enable better sensitivities than others when using FLD detection. Conversely, for MS detection, there are significant differences between them. Focusing on 2-AB, although it is relatively highly fluorescent, its ionization power is
low, making it not the optimal choice for MS detection. Table 1 provides the preferred MS mode, mass shift, and fluorescence wavelengths of these commonly used labeling agents. The tertiary amine in Proc and ProcA makes them better labeling agents for MS detection because it can be easily protonated, resulting in a positive charge. 2-AA has also been used for its ease of deprotonation, allowing for the generation of a negative charge. However, positive mode MS is typically preferred for sensitivity. When negative mode is required for labeled N-glycans, the preferred labeling agents are 2-AA and APTS. These agents can readily acquire a negative charge. Although other labeling agents such as ANTS could also be used [57], 2-AA has been used for its ease of deprotonation, allowing for the generation of a single negative charge. APTS with the three negative charges has been used less frequently, but recently it was shown that it could be of interest for N-glycan analysis using MS detection [20]. APTS has three negative charges due to the presence of three sulfonic acid groups. This makes APTS the preferred labeling agent for CE separation because in CE, the charge leads the separation. However, APTS is primarily used as a labeling reagent for the fluorescence detection of N-glycans (CE-fluorescence), with limited descriptions of its application in CE-MS analysis [63, 64].

Before the revolution of mass spectrometry, assigning N-glycans to each peak based on HILIC-FLD analysis was a laborious task. As a simple example, in Figure 3 highlighted in orange rectangle, we can observe that G1E-FA2G1 has two distinct isomers that are poorly separated and that co-elute with FA2BG1. One isomer of FA2G1 has the galactose attached to the 6-arm, while the other isomer has it attached to the 3-arm. Thorough characterization with exoglycosidase digestion [65, 66] and MS analyses have shown that the one with galactose on the 6-arm elutes first under specific HILIC conditions. This issue arises when employing FLD detection also for other co-eluting N-glycans, complicating the interpretation of FLD data. However, in this context of FLD detection, to perform this complete analysis for each peak (= each N-glycan), it requires a very long and complex data treatment using the exoglycosidase digestion [66]. Furthermore, the LC conditions and type of labeling agent are key parameters that can result in differences in retention or migration between different techniques, even when the conditions are relatively similar. To simplify the task of N-glycan annotation, Rudd and co-workers developed a glucose unit (GU) library called GlycoBase [67]. The latter was first constructed
based on the retention of peaks of a dextran ladder, which was itself labeled with 2-AB [58]. Each 2-AB labeled N-glycan was then assigned a GU value. With advancements and the development of various separation techniques and labeling reagents, GlycoBase has transitioned to the GlycoStore database. This extended version of the library includes HILIC separation with HPLC-FLD and UHPLC-FLD, RPLC-FLD, PGC MS, and CE-LIF retention data [68, 69]. This evolution to GlycoStore enabled analysts to address the limitations of GlycoBase. At its launch in 2017, the database contained more than 850 glycans with over 8500 retention/migration information.

Furthermore, to assist analysts in performing N-glycan profiling, several companies have developed kits, such as LudgerTag™ 2-AB, GlycoProfile™ 2-AB (Sigma Aldrich), Agilent 2-AB, and GlycoWorks 2-AB (Waters). These kits include various steps for sample preparation, such as unfolding, N-glycan release, labeling, SPE purification, and inclusion of ladder/standard samples. They are also linked to specific separation and detection conditions, such as UHPLC HILIC FLD/MS. If the kits are quite expensive, they usually include all the necessary reactants, materials, and complete step-by-step protocols that enable easy use. However, these kits are typically designed using standard glycoproteins such as IgG, mAb, fetuin, etc. They can also be modified to accommodate more complex proteins or samples as N-glycan from complete glycoproteins in blood, erythropoietin, immunoglobulins that target a specific antigen purified from blood, etc. If the kits mentioned above are for 2-AB labeling, nowadays there are also kits available for APTS, procainProc, procainamideProcA, and other labeling agents.

4.2. Agents for rapid labeling

More recently, rapid chemistry has been used for N-glycan labeling with reagents intended for either FLD or MS detections. Combining this with the rapid release (rapid PNGase F release) enables the preparation of several dozen samples in less than 30 min. Rapid labeling chemistry is based on N-hydroxysuccinimide (NHS) carbamate chemistry. This method has been used in the past to derivatize free amino acids. One advantage of this method is that when the NHS-carbamate group hydrolyzes, it forms amine byproducts which can also self-quench [70, 71]. For N-glycan labeling, the NHS carbamate labeling agent reacts with the glycosylamine moiety that is present on the N-glycans, which have been freshly released from the protein backbone. This reaction results in the formation of the labeled N-glycan.
[26, 72, 73] (Figure 7A). The reaction is considered complete in a few minutes, unlike reductive amination, where protocols recommend a labeling step of 1 to 2 hours to assure a complete labeling. In this context, a rapid 2-AB labeling reagent called InstantAB has been developed, followed by Instant procaine (InstantPC) and RapiFluo-MS (RFMS) labeling reagents (Figure 7B). All of them utilize fast chemistry involving NHS carbamate, but they vary in terms of effectiveness and sensitivity due to the labeling group. The next section will cover this topic.

4.3. Comparison of labeling strategy for sensitivity

Comparing the different labeling agents in terms of signal sensitivity for MS detection, it was shown that the signal with ProcA was 10-50 times higher than with 2-AB labeling. To a lesser extent, the FLD signals are also higher with ProcA than 2-AB. This higher sensitivity of ProcA than 2-AB enabled the detection of additional minor peaks [74].

The advantage of InstantPC and RFMS is that they contain a tertiary amine, which allows for higher ionization rates in positive mode compared to 2-AB. This leads to increased sensitivity for MS detection [74]. The acidic conditions usually present in LC (HILIC) mobile phase for the separation of N-glycans facilitate the positive ionization of the tertiary amine. It was shown that InstantPC (procaine-based instant labeling) has better sensitivity than ProcA (with 1/3 lower sensitivity) and RFMS (with 2/3 lower

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<table>
<thead>
<tr>
<th>Labeling agent</th>
<th>Preferred MS mode</th>
<th>Mass shift (Da)</th>
<th>FLD excitation λ (nm)</th>
<th>FLD emission λ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-aminobenzamide (2-AB)</td>
<td>-</td>
<td>120.0687</td>
<td>330</td>
<td>419</td>
</tr>
<tr>
<td>2-aminobenzoic acid (2-AA)</td>
<td>Negative</td>
<td>121.0528</td>
<td>350</td>
<td>425</td>
</tr>
<tr>
<td>2-aminopyridine (PA)</td>
<td>Positive</td>
<td>78.05818</td>
<td>310-320</td>
<td>375</td>
</tr>
<tr>
<td>8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)</td>
<td>Negative</td>
<td>366.9490</td>
<td>353</td>
<td>535</td>
</tr>
<tr>
<td>8-aminopyrene-3,6,8-trisulfonic acid (APTS)</td>
<td>Negative</td>
<td>440.9647</td>
<td>455</td>
<td>512</td>
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<tr>
<td>Procaine (Proc)</td>
<td>Positive</td>
<td>220.1576</td>
<td>285</td>
<td>345</td>
</tr>
<tr>
<td>Procainamide (ProcA)</td>
<td>Positive</td>
<td>219.1735</td>
<td>310</td>
<td>370</td>
</tr>
<tr>
<td>RapiFluo-MS (RFMS)</td>
<td>Positive</td>
<td>312.3663*</td>
<td>265</td>
<td>425</td>
</tr>
</tbody>
</table>

Table 1: Labeling agents and their preferred MS mode, mass shift after reductive amination, and FLD wavelength (λ)

*from glycosylamine
sensitivity) labelings for FLD detection. It was also shown that InstantPC and RFMS have similar MS detection sensitivity, which is 5 times higher than ProcA [75]. It should be noted that the ProcA labeling in the Prozyme application note was not conducted using an NHS carbamate chemistry, and the sample preparation for ProcA was not described in detail. Another comparison by Lauber et al. [26] yielded different results. They showed that for FLD, RFMS was twice more sensitive than InstantAB (Prozyme) and 14 times more sensitive than ProcA. For ProcA, the authors extrapolated data from previous literature [58]. Lauber et al. also observed that RFMS was three times more sensitive than ProcA for MS, but again with extrapolation of the signal from literature [58]. The sensitivity is highly dependent on the entire procedure of sample preparation, including the choice of PNGase F provider, digestion conditions, labeling agent/reaction, and sample purification analysis method. This can make the comparison difficult, especially if it is not done under the same conditions. Our experience with online SPE purification of ProcA labeled N-glycans has shown higher sensitivity compared to RFMS and InstantPC for HILIC-FLD analysis [30]. However, this was observed only when the procedure involved overnight digestion followed by ProcA labeling, as opposed to the rapid digestion in the RFMS protocols. Enhancing a protocol is crucial when sensitivity is needed. This applies to cases where samples have small amounts of glycoproteins and/or to identify N-glycans with limited presence in glycoproteins. Furthermore, some kits have been optimized for a certain quantity of specific glycoproteins. For the RFMS kit, the recommended quantity is 15 µg of mAb like proteins in a simple matrix. Some contaminants, such as nucleophilic compounds and SDS, should also be avoided for optimal sensitivity for this kit applications. In the presence of such contaminants, a buffer exchange can be performed before the unfolding step. Furthermore, when using MS detection, the sensitivity can be more influenced by the configuration of the mass spectrometers than by the labeling agent itself has it was previously shown [20]. Finally, Table 2 summarizes the most used approaches based on the labeling agent describing the recommended glycoprotein amount, the denaturing and reductive agents, clean-up strategy, total preparation time and the most suitable analysis method.
When MS/MS is used for detection, one advantage is that the fragmentation provides more information for structural elucidation [20, 80, 81]. The fragment nomenclature has been described a long time ago and is still used [82]. Glycosidic cleavages provide information about the monosaccharide sequence, while cross-ring information reveals details about the linkage and branching. The best ionization mode to obtain more detailed information is the negative mode. Indeed, it has been shown that the negative mode provides more cross-ring fragments than the positive mode, enabling better structure determination [83, 84]. A development of PGC microchip LC coupled to ESI QTOF with negative ionization and the addition of fluoride in the mobile phase has shown to provide a comprehensive characterization of N-glycans, thanks to the specific additional cross-ring fragments. However, this was based on reduced N-glycans only, and not labeled ones. When negative mode is required for labeled N-glycans, the preferred labeling agents are 2-AA and APTS. These agents can readily acquire a negative charge. Although other labeling agents such as 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) could also be used [57]. APTS with the three negative charges has been used less frequently, but recently it was shown that it could be of interest for N-glycan analysis using MS detection [20].

### Table 2: Summary of sample preparation using 5 labeling agentkits

<table>
<thead>
<tr>
<th>Labeling agent/kit</th>
<th>Glycoprotein amount</th>
<th>Denaturing agent</th>
<th>Reductive agent</th>
<th>Clean-up</th>
<th>Prep. time</th>
<th>Recommended Analysis method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AB</td>
<td>10-50 µg</td>
<td>RapGest SF, Nonidet P-40, SDS</td>
<td>cyanoborohydride or 2-picolineborane</td>
<td>HILIC or normal phase SPE tubes, tips, plates</td>
<td>&gt;1 day</td>
<td>HILIC-FLD</td>
<td>[36, 76]</td>
</tr>
<tr>
<td>Aprox</td>
<td>0.05-40 µg</td>
<td>Nonidet P-40, SDS</td>
<td>cyanoborohydride or 2-picolineborane</td>
<td>HILIC or normal phase SPE tubes, tips, plates</td>
<td>4 h to 1 day</td>
<td>HILIC-FLD-MS</td>
<td>[29, 30, 74]</td>
</tr>
<tr>
<td>InstantAB</td>
<td>10-50 µg</td>
<td>Guanidine</td>
<td></td>
<td>GlvkoPrep Cleanup (CU) Cartridges (hydrophilic interaction)</td>
<td>1 h-6 h</td>
<td>HILIC-FLD</td>
<td>[77]</td>
</tr>
<tr>
<td>InstantPC</td>
<td>10-50 µg</td>
<td>Guanidine</td>
<td></td>
<td>Cleanup (CU) Cartridges (hydrophilic interaction)</td>
<td>1 h-6 h</td>
<td>HILIC-FLD-MS</td>
<td>[73, 78]</td>
</tr>
<tr>
<td>RFMS</td>
<td>15 µg</td>
<td>RapGest SF</td>
<td></td>
<td>HILIC SPE plate</td>
<td>&gt;30 min</td>
<td>HILIC-FLD-MS</td>
<td>[26, 79]</td>
</tr>
</tbody>
</table>
Nevertheless, due to the availability of retention/migration libraries for N-glycans such as GlycoSuite, as well as instrument/kit company libraries, MS/MS fragmentation is not widely used for orthogonal approaches, unlike glycopeptide analysis where fragmentation is used for the reliable identification of the peptide backbone and the glycan. This is because the identity of N-glycans can be determined without the need for fragmentation. However, fragmentation is commonly used for the elucidation of specific and complex N-glycans and of glycopeptides.

4.5. Multiplex labeling agents

One major drawback of N-glycan analysis using LC-FLD/MS is the lengthy analysis time required for each sample. Additionally, there is no option to analyze multiple samples simultaneously, as is possible with the DNA sequencer system (CE-based) mentioned above [54]. In this context, multiplex approaches have been developed for LC-MS.

Multiplex analysis is widely used in proteomics, especially for relative or absolute quantification. It has the main advantage of analyzing multiple samples in a single run. Additionally, thanks to MS fragmentation, it can differentiate and quantify peptides from the different samples [85-88]. For glycomics, multiplex analyses have also been reported [89, 90]. Isotopic and isobaric approaches are the main methods for multiplexing. In the isotopic strategy, also known as the mass shift strategy, different heavy isotopes of the labeling agent are used to label N-glycans. One isotope is used for each sample. The samples are then mixed and analyzed. The advantage of isotopic strategies is that it requires only MS1, as fragmentation is not required. However, the main disadvantage is that it is often limited to triplex comparison due to the complexity of spectra [89]. The isobaric approach is different. The isobaric labeling agents have all the same mass, but they consist of three components: a reporter, a balancer, and a reactive group. Thanks to MS fragmentation (MS2), we can easily differentiate the samples based on the reporter [89]. One advantage is that this technique enables a higher level of multiplexing (larger than triplex).

Among the isotopic approaches, 2-AA and aniline heavy isotopes have been used either with HILIC-MS and CE-MS to compare batches of therapeutic glycoproteins, but they might also be used for the development of glycoprotein production or glycan profiling [90-93]. Other labeling agents, such as 3-
benzoyl-2-oxothiazolidine-4-carboxylic acid (BOTC), have also been used [94]. Furthermore, a similar isotopic approach has been used with the permethylation of glycans. Permethylation is not discussed in the present review and this approach is described here [95]. More recently, a labeling strategy called Individuality Normalization when Labeling with Isotopic Glycan Hydrazide Tags (INLIGHT™) [96-100] has been developed using 4-phenethylbenzohydrazide (P2GPN) [101]. This strategy includes a reactive hydrazide group that allows for the rapid derivatization of released N-glycans without the need for reductive amination. Other agents with rapid chemistry based on the NHS carbamate group have also been developed, such as the one by Gong et al. [102]. As mentioned above, this isotopic approach remains limited, often only applicable under duplex conditions. We have developed a similar approach using ProcA with two additional synthetized heavy isotopes (+5 and +10) to enable triplex analysis.

Isobaric strategies were also developed for quantitative glycomics, even though there are fewer available isobaric labeling reagents compared to proteomics or isotopic glycan labeling strategies. Only a few approaches were developed. Among them, there are aminooxyTMT [103], iART [104], QUANTITY [105], and SUGAR. If MS fragmentation is necessary for this approach (at least MS2), the main advantage of this method is its high level of multiplexing, similar to the SUGAR tag approach with a 12-plex [106]. In this case, 12 samples can be gathered and analyzed in a single run from a quantitative perspective. This also decreases the problem of ionization variation during long injection run lists, which can lead to variations in the quantification and comparison of N-glycan profiles between samples.

5. Perspectives

Nowadays, many strategies are used for the characterization of N-glycans. Released N-glycans are one of the major approaches and is considered as the gold standard. The derivatization and analysis protocols have evolved, enabling rapid release, labeling, analysis of N-glycans, and obtaining quantitative results. The available labeling agents have evolved, and the newer ones enable high sensitivity for both fluorescence and mass spectrometry detection. Commercial kits for sample preparation combine rapid PNGase F release and rapid derivatization chemistry, enabling results to be obtained within 1h. Mass spectrometry has also revolutionized the possibilities for the identification of labeled N-glycans. With
the retention database coupled to exoglycosidase strategies, the process is now easier than before. Furthermore, thanks to the availability of isotopic and isobaric labeling agents, the relatively long analysis time of samples by (U)HPLC-FLD has now decreased due to the possibilities of multiplexing. CE-LIF/MS is also a good alternative for rapid analysis. Commercial kits for sample preparation are also available for CE analysis of released N-glycans, which facilitate easy analysis. The major drawbacks of all commercial kits are their high costs and the relatively restricted concentration range. This is not a limitation for the analysis of N-glycans from therapeutic glycoproteins but is a really important limitation when only small amounts of glycoproteins are available, such as in specific marker research. In this case, homemade approaches are still used, but the evolution of labeling agents and available protocols enables analysts to optimize the protocols for sample preparation and analysis strategies, making the method capable of producing sensitive results. The evolution of mass spectrometry will continue to assist analysts in making the analysis of N-glycans easier and more sensitive.
Figure legends:

**Figure 1**: (A) Major monosaccharides present in N-glycans, along with their structure, name, abbreviation, and symbol. The symbols used are based on the Symbol Nomenclature for Glycans (SNFG). (B) Color Oxford representations of a high-mannose, a hybrid, and two complex N-glycans.

**Figure 2**: (A) Cleavage sites of Endo H, PNGase F, and PNGase A. (B) PNGase F facilitates the detailed release of an N-glycan attached to a protein.

**Figure 3**: Typical chromatogram of HILIC separation of released N-glycan mixture labeled with procainamide: (A) MS profile, (B) FLD profile (both in relative intensities). Annotation of the N-glycans is in the Color Oxford nomenclature based on the SNFG.

**Figure 4**: Typical protocol for preparing released N-glycans, which includes denaturation, digestion, labeling, and clean-up of the released and labeled N-glycans. Denaturation is commonly achieved using surfactants (such as RapiGest SF© (Waters) or sodium dodecyl sulfate, SDS), along with heating (60-100 °C). Digestion can be performed using enzymes (such as PNGase F, PNGase A, etc.) or chemical release (β-elimination, hydrazinolysis). The labeling step may use a large panel of different labeling reagents such as 2-AB, ProcA, RFMS, etc. Finally, the clean-up is achieved through solid-phase extraction (SPE) usually under HILIC chemistry using either SPE tubes, SPE plates, or online-SPE before analysis.

**Figure 5**: Reductive amination reaction and the structures of the two major reduction agents: sodium cyanoborohydride and 2-picolineborane.

**Figure 6**: Structure of the major labeling agents for N-glycans: 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 2-aminopyridine (PA), procaine (Proc), procainamide (ProcA), and 8-aminopyrene-3,6,8-trisulfonic acid (APTS).

**Figure 7**: (A) N-hydroxysuccinimide (NHS) carbamate reaction with terminal N-AcetylGlucosamine (GlucNAc) from a freshly released N-glycan. (B) Structure of the three major labeling agents for rapid
chemistry labeling: Instant 2-aminobenzamide (InstantAB), Instant procaine (InstantPC), and RapiFluo-MS (RFMS)
References:


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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: