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High-performance liquid chromatographic mapping of the oligosaccharides released from the humanised immunoglobulin, CAMPATH ™1H

Anne C. Lines

The Wellcome Foundation, Bldg. 74, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS, UK

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Abstract

A sensitive and reproducible method for the routine mapping of oligosaccharides in a humanised immunoglobulin (IgG) is described.

The method involves the enzymic release of intact glycans using the endoglycosidase glycopeptidase-F, and subsequent derivatisation with 1-phenyl-3-methyl-5-pyrazolone to facilitate analysis by high-performance liquid chromatography (HPLC). The heterogeneous oligosaccharide chains are separated by a phosphate buffer–acetonitrile gradient reversed-phase HPLC method and monitored by ultraviolet detection at 245 nm, allowing the detection of picomole amounts. A number of standard oligosaccharides are similarly derivatised to enable classification of the types of structures present from a comparison of retention times.

Keywords: Reversed-phase HPLC; Humanised immunoglobulin; Oligosaccharide mapping; Glycopeptidase-F; *N*-Glycans

1. Introduction

Glycobiology is experiencing a tremendous rate of growth which is being fuelled by the emergence of recombinant glycoproteins and proteoglycans as potential pharmaceutical products.

The carbohydrate structures of these recombinant glycoproteins are of particular interest because of their possible influence on activity, clearance from circulation, solubility and stability of the proteins [1]. Different cell cultures are unique in their post translational modifications of glycosylation which occur as a result of the sequential actions of several enzymes. The cells' ability to perform glycosylation will, therefore, be directly affected by the cell culture environment. For this reason, glycosylation of proteins is now coming under scrutiny in terms of production variability. Changes in glycosylation between production batches of recombinant glycoproteins that are intended for human pharmaceutical use need to be routinely monitored.

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Described here is a method developed to monitor the carbohydrate content of glycoproteins as released oligosaccharides. Speed, resolution and sensitive detection at the picomole level are prerequisites for developing such a technique. A reversed-phase HPLC technique is suited to this application as a valuable method for producing an oligosaccharide profile. The target is a "carbohydrate map" that can be used as part of routine testing to rapidly ascertain batch-to-batch consistency in the glycosylation of the protein product.

Derivatisation of the oligosaccharides affords the sensitivity required, and numerous papers have been published on the subject. Common derivatisation techniques include perbenzoylation [2,3], which introduces chromophores on to the hydroxyl groups, pyridylamination [4–6], and reductive amination with *p*-aminobenzoic acid ethyl ester (ABEE) [7]; these techniques derivatise the reducing end of the *N*-acetylglucosamine cleaved from the asparagine. More recently, 8-amino-2-naphthalenesulphonic acid (ANS) has been used as a fluorescent label prior to high-performnce liquid chromatographic analysis [8]. These techniques generally involve complex procedures and consequently are not considered suitable for routine mapping.

This report describes the use of a rapid and sensitive derivatisation technique based on a method for monosaccharide analysis [9] which introduces a UV-absorbing chromophore, 1-phenyl-3-methyl-5-pyrazolone (PMP), on to the reducing end of oligosaccharides.

The derivatised oligosaccharides are subsequently separated using a gradient reversed-phase HPLC method. The conditions of enzymic release of oligosaccharides, their subsequent isolation, derivatisation and HPLC separation have been evaluated and optimised to study the oligosaccharide structures released from CAMPATH™ 1H, a humanised immunoglobulin (IgG) monoclonal antibody (Mab) which has been expressed in a Chinese hamster ovary (CHO) cell line. Comparative tests for the evaluation of the method used transferrin (human serum) and the alternative oligosaccharide release technique of hydrazinolysis.

Recently, the carbohydrate content and structures of various IgGs have been investigated and reported in the literature in detail [10,11]. In general, it has been shown that IgG structures contain one N-linked oligosaccharide chain attached to the CH_2 domain of each of the heavy chain subunits and can be highly heterogeneous in structure. CAMPATH-1H has an N-linked glycosylation site on each heavy chain, at Asn_{301}, within the sequence Asn-Ser-Thr. The characterisation of the types of N-linked oligosaccharides on CAMPATH-1H was made possible from the carbohydrate map method described in this study.

2. Experimental

2.1. Chemicals/reagents

Human serum transferrin, and its corresponding oligosaccharide library (oligosacchrides derived from the glycoprotein which have been released by large-scale hydrazinolysis), supplied as a lyophilised solid, native human IgG (polyclonal) and its corresponding oligosaccharide library, and individual biantennary sugars were purchased from Oxford GlycoSystems (Abingdon, UK).

PMP (1-phenyl-3-methyl-2-pyrazolin-5-one) was purchased from Sigma, glycopeptidase-F (GPF) from Boehringer Mannheim (Lewes, UK), and acetonitrile, super-quality HPLC grade, from Romil (Cambridge, UK). All other chemicals were of analytical reagent grade from BDH (Poole, UK). Water was purified through a Milli-Q system (Millipore, Chester, UK).

2.2. Apparatus and HPLC conditions

The HPLC apparatus comprised a Spectra Physics SP8000 ternary pump with an SP8880 autosampler and an LDC Milton Roy Spectromonitor D variable-wavelength UV detector set at 245 nm. This was connected to a DEC microvax with VG Data Systems Multichrom software. A Rheodyne injection valve was used with a 100 μl sample loop (model 7413, Microvalve).

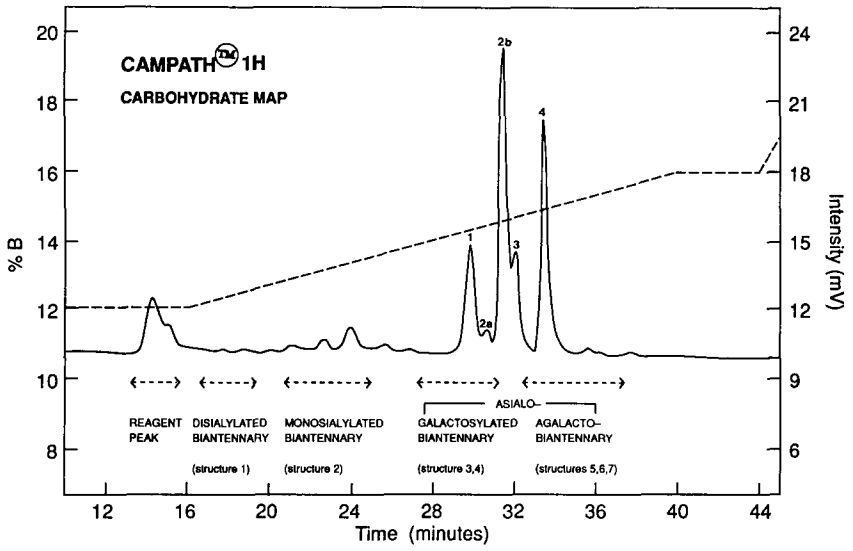

Fig. 1. Reversed-phase HPLC map of oligosaccharides released from CAMPATH-1H. Conditions: column, Spherisorb S3-ODS 2 (15 cm × 4.6 mm; Phase Sep); mobile phase A, 0.1 M sodium phosphate (pH 7); mobile phase B, acetonitrile; mobile phase gradient as indicated; flow rate, 0.7 ml min^{-1}; wavelength, 245 nm; room temperature.

Table 1
Averaged retention times of derivatised oligosaccharide standards[a]

Description[b]	Structure no.[b]	Oligosaccharide[c]	Retention time (min)
Disialylated biantennary	1	224300	20.5
1 with fucose	-	224301	19.5
Monosialylated biantennary	2	124300	23.0
Asialo, galatosylated, biantennary	3	024300	29.4
3 bisected with fucose	-	024311	30.4
3 with fucose	-	024301	28.0
3 bisected	-	024310	29.5
Agalacto-biantennary	5	004300	34.7
5 with fucose	-	004301	33.3
5 bisected with fucose	-	004311	35.6
Trimannosyl core	7	002300	37.5
7 with fucose	-	002301	36.0

[a]Purchased from Oxford Glycosystems. Structures 4 and 6 (see Fig. 2) are not available.
[b]See Fig. 2.
[c]Following the coding system of Oxford GlycoSystems. In order, the digits represent the number of residues of sialic acid, galactose, *N*-acetylglucosamine, mannose, bisecting *N*-acetylglucosamine and fucose.

Samples were concentrated and buffer exchanged using Centricon-10 microconcentrators (Amicon, Gloucestershire, UK).

Excess derivatising reagent was removed by dialysis using a SpectroPor-6 membrane with a molecular weight cut-off of 1000 (Pierce and Warriner (UK) Ltd., Chester, UK).

The PMP derivatives were separated on a 15 cm × 4.6 mm i.d. Spherisorb S3-ODS 2 reversed-phase C_{18} column, particle size 3 μm (Phase Separations Ltd., Deeside, UK), and eluted at a flow rate of 0.7 ml min^{-1}, using a gradient of acetonitrile and sodium phosphate buffer (pH 7; 0.1 M) as descibed in the Fig. 1 caption.

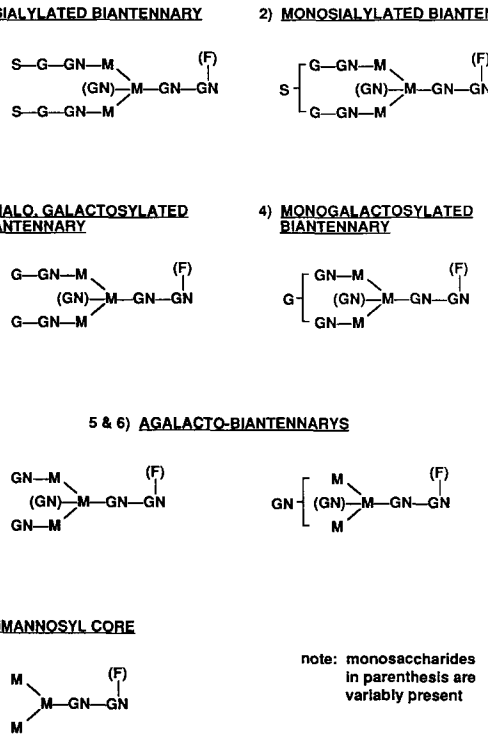

Fig. 2. Typical biantennary structures found in humanised immunoglobulins, any number of which may be present. S, sialic acid; G, galactose; M, mannose; F, fucose; GN, *N*-acetylglucosamine.

Samples were taken to dryness using a centrifugal rotary evaporator (Univap, Uniscience, UK).

2.3. Sample preparation

The glycoprotein samples (0.5 mg) were concentrated to approximately 40 μl by ultrafiltration using the Centricon-10 microconcentrators, and the buffer was exchanged to sodium phosphate (pH 8.5; 0.2 M). This creates optimum conditions for the enzyme glycopeptidase-F.

The concentrated samples were transferred into 1 ml Reactivials (Pierce) along with 20 units of GPF, 200 μl of sodium phosphate buffer (pH 8.5; 0.2 M) and incubated for 42 h at 37°C [12].

GPF is supplied as a 50% glycerol solution which will cause interference in the reaction. The glycerol was removed by ultrafiltration prior to the enzyme reaction.

The released carbohydrates were isolated via ethanol precipitation of the protein using three volumes of ice-cold ethanol [13], and were separated from the protein precipitate by centrifugation at 10000*g* for 10 min at 4°C. The supernatant was taken to dryness by centrifugal evaporation.

Oligosaccharide standards and library standards were introduced into the procedure at this stage. Aliquots of these were evaporated to dryness prior to derivatisation. Generally 20 μg (about 10 nmol) of oligosaccharide library and 5 μg (about 2 nmol) of an individual biantennary sugar were derivatised.

Table 2
Reproducibility of peak ratios obtained from one sample assayed on six separate occasions

Day	Peak 1 (% of total peak area)	Peak 2 (2a + 2b) (% of total peak area)	Peak 3 (% of total peak area)	Peak 4 (% of total peak area)
1	16.6	38.1	10.4	34.9
2	14.9	37.8	11.5	35.8
3	14.1	36.8	14.6	34.5
4	14.1	38.2	13.9	33.8
5	14.4	37.5	14.5	33.6
6	14.4	39.3	13.2	33.0
Mean	14.8	33	13	34.3
SD[a]	0.9	0.8	1.6	0.9
RSD[b]	6.1	2.1	12.3	2.6

[a]Standard deviation.
[b]Relative standard deviation (%).

2.4. Derivatisation procedure

PMP derivatisation was achieved by modification of the method described by Honda et al. [9]. A 20 μl amount of methanolic PMP (0.5 M) solution and 20 μl of sodium hydroxide solution (0.3 M) were added to the dried oligosaccharides (5–20 μg) and the mixture was heated at 70°C for 30 min. After the reaction, the mixture was cooled to room temperature.

Samples were then diluted to 250 μl with purified (Milli-Q) water and dialysed for 4 h using the 1000 molecular weight cut-off dialysis membrane to remove excess reagents.

The samples were transferred to Reactivials and taken to dryness by centrifugal evaporation, and the residues were redissolved in 200 μl of purified water and transferred to HPLC vials ready for HPLC analysis. Aliquots of 50 μl were then injected (500–2500 pmol).

3. Results and discussion

The reducing end of the oligosaccharides react with the PMP reagent in the ratio of 1:2 [9] to form stable products with a highly absorbing chromophore. IgGs are known to contain approximately 2% oligosaccharide, so a 0.5 mg sample of CAMPATH- 1H may be expected to release approximately 10 μg of oligosaccharide. Assuming 100% recovery, 2.5 μg of the oligosaccharide would be loaded on to the column (1000 pmol).

A series of biantennary standards were derivatised and run, in order to give retention times corresponding to the individual structures. It was found that the structures eluted in order of decreasing molecular weight and polarity; thus, the disialylated biantennary structures eluted first, followed successively by the monosialylated, non-sialylated and agalacto-biantennary sugars. Some structures possessing the same polarity will coelute and so absolute identification is not possible.

The PMP-derivatised oligosaccharides of CAMPATH-1H gave rise to four major peaks together with a series of smaller peaks corresponding to monosialylated oligosaccharides (see Fig. 1). A distinctive pattern was observed between the areas of the four main peaks for all batches of CAMPATH-1H (peaks 2a and 2b were combined, see Fig. 1). From a comparison of retention times between the individual oligosaccharide standards (see Table 1) and the released oligosaccharide structures from CAMPATH-1H, identification of the types of structures present in CAMPATH-1H was possible (see Fig. 2).

3.1. Reproducibility of the method

The corresponding peak areas were recorded for one batch of CAMPATH-1H on six separate occasions to show day-to-day reproducibility of the method (see Table 2). From the results it can be seen that the peak area ratio between the four peaks is consistent over the six occasions.

The four main peak areas were also recorded for 22 different batches of CAMPATH-1H tested on separate occasions (see Table 3).

Table 3
Reproducibility of peak areas for 22 batches of CAMPATH-1H

Batch no.	% Peak 1	% Peak 2, 2a + 2b	% Peak 3	% Peak 4
1	18.9	35.3	10.7	34.8
2	16.9	34.6	12.1	36.3
3	14.5	36.3	11.8	37.3
4	21.9	32.2	10.1	35.8
5	19.2	32.5	10.4	33.9
6	17.3	33.7	11.4	37.7
7	13.3	35.6	13.7	37.4
8	14.1	36.3	12.2	37.4
9	19.5	33.3	13.6	33.7
10	18.0	35.6	12.4	34.0
11	20.4	32.8	11.9	34.8
12	19.5	36.4	11.2	32.8
13	18.3	35.6	11.5	34.6
14	18.4	35.3	12.2	34.0
15	18.1	35.5	11.4	34.9
16	17.4	35.1	11.6	33.5
17	18.6	36.1	11.7	33.7
18	17.9	40.5	10.6	31.0
19	17.1	41.0	11.5	30.4
20	16.7	39.3	11.6	32.3
21	18.5	38.1	11.6	31.5
22	18.3	37.4	10.7	33.5
Mean	17.8	35.5	11.6	34.3
SD	1.9	2.3	0.8	2.0
RSD (%)	10.6	6.5	7.7	5.9

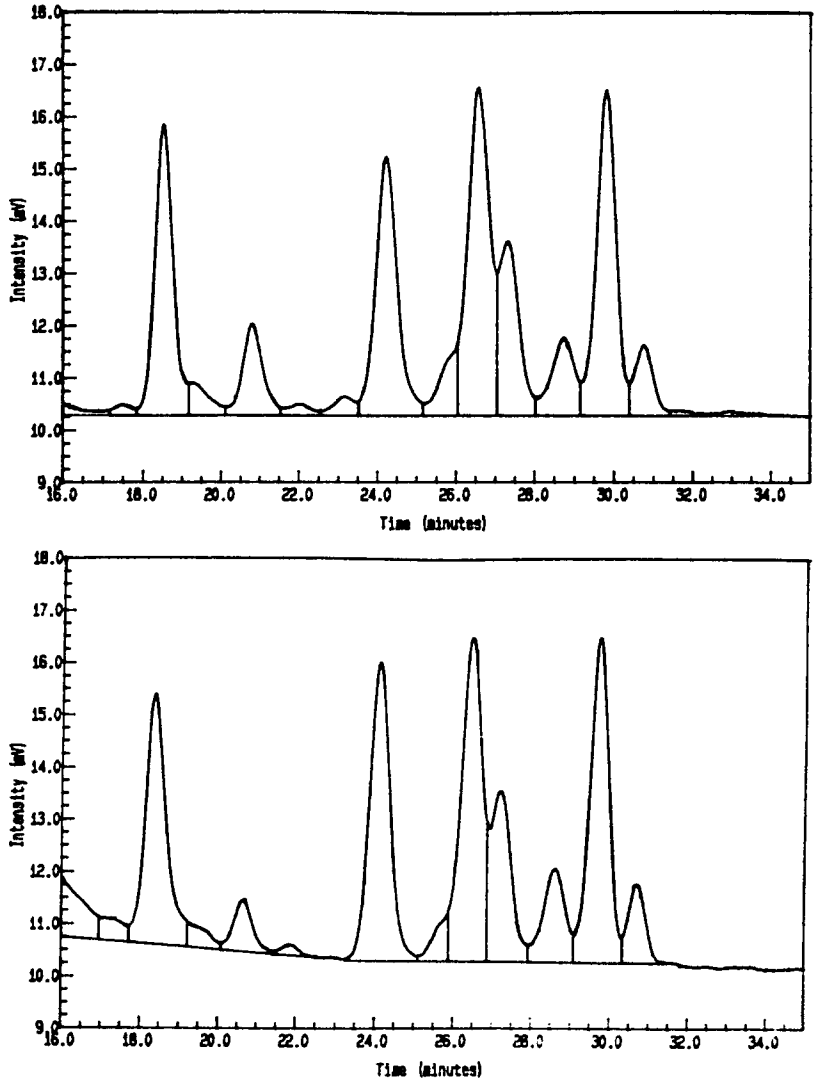

Fig. 3. (a) Carbohydrate map of GPF-released sugars from human IgG (0.125 mg); (b) carbohydrate map of purchased library of released sugars (by hydrazinolysis) from human IgG (5 μg).

The results show that the peak area ratio between the four main peaks remains consistent from batch to batch, giving good between-day reproducibility of 6.5–10.6%. These resulting data include the variables of column and mobile phase batches and room temperature changes.

3.2. *Efficiency of enzymic release*

Glycopeptidase-F (GPF) is an enzyme with great potential for structural analysis of glycoproteins and glycopeptides. Its specificity will accommodate carbohydrate structures such as high mannose and complex oligosaccharide chains.

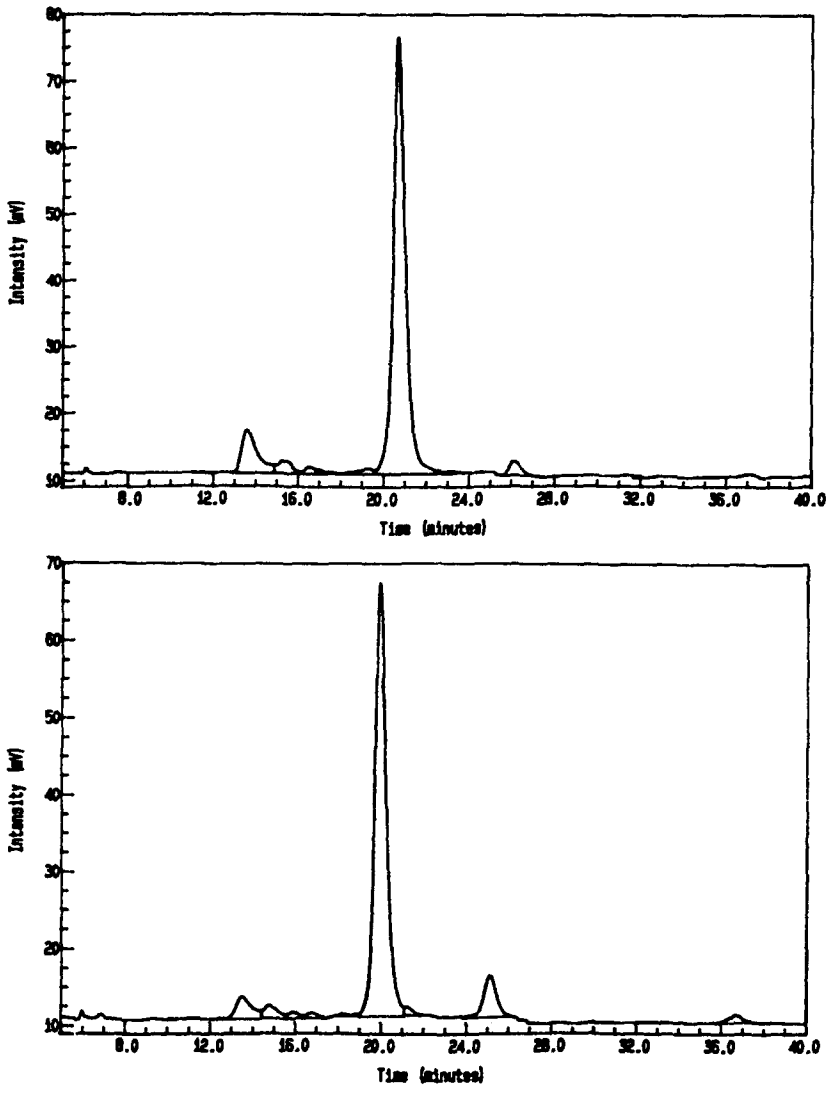

Fig. 4. (a) Carbohydrate map of GPF-released sugars from human transferrin (0.125 mg); (b) carbohydrate map of purchased library of released sugars (by hydrazinolysis) from human transferrin (5 mg).

The presence of fucose on the *N*-acetylglucosamine residue proximal to asparagine does not hinder the cleavage [14]. Release of intact oligosaccharide chains from glycoproteins using GPF is an uncomplicated and "user friendly" method, which facilitates structural elucidation. With a routine method in mind, GPF was chosen for our purposes in preference to hydrazinolysis, which is more suited to large-scale oligosaccharide release.

To demonstrate the efficiency of the carbohydrate release, a sample of GPF-digested CAMPATH-1H was dot-blotted onto Trans-Blot nitrocellulose membrane (0.4 μm; Bio-Rad) along with serial dilutions of a control sample of untreated CAMPATH-1H.

The portion of glycosylated material remaining in the digested sample was estimated by oxidation and digoxigen labelling, using a glycan detection kit purchased from Boehringer Mannheim. The

results showed that 10–15% of the glycosylated material remained, i.e. 85–90% of the glycoprotein had been deglycosylated (results not shown).

A comparison was made between the GPF-released oligosaccharides of the purchased glycoproteins, namely human IgG (polyclonal) and human transferrin and their hydrazinolysis-released libraries (see Figs. 3 and 4).

From a comparison of chemically released oligosaccharides from human IgG and transferrin and their corresponding libraries, the GPF cleavage of oligosaccharides appears to be as effective as hydrazinolysis in these examples. The two carbohydrate maps of enzyme-released and hydrazine-released glycans compare favourably, giving the same "fingerprint" of sugars.

The extent and rate of deglycosylation of glycoproteins depend on the nature of the protein, its carbohydrate structure and whether prior denaturation is required. CAMPATH-1H did not require denaturation, as GPF deglycosylates from its native form.

4. Conclusion

A sensitive and reproducible HPLC method for the profiling of oligosaccharides at the picomole level has been described, giving a "carbohydrate map" to monitor the glycosylation present in glycoproteins. The simplicity and ease of the method lends itself to the routine monitoring of samples to determine the consistency between production batches of recombinant glycoproteins.

No one technique can give a comprehensive characterisation of oligosaccharides. Other complementary methods such as electrospray-mass spectrometry (ES-MS) and sequential enzyme digestion need to be employed in order to obtain full structural infomation [15,16].

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