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# Comparison of ZIC-HILIC and graphitized carbon-based analytical approaches combined with exoglycosidase digestions for analysis of glycans from monoclonal antibodies

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ABSTRACT

Two LC approaches for analysis of therapeutic monoclonal antibodies (MAbs) are presented and compared. In the first approach, zwitterionic-type hydrophilic interaction chromatography (ZIC-HILIC) of 2-aminobenzamide-labelled glycans was coupled with fluorescence or electrospray ionisation mass spectrometric (ESI-MS) detection. The ZIC-HILIC method enabled relative quantification and identification of major glycan species. The sensitivity of fluorescence detection was higher compared to ESI-MS; however, MS detection enabled identification of co-eluted peaks. The new ZIC-HILIC approach was compared with porous graphitized carbon (PGC) separation of reduced glycans coupled with ESI-MS. Using PGC higher sensitivity was achieved compared to ZIC-HILIC due to the lower chemical background originating from the mobile phase and the derivatisation step, providing detailed information on minor glycan species. Furthermore, PGC exhibited excellent capability for separation of isobaric glycans with various degrees of mannosylation and galactosylation. The structures of glycans from MAbs used in this study were confirmed by exoglycosidase digestions. The two methods were applied to two monoclonal antibodies expressed in Chinese Hamster ovary cell lines and a monoclonal antibody expressed in a murine NS0 cell line. While the fluorescence-based approach is more suitable for routine glycan profiling due to the simplicity of data analysis, MS-based approaches were shown to provide detailed glycosylation analysis of complex glycoprotein samples.



## 1. Introduction

Monoclonal antibodies (MAbs) are a rapidly growing group of pharmaceuticals, used primarily in anti-cancer and anti-rheumatic therapies [1–3]. Most currently used MAbs belong to the IgG structural class, containing two variable regions (Fv) with an antigen-binding specific site and a conserved region (Fc) that determines interaction with the effector molecules resulting in the elimination of the antigen [4]. Glycosylation in the Fc region plays an important role in the stability and therapeutic efficacy of the drugs [5,6]. The expression of therapeutic MAbs in non-human systems can also introduce different glycosylation patterns that can cause immune responses in patients [7,8]. Due to the structural complexity of glycans, glycosylation analysis remains a challenge and the main focus of current method development lies in the simplification of sample preparation, new separation approaches, and wide application of mass spectrometry (MS), either coupled with prior separation or on its own.

Common strategies in glycosylation analysis include liquid chromatography including anion-exchange chromatography, and capillary electrophoresis (CE), usually coupled with fluorescence or MS detection. Although fluorescence detection does not provide direct information on glycan structure or composition, it remains the most widely used approach for glycan profiling in the pharmaceutical industry. This is due to the straightforward data analysis, high sensitivity and capability of relative quantification of glycan species. Confirmation of the glycan structures usually requires chromatography of labelled standards, collection of fractions with offline MS analysis, or exoglycosidase digestions. In addition, the analysis may be extremely tedious and standards for less common glycans can be very expensive or are usually not available.

*Abbreviations:* ESI-MS, electrospray ionisation mass spectrometry; ZIC-HILIC, zwitterionic type hydrophilic interaction chromatography; 2-AB, 2-aminobenzamide; HILIC, hydrophilic interaction chromatography; PGC, porous graphitized carbon; MAbs, monoclonal antibodies; GlcNAc, N-acetylglucosamine; Fuc, fucose; Gal, galactose; NeuGc, N-glycolylneuraminic acid; Man, mannose; dHex, deoxyhexose; Hex, hexose; HexNAc, N-acetylhexosamine.

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Furthermore, fluorescence detection is less suitable for the analysis of highly complex samples because overlapping of peaks can yield misleading results [9].

PNGase F released glycans are usually fluorescently labelled via reductive amination and the choice of the label depends on the separation technique used. Widely used fluorescent tags are 2-aminobenzamide (2-AB) and 2-aminobenzoic acid (2-AA) which are readily available in commercial kits and have been applied in combination with a range of techniques, including hydrophilic interaction liquid chromatography (HILIC), reversed phase liquid chromatography (RPLC) LC, anion-exchange chromatography, or CE [10–16]. Despite lower sensitivity and non-quantitative yields, 2-aminopyridine (2-AP) has been frequently used in the literature since it was introduced as a tag for sugar analysis much earlier than 2-AA and 2-AB [17,18]. Introducing tags with more charged groups, such as 8-aminopyrene-1,3,6-trisulfonic acid (APTS), is frequently used for CE analysis, both increasing resolution and reducing the time of the analysis [19]. In addition, sialylated glycans can be effectively separated by CE without prior labelling as demonstrated on recombinant erythropoietin [20].

HILIC columns with amide functionality have been used extensively for the separation of 2-AA and 2-AB labelled glycans coupled with fluorescence detection [11] and a database of 2-AB labelled glycans based on the HILIC retention properties has been established. Software has been developed that is linked to the database to automatically assign possible structures to a chromatographic peak [21]. Amide HILIC has also been coupled to ESI-MS. Since downscaling to capillary and nano format greatly enhances ESI-MS sensitivity, fluorescent labelling is no longer required and glycans can be analysed in the native form, thereby reducing the time and cost of sample preparation [22,23].

Recently, glycan profiling using HILIC columns with zwitterionic functionality (ZIC-HILIC, Merck SeQuant) has been introduced. This approach showed good capability for structural recognition of sialylated glycopeptides [24] and 2-aminopyridine derivatised glycans from human serum IgG [25]. Recently, we reported the separation of reduced glycans from monoclonal antibodies by ZIC-HILIC coupled with ESI-MS [26]. Similarly to amide HILIC, the sensitivity of the analysis was improved when a ZIC-HILIC nano column was employed. This allowed for the successful analysis of a range of neutral and sialylated glycans in the reduced form [27].

RPLC of glycans provides an orthogonal approach to HILIC, but requires a derivatisation step due to the poor retention of native glycans on C18 stationary phases. The same fluorescent tags as used in the HILIC mode can be employed in RPLC [15,16,28]. Since RPLC approaches allow the separation of permethylated glycans with or without fluorescent tag, permethylation may be employed to improve the quality of fragmentation data when the method is coupled to tandem MS [29]. Another stationary phase commonly employed in glycan separations is porous graphitized carbon (PGC), which exhibits an enhanced separation of isobaric species compared to HILIC and RPLC [30–32]. Glycans are commonly determined in the reduced form using PGC to avoid separation of anomers, which makes the method compatible with MS. The use of PGC has also been downscaled successfully to improve the sensitivity, either in nano column or microchip formats [33,34].

Due to the large number and complexity of glycan structures, various approaches for structural elucidation have been developed. One of the common approaches involves the HILIC separation of labelled glycans in combination with digestions using highly specific exoglycosidases, either simultaneously or sequentially [11,35]. Chromatography of standard glycans or MS is usually required for confirmation of the structures. The second approach for structural elucidation of glycans uses low-energy collision induced dissociation CID-MS/MS, either on its own or in combination with LC or other separation techniques [36,37]. The quality of structural information obtained depends largely on the ions selected for fragmentation. Glycans can be fragmented in native, labelled, or permethylated forms. Permethylation increases sensitivity and the quality of fragmentation data and is highly suitable for high-energy MALDI-CID-MS/MS analysis of glycans without prior separation and can also involve exoglycosidase digestions [36,37]. However, when combined with PGC, permethylation of glycans can represent an additional analytical challenge due to the loss of chromatographic resolution [38,39].

In this paper we demonstrate the use of a ZIC-HILIC column for the separation of 2-AB labelled glycans released enzymatically from monoclonal antibodies. The ZIC-HILIC separation was coupled to fluorescence detection to obtain relative quantitative data and the method was optimised for direct coupling with ESI-MS for direct identification of glycans previously observed by fluorescence detection. The new method is compared to the PGC separation of reduced glycans coupled with ESI-MS detection, and the suitability of both methods for potential use in glycan profiling of MAbs is discussed. Three MAbs produced with recombinant technology were used in this study; MAbs denoted as MAb1 and MAb2 were expressed in Chinese Hamster ovary (CHO) cell lines and MAb3 was expressed in a murine NS0 cell line. Exoglycosidase digestions were used for the confirmation of the proposed structures.

## 2. Experimental

### 2.1. Reagents and chemicals

Unless otherwise noted, all chemicals were of analytical grade. Acetonitrile was obtained from VWR International (Poole, UK). Acetic acid, ethanol, sodium borohydride, 2-aminobenzamide labelling kit, PNGase F (from Elizabethkingia meningoseptica), α-galactosidase (from green coffee beans), α(2→3,6,8,9) neuraminidase (from *Arthrobacter ureafaciens*) and α-mannosidase (from jack bean) were obtained from Sigma (St. Louis, MO, USA). β-N-acetylhexosaminidase (from jack bean) was purchased from Prozyme (CA, USA).

Samples of MAbs (denoted as MAb1, MAb2 and MAb3) prepared by recombinant DNA technology were kindly donated by Pfizer Inc. (Chesterfield, MO, USA).

### 2.2. Sample preparation

Glycoprotein samples (250–590 μg) were desalted prior to digestion using centrifugal filter units (Amicon Ultra, 10.000 MWCO, Millipore, Carrigtwohill, Ireland). Glycans were released from glycoproteins by digestion with 3 μL of PNGase F (500 units/mL) per 100 μL of glycoprotein solution in 50 mM ammonium bicarbonate buffer, pH 8.0, overnight at 37 °C. Proteins were removed by precipitation with 400 μL of ice-cold ethanol and the released glycans were dried under vacuum oven overnight at 40 °C. The supernatants were reduced with 30 μL of 0.5 M sodium borohydride in 0.025 mM sodium hydroxide in 50% ethanol (v/v) at room temperature overnight. The reaction was terminated by adding 5 μL of glacial acetic acid and samples were desalted by ion-exchange chromatography (Dowex MR-3 mixed bed, Sigma). The samples were then dried prior to the exoglycosidase treatment.

The exoglycosidase digestions were carried out using the following enzymes and conditions: α-galactosidase: 20 mU in 80 μL of 50 mM ammonium acetate pH 6.5, for 48 h, at room temperature; β-N-acetylhexosaminidase: 250 mU in 80 μL of 50 mM ammonium formate pH 4.8 for 48 h, at 37 °C; neuraminidase: 2.5 mU in 80 μL of 50 mM ammonium formate pH 4.8 for 48 h, at 37 °C; α-mannosidase: 680 mU in 80 μL of 250 mM ammonium formate pH