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A rapid mass spectrometric strategy suitable for the investigation of glycan alterations in knockout mice

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Abstract

A method for the rapid screening of mouse tissues using fast atom bombardment mass spectrometry in combination with linkage analysis and specific enzymatic and chemical digests is described. This strategy has been successfully employed in the analysis of a wide range of murine organs and data from brain, kidney and lung tissue are presented.

1. Introduction

N- and O-linked glycans are implicated in many cell–cell recognition events in mammalian systems but the molecular foundations for these interactions are still poorly understood. Knockout mouse technology and glycoprotein engineering provide a means of investigating the importance of specific glycan epitopes via ablation of individual glycosyltransferases or targeted changes of glycan epitopes. Rigorous characterisation of any glycosylation changes occurring as a result of these experiments is essential if functional conclusions are to be derived from these experimental systems.

Investigations of knockout mice are revealing important aspects of glycan functions in mammalian systems.[1–3] However, studies are limited in that the precise nature of glycan alterations are essentially unknown. In addition, the glycan repertoire of the normal mouse is poorly defined. Structural elucidation of glycans from normal murine tissues and organs is therefore an essential pre-requisite to investigating changes occurring in knockout mice. In this paper we describe a strategy based on fast atom bombardment (FAB-MS) and electron impact (EI-MS) mass spectrometry, complemented by enzymatic and chemical protocols, for rapidly screening murine organs for their *N*- and *O*-glycan content. The glycosylation profiles of brain, kidney and lung are reported.

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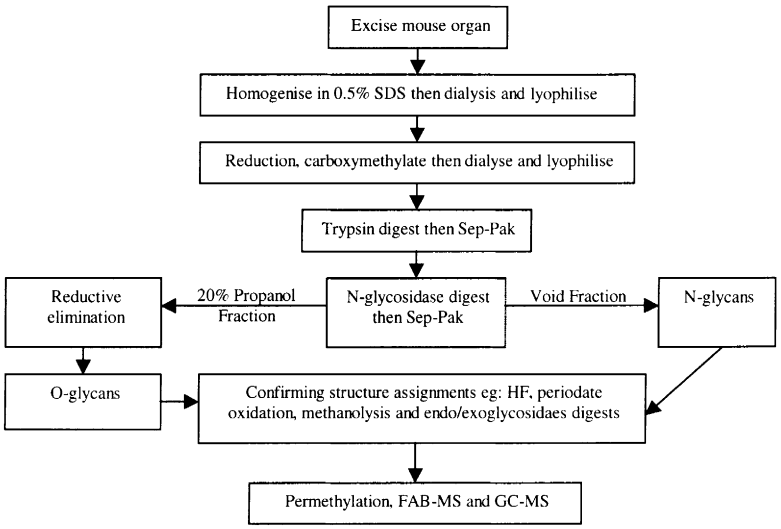
2. Results and discussion

2.1. N- *and* O-*Glycan preparation*

The protocols that we have developed for preparing *N*- and *O*-glycans from glycoproteins for mass spectrometric analysis[4] have been incorporated into a strategy (Scheme 1) to screen mouse tissues and organs rapidly. Firstly, the tissue to be studied is homogenised in Tris buffer (50 mM, pH 7.4) with 0.5% w/v SDS. The homogenate is dialysed in 12–14 kDa cut-off dialysis tubing in an ammonium hydrogen carbonate solution (50 mM, pH 7.4) during 48 h, and lyophilised. Following lypholisation the sample is carboxymethylated by first incubating it for 45 min at 37°C with 1 ml of 2 mg/ml dithiothreitol in deoxygenated Tris buffer, followed by addition of 12 mg of iodoacetic acid and incubation in the dark for 90 mins. The carboxymethylation reaction is terminated by dialysing the reaction mixture for 16 h. The sample is lyophilised and digested with approximately 2 mg of TPCK treated bovine pancreas trypsin at 37°C in 1 ml of an ammonium hyodrogen carbonate solution (50 mM, pH 8.4) for 8 h. The digested sample is purified by passing through a Sep-Pak C_{18} eluted with 30 ml of 5% acetic acid followed by a collection of 3 ml elutions of 20 and 40% propanol in 5% acetic acid. The collected fractions are pooled, evaporated to dryness and digested with 3 units of *N*-glycosidase F in 200 μl of an ammonium hydrogen carbonate solution (50 mM, pH 8.4) at 37°C for 20 h. The digested sample is loaded on a pre-conditioned Sep-Pak C_{18} and eluted with 5 ml of 5% acetic (the *N*-glycan fraction) followed by elution with 4 ml of 20% propanol in 5% acetic acid (the peptide/O-linked glycopeptide fraction). The *O*-glycans are released from the glycopeptides by reductive elimination (400 μl of 1 M 1$NaBH_4$ in 0.05 M NaOH at 45°C for 16 h).



Scheme 1. The rapid screening strategy used to screen mouse organs

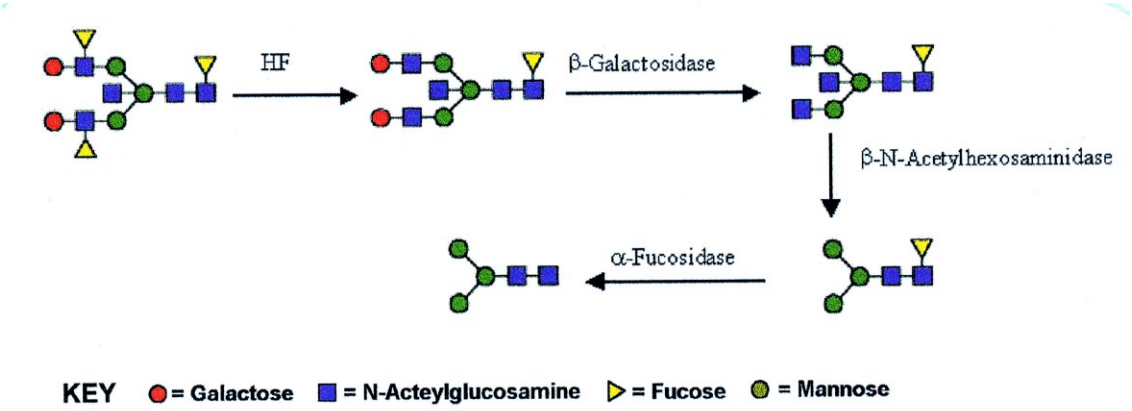
2.2. *Screening for glycan content*

A portion of each pool of isolated *N*- or *O*-glycans is then derivatised by permethylation before being analysed by FAB-MS.[4] Partially methylated alditol acetates are prepared from the permethylated samples for linkage analysis by EI GC–MS.[5] These initial screening experiments provide information on glycan compositions (via the molecular ions), sequence (via the A-type fragment ions) and linkages. Representative FAB data of fragment and molecular ion regions of permethylated *N*-glycans derived from mouse brain, kidney and lung are shown in Fig. 1. The putative structure assignments are based

on the usually unique glycan composition for a given mass (Fig. 1b, D–F), the A-type fragment ions present in the low mass region (Fig. 1a, A–C), which aid determination of the non-reducing epitopes, and knowledge of *N*-glycan biosynthesis. FAB data from analysis of the *O*-glycans present in brain, kidney and lung are shown for the brain, kidney and lung (Fig. 2, A–C), together with putative structures which are assigned in a similar manner to the *N*-glycans.

2.3. Confirming structural assignments

In order to confirm tentative assignments additional experiments are required. This is achieved by partial chemical and enzymatic degradations monitored by FAB-MS and linkage analysis. Scheme 2 shows a typical sequential degradation experiment performed on a bisected bi-antennary *N*-glycan. At each degradative step a small aliquot is permethylated and analysed by FAB-MS. The resulting mass shift for a given molecular ion is indicative of the residue/s removed. Bisecting GlcNAc is not cleaved from this class of *N*-glycan until the galactose residues have been removed and fucosylated lactosamine structures are not substrates for β-galactosidase and therefore defucosylation is required as a first step in the antennae degradation.



Scheme 2. A typical sequential degradation experiment used to confirm structure assignments made from the FAB-MS experiment and linkage analysis. Neither *N*-acetylhexosaminidase nor β-galactosidase can degrade the original *N*-glycan and, therefore, HF treatment is used to perform a specific defucosylation reaction

The most useful chemical procedures for aiding the assignment of glycan structures are acid-catalysed cleavages of labile glycosidic linkages and periodate cleavage of sugar residues that contain vicinal hydroxyl groups. A particularly important example of the former is hydrofluoric acid (HF). Experiments on a variety of fucosylated oligosaccharides have revealed (data not shown) that incubation of glycans with 50 μl of 48% HF at 0°C for 14 h results in loss of fucose residues that are either 2-linked to galactose or 3- or 4-linked to GlcNAc (as in blood group antigen and Lewis-type structures). However, fucose residues that are 6-linked to GlcNAc (a linkage that occurs in fucosylated core *N*-glycans) are not lost under these conditions. This can be particularly useful when performing structural analysis of *N*-glycan mixtures that contain *N*-glycans with and without fucosylated core structures.

Fig. 1 (pages 366 and 367). FAB mass spectra of fragment and molecular ion regions of permethylated *N*-glycans from normal mouse brain (A and D), kidney (B and E) and lung (C and F). All three tissues contain high mannose structures, $Man_{5-9}GlcNAc_2$, in addition to the labelled complex-type structures. The mass range shown here shows molecular ions at 2193 and 2397 corresponding to high mannose structures with eight and nine mannose residues, respectively

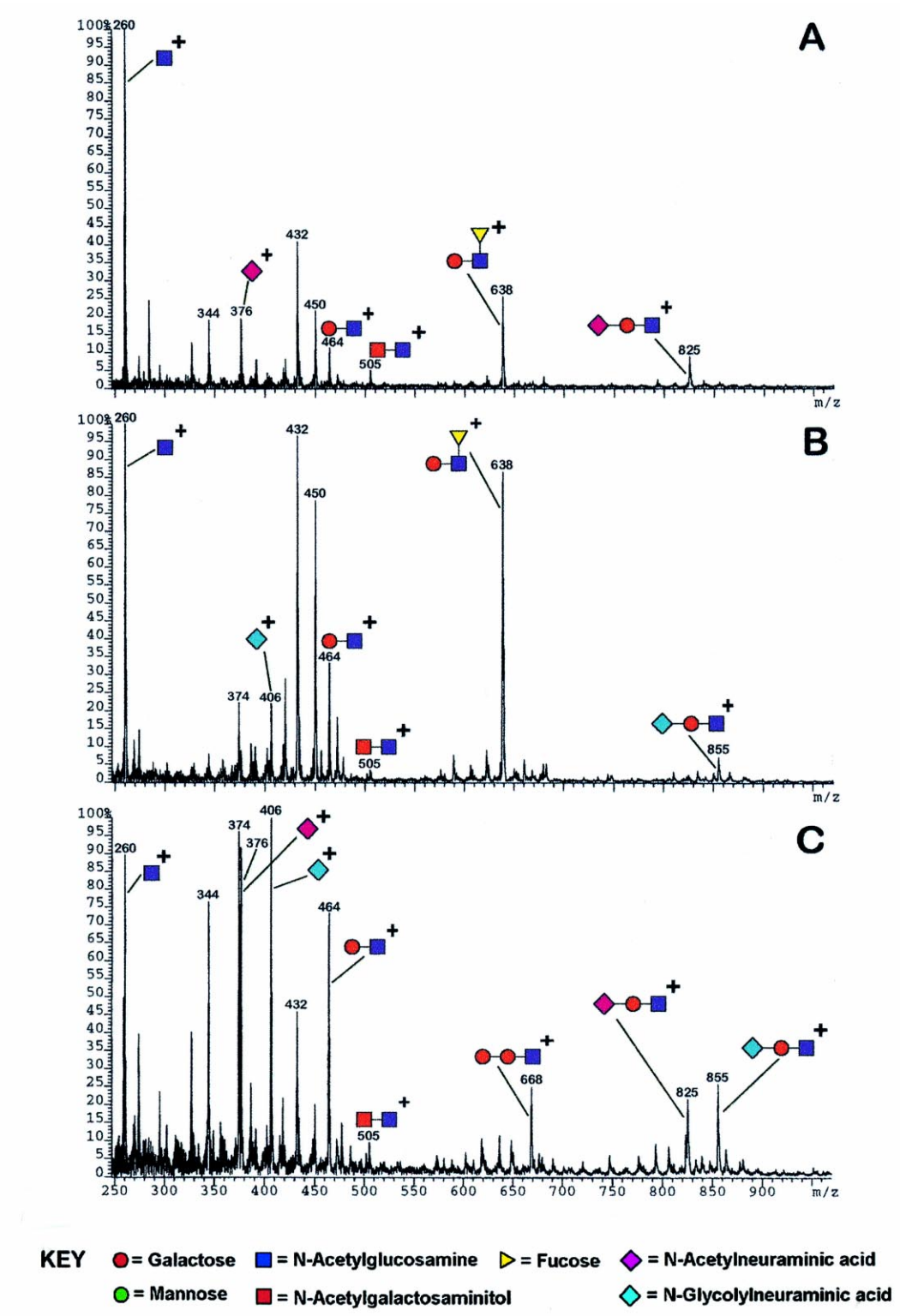
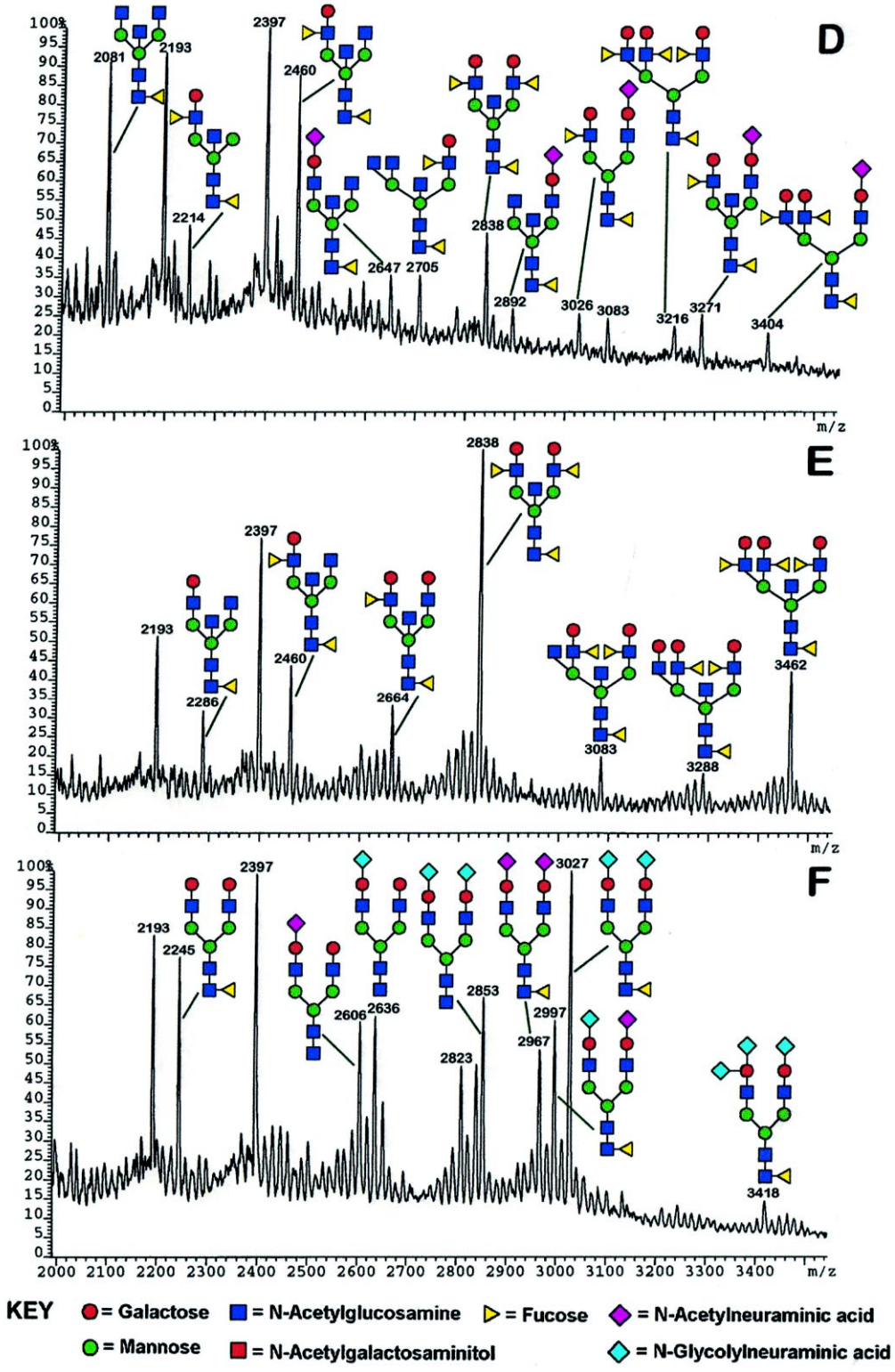

A
B
C
m/z
260
344
376
432
450
464
505
638
825
374
406
855
668
KEY
= Galactose
= N-Acetylglucosamine
= Fucose
= N-Acetylneuraminic acid
= Mannose
= N-Acetylgalactosaminitol
= N-Glycolylneuraminic acid

Fig. 1a.


D
2081
2193
2214
2397
2460
2647
2705
2838
2892
3026
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3216
3271
3404
m/z
E
2193
2286
2397
2460
2664
2838
3083
3288
3462
m/z
F
2193
2245
2397
2606
2636
2823
2853
2967
2997
3027
3418
2000 2100 2200 2300 2400 2500 2600 2700 2800 2900 3000 3100 3200 3300 3400 m/z
KEY
= Galactose
= N-Acetylglucosamine
= Fucose
= N-Acetylneuraminic acid
= Mannose
= N-Acetylgalactosaminitol
= N-Glycolylneuraminic acid

Fig. 1b.

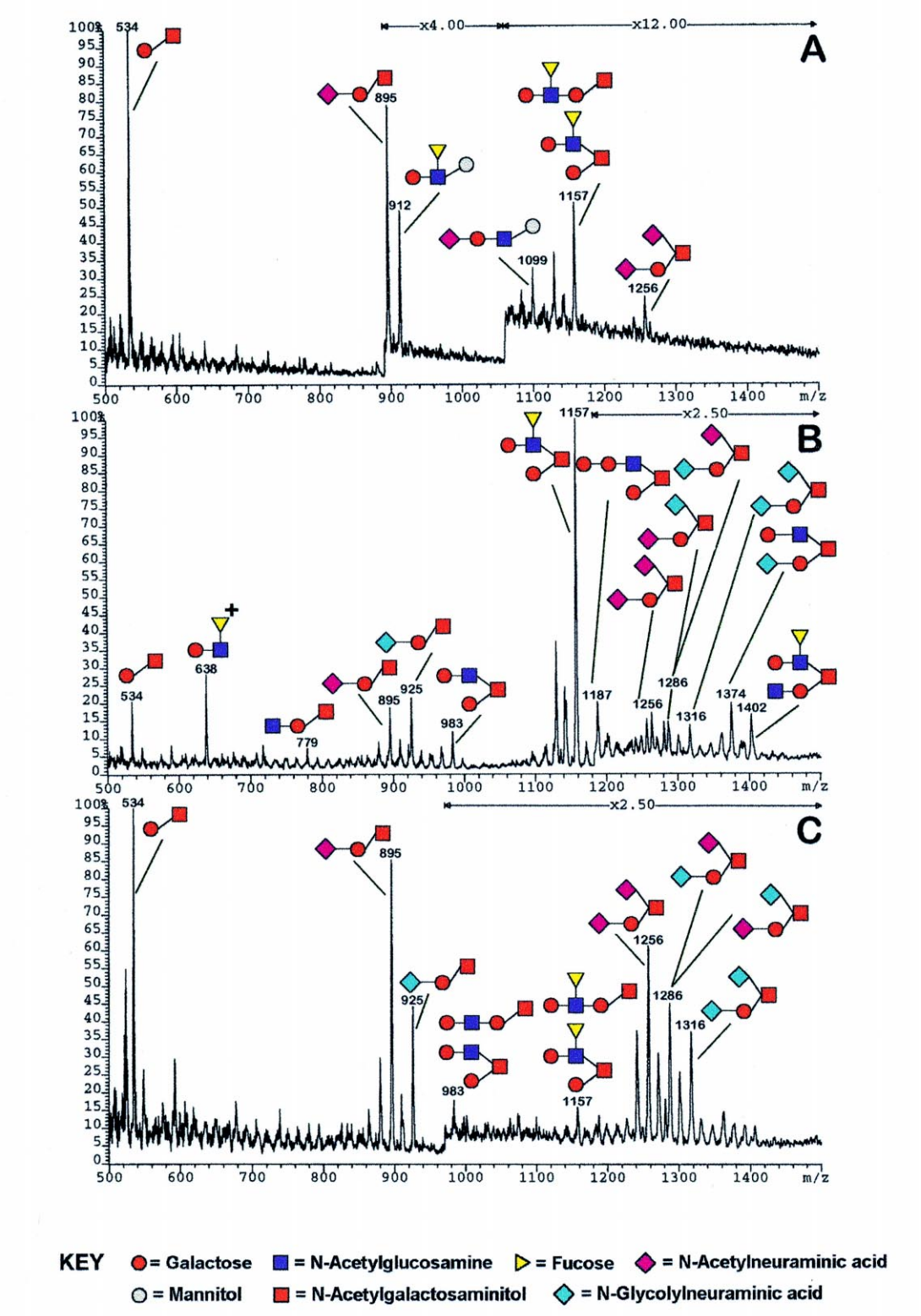

Fig. 2. FAB mass spectra of permethylated *O*-glycans from normal mouse brain (A), kidney (B) and lung (C)

Periodate oxidation is extremely useful for *O*-glycan structure determination because under mild conditions cleavage occurs specifically between the core *N*-acetylgalactosaminitol C_4 and C_5 carbons, allowing the core type of *O*-glycans to be identified.[6] Thus, periodate degradation allows the tentative structures shown in Fig. 2 to be confirmed.

3. Conclusion

The screening methodology described above is applicable to any organ, tissue, mucus, fluid etc. provided the glycan content that can be extracted is a minimum of about 10 μg. Confirmation of tentative sequences requires more material but most experiments can be carried out on a few microgrammes. We are currently using these strategies to examine organs from knockout mice with targeted deletion of genes involved in key steps of *N*- and *O*-glycan biosynthesis including α-mannosidase II,[7] which is responsible for removing the 3- and 6-linked mannoses from the antennae of the *N*-glycan biosynthetic precursor, and enzymes involved in initiating and elongating O-linked glycans[8].

Acknowledgements

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