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High resolution of oligosaccharide mixtures by ultrahigh voltage micellar electrokinetic capillary chromatography

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Abstract

Oligosaccharide mixtures released from ribonuclease B and human IgG have been separated using micellar electrokinetic capillary chromatography operated at 100 kV. The resolution of these closely related analytes at this high voltage was found to be superior to that obtained at 20 kV, a voltage which is ordinarily used in most capillary electrophoresis separations.
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1. Introduction

Polysaccharide moieties covalently attached to proteins can be highly branched, and the individual sugar residues can be connected to one another by a number of linkage types. These large variations make carbohydrates much more structurally complex than other biopolymers such as proteins and nucleic acids, which are almost entirely linear.

One of the most common types of glycosylation involves carbohydrate N-linked to asparagine. It is normally the case that several glycan types can be linked to a particular asparagine. Thus, glycoproteins are usually mixtures of components that have the same amino acid sequence, but differ from one another by their oligosaccharide composition. These changes in the composition of the mixture of glycans in a glycoprotein can alter the physico–chemical and biological properties of the protein, affecting protection of the polypeptide chain from recognition by proteases, altering folding and modifying the overall solubility of the protein [1,2].

In the case of therapeutic proteins, it is essential that the same mixture of glycans are post-translationally expressed from one fermentation to another. Unequivocal reproducibility in glycan diversity can only be verified by using high resolution separation technology. As most common oligosaccharides do not absorb ultra-violet light, direct analysis is not simple. However, reductive amination of the reducing end of these molecules takes place with ease, so that the use of highly fluorescent aromatic amines is now the preferred methodology for the detection of picomole quantities of oligosaccharides [3].

The use of 2-aminoacridone (2-AMAC) for the analysis of complex glycan mixtures has been reported over the past few years [4–7]. This reagent is preferable to other reagents because of the versatility

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in analytical methodology that can be used in resolving and identifying glycan mixtures. Thus, capillary electrophoresis, reverse- and normal-phase high-performance liquid chromatography separation methods were developed for these derivatives, with detection by ultra-violet absorbence, fluorescence, matrix assisted laser desorption ionization and electrospray ionization mass spectrometry.

It was recently shown that the resolving power in capillary zone electrophoresis (CZE) increases considerably at potentials of the order of 100 kV [8]. We have now applied these high voltages using micellar electrokinetic capillary chromatography (MECC) to resolve mixtures of 'high' mannose and 'complex' type oligosaccharides released from ribonuclease B and human IgG, respectively.

2. Experimental section

2.1. Materials

Carbohydrates were purchased from Oxford GlycoSystems (Abingdon, U.K.). All reagents and solvents used in the derivatization with 2-AMAC were supplied by Sigma-Aldrich (Poole, U.K.). Taurodeoxycholate and buffers used in the MECC experiments were also obtained from Sigma-Aldrich. The derivatization of carbohydrates was carried out using a procedure detailed in previous studies [4–7].

2.2. Enzymic digestion of 2-AMAC glycans

The lyophilized 2-AMAC derivatized glycan pool from IgG was treated with a two enzyme array sialidase (*Arthrobacter ureafaciens*) and α-fucosidase (*bovine epididymis*) according to the following procedure: An aliquot (20 μl) of sialidase solution [prepared by dissolving 0.2 U of dried enzyme in 100 μl of 100 m*M* sodium acetate (pH 5.0)] and an aliquot (20 μl) of α-fucosidase solution [prepared by dissolving 0.1 U of dried enzyme in 100 μl of 100 m*M* sodium citrate (pH 6.0)] were added to the glycan residue and incubated at 37°C for 18 h. The reaction solution was then freeze dried.

2.3. MECC system

The fused-silica capillary (Polymicro Technologies, Phoenix, AZ) was 25 μm I.D., 360 μm O.D., 432 cm long with a 418 cm length to the detector for the experiments at 100 kV, and 100 cm with a 90 cm length to the detector for the experiments at 20 kV. The capillary inner surface was not treated or coated in any manner. Injections were performed hydrodynamically, with a 60 s misleveling of 30 cm for the 100 kV experiments and a 10 s misleveling of 10 cm for the 20 kV experiments. This should produce injection volumes of approximately 400 pl for the 100 kV experiment, and 90 pl for the 20 kV experiment. The concentration of the sample and buffer were such that stacking occurred, causing the variance due to the injection to be negligible compared to the overall variance. The buffer used in all experiments was prepared from taurodeoxycholic acid and sodium borate (obtained from Sigma-Aldrich) to be 100 m*M* borate and 34 m*M* taurodeoxycholic acid, and the pH was adjusted to 9.0. A 30 kV commercial power supply (Spellman, Yonkers, NY) was used for the experiments at 20 kV, while a homebuilt system was used for the experiments at 100 kV.

The ultrahigh voltage system used for this work is a slightly modified version of that described previously [8]. Briefly, a high voltage power amplifier (TREK, Inc., Medina, NY) with a 2.5 kHz square wave input signal from a Stanford Research Systems waveform generator (Sunnyvale, CA) was used to drive a rectification/multiplication stack. The amplitude of the square wave from the signal generator determined the DC output voltage of the multiplier stack. The multiplier is a 26-fold multiplier of the Cockroft-Walton type made of ceramic capacitors (2400 pF, rated to 20 kV DC; Newark Electronics, Chicago, IL) and diodes (ED2139, 25 kV maximum reverse operating voltage, 40 mA maximum forward current; Electronic Devices, Inc., Yonkers, NY). The capillary was protected from dielectric breakdown by means of a metal shielding system consisting of a set of aluminum cylinders. Injection was performed in an air-tight region at the high-voltage end of the apparatus. The multiplier stack, shielding and capillary, were immersed in transformer oil (Diala AX,

Shell Corp., Houston, TX, dielectric strength of 280 kV/cm).

2.4. Laser-induced fluorescence detector

Laser-induced fluorescence was achieved with the use of a 442 nm HeCd laser (LiCONiX, Santa Clara, CA) filtered through a 440±30 nm bandpass filter and focused onto the capillary with a 10× microscope objective (Melles Griot, Irvine, CA). The induced fluorescence was measured by collecting the light through a 60× microscope objective (Edmund Scientific, Barrington, NJ), passing the light through a bandpass filter with a 530 nm centre wavelength and 30 nm bandpass (Omega Optical, Brattleboro, VT) to remove scattered light, and focusing the light onto a photomultiplier tube (PMT) (Hamamatsu Model R1477, Bridgewater, NJ). The PMT voltage was set to 1 kV. The PMT current was sent to an amplifier (Stanford Research System, Sunnyvale, CA) set at a gain of 20 μA/V and with a lowpass filter cutoff setting of 10 Hz (−3 dB point) and a 12 dB/octave rolloff. Data was acquired at a rate of 20 Hz on a 16 bit ADC board using an in-house data acquisition program written in LabVIEW (National Instruments, Austin, TX).

3. Results and discussion

Separation efficiency in CZE increases linearly with the applied potential, whereas resolution increases with a square root dependence on applied potential [8]. The use of ultrahigh voltage CZE has been shown to give considerably better resolution of complex mixtures of peptides obtained from a protein digest. We now report the application of ultrahigh voltages in the separation of glycans. As mixtures of oligosaccharides released from the same glycoprotein are usually closely related molecules they are difficult to separate. Any increase in efficiency of the peaks in the analytical system used is useful in resolving co-migrating peaks.

2-AMAC derivatization of carbohydrates has a number of advantages: the intense fluorescence of 2-AMAC allows facile detection at excitation wavelengths which are accessible by using either an argon or, preferably a HeCd laser; large differences in the hydrophobicity of 2-AMAC and its carbohydrate derivatives makes MECC a most suitable technique for the resolution of a oligosaccharides without interference by excess reagent, as the excess 2-AMAC is associated almost completely with the micelles.

Oligosaccharide structures released from ribonuclease B are of the high mannose type as shown in Fig. 1, and differ from one another by the number of mannose residues attached to the non-reducing end of each branched oligosaccharide. In the case of two of these glycans, Man 7 and Man 8, isomeric species are also possible.

Fig. 2 compares MECC traces obtained for the same glycan mixture after electrophoresis at 20 and 100 kV. The order of migration of these neutral glycans is related to their hydrophobicity. Thus the least hydrophobic molecule, Man 9, is the first to migrate whereas the most hydrophobic glycan, Man 5, has the slowest migration time. Excess 2-AMAC is trapped by the deoxycholate micelles and therefore migrates at a much longer time (not shown).

A striking difference between the electropherograms in Fig. 2 is the increase in the resolution achieved for this mixture of 2-AMAC glycans at the higher potential of 100 kV. According to theory, since the potential used has increased by a factor of 5 (from 20 to 100 kV) the number of theoretical plates should also increase by a factor of 5 and the

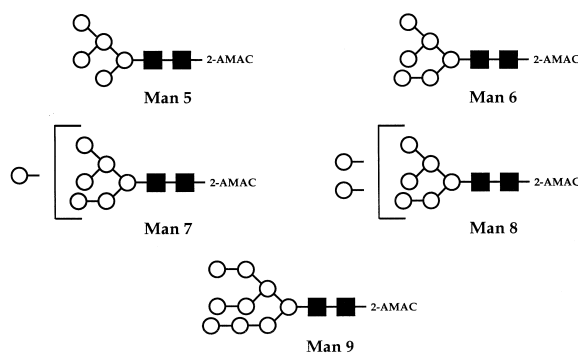

Fig. 1. Structures of high mannose oligosaccharides released from ribonuclease B and labelled with 2-AMAC. Symbols used to identify sugar residues are as follows: (■) *N*-acetylglucosamine, (○) mannose. Linkages between sugar residues have been omitted for simplicity.

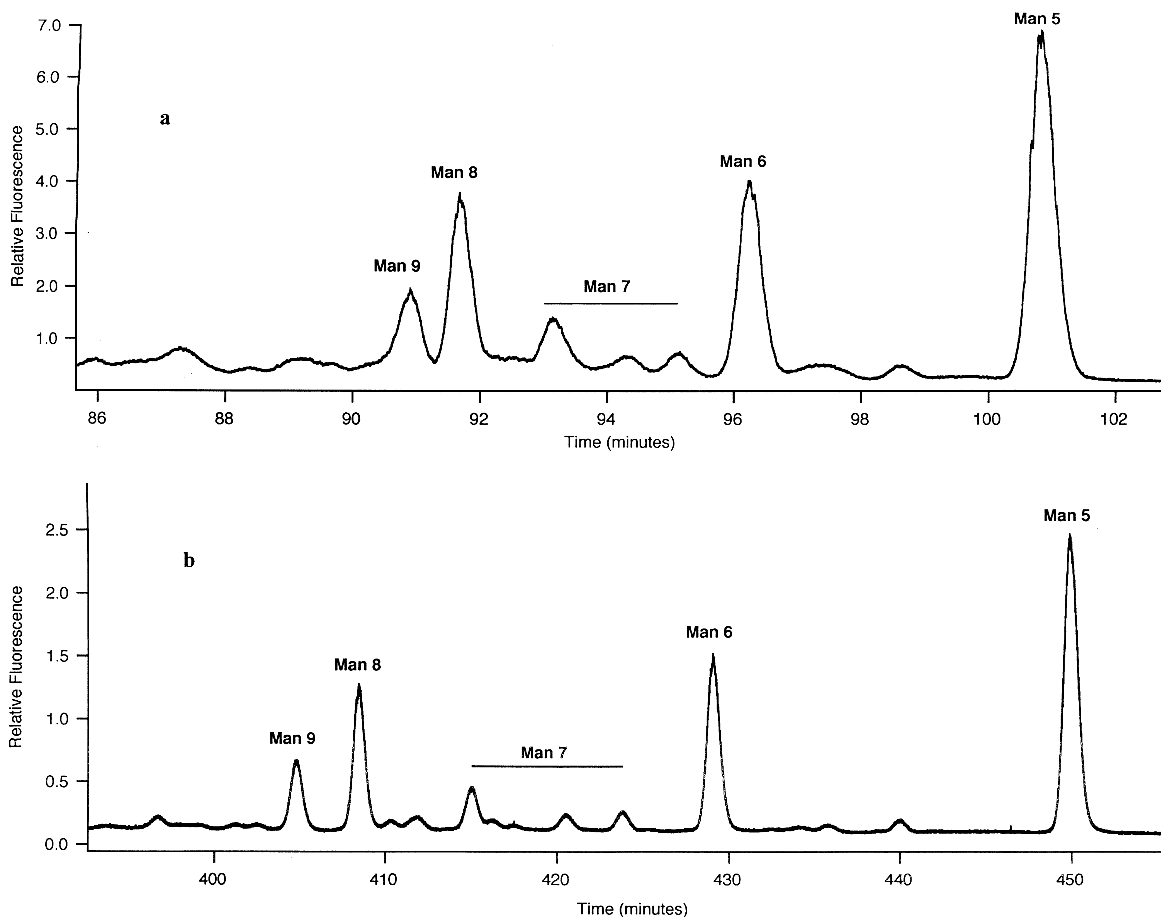

Fig. 2. Electropherograms of 2-AMAC derivatized high-mannose glycans (a) on an ultra-high voltage capillary electrophoresis instrument at 20 kV and (b) on the same instrument at 100 kV.

resolution should increase by $5^{1/2}$, or 2.2. In fact, the number of plates increases by a factor of 4.1±0.2, from typical values of 320 000 to 1 300 000 theoretical plates, as indicated in Table 1. The resolution increases by a factor of 2.1±0.1 as measured by pairs of adjacent peaks and is in good agreement with theory. The difference between the expected and measured increase in theoretical plates is due to variations in electro–osmotic flow. This factor of 4 improvement in performance provides markedly better separation, and several peaks which were unresolved in the low voltage experiment are now separated from their neighbours. This improvement is especially evident in the region near the Man 7 fragment.

Immunoglobulins (IgGs) are glycoproteins which contain carbohydrate covalently linked to asparagine-297 in the Fc (C-terminal) region. Oligosaccharides in IgG are of the complex type (Fig. 3), and are characterised by the presence or absence of a fucose residue (attached to *N*-acetylglucosamine at the reducing end), of an *N*-acetylglucosamine residue (bisecting the mannose core), and of galactose residues (at the non-reducing end). Glycans containing one or two galactoses can be acidic due to the attachment of sialic acid to these sugar residues. Despite the number of glycans in this widely distributed protein, the relative distribution of the various classes (for example, the ratio of sialylated to non-sialylated) in preparations from healthy individuals is remarkably similar [9].

The extensive micro-heterogeneity of glycans in

Table 1
Glycan pool from ribonuclease B: Efficiency and resolution

Peak ID	Millions of plates		Plate ratio	Neighbouring peak pair	Resolution		R_s ratio
	100 kV	20 kV			100 kV	20 kV	
Man 9	1.2	0.31	3.9				
Man 8	1.5	0.36	4.2	Man9/Man8	2.6	1.3	2.0
Man 7	1.3	0.33	3.9	Man8/Man7	4.6	2.3	2.0
Man 6	1.3	0.30	4.3	Man7/Man6	9.4	4.6	2.1
Man 5	1.3	0.29	4.5	Man6/Man5	13.5	6.4	2.1
			average=4.2 SD=0.2 expected=5.0				average=2.1 SD=0.1 expected=2.2

IgG was a good test to demonstrate the resolving power of ultra-high voltage capillary electrophoresis. Fig. 4 shows a comparison of electropherograms of 2-AMAC glycans at 20 and 100 kV. The number of clearly resolved peaks is considerably greater at the higher voltage. In fact, the plate count improves by a factor of 4.7±0.7, and the resolution improves by a factor of 2.3±0.9 for pairs of peaks (data shown in Table 2). This dramatic improvement in resolution allows peaks which are entirely unresolved in the low voltage electropherogram, such as the G2F and A1 peaks, to be nearly baseline resolved in the high voltage electropherogram.

The presence of both neutral and acidic glycans from IgG makes the assignment of peaks in Fig. 4 more difficult than that for the 'high' mannose structures from ribonuclease B. From prior experience in separating IgG glycans using other MECC conditions and chromatographic methodology, we tentatively labelled the major peaks in this figure. A glycan mixture after treatment with the two enzyme array, sialidase and fucosidase, was also analysed to increase the certainty in the identification of the 2-AMAC glycans. The ratio of neutral to acidic glycans is close to 4 and is of the same order of magnitude as that reported by Furukawa and Kobata [9]. The ratio of mono- (A1F) to di-sialylated (A2F) glycans is about 3, also in agreement with that reported by these authors. Lower-level peaks are due to the less abundant 'bisected' and non-core fucosylated glycans [9].

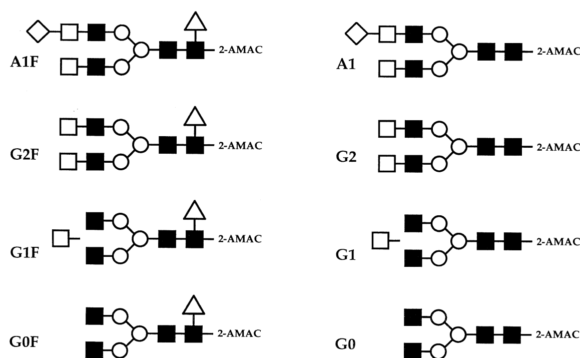

Fig. 3. Structures of some of the complex type glycans released from human IgG and labelled with 2-AMAC. Symbols used to identify sugar residues are as follows: (■) *N*-acetylglucosamine, (○) mannose, (□) galactose, (△) fucose and (◇) sialic acid.

Fig. 5 shows electropherograms of the original mixture of IgG glycans after treatment with both sialidase and fucosidase. Results from the analyses performed at 20 and 100 kV are shown in Table 3. Again resolution at the higher voltage was far superior, baseline resolution being obtained for a number of minor components in this mixture. The relative distribution of the major components G0, and the mono-galactosylated isomers, G1 and G1*, after enzymatic treatment (Fig. 5) is similar to that of G0F, G1F and G1F* in the untreated mixture (Fig. 4). As expected, the level of the di-galactosylated species, G2 has increased markedly due to the desialylation and defucosylation of A1F and A2F.

In conclusion, we have shown that the use of ultra-high voltage MECC provides more analytical information in the analysis of complex glycan mixtures derivatized with 2-AMAC. The increase in theoretical plate numbers has led to a considerable improvement in the overall resolution of a number

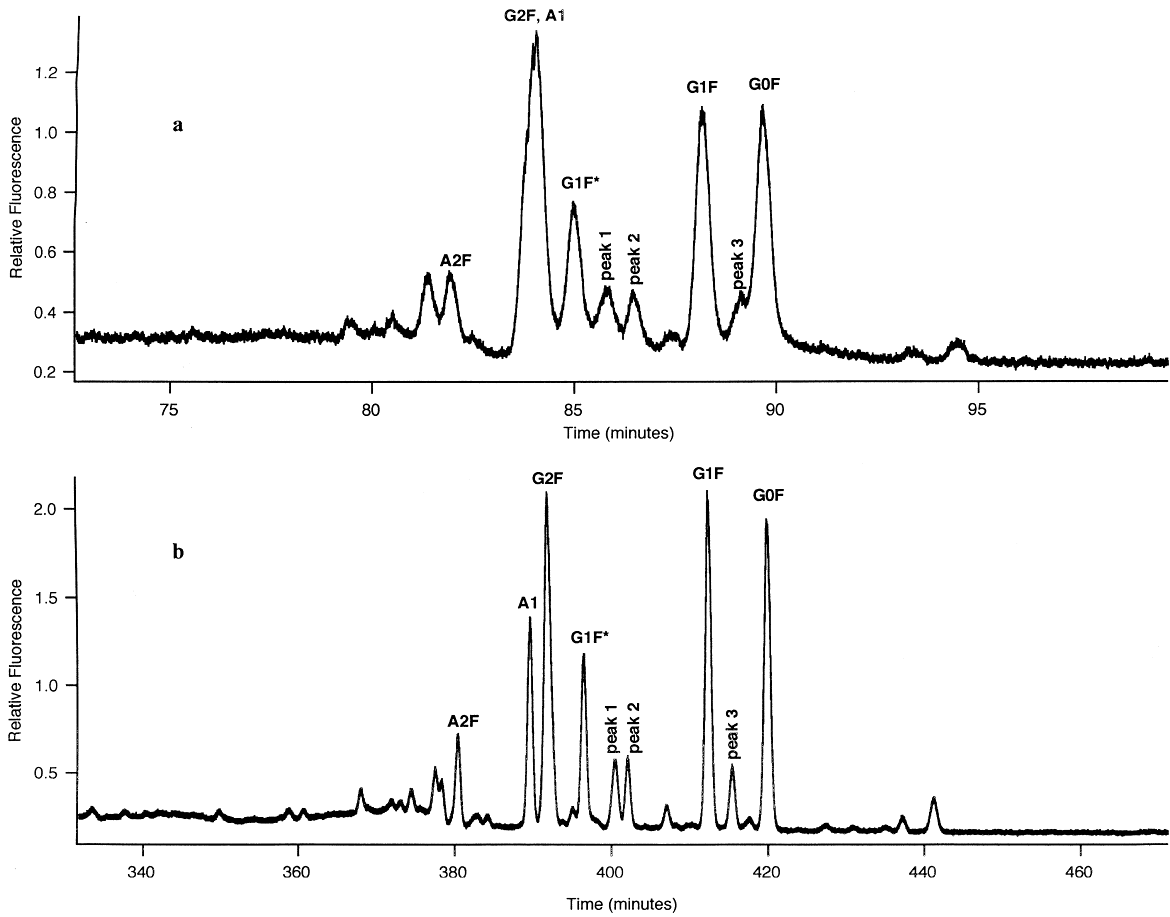

Fig. 4. Electropherograms of glycans released from IgG and derivatized with 2-AMAC analysed (a) using an ultrahigh voltage capillary electrophoresis instrument at 20 kV and (b) on the same instrument at 100 kV.

Table 2
Glycan pool from IgG: efficiency and resolution

Peak ID	Millions of plates		Plate ratio	Neighbouring peak pair	Resolution		R_s ratio
	100 kV	20 kV			100 kV	20 kV	
A2F	1.6	0.34	4.8				
G1F*	1.4	0.27	5.3	A2F/G1F*	12.8	5.0	2.5
peak1	1.2	0.22	3.7	G1F*/peak1	2.9	1.2	2.5
peak2	1.8	0.49	4.8	peak1/peak2	1.2	1.1	1.1
G1F	1.4	0.29	4.2	peak2/G1F	7.9	3.0	2.7
peak3	1.5	0.35	4.4	G1F/peak3	2.2	1.5	1.5
G0F	1.3	0.29	4.7	peak3/G0F	3.1	0.9	3.5
			average=4.7				average=2.3
			SD=0.7				SD=0.9
			expected=5.0				expected=2.2

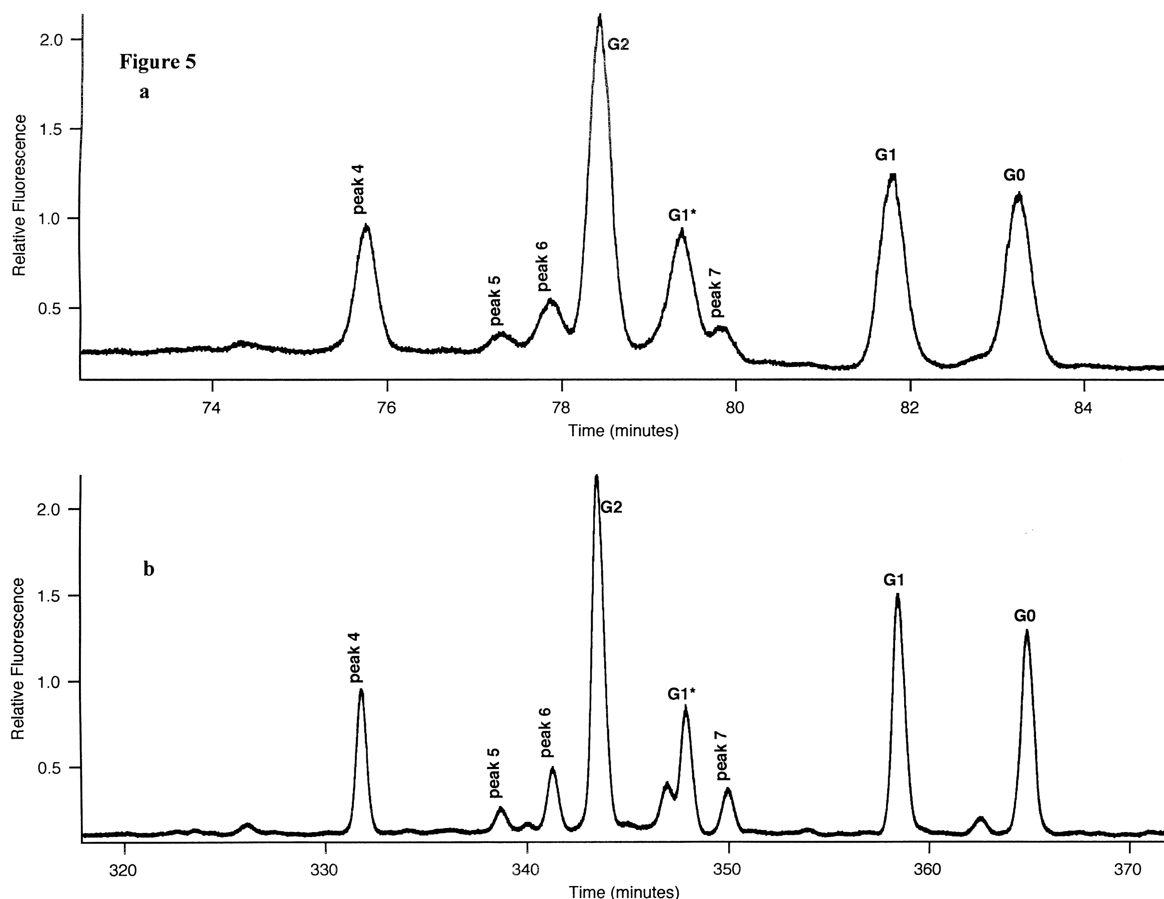

Fig. 5. Electropherograms of the glycan mixture in Fig. 4 after sialidase and fucosidase treatment. Desialylated defucosilated mixtures were analysed using the same electrophoresis conditions in (a) and (b) in Fig. 5.

Table 3
Glycan pool from sialidase and fucosidase treated IgG: efficiency and resolution

Peak ID	Millions of plates		Plate ratio	Neighbouring peak pair	Resolution		R_s ratio
	100 kV	20 kV			100 kV	20 kV	
peak4	1.9	0.34	4.8				
peak5	1.5	0.27	5.3	peak4/peak5	6.7	3.1	2.2
peak6	1.7	0.22	3.7	peak5/peak6	2.4	1.1	2.3
G2	1.5	0.49	4.8	peak6/G2	2.1	1.1	1.8
G1*	1.7	0.29	4.2	G2/G1*	4.0	1.9	2.1
peak7	1.5	0.35	4.4	G1*/peak7	1.8	0.7	2.6
G1	1.6	0.29	4.7	peak7/G1	7.4	3.1	2.4
G0	1.5	0.30	4.4	G1/G0	5.5	2.6	2.1
			average=4.8				average=2.2
			SD=1.3				SD=0.2
			expected=5.0				expected=2.2

components. Information on the composition of complex glycan mixtures was found to be more comprehensive than that at voltages commonly used in commercial CE instrumentation. This methodology should be invaluable in the fingerprinting of oligosaccharide mixtures released from therapeutic glycoproteins produced under different fermentation conditions.

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