Chapter 4
Carbohydrates

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1 Introduction

Structural analysis of carbohydrates is highly complicated. In nature a series of monosaccharides occur, both in free form and as part of linear or branched (poly)saccharides. They are also constituents of e. g. lipopolysaccharides, proteoglycans, glycoproteins and glycolipids.

The primary structure of carbohydrate chains is defined by several parameters: 1. nature and number of the constituent monosaccharides; 2. sequence and ring size of the monosaccharides and 3. type and anomeric configuration of the glycosidic linkages (Figure 1). In the case of proteoglycans and glycoproteins two additional parameters have to be determined, namely the type of the carbohydrate-peptide linkage and the nature and position in the polypeptide backbone of the amino acids involved (Figure 2). For the determination of these parameters mass spectrometry (MS) frequently combined with gas-liquid chromatography (GC) plays a key role. Several comprehensive reviews on MS and GC of carbohydrates have already appeared (1 – 8). For this reason the present chapter is focused on those aspects of the use of GC-MS which are of relevance in (clinical) biochemistry, biomedicine and biology.

Carbohydrates are of importance in several contexts e. g. in hereditary metabolic diseases, recognition phenomena on the cellular level, cancer and immunological features. Saccharides isolated from physiological fluids, tissues, bacteria, etc., are subjected to structural analysis in several ways. Most of the oligo- and polysaccharides and the glycolipids can be analysed directly. However, in general it is not possible to study the structures of high molecular weight carbohydrate chains directly in intact lipopoly-saccharides, proteoglycans and glycoproteins. In lipopolysaccharides the lipid A part has to split off; if present the *O*-antigen and core oligosaccharide are also split (9). Proteoglycans and glycoproteins often contain more than one carbohydrate chain attached to separate amino acids in the polypeptide backbone. Frequently the chains show (micro-)heterogeneity. The presence of the polypeptide backbone as such can interfere with the analysis of carbohydrates. To obtain suitable products for the analysis of the carbohydrate chains of these glycoconjugates a number of degradation methods

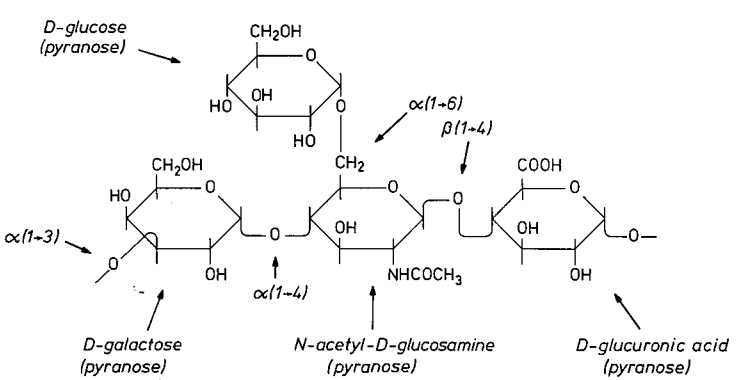


Figure 1. Schematic presentation of the parameters, which define the primary structure of a carbohydrate chain.

are commonly applied: 1. degradation of glycoproteins by exhaustive protease (pronase) digestion to glycopeptides having only a few amino acids; 2. cleavage of the *N*-acetylgalactosamine-serine/threonine linkage and formation of oligosaccharide-alditols using alkaline borohydride (*β*-elimination reaction); 3. cleavage of the *N*-acetylglucosamine-asparagine linkage by hydrazinolysis followed by *N*-acetylation and reduction, yielding oligosaccharide-alditols; 4. cleavage by endoglycosidases like endo-*β*-*N*-acetylglucosaminidases and endo-*α*-*N*-acetylgalactosaminidase or by peptide-N^4-(*N*-acetyl-*β*-glucosaminyl)-asparagine amidases, releasing oligosaccharides. For the preparation of carbohydrate oligomers, polymeric structures are frequently subjected to further chemical degradations such as periodate oxidation, partial acetolysis, partial hydrolysis or hydrazinolysis/nitrous acid deamination, and to enzymic degradations with exo- or endo-glycosidases (10,11).

In the following, experimental procedures and mass spectrometric data in relation to sugar analysis and methylation analysis, and MS of oligosaccharides, oligosaccharide-alditols, glycopeptides and glycolipids will be presented.

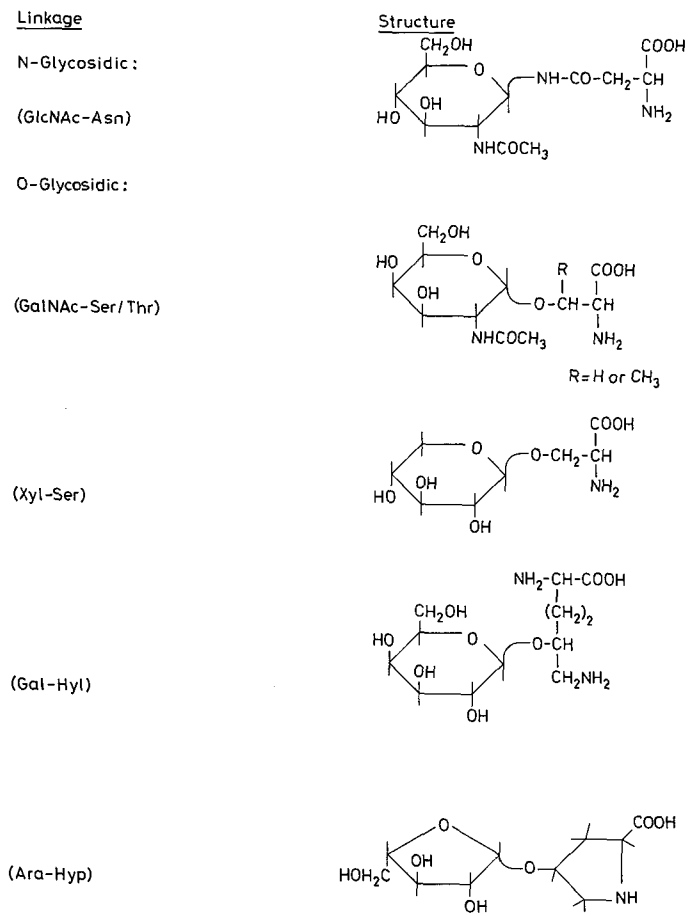


Figure 2. Major types of carbohydrate-peptide linkages in glycoproteins.

2 Sugar Analysis

The approaches to the determination of the sugar composition of complex carbohydrates are mainly based on GC-(MS) procedures. Sugar analysis of oligosaccharides, oligosaccharide-alditols, polysaccharides, glycoproteins, glycopeptides, glycolipids and proteoglycans is currently established after hydrolysis or methanolysis. Monosaccharides, occurring as such in biological sources, can be analysed directly or after treatment with methanolic HCl. To obtain volatile derivatives, many derivatisation procedures have been reported (4).

The hydrolysis procedure involves cleavage of the glycosidic linkages by mineral acid and, after conversion into suitable derivatives, analysis of the resulting monosaccharide mixture by GC-(MS). The conditions for complete hydrolysis of all glycosidic bonds with minimal destruction of the constituents have to be determined experimentally in view of the different stabilities of the monomers. For many polysaccharides the use of 0.25 $mol \cdot l^{-1}$ H_2SO_4 for 17 h at 100 °C seems to be a good compromise (12). Glycoproteins have been hydrolysed using 4 $mol \cdot l^{-1}$ HCl for 4–6 h at 100 °C (13). However, for quantification of sialic acid, very mild acidic conditions have to be applied (14). The hydrolysis of glycosidic bonds of 2-acetamido sugars and uronic acids can give rise to severe problems due to incomplete splitting. Acetamido sugar-containing polysaccharides can be hydrolysed in very good yields with anhydrous HF (2 h, room temperature) (15). Also 0.5 $mol \cdot l^{-1}$ trifluoroacetic acid (19 h, 100 °C) and 4 $mol \cdot l^{-1}$ trifluoroacetic acid (1 h, 125 °C /4 h, 100 °C) have been shown to give good results (16, 17). Ketose-containing carbohydrates have to be hydrolysed under extremely mild conditions (18).

Before analysis by GC-(MS) neutral monosaccharides and amino sugars are most commonly converted into the corresponding alditol acetates (4, 6, 13, 19–23). Other applied derivatives are i.a. trimethylsilylated alditols (4, 6, 24), trifluoroacetylated alditols (4, 6, 25), aldononitrile acetates (4, 6, 7, 26–29) and oxime derivatives (4, 6, 7, 17, 28, 30–35). Also direct derivatisations such as pertrimethylsilylation, peracetylation and pertrifluoroacetylation are sometimes used (3, 4, 6). *N,O*-acylneuraminic acids can be analysed by GC(-MS) after trimethylsilylation of the free acids or methyl esters (36).

Methanolysis, usually carried out with methanolic 1 $mol \cdot l^{-1}$ HCl for 24 h at 85 °C, is applied to the simultaneous analysis of neutral monosaccharides, acetamido sugars, uronic acids, octulosonic acids and sialic acids (37). Carboxyl groups of the acidic sugars are blocked by methyl ester formation. Owing to anomerisation, each monosaccharide gives rise to a characteristic GC peak pattern of methyl glycosides, facilitating the identification of a sugar. This procedure causes less destruction of the monosaccharides than hydrolysis, but it is very effective in cleaving glycosidic linkages. The mixture of methyl glycosides of the different types of sugars can be determined by GC(-MS) after *N*-(re)acetylation/trimethylsilylation (4, 36–49) or trifluoroacetylation (4, 50–54). It has to be noted that in glycolipid analysis the ceramide residues are degraded to sphingosine and fatty acid methyl esters. Similar procedures based on the glycosidation with (−)-2-butanol or (+)-2-octanol are in use for the GC(-MS) determination of the absolute configuration of monosaccharides (55–57).

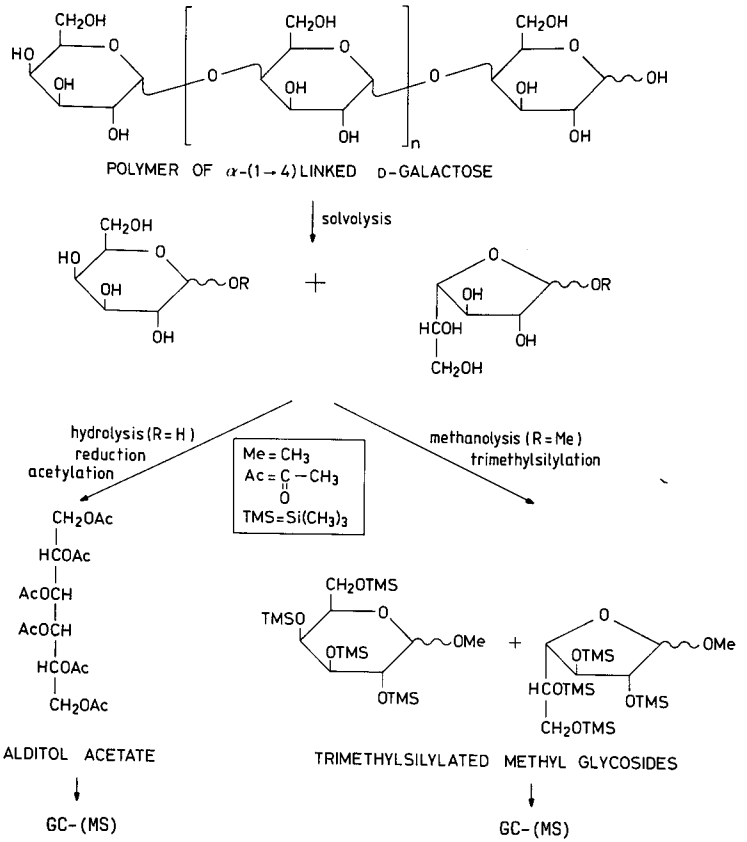


Figure 3. Schematic hydrolysis and methanolysis procedures for the sugar analysis.

In Figure 3 the sugar analysis procedures based on the preparation of alditol acetates and of trimethylsilylated methyl glycosides (methyl esters) are presented schematically. These procedures will be discussed in more detail, including the mass spectrometric characteristics of both types of derivatives. The same holds for the already mentioned analysis of *N,O*-acylneuraminic acids and the absolute configuration determinations.

2.1 Hydrolysis procedure/alditol acetates

In an ampoule the carbohydrate material (0.5 mg) is dissolved in 0.2 ml 4 mol · l^{-1} trifluoroacetic acid and heated for 4 h at 100 °C. Any alditol which does not interfere with the analysis of the constituting monosaccharides can be used as internal standard. The solution is evaporated with a stream of nitrogen and the residue washed twice with methanol followed by evaporation. The residue is taken up in 0.2 ml water and treated with 5 mg $NaBH_4$ for 2 h at room temperature. After decomposition of the excess of $NaBH_4$ with a few drops of 20% acetic acid (to pH 4), the solution is

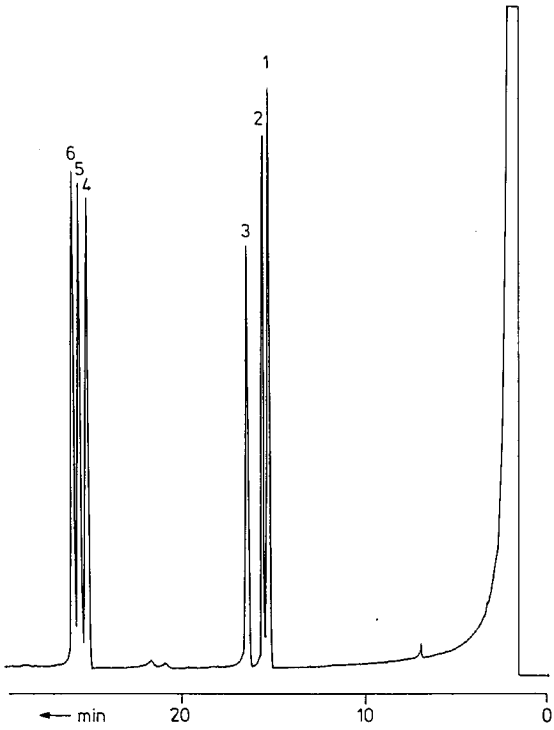


Figure 4. Gas chromatogram of alditol acetates on a CP-Sil 5 WCOT fused-silica capillary column (25 m × 0.32 mm). Oven temperature program: 130 °C to 220 °C at 2 °C · min^{-1}; 1 min at 220 °C. The peaks are numbered in their order of elution and are assigned as follows: (1) rhamnose; (2) fucose; (3) xylose; (4) mannose; (5) glucose and (6) galactose.

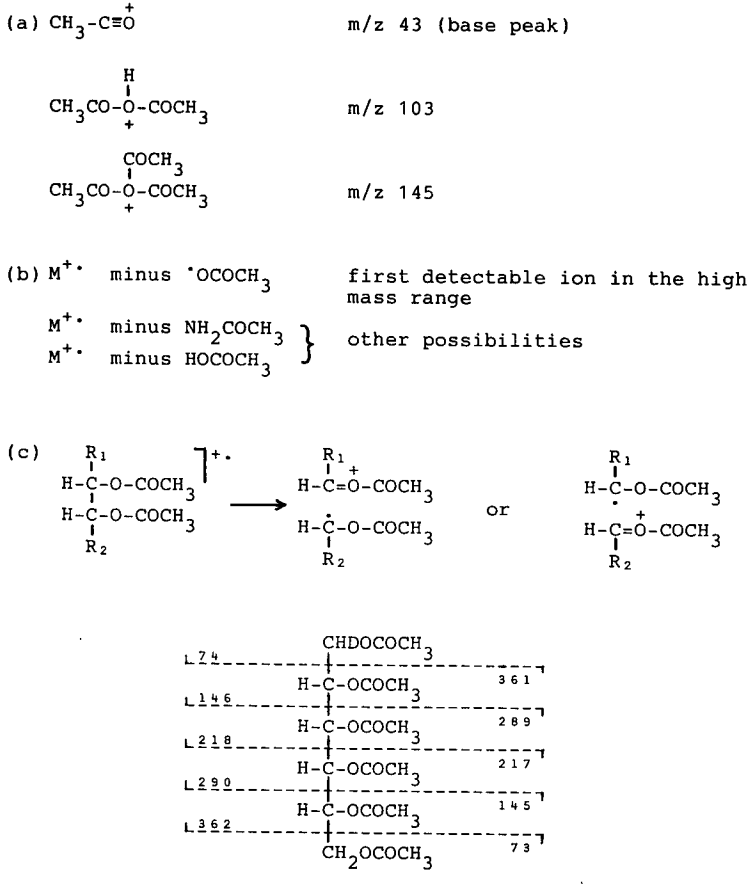
evaporated *in vacuo*. Boric acid is removed as trimethylborate by co-evaporation with 0.2 ml 1% methanolic acetic acid (five times). The mixture of alditols is acetylated with 0.2 ml acetic anhydride/pyridine (1:1) for 1 h at 100 °C. Finally the solution is evaporated with a stream of nitrogen in the presence of toluene. For analysis by GC (-MS) the residue is dissolved in 0.1 ml methylene chloride.

The sugar analysis can be performed on a CP-Sil 5 WCOT fused-silica capillary column (25 m × 0.32 mm) using flame-ionisation detection (58) and the following conditions: carrier gas nitrogen flow-rate 1.5 ml · min^{-1} and the make-up gas nitrogen flow-rate 35 ml · min^{-1}; split-ratio of 1:10; injection-port temperature 210 °C, and the detector temperature 230 °C; oven temperature programmed from 130 °C to 220 °C at 2 °C · min^{-1} and kept isothermally at 220 °C for 1 min. A typical gas chromatogram of a mixture of neutral monosaccharides is presented in Figure 4.

For the alditol acetate analysis several other types of stationary phases have been recommended, e. g. SP-1000 (12), Silar 10C (13), OV-1 (21), SE-54 (21), Carbowax 20M (21), SP-2330 (23), Dexsil 410 (59), SE-30 (59), OV-101 (59), and OV-275 (60). The more polar column materials are not suitable for the analysis of the amino sugar alditols.

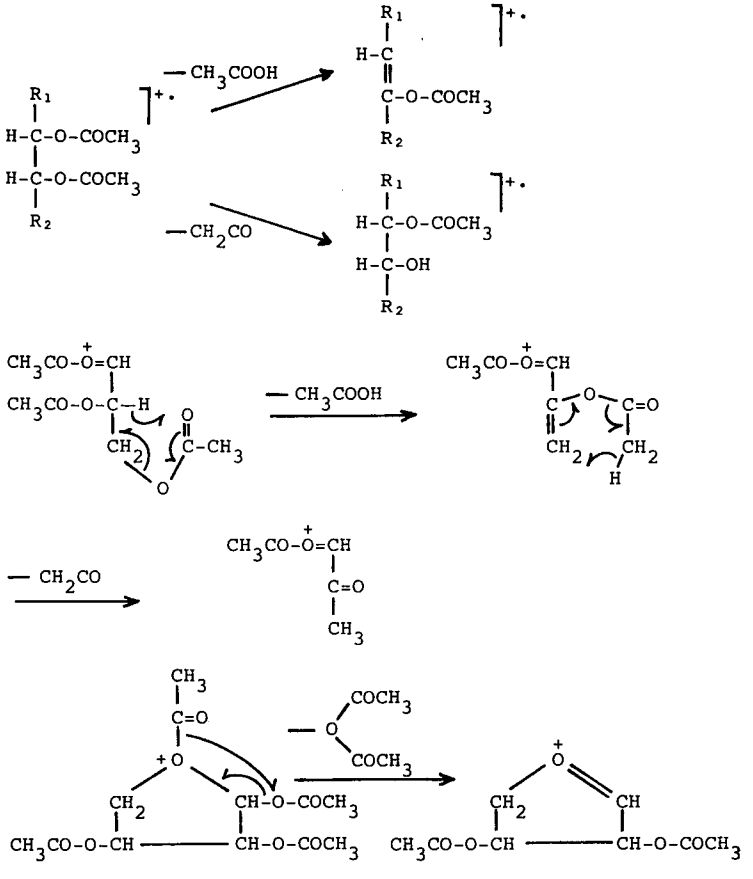
2.2 Mass spectrometry of alditol acetates

The EI fragmentation patterns of alditol acetates have been extensively described in the literature (1–3, 6, 61–64). Some basic information is presented in Scheme 1. In Figures 5–8 the EI mass spectra of a series of acetylated alditols obtained from different classes of monosaccharides are depicted: xylitol-[1-^{2}H], glucitol, fucitol-[1-^{2}H] and 2-acetamido-2-deoxy-glucitol-[1-^{2}H]. Under the usual EI mass spectrometric conditions, it is almost impossible to discriminate between stereochemical differences within one class of alditol acetates like pentitols, hexitols, heptitols, etc. Combination of the mass spectrometric data with the GC retention times enhances the reliability of the assignments. However, it has to be noted that owing to symmetry properties two different aldoses can yield the same alditol upon reduction (e. g. glucose and gulose or arabinose and lyxose), whereas ketoses are converted into two epimeric alditol structures. To differentiate between alditols obtained from aldoses and ketoses, the reduction of these monosaccharides can be carried out with NaB^2H_4 leading to deuterium-labeled C1 or C2 atoms, respectively (see also Figs. 5, 7 and 8).



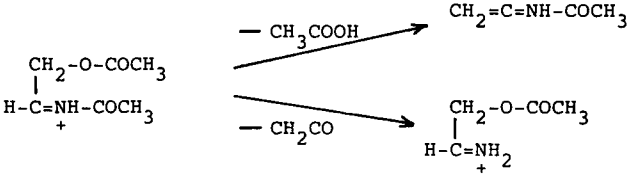
Scheme 1. General information on alditol acetate EI fragmentation (a–f).

(d)

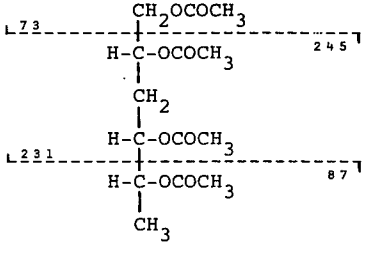


(cyclic form of a primary fragment)

(e)



(f)



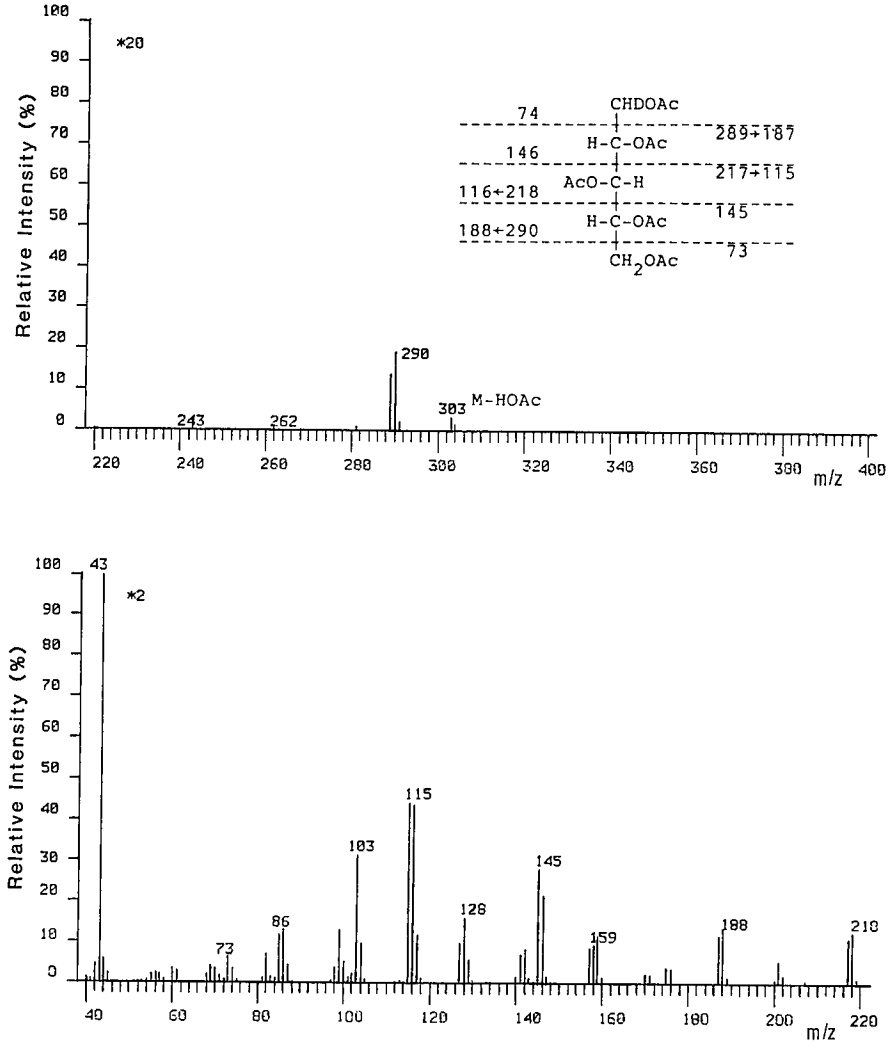


Figure 5. 70 eV EI mass spectrum of peracetylated xylitol-[1-^{2}H], as an example of a pentitol acetate.

Molecular ions are rarely observed in EI-MS. The first detectable fragment ion in the high-mass-region corresponds to the elimination of an acetoxyl radical or acetic acid from the molecular ion. For acetamido-alditols the elimination of acetamide cannot be excluded without high-resolution exact mass measurements (Fig. 8). As indicated in Scheme 1 and Figures 5–8, several primary fragmentations do occur, either by elimination of an acetoxyl group, e. g. as acetic acid, or by cleavage of the alditol chain. The primary fragments are degraded by elimination(s) of acetic acid, ketene, acetic anhydride or acetamide. The presence of methylene groups (deoxy functions) in the alditol chain as well as acetamido and methoxyl substituents have a

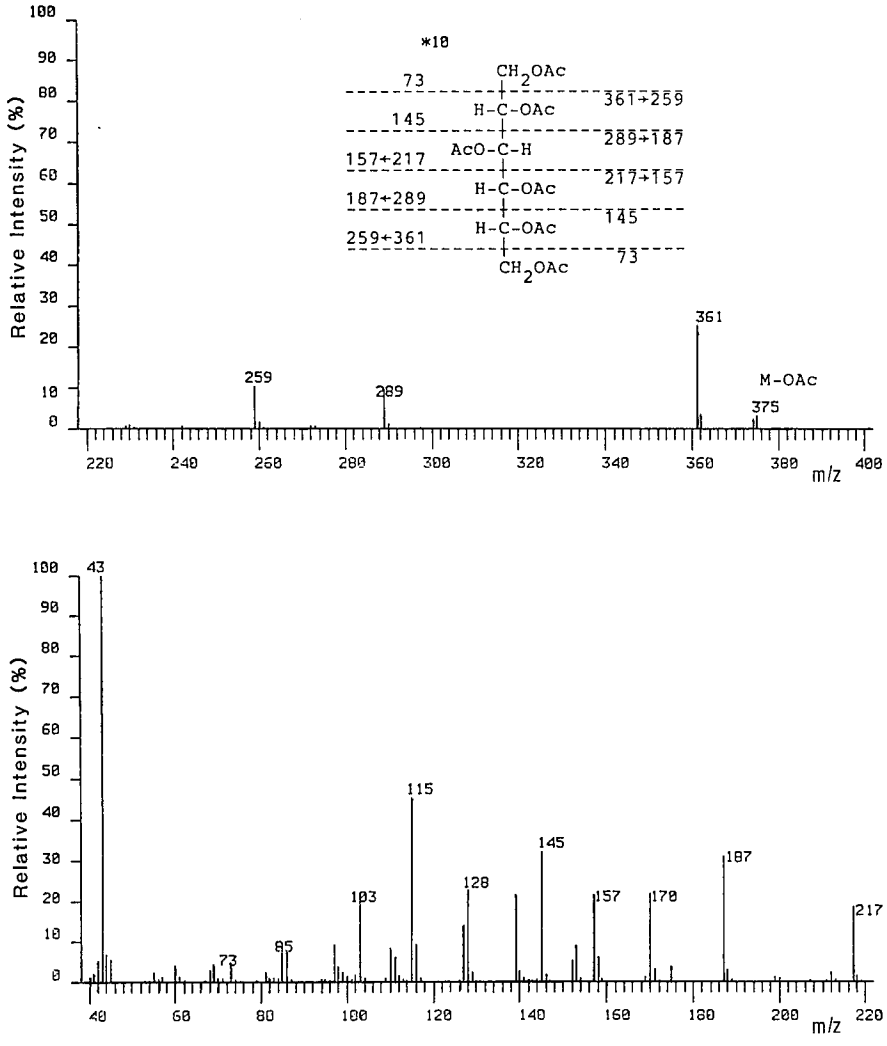


Figure 6. 70 eV EI mass spectrum of peracetylated glucitol, as an example of a hexitol acetate.

significant influence on the peak patterns of the mass spectra. The alditol chain of an acetylated deoxyalditol is little cleaved next to the methylene group. The occurrence of acetamido or methoxyl functions give rise to a high preference of cleavage next to the carbon atoms at which the specific functions are bound (see also methylation analysis section).

The use of NaB^2H_4 has already been mentioned above for discrimination purposes. When the carbohydrate chain contains *O*-methylated monosaccharides (12), it is also advisable to carry out the reduction with NaB^2H_4, making assignment of the C1 atom possible. Reduction of uronic acids, carried out with NaB^2H_4 in the presence of a

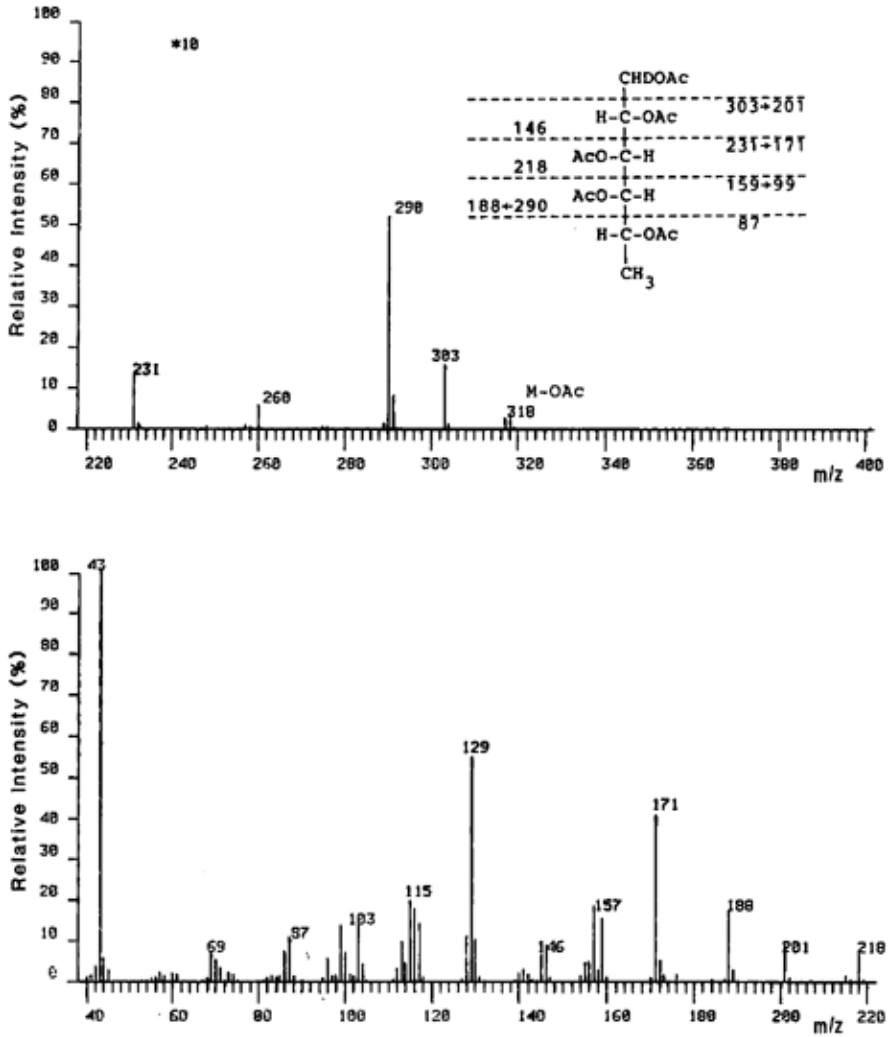


Figure 7. 70 eV EI mass spectrum of peracetylated fucitol-[1-^{2}H], as an example of a 6-deoxyhexitol acetate.

carbodiimide before hydrolysis (65, 66), yields an the alditol acetate analysis products with two deuterium atoms at C6. For the identification of *N*-acetyl groups of amino sugars and the determination of the degree of *N*-deacetylation in these sugars during acid hydrolysis, the acetylation should be carried out with trideuteroacetic anhydride (67).

Biological fluids contain several free alditols. To analyse these alditols by GC(-MS) use has been made of the corresponding peracetylated derivatives. A selected ion monitoring assay has been reported for the analysis of peracetylated mannitol, gal-

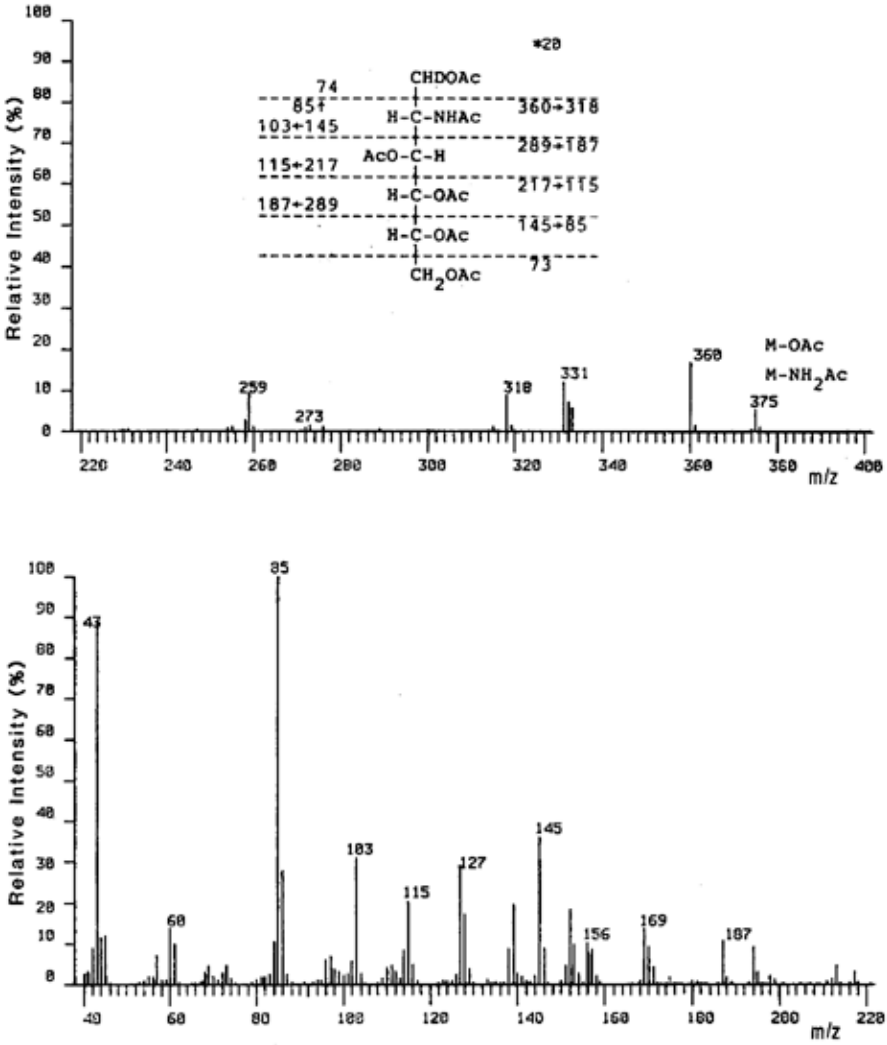


Figure 8. 70 eV EI mass spectrum of peracetylated 2-acetamido-2-deoxy-glucitol-[1-^{2}H], as an example of a *N*-acetyl-aminohexitol acetate.

actitol and glucitol (*m/z* 217 and *m/z* 259) as well as inositol (*m/z* 210) in serum, in urine and in amniotic fluid from pregnant women, applying iditol as an internal standard (68). A similar approach has been described for the determination of glucitol (*m/z* 361) in erythrocytes of diabetic and healthy subjects (58). For the determination of galactitol in amniotic fluid a selected ion monitoring method has been developed, utilizing GC-CI(NH_3)MS with galactitol-[6,6'-$^2H_2$] as internal standard (*m/z* 452 / *m/z* 454 ≡ $[M + NH_4]^+$) (69).

2.3 Methanolysis procedure/trimethylsilylated methyl glycosides

In an ampoule the carbohydrate material (0.1 – 2 mg) is mixed with a mannitol solution (internal standard; 10 – 100 nmol). After lyophilisation and drying over P_2O_5 in a vacuum desiccator for 18 h, the residue is dissolved in 1.0 mol · l^{-1} methanolic HCl (0.5 ml). Nitrogen is bubbled through the solution for 30 s, and then the ampoule is sealed. The solution is heated for 24 h at 85 °C; subsequently, neutralisation is carried out by addition of solid silver carbonate (pH-paper). *N*-(Re)acetylation is performed by the addition of acetic anhydride (10 – 50 µl). After mixing, the resulting suspension is kept at room temperature for 24 h in the dark. The precipitate is then triturated thoroughly and after centrifugation, the supernatant is collected. The residue of silver salts is washed twice with 0.5 ml dry methanol.

The pooled supernatants are evaporated under reduced pressure at 35 °C. In the case of glycolipids, fatty acid methyl esters can be removed by hexane extraction of the residue. The final residue is dried for 12 h in a vacuum desiccator over P_2O_5. Before GC-analysis, the sample is trimethylsilylated with a mixture of pyridine-hexamethyldisilazane-chlorotrimethylsilane (5:1:1; 100 µl) for 30 min at room temperature.

The quantitative sugar analysis is carried out on a CP-Sil 5 WCOT fused-silica capillary column (25 m × 0.32 mm) using flame-ionization detection with carrier gas nitrogen flow-rate 1.5 ml · min^{-1}, make-up gas nitrogen flow-rate 35 ml · min^{-1}, split-ratio of 1:10, injection-port temperature 210 °C, and the detector temperature 230 °C. The oven temperature is programmed from 130 °C to 220 °C at 2 °C · min^{-1} and kept isothermally at 220 °C for 1 min. A typical gas chromatogram of a standard mixture is presented in Figure 9.

Notes:

1. Methanolic HCl is prepared by bubbling HCl gas (obtained by mixing conc. HCl with conc. H_2SO_4) through cooled dry methanol. After determination of the substance concentration by titration, the solution is diluted with dry methanol until 1.0 mol · l^{-1} HCl, and stored in a desiccator at −18 °C.
2. The amount of added internal standard depends on the carbohydrate-content of the material analysed.
3. Under the applied conditions of methanolysis, the *N*-acyl group in amino sugars is cleaved nearly completely, giving rise to additional peaks in the neutral hexose region of the gas chromatogram. Therefore a *N*-(re)acetylation step is incorporated in the procedure. However, when too much acetic anhydride is added, one of the primary hydroxyl functions of mannitol is *O*-acetylated giving rise to an additional small peak in the gas chromatogram. The same holds for other alditols and for the primary hydroxyl function of the methyl ester methyl glycoside of *N*-acetylneuraminic acid.
4. The internal standard used should not occur naturally in the material being investigated. In this connexion it has to be noted that human urine and other physiological fluids contain mannitol (68 – 70).
5. Molar adjustment factors of monosaccharides, except *N*-acetylneuraminic acid, are determined by application of the methanolysis procedure on standard mixtures of free sugars and internal standard. For *N*-acetylneuraminic acid the molar adjustment factor is determined by subjecting a known sialo-oligosaccharide to methanolysis.

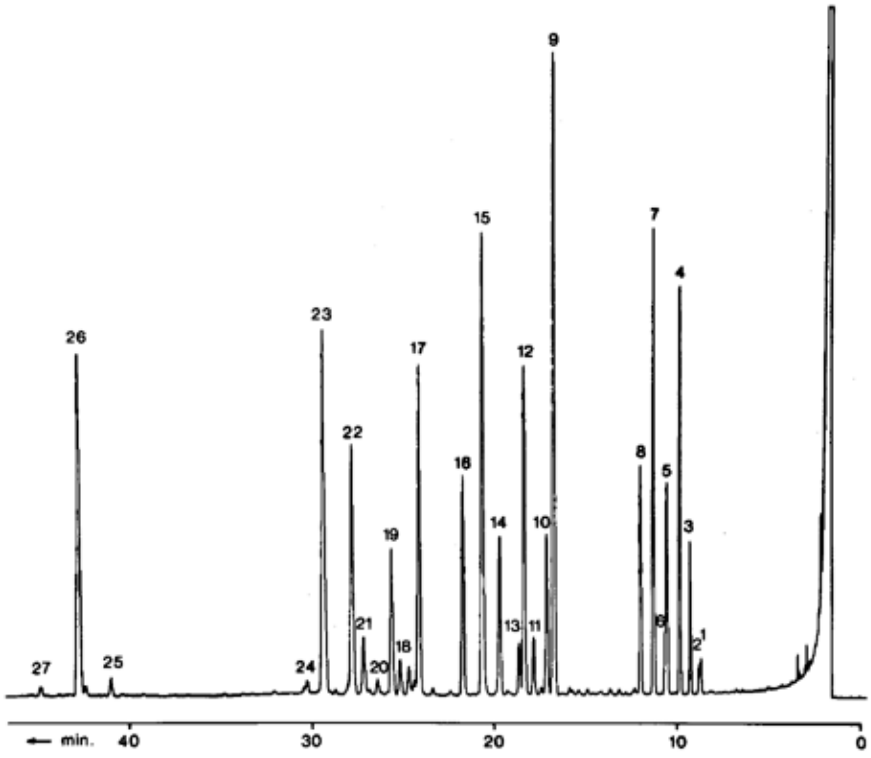
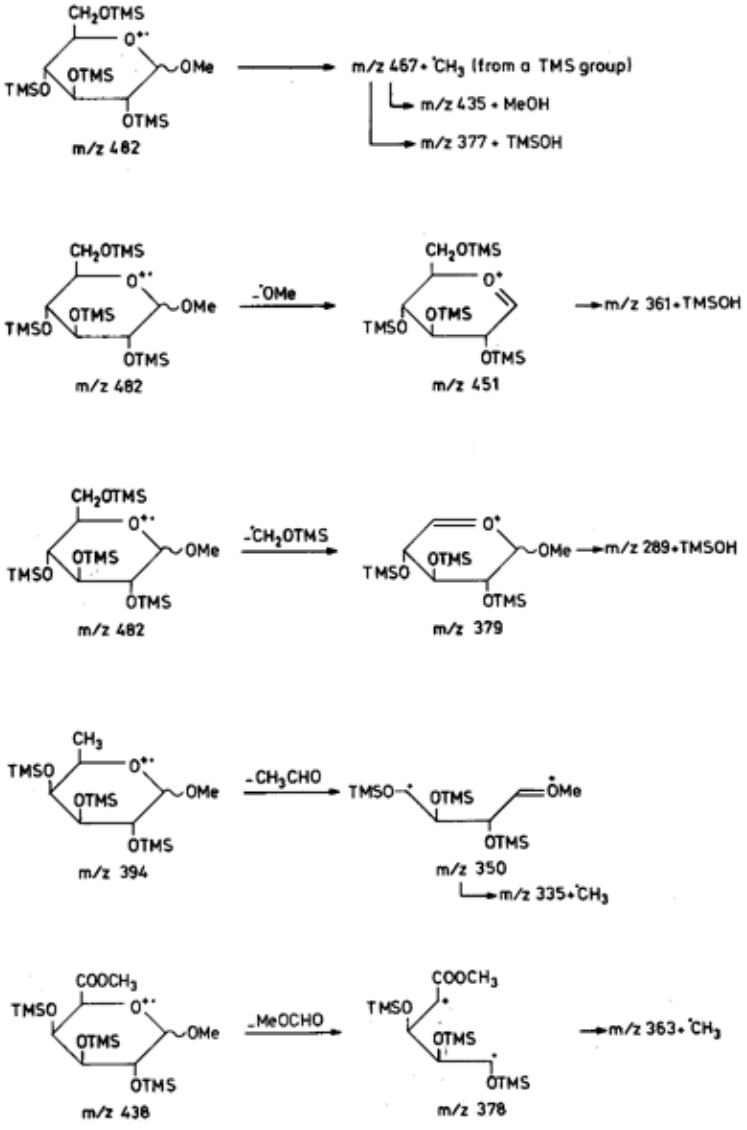
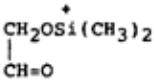


Figure 9. Gas chromatogram of trimethylsilylated (methyl ester) methyl glycosides on a CP-Sil 5 WCOT fused-silica capillary column (25 m × 0.32 mm). Oven temperature program: 130 °C to 220 °C at 2 °C · min^{-1}; 1 min at 220 °C. The peaks are numbered in their order of elution and are assigned as follows: (1) xylose (*β-f*); (2) xylose (*α-f*); (3) fucose (*β-f*); (4) fucose (*α-p*); (5) fucose (*β-p*); (6) fucose (*α-f*); (7) xylose (*α-p*); (8) xylose (*β-p*); (9) mannose (*α-p*); (10) galactose (*β-f*); (11) mannose (*β-p*); (12) galactose (*α-p*); (13) galactose (*α-f*); (14) galactose (*β-p*); (15) glucose (*α-p*); (16) glucose (*β-p*); (17) mannitol (internal standard); (18) *N*-acetylglucosamine (*α-f*); (19) *N*-acetylgalactosamine (*α,β-f*); (20) mono-*O*-acetyl-mannitol; (21) *N*-acetylglucosamine (*β-p*); (22) *N*-acetylgalactosamine (*α,β-p*); (23) *N*-acetylglucosamine (*α-p*); (24) *N*-acetylglucosamine (*α,β-p*; no methyl glycoside); (25) *N*-acetylneuraminic acid (*α*); (26) *N*-acetylneuraminic acid (*β*); (27) 9-*O*-acetyl-*N*-acetylneuraminic acid. *f*, furanoside; *p*, pyranoside.

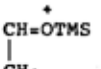
6. The sugar analysis of *N*-glycosidically bound carbohydrate chains of glycoproteins and glycopeptides (GlcNAc-Asn type) has shown that, under the usual conditions, the linkage between *N*-acetylglucosamine (GlcNAc) and asparagine (Asn) is split only to a very limited extent and mainly the free monosaccharide is liberated instead of its methyl glycoside. This has to be taken into account when calculating molar ratios.
7. Under the applied conditions of methanolysis several alditols give rise to anhydro derivatives, e. g. xylitol (23%), arabinitol (5%), fucitol (10%), glucitol (20%), galactitol (14%), 2-acetamido-2-deoxy-galactitol (35%) and the epimeric alditols



Scheme 2. General information on the fragmentation of trimethylsilylated methyl glycosides.



m/z 117



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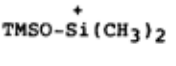
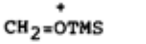
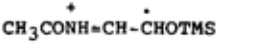


m/z 147



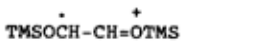
m/z 103

(is not specific for the presence of a primary hydroxyl function)



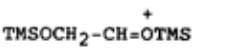
m/z 173

m/z 186



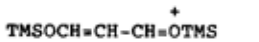
m/z 204

(major C2-C3, minor C3-C4 in pyranose ringforms)



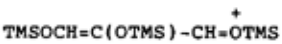
m/z 205

(C5-C6 fragment in furanose ringforms)



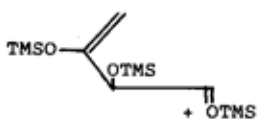
m/z 217

(mainly C2-C3-C4 in pyranose ringforms)



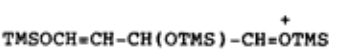
m/z 305

(mainly C2-C3-C4 in pyranose ringforms)



m/z 319

(major contribution in pyranose ringforms)



m/z 319

(furanose ringforms)

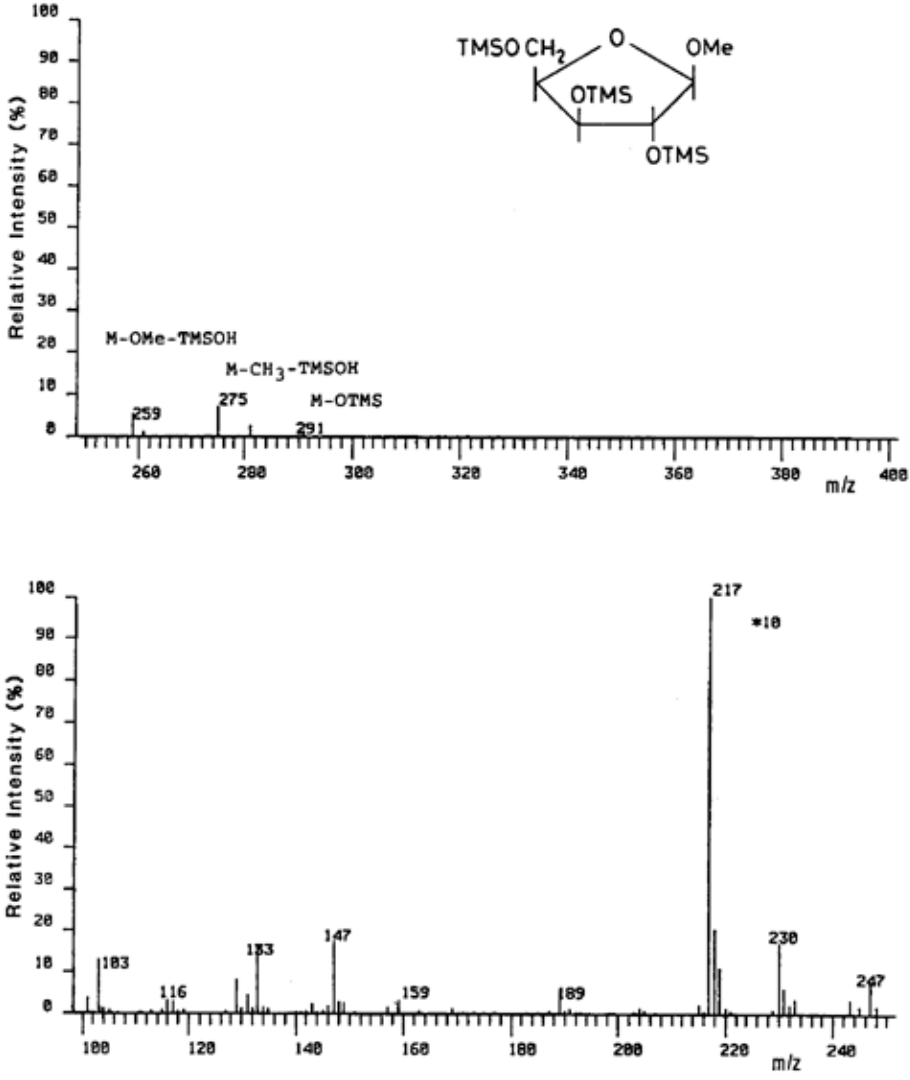


Figure 10. 70 eV EI mass spectrum of trimethylsilylated methyl β-D-xylofuranoside.

of *N*-acetylneuraminic acid (43%). Mannitol and 2-acetamido-2-deoxy-glucitol form little anhydride (<1%) (49). These findings are of importance for the structural analysis of *O*-glycosidic carbohydrate chains, which are linked to serine (Ser) or threonine (Thr) through *N*-acetylgalactosamine (GalNAc), and for the analysis of alditols together with aldoses from for instance physiological fluids. Mass spectra of trimethylsilylated 1,4-anhydro-D-xylitol-[1-^{2}H], 2,5-anhydro-D-xylitol-[1-^{2}H], 1,4-anhydro-L-fucitol-[1-^{2}H], 1,4-anhydro-D-galactitol-[1-^{2}H], 3,6-anhydro-D-galactitol-[1-^{2}H], 2-acetamido-3,6-anhydro-2-deoxy-D-galactitol-[1-^{2}H] and 2-acetamido-1,4-anhydro-2-deoxy-D-galactitol-[1-^{2}H] are included in (49).

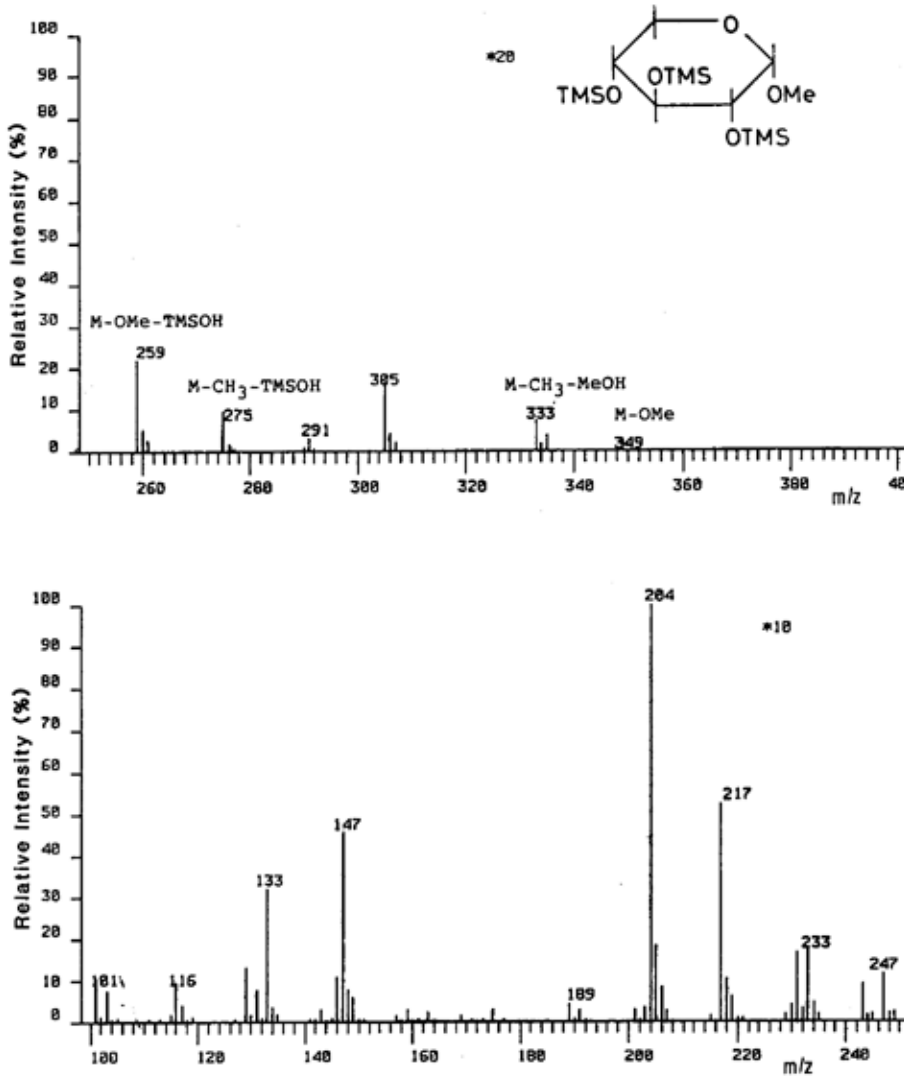


Figure 11. 70 eV EI mass spectrum of trimethylsilylated methyl α-D-xylopyranoside.

8. The applied methanolysis conditions lead to total degradation of fructose (2-ketohexose). Several uronic acids give rise to 3,6-lactone formation. Gulose yields also the 1,6-anhydride (30%) (71), whereas *N*-acetylneuraminic acid gives a little 2,7-anhydride (3%). Anhydro-formation has also been observed for heptoses (72). The cleavage of the rather stable glycosidic linkages of uronic acid residues may be incomplete, as in the hydrolysis procedure.
9. For data on 3-deoxy-D-*manno*-2-octulosonic acid (KDO), see (54, 73, 74).

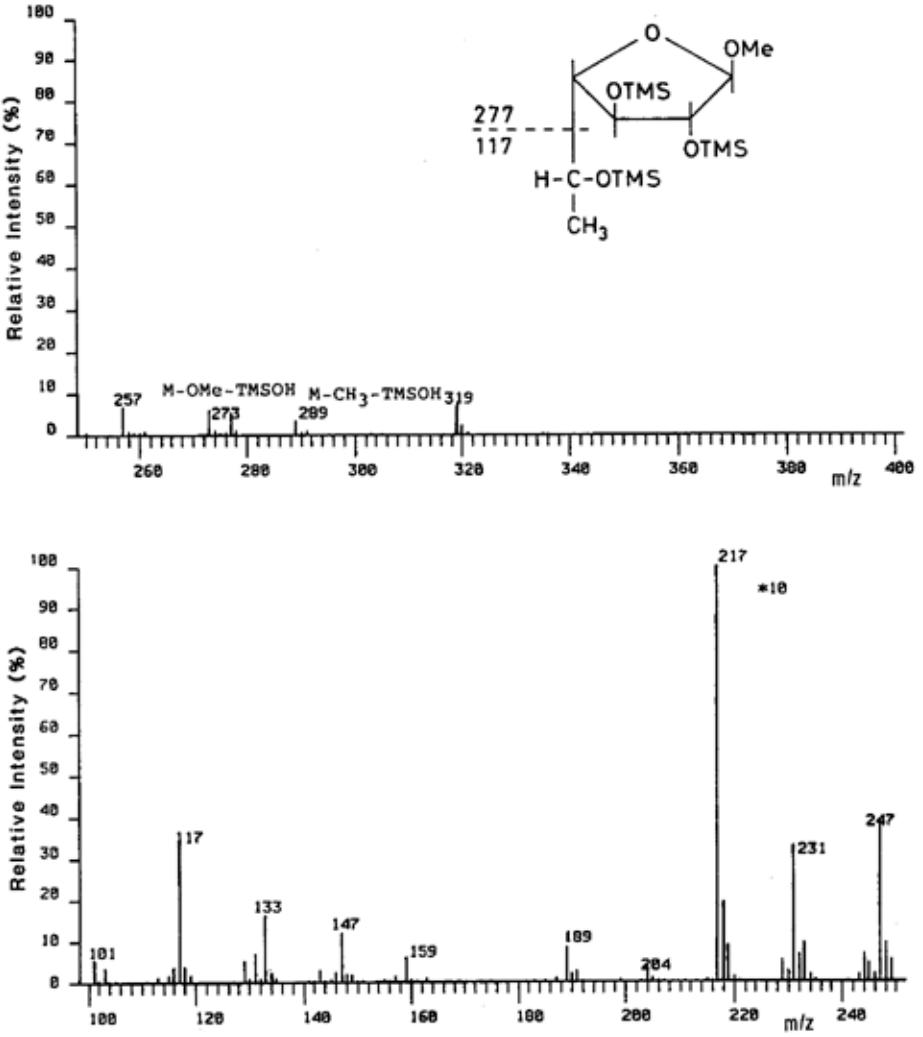


Figure 12. 70 eV EI mass spectrum of trimethylsilylated methyl β-D-fucofuranoside.

2.4 Mass spectrometry of trimethylsilylated methyl glycosides

The EI mass spectra of trimethylsilylated methyl glycosides have been studied in great detail (2, 3, 6, 7, 40, 75–82). Some basic fragmentation patterns are presented in Scheme 2. A series of examples of mass spectra obtained from different types of monosaccharides in furanose, pyranose and lactone forms is depicted in Figures 10–21. In the high-mass-region the spectra are usually characterised by [M – CH_3] fragment ions. Sometimes, the molecular ion itself is observed in low intensity. Just as for the alditol acetates, differences in stereochemistry within one class of monosaccharides are not reflected in the mass spectra.

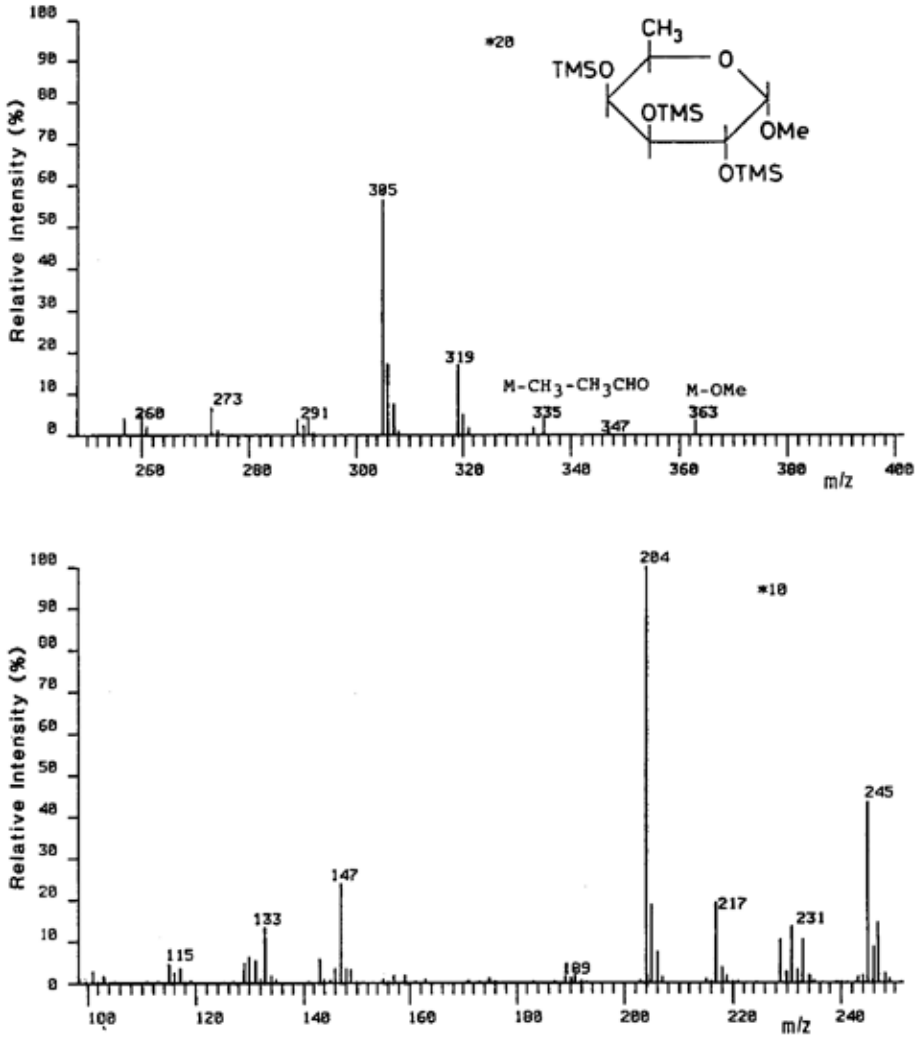


Figure 13. 70 eV EI mass spectrum of trimethylsilylated methyl α-D-fucopyranoside.

In general it is possible to discriminate between pyranose and furanose ring forms in one class of monosaccharides. For trimethylsilylated methyl aldohexosides it has been found that the ratio of the intensities of the peaks at m/z 204 and m/z 217 is useful (40, 76, 77). In pyranose ring forms the ion m/z 204 corresponds with the fragments $TMSO\overset{2}{C}H-\overset{3}{C}HOTMS$ (major) and $TMSO\overset{3}{C}H-\overset{4}{C}HOTMS$ (minor); m/z 217 corresponds mainly with $TMSO\overset{2}{C}H-\overset{3}{C}H-\overset{4}{C}HOTMS$. In furanose ring forms m/z 204 can be explained as $TMSO\overset{2}{C}H-\overset{3}{C}HOTMS$ and m/z 217 as $TMSO\overset{2}{C}H-\overset{3}{C}HOTMS-\overset{4}{C}H$ (83). The studied pyranose rings give rise to a ratio of $I_{204}/I_{217} > 1$,

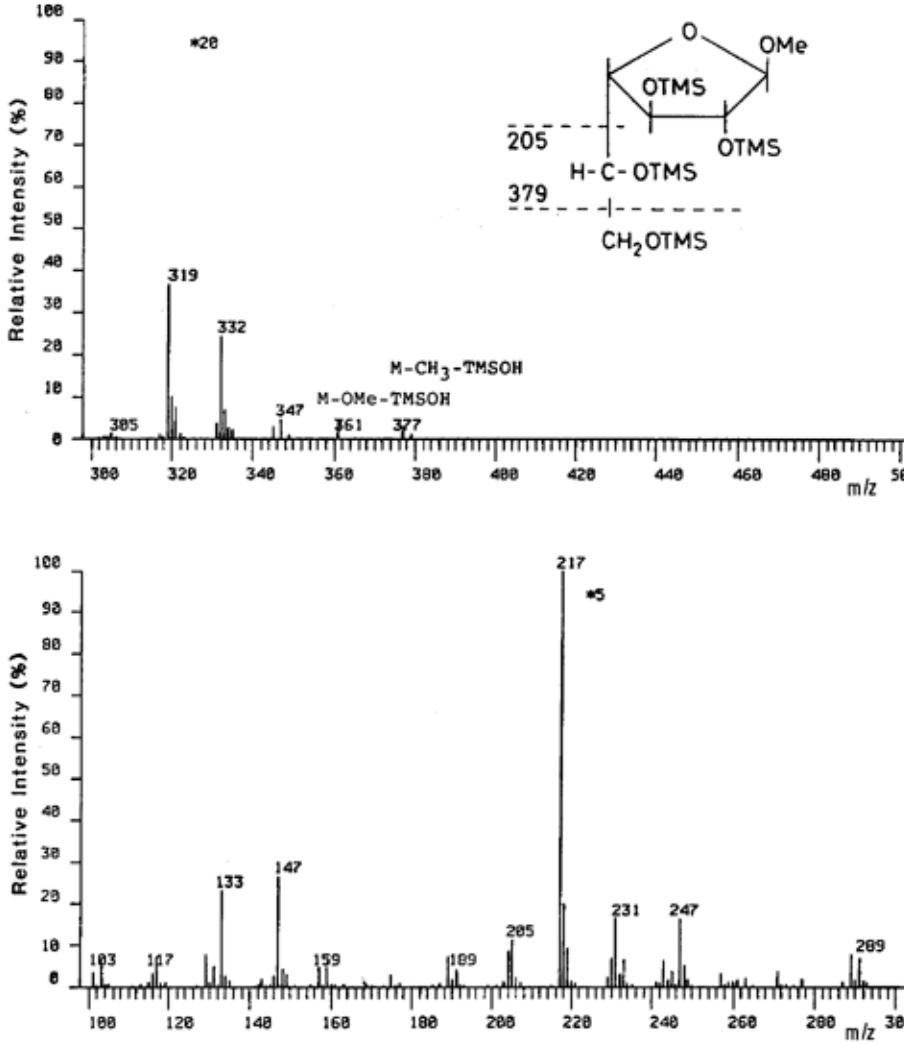


Figure 14. 70 eV EI mass spectrum of trimethylsilylated methyl β-D-galactofuranoside.

whereas the furanose rings show a ratio $\ll 1$. In addition the presence of a relatively intense peak at m/z 205 ($TMSO\overset{6}{C}H_2-\overset{5}{C}HOTMS$) is characteristic of a furanose ring. The concept of discrimination between pyranose and furanose ring forms on the basis of the intensities of m/z 204 and m/z 217 is also applicable to trimethylsilylated methyl glycosides of uronic acid methyl esters (78) and to most of the aldopentosides (40, 75, 77). In the same way for trimethylsilylated methyl 2-acetamido-2-deoxy-aldohexosides use can be made of the intensities of the fragment ions at m/z 173 ($CH_3CONH\overset{2}{C}H-\overset{3}{C}HOTMS$) and m/z 186 ($CH_3CONH\overset{2}{C}H-\overset{3}{C}H-\overset{4}{C}HOTMS$; $CH_3CONH\overset{2}{C}H-\overset{3}{C}HOTMS-\overset{4}{C}H$) (40, 77, 79, 81). A ratio of $I_{173}/I_{186} > 1$ points to

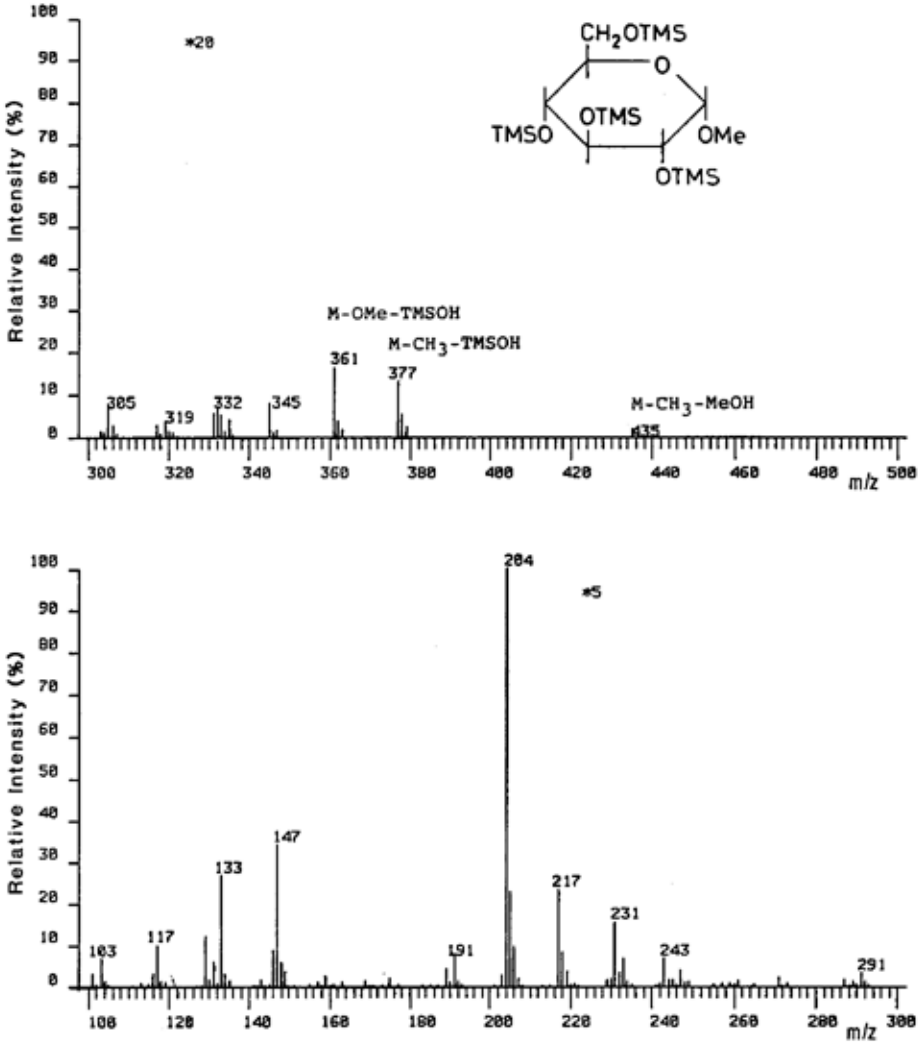


Figure 15. 70 eV EI mass spectrum of trimethylsilylated methyl α-D-glucopyranoside.

a pyranose ring form and a ratio of $I_{173}/I_{186} \ll 1$ to a furanose ring form. In the latter case also an intense peak at m/z 205 is present.

Naturally occurring *O*-methylated monosaccharides have also been analysed after applying the methanolysis procedure. The presence of *O*-methyl groups at specific positions have a distinct influence on the EI mass spectrometric fragmentation patterns (12, 71, 75–77, 79, 84). Especially the fragment ions mentioned above together with those in the high-mass-region are very diagnostic. Frequently, the introduction of deuterated trimethylsilyl, *N*-acetyl and/or methyl groups in the monosaccharide is very helpful for the interpretation of mass spectra of unknown monosaccharide residues.

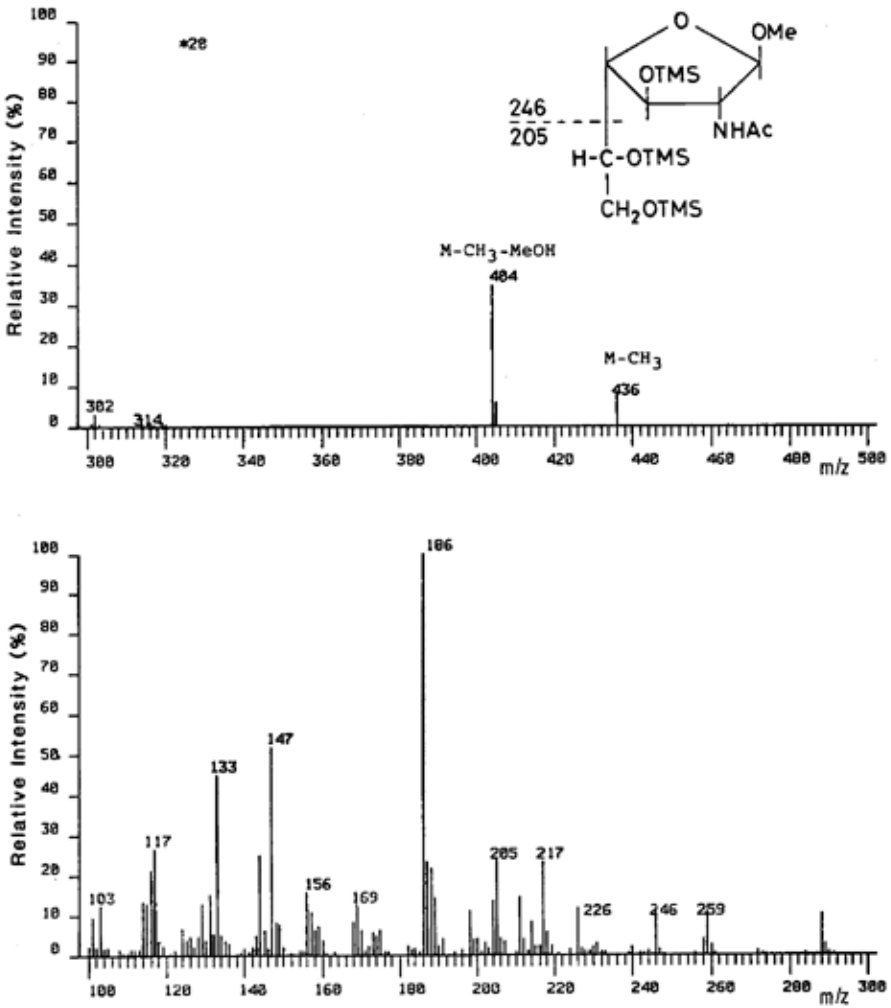


Figure 16. 70 eV EI mass spectrum of trimethylsilylated methyl β-*N*-acetyl-D-galactofuranoside.

2.5 Mass spectrometry of trimethylsilylated alditols and anhydro-alditols

The EI mass spectra of a trimethylsilylated hexitol and a trimethylsilylated 2-acetamido-2-deoxy-hexitol are presented in Figures 22 and 23, respectively. The amino hexitol derivative is frequently observed when analysing carbohydrate chains derived from GlcNAc-Asn type glycoproteins subjected to the hydrazinolysis procedure and from GalNAc-Ser/Thr type glycoproteins subjected to the β-elimination procedure, respectively. Some specific framentations have been reported (85). As indicated already above, a series of EI mass spectra of anhydro-alditols obtained from different alditols have been discussed (49).

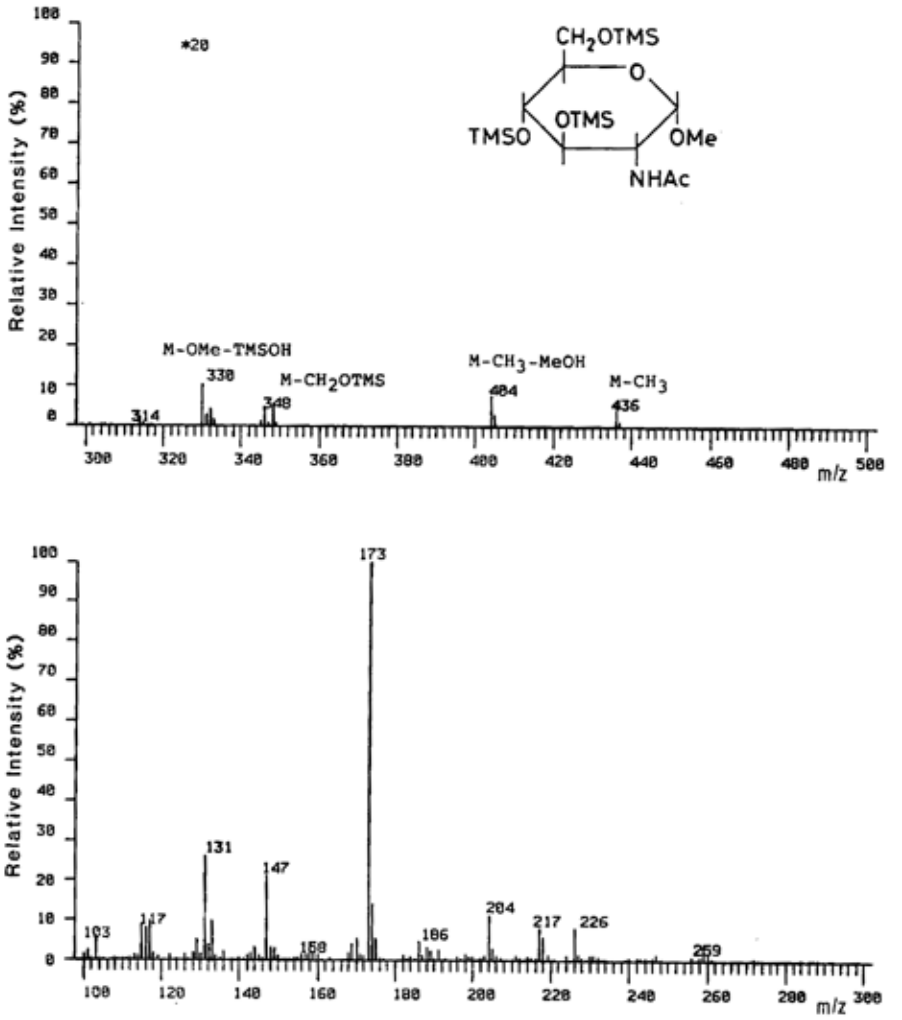


Figure 17. 70 eV EI mass spectrum of trimethylsilylated methyl α-*N*-acetyl-D-glucopyranoside.

In connexion with the analysis of alditols in uremic serum by GC-MS, attention has been given to the use of CI-MS with isobutane as reagent gas (86). The latter study also describes the analysis of aldoses and aldonic acids as their trimethylsilylated derivatives. For additional CI studies of trimethylsilylated aldohexoses (NH_3 as reagent gas) and amino sugars (CH_4 as reagent gas), see (87) and (88), respectively. Another GC-MS study of the polyols present in urine and serum of healthy subjects and uremic patients has led to the finding of several new deoxyalditols and inositol isomers. Both EI- and CI-MS were used for the analysis of the trimethylsilylated derivatives (89). For additional MS data of trimethylsilylated (and acetylated) inositols and trimethylsilylated naturally occurring *O*-methylinositols, see (90) and (91), respectively.

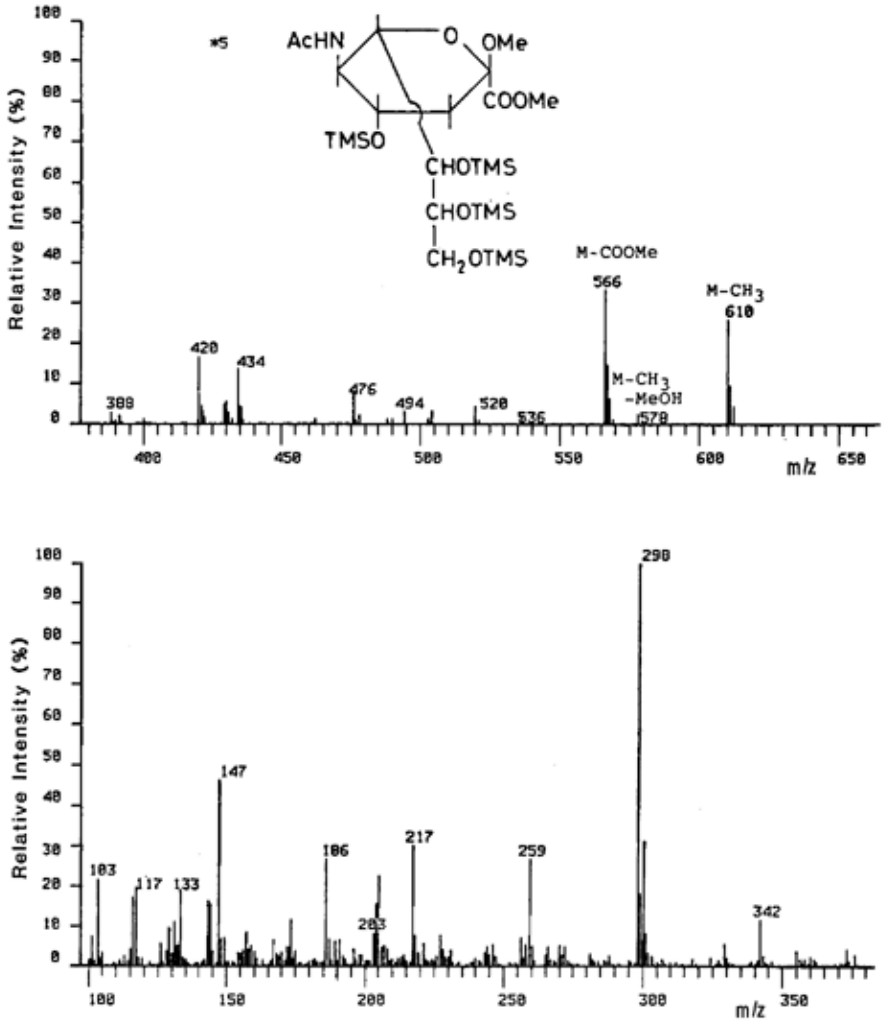


Figure 18. 70 eV EI mass spectrum of trimethylsilylated methyl ester β-methyl glycoside of *N*-acetylneuraminic acid.

2.6 GC-MS of *N,O*-acylneuraminic acids

The analysis of *N,O*-acylneuraminic acids (sialic acids) requires special attention to retain the *N*- and *O*-acyl substituents during isolation and analysis procedures. Routine sugar analysis methods as described above lead to a complete *N*- and *O*-deacylation of sialic acids. For this reason several attempts have been made to develop analytical methods keeping the specific sialic acids unimpaired (36, 92). Sialic acids are released in good yield under mild acidic conditions like formic acid, pH 2 (60 min, 70 °C). These conditions do not cause *N*-deacylation, whereas only 20–30% of the *O*-acyl groups are lost. Also the possibility of *O*-acyl migration has to be taken into account.

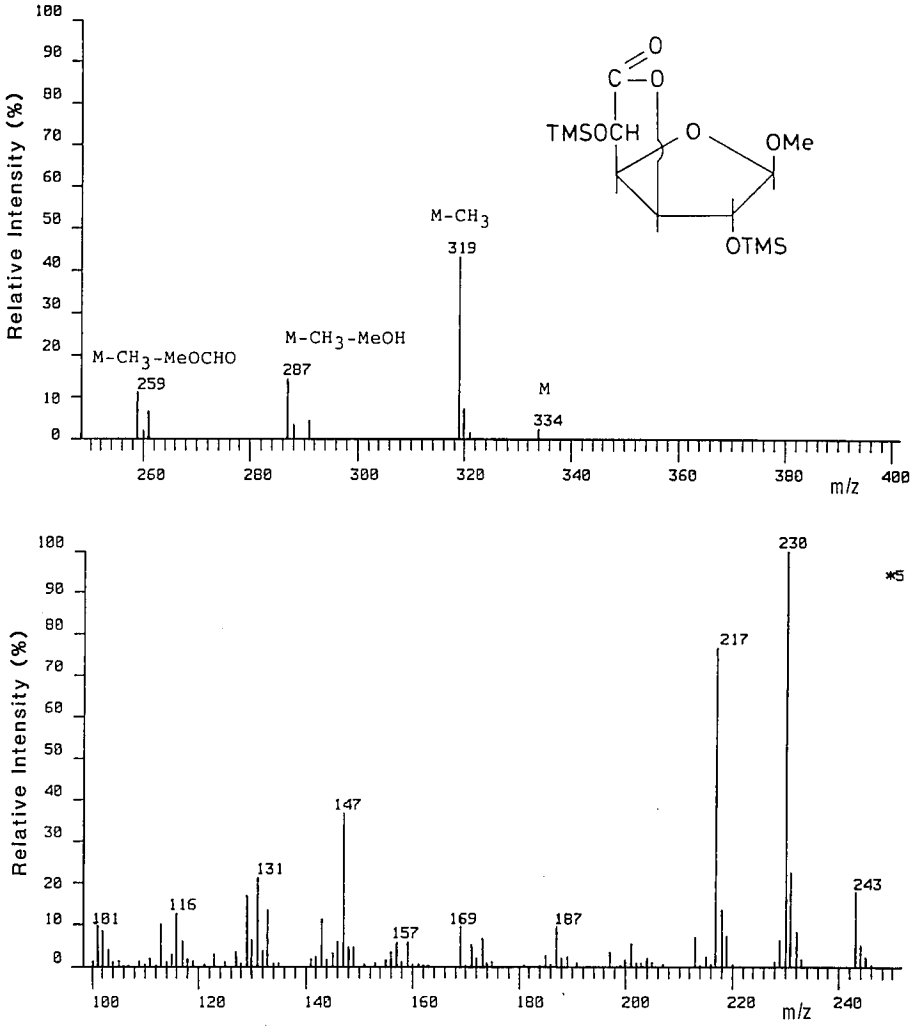


Figure 19. 70 eV EI mass spectrum of trimethylsilylated methyl β-D-glucofuranurono-6,3-lactone.

In several cases enzymic cleavages by neuraminidase have been applied (92). The released sialic acids are isolated by ultrafiltration or dialysis at 2–4 °C. Purification and separation of the isolated sialic acid fraction is based on various chromatographic techniques (14, 92).

Sialic acids are currently analysed by GC-MS after esterification with diazomethane followed by trimethylsilylation of the free hydroxyl groups, or after trimethylsilylation of carboxyl and free hydroxyl groups (36, 93–95). Depending on the applied silylation reagent, also the secondary amide group (*N*-acyl group) can be trimethylsilylated (96). Because of partial *O*-deacylation of the *N,O*-acylneuraminic acids and/or migration of the *O*-acyl groups in the glycerol side chain (7-*O*-acetyl → 9-*O*-acetyl; 7,9-di-*O*-acetyl → 8,9-di-*O*-acetyl) (96a) which can occur during isolation procedures, quanti-

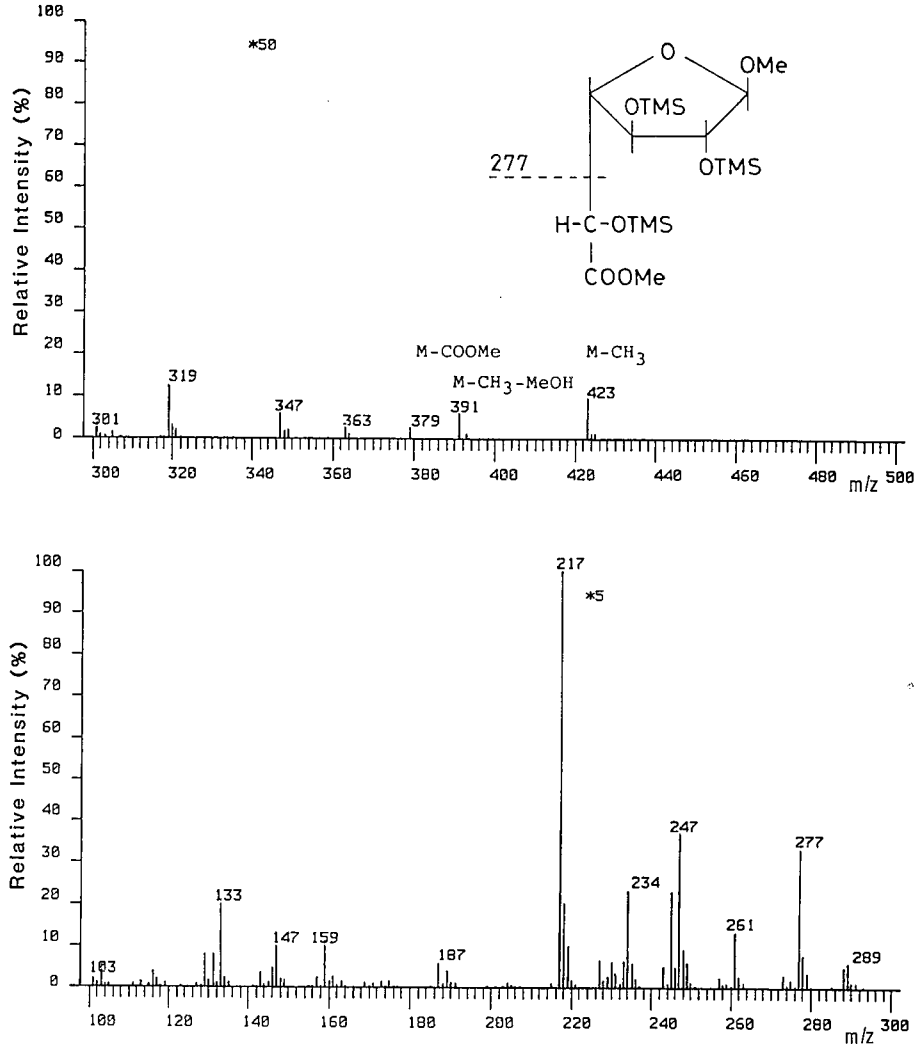


Figure 20. 70 eV EI mass spectrum of trimethylsilylated methyl ester methyl β-D-galactofuranosiduronic acid.

tative analysis of the various *N,O*-acylneuraminic acids present in native biological material is still a serious problem. For quantitative determinations based on GC-MS, see (36, 97).

The determination of the number, type and position of *O*-acyl groups (and also of *O*-alkyl groups) as well as of the type of the *N*-acyl group in sialic acids is facilitated by the highly specific EI mass spectra of these derivatised compounds. Based on the spectra of the trimethylsilylated methyl esters a micromethod using seven selected fragment ions A–G has been reported, which furnish the information (abundances and *m/z* values of the ions) necessary to deduce the complete structure of the sialic

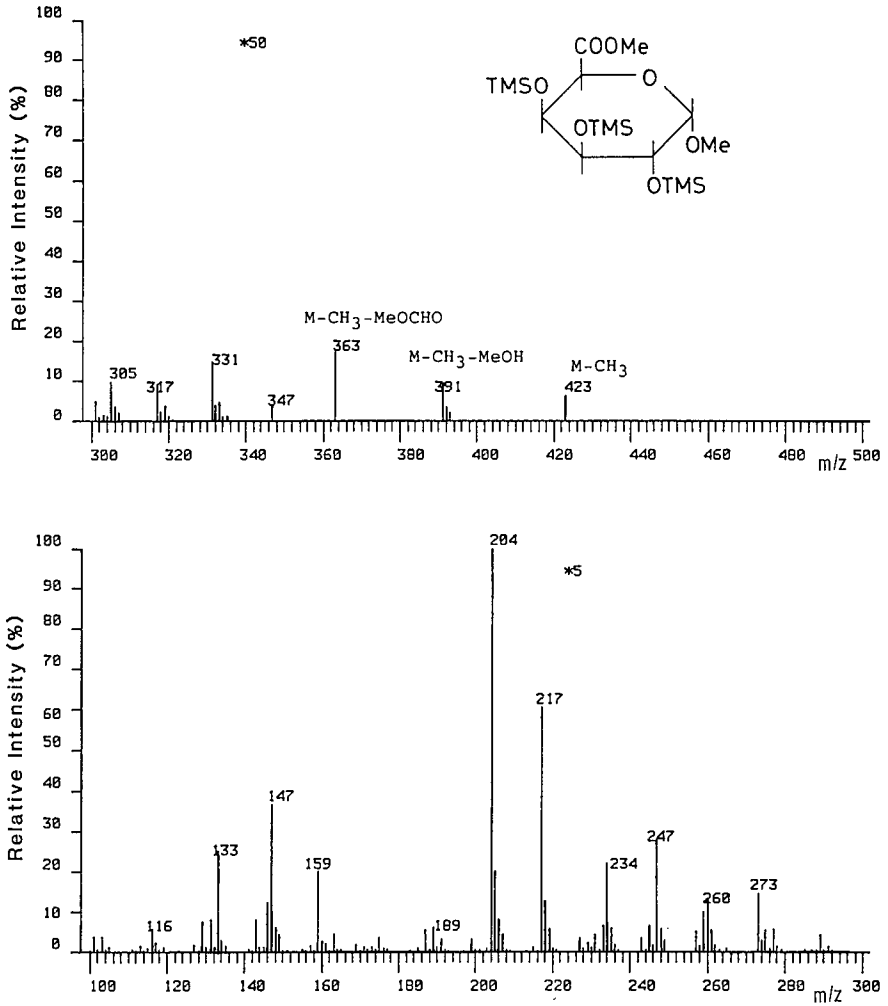


Figure 21. 70 eV EI mass spectrum of trimethylsilylated methyl ester methyl α-D-galactopyranosiduronic acid.

acids (Scheme 3). In Figure 24 the mass spectrum of one example, namely the trimethylsilylated methyl ester of *β-N*-acetylneuraminic acid, is depicted. Although it has not been checked in detail, it is highly probable that the use of trimethylsilyl esters instead of methyl esters do not change the identification procedure. As will be discussed in the methylation analysis part of this chapter, the scheme is also of value in determining substitution patterns of sialic acids in carbohydrate chains. In the latter case an additional fragment H is included (36).

Fragments A and B indicate the molecular weight of the sialic acid derivatives and thereby the number and type of substituents. Fragments C-H can be used for determination of the positions of the different substituents (36).

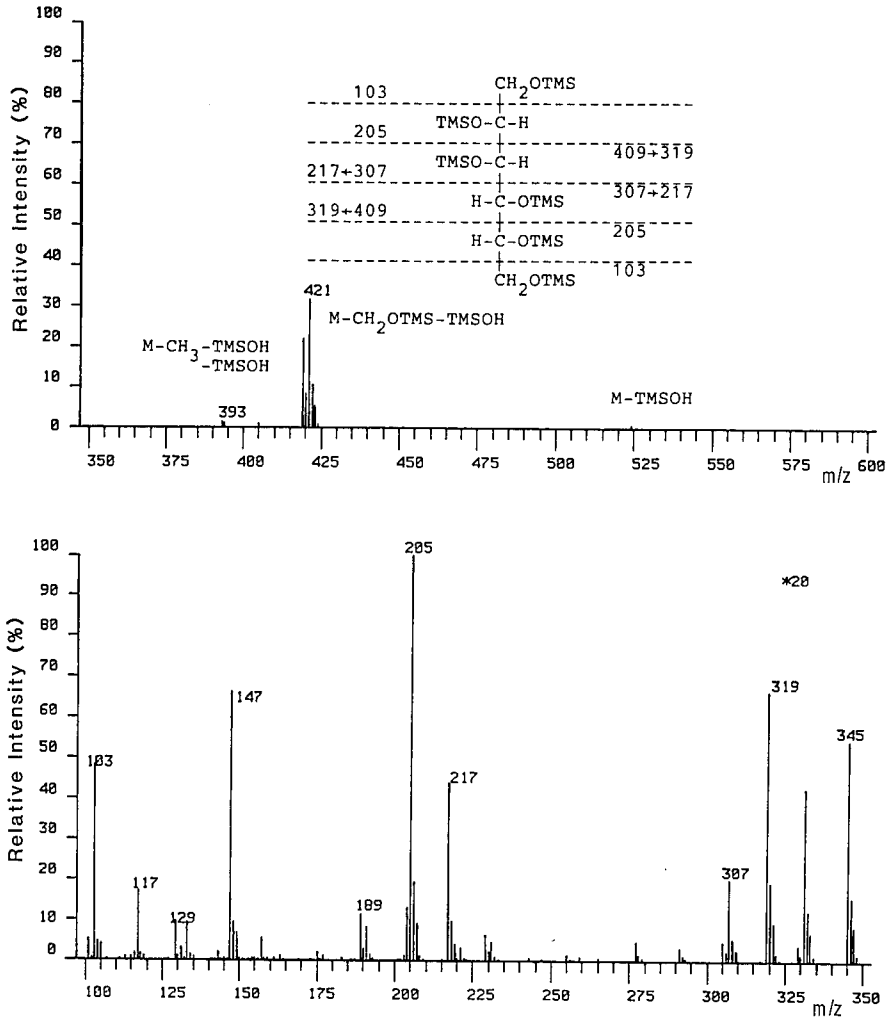


Figure 22. 70 eV EI mass spectrum of trimethylsilylated mannitol.

Fragment A is formed from the molecular ion by the elimination of a methyl group originating from a trimethylsilyl substituent in trimethylsilylated (*O*-acylated) *N*-acylneuraminic acid derivatives. When $R_{5'} = CH_3$ (methylation analysis procedure), the eliminated methyl group can also originate from the *N,N*-acyl,methyl group.

Fragment B is formed by elimination of the C1 part of the molecule. Eliminations of $OCOCH_3$ in *O*-acetylated sialic acid derivatives and of NH_2COCH_3 in *N*-acetylneuraminic acid derivatives, which in principle give rise to the same *m/z* value as fragment B in the case of $R_1 = CH_3$, can be neglected.

Fragment C is formed by elimination of C8-C9, with localisation of the charge on position 7. In general, cleavage occurs between two alkoxylated carbon atoms, or between an acetoxylated and an alkoxylated carbon atom, rather than between two

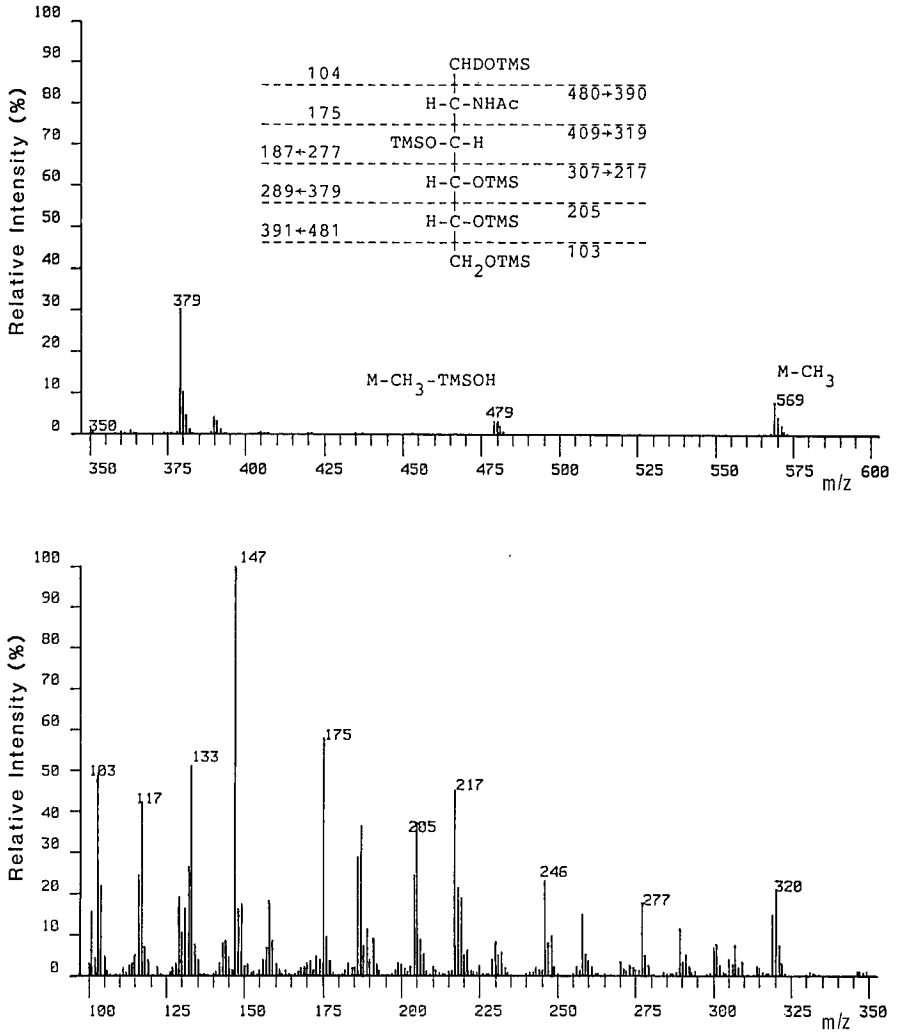
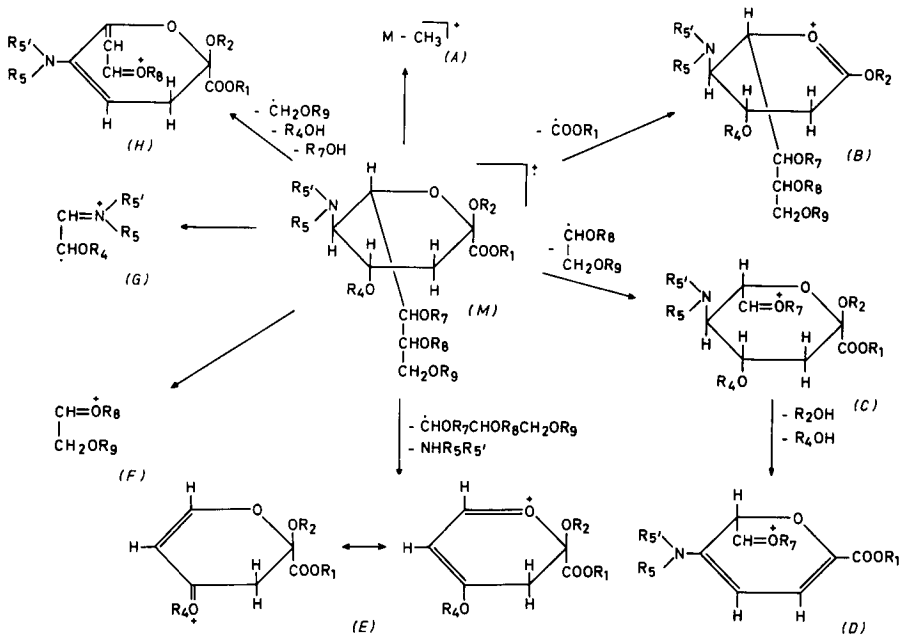


Figure 23. 70 eV EI mass spectrum of trimethylsilylated 2-acetamido-2-deoxy-glucitol-[1-^{2}H].

acetoxylated carbon atoms. It has been found that the charge is preferentially located on an ether oxygen instead of on an ester oxygen (6). Therefore fragment C has only significant abundance if C7 bears an ether group. When an ester group is present at C7, this fragment ion is absent or hardly observable.

Fragment D is formed from fragment C by consecutive eliminations of R_2OH and R_4OH. It is evident that the occurrence of this fragment ion is dependent on the presence of fragment C.

Fragment E is formed by elimination of the whole side-chain C7-C8-C9 and the substituent at C5. This fragment ion is not observed if an *O*-acyl group is attached to C4, illustrating that the transition state in the McLafferty rearrangement is more favoured when the substituent at C4 is an ether group rather than an ester group.



R_1 = CH_3

R_2 = CH_3 or $Si(CH_3)_3$

R_4, R_7, R_8, R_9 = CH_3, $Si(CH_3)_3$, $COCH_3$ and / or $COCH[OSi(CH_3)_3]CH_3$

R_5 = $COCH_3$ or $COCH_2OSi(CH_3)_3$

$R_{5'}$ = H or CH_3

Scheme 3. Survey of the selected fragment ions A-H determined for trimethylsilylated (*O*-acylated) *N*-acylneuraminic acid methyl ester(s) (methyl glycosides) and for (partially) *O*-methylated *N,N*-acetyl,methyl-neuraminic acid methyl ester methyl glycosides. Partially *O*-methylated compounds are trimethylsilylated or acetylated.

Fragment F contains C8-C9. Based on the same fragmentation rules as mentioned above for fragments C and D, this ion can only readily be formed if an ether group is attached to C8.

Fragment G consists of the C4-C5 part of the molecule.

Fragment H, only necessary to use in methylation analysis studies, is formed by elimination of the C9 part, followed by elimination of R_4OH and R_7OH. The fragment is useful to discriminate between an OTMS group at C8 or C9 in trimethylsilylated partially methylated *N*-acylneuraminic acids.

A series of naturally occurring *N,O*-acylneuraminic acids analysed by GC-MS as their trimethylsilylated methyl ester derivatives is summarized in Table 1. The Table includes retention times on 3.8% SE-30 and the *m/z* values of the diagnostic fragments A-G. For more detailed information including high-resolution exact mass measurements and the EI mass spectra of the various sialic acid derivatives, see (36).

Chemical ionization mass spectra of derivatised and underivatised sialic acids have also been reported (97a, 97b).

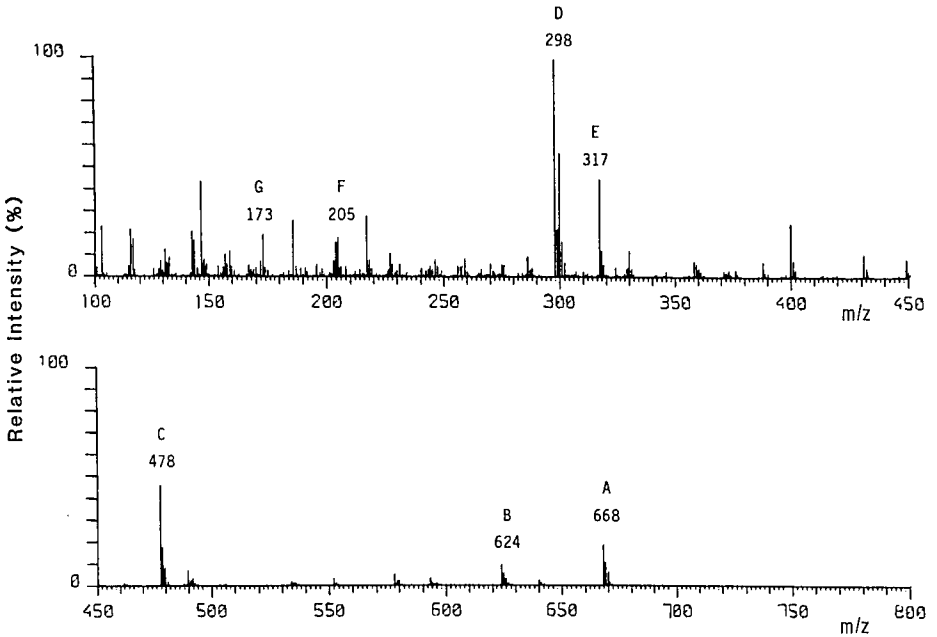


Figure 24. 70 eV EI mass spectrum of the trimethylsilylated methyl ester of *β-N*-acetylneuraminic acid.

2.7 Absolute configuration determination of monosaccharides

Monosaccharides can occur both as D- and L-enantiomers. It is even possible that the D- and L-enantiomers of one sugar form part of one carbohydrate chain (12). The resolution of racemic mixtures on GC can be achieved on a non-chiral phase after conversion of the enantiomers into diastereomers by glycosidation with a chiral alcohol, and subsequent protection of additional polar functions. Two approaches have been reported for the resolution of D- and L-monosaccharides: 1. GC(-MS) of the trimethylsilylated (−)-2-butyl glycosides (55, 57), and 2. GC of the acetylated (+)-2-octyl glycosides (56). In the case of the trimethylsilylated (−)-2-butyl glycosides attention has been given to the EI mass spectral data in relation to pyranose and furanose ring forms (55, 57). These GC(-MS) approaches can be applied directly to mixtures of monosaccharides, and can be incorporated easily in the sugar analysis procedure using methanolysis (see above).

2.8 Miscellaneous data

By using the trimethylsilylated methyloxime derivatives of glucose and glucose-[$^2H_7$], a mass fragmentographic reference method has been developed for the determination of serum glucose (98). *n*-Butyldiboronate derivatives of mannitol and glucitol have been applied for the simultaneous determination of these alditols in plasma with GC-

Table 1. GLC and MS data of trimethylsilylated (*O*-acylated) *N*-acylneuraminic acid methyl esters. The R_{Neu5Ac}-values on 3.8% SE-30 at 215 °C are given relative to the trimethylsilylated methyl ester of Neu5Ac (β-anomeric form). For an explanation of the '-' signs, see text. EI-mass spectra are depicted in (36).

Sialic acid*	R_{Neu5Ac} β-anomer	R_{Neu5Ac} α-anomer	*m/z* values						
			A	B	C	D	E	F	G
Neu5Ac	1.00	1.05	668	624	478	298	317	205	173
Neu4,5Ac_2	1.18	1.21	638	594	448	298	–	205	143
Neu5,7Ac_2	1.04	1.00	638	594	–	–	317	205	173
Neu5,8Ac_2	1.05		638	594	478	298	317	–	173
Neu5,9Ac_2	1.13		638	594	478	298	317	175	173
Neu4,5,9Ac_3	1.31		608	564	448	298	–	175	143
Neu5,7,9Ac_3	1.14	1.07	608	564	–	–	317	175	173
Neu5,8,9Ac_3	1.19		608	564	478	298	317	–#	173
Neu5,7,8,9Ac_4	1.15		578	534	–	–	317	–	173
Neu5Ac9Lt	2.55		740	696	478	298	317	277	173
Neu4,5$Ac_2$9Lt	3.01		710	666	448	298	–	277	143
Neu5Gc	1.81	1.90	756	712	566	386	317	205	261
Neu4Ac5Gc	2.02		726	682	536	386	–	205	231
Neu7Ac5Gc	1.83		726	682	–	–	317	205	261
Neu9Ac5Gc	2.04		726	682	566	386	317	175	261
Neu7,9$Ac_2$5Gc	2.01		696	652	–	–	317	175	261
Neu8,9$Ac_2$5Gc	1.99		696	652	566	386	317	–#	261
Neu7,8,9$Ac_3$5Gc	1.93		666	622	–	–	317	–	261

* Neu5Ac = *N*-acetylneuraminic acid; Neu5Gc = *N*-glycolylneuraminic acid; Neu4,5Ac_2 = 4-*O*-acetyl-*N*-acetylneuraminic acid; Neu5Ac9Lt = 9-*O*-lactyl-*N*-acetylneuraminic acid; etc.

\# The small peak at *m/z* 145, also present in the mass spectra of other sialic acids, has not been checked by exact mass measurements (93).

mass fragmentography (99). The isotopic dilution technique was used for determining the content of myo-inositol (as hexa-acetate) in human urine, plasma, and haemolysed erythrocyte samples using myoinositol-[$^2H_6$] as standard (100). Application of EI- and CI-MS for the GC-analysis of sugar components in human eye lenses has led to the identification of a heptitol and an octitol besides several other alditols (101).

3 Methylation Analysis

To gain insight into the substitution pattern (positions of glycosidic linkages, terminal units, branching points) and into the ring size of the monomeric units of a carbohydrate chain, methylation analysis including GC-MS has proved to be of great importance. A frequently applied procedure for neutral and acetamido sugars is presented in Figure 25. The first step involves the methylation of the free hydroxyl groups and of

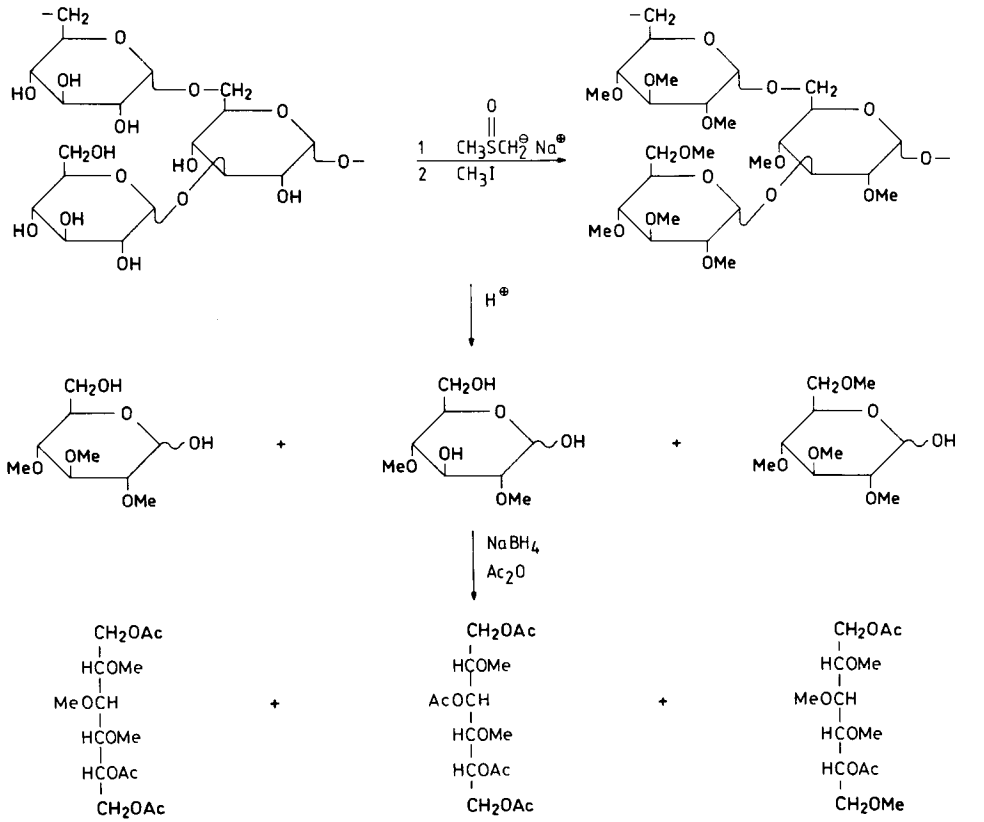
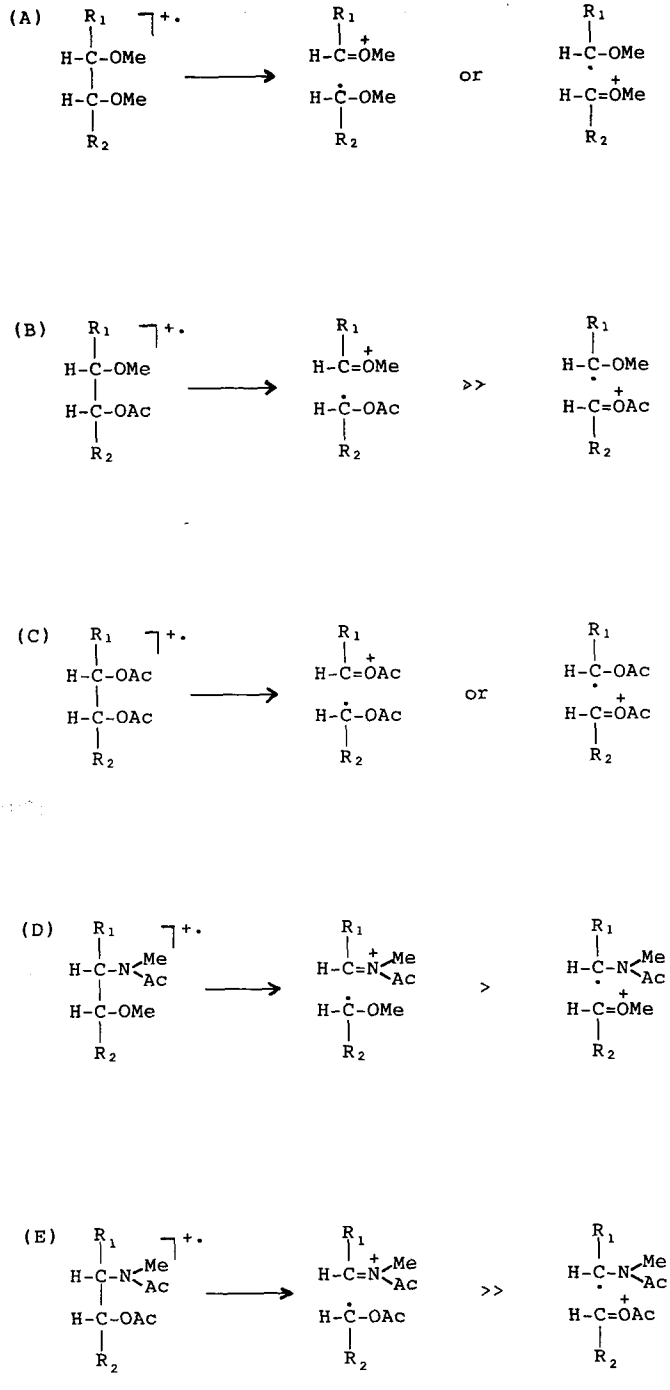


Figure 25. Schematic presentation of the methylation analysis of an artificial polysaccharide.

the acetamido groups. Carboxyl functions of uronic acids, sialic acids, etc. are esterified. For a review of the various developed methylation methods, see (5). This reaction is carried out most often with methyl iodide using sodium methylsulfinylmethanide in dimethylsulfoxide as base (102–106). Recently other bases have been applied to this methylation, e. g. potassium *tert*-butoxide/potassium methylsulfinylmethanide in dimethylsulfoxide (107), and potassium (108, 109) and lithium (110, 110a, 110b) methylsulfinylmethanide in dimethylsulfoxide, mainly because the latter reagents seem to suffer less from impurities when compared with the sodium salt preparation. In addition, the use of methyl trifluoromethanesulfonate in trimethylphosphate with 2,6-di-(*tert*-butyl)pyridine as proton scavenger has been mentioned (111). Although a complete methylation in a single operation is essential for reliable results, it is sometimes impossible to overcome a certain degree of undermethylation, even after several successive methylation steps. When uronic acid-containing carbohydrate chains are investigated, repeated treatment with base leads to β-elimination reactions in the carbohydrate chain (112). Such alkaline degradations can also be expected if reducing carbohydrates are methylated in the presence of strong bases as mentioned above.

After permethylation the material is hydrolysed, yielding a mixture of partially methylated monosaccharides. Routine solvolysis conditions are e. g. 90% formic acid/



Scheme 4. Genral information on the fragmentation of partially methylated alditol acetates.

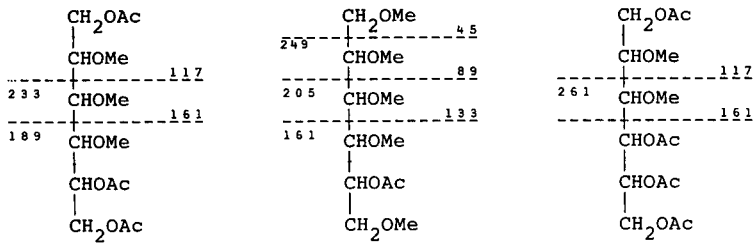
(F) $CH_3-C\equiv\overset{+}{O}$ (m/z 43)

Generally the most intense ion, derived from the acetyl function by α-cleavage

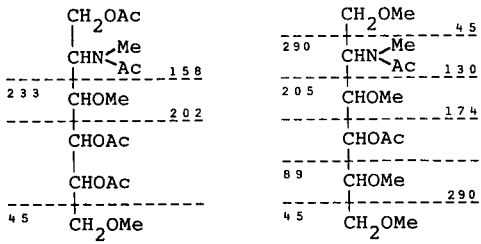
(G) $CH_2=\overset{+}{O}Me$ (m/z 45)

Not characteristic for a primary methoxyl group, but can also be formed by migration of a hydrogen atom to any charged one-carbon fragment bearing an OMe group

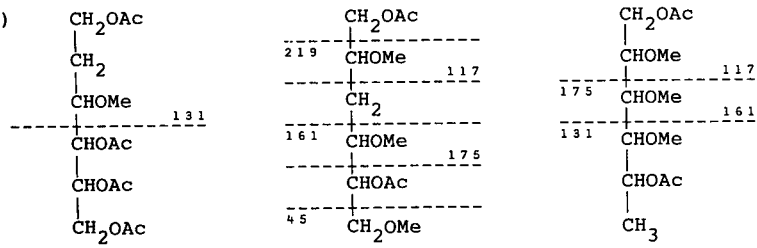
(H)

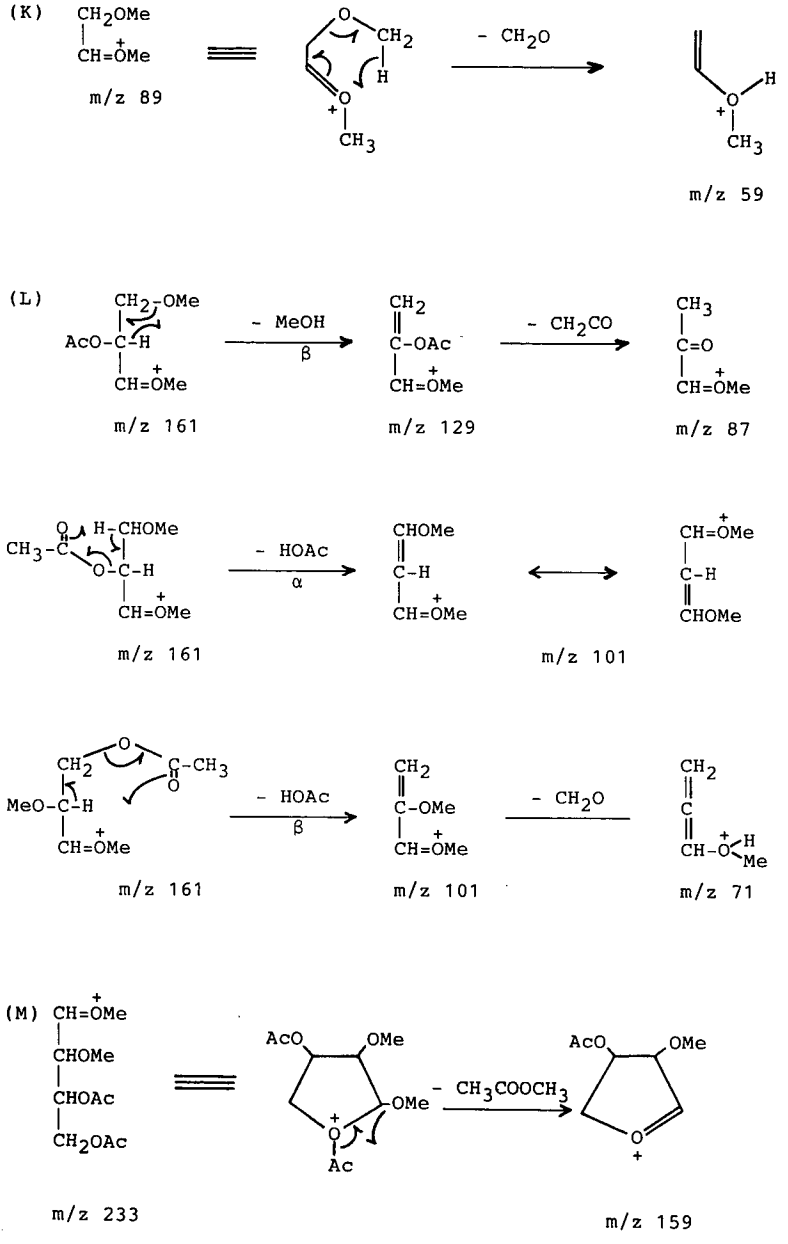


(I)



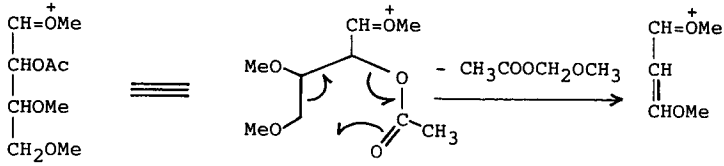
(J)





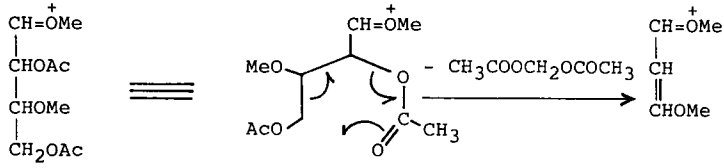
Elimination of methanol is only observed when the methoxyl group is situated at the β-position to the carbon atom having the formal charge. Elimination of acetic acid is possible from β- and α-positions, in the latter case provided that the resulting ion is stabilized by resonance. When an acetoxyl group is linked to an unsaturated carbon atom, ketene is eliminated with the formation of a carbonyl group.

(N)



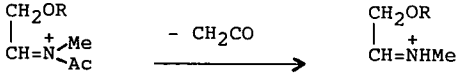
m/z 205 m/z 101

(O)



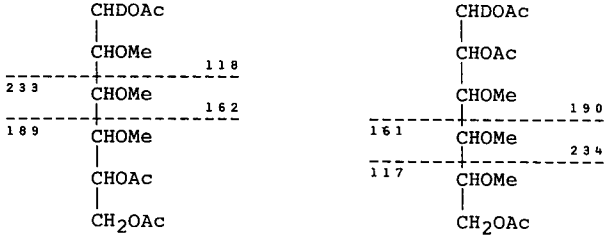
m/z 233 m/z 101

(P)



R=Ac : m/z 158 → m/z 116
R=Me : m/z 130 → m/z 88
Intense and highly characteristic peaks for assignments of partially methylated N-methyl-acetamido alditol acetates

(Q)



0.13 mol·l^{-1} sulfuric acid (103), 2–4 mol·l^{-1} trifluoroacetic acid (106), and 0.25 mol·l^{-1} sulfuric acid in 90% acetic acid (113–115). The mixture is reduced with $NaBH_4$ or NaB^2H_4, followed by acetylation of the liberated hydroxyl groups with acetic anhydride. The use of NaB^2H_4 facilitates the discrimination between primary hydroxyl groups. The partially methylated alditol acetates derived from neutral and amino sugars are analysed by GC-MS (4, 6, 8, 18, 59, 62, 103–106, 113, 116–122). Uronic acids, sialic acids and KDO can not be analysed in this way because of hydrolysis of their methyl ester groups.

3.1 EI-MS of partially methylated alditol acetates

EI-MS of partially methylated alditol acetates gives rise to very characteristic mass spectra, mostly without molecular ion peaks. The peak patterns obtained from the various derivatives yield information on the positions of the *O*-methyl and *O*-acetyl groups in the alditol chains. The *O*-methyl groups reflect the free hydroxyl groups in the corresponding monosaccharides of the native material. The mass spectra of stereoisomeric, partially methylated alditol acetates show only minor differences making an assignment of the sugar configuration (*gluco, galacto*, etc.) impossible. However, the nature of the parent monosaccharides can be derived from the retention times of the alditol derivatives on GC.

Comprehensive data concerning the mass spectra of partially methylated alditol acetates are available in the literature (6, 62, 103, 113, 116–121, 123–129). The relevant possibilities for the primary fragmentation of these derivatives have been summarized in Scheme 4 (A-J). Primary fragments are formed by α-cleavage, resulting in fission between the carbon atoms in the alditol chain. In principle, either of the two fragments formed can carry the positive charge. In the case of alditols derived from neutral sugars the charge is preferentially located on an ether oxygen instead of on an ester oxygen. The following rules can be formulated: 1. formation of ions of lower mass is preferred; 2. formation of ions from cleavage between two methoxylated carbon atoms is predominant, with no marked preference for one of the two possible cations (Scheme 4A); 3. in the formation of ions from cleavage between a methoxylated and an acetoxylated carbon atom, there is a high preference for the methoxyl-bearing cations (Scheme 4B), (in general the fission as shown in Scheme 4A is preferred over that in Scheme 4B, because the methoxyl radical formed seems to be better stabilised than the acetoxyl radical) and 4. ions formed by cleavage between two acetoxylated carbon atoms are generally of low abundance (Scheme 4C).

For alditols derived from *N,N*-methyl,acetyl amino sugars the same rules hold as mentioned above. However, the most preferable α-cleavage in partially methylated alditol acetates derived from 2-acetamido-2-deoxy sugars stems from the scission between C2, bearing the *N,N*-methyl,acetyl amino group, and C3, bearing the methoxyl or acetoxyl function, with the predominant localisation of the charge at the amino fragment (Scheme 4D–4E). α-Cleavages adjacent to deoxygenated carbon atoms are only significant when the neighbouring carbon atom bears a methoxy group (Scheme 4J).

The primary fragments give rise to secondary fragments, generally by single or successive eliminations of formaldehyde, methanol, ketene, acetic acid, methyl acetate,

methoxymethyl acetate or acetoxymethyl acetate (Scheme 4K-4P). It is advantageous to use NaB^2H_4 for the preparation of the alditols from the mass spectrometric point of view, because it enables the differentiation between primary hydroxyl groups. The primary fragmentation patterns of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-hexitol-[1-^{2}H] and 1,2,6-tri-*O*-acetyl-3,4,5-tri-*O*-methyl-hexitol-[1-^{2}H] are given in Scheme 4Q to illustrate this feature.

To determine the substitution patterns and ring sizes of uronic acid moieties it is necessary to reduce these residues to the corresponding aldoses. The reduction can be carried out with $NaBH_4$ after complexation of the carboxyl group(s) with a carbodiimide in water (65). It is also possible to reduce the methyl ester groups in the permethylated carbohydrate material with $LiAlH_4$ in diethyl ether or tetrahydrofuran (130), or with $NaBH_4$ in 95% ethanol : oxolane (27:73, v/v) (106). For microgram scale procedures it has been advised to reduce the methyl esters of uronic acid residues in permethylated polysaccharides instead of to carboxyl-reduce the native polysaccharide followed by permethylation (106). The application of NaB^2H_4 and $LiAl^2H_4$ as reducing agents lead to the incorporation of two deuterium atoms at the carboxyl-group derived primary hydroxyl functions. In this way alditols derived from uronic acids can be distinguished by MS from the corresponding native aldoses.

If the carbohydrate chain contains methoxyl groups initially, the use of trideuteromethyl iodide is important to discriminate between originally present and chemically introduced methyl groups (131). For several specific degradation procedures in which methylation analysis plays a role, the use of trideuteromethyl iodide and/or ethyl iodide have been reported (132–135). The location of a methoxyl function at C4 or C5 in aldoses defines the ring size of the sugar residue. When acetoxyl groups are present at C4 and C5, 4-linked aldopyranosyl or 5-linked aldofuranosyl residues cannot be distinguished from each other. To this end specific degradation methods in combination with alkylation analysis have been developed (136).

Since the use of methylsulfinylmethanide causes only marginal desulfation in the methylation procedure, the location of ester sulfate groups can be established by application of the methylation technique before and after desulfation. The same holds for acetals. For phosphate substituents it has to be taken into account that they may migrate under alkaline conditions via intramolecular cyclic esters. Because *O*-acyl groups are cleaved during base treatment, other procedures have to be followed e. g. as described in (137), applying methylvinyl ether.

As was already indicated, a large series of EI mass spectra of partially methylated alditol acetates have been depicted in several references, e. g. in (103, 113, 116–121, 123–127, 120a). To give an impression of the highly characteristic peak patterns, the mass spectra of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-mannitol-[1-^{2}H], 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-mannitol-[1-^{2}H] and 4-mono-*O*-acetyl-1,3,5,6-tetra-*O*-methyl-2-*N*-methyl-acetamido-2-deoxy-glucitol-[1-^{2}H] are shown in Figures 26–28. In Tables 2 and 3 fragment ions of several partially methylated alditol acetates of neutral and amino sugars, respectively, are summarized. Mass fragmentography has been shown to be of value for the analysis of complex mixtures.

Mass spectra of partially methylated alditol acetates of neutral sugars are not greatly influenced by the mass spectrometer used. However, for amino sugar derivatives the spectra can be affected by the type of instrument. The intensities of the diagnostic fragments of larger mass tend to be higher in the mass spectra of magnetically scanning

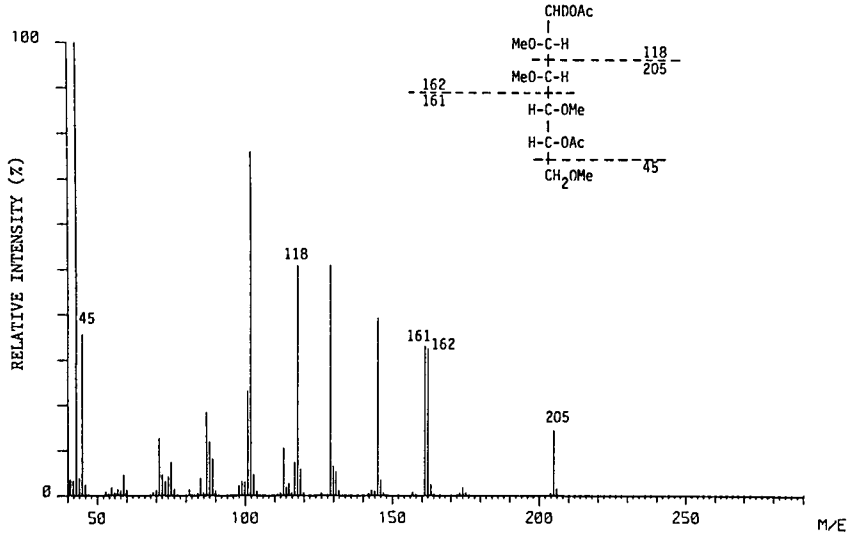


Figure 26. 70 eV EI mass spectrum of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-mannitol-[1-^{2}H].

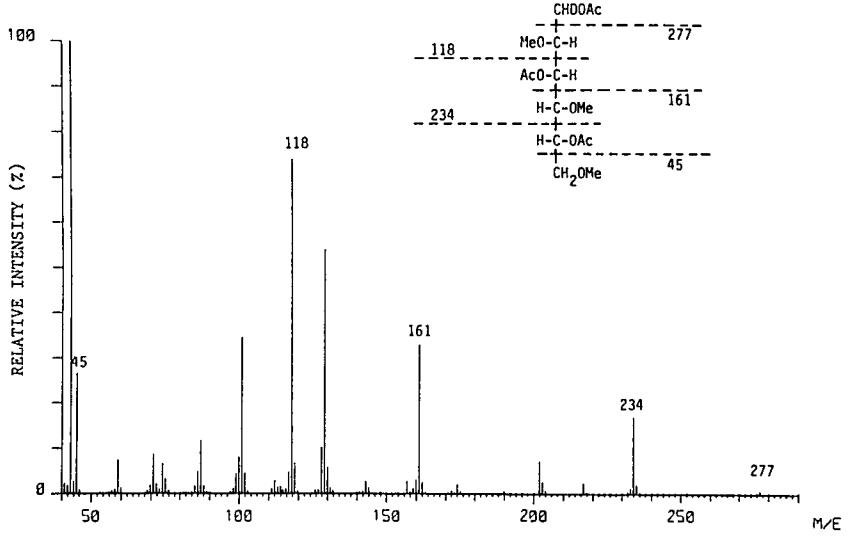


Figure 27. 70 eV EI mass spectrum of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-mannitol-[1-^{2}H].

instruments than in the mass spectra obtained with quadrupole instruments (8, 119). The temperature of the separator can also influence the peak pattern, probably because of pyrolysis (138). Furthermore it has been reported that the GC column phase can have an influence on the mass spectrometric result (121). Therefore, the availability of reference spectra of authentic amino sugar derivatives recorded with the same GC-MS combination is useful.

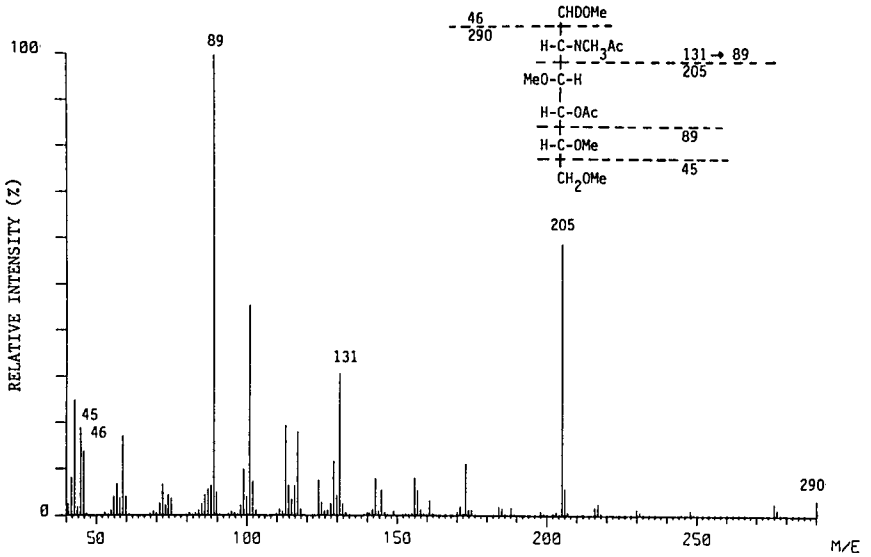


Figure 28. 70 eV EI mass spectrum of 4-mono-*O*-acetyl-1,3,5,6-tetra-*O*-methyl-2-*N*-methyl-acetamido-2-deoxy-glucitol-[1-^{2}H].

3.2 CI-MS of partially methylated alditol acetates

Using isobutane as the ionizing gas, a series of partially methylated alditol acetates of glucose, galactose and mannose has been investigated (139). The various derivatives give rise to abundant MH^+, $[MH-MeOH]^+$ and $[MH-HOAc]^+$ ions. The spectra yield direct information on the molecular weight of the substances and therefore also on the number of *O*-methyl and *O*-acetyl substituents in the alditol chain. Fragmentations of the alditol chain are very limited. The relative intensities of the ions are found to be sensitive to the source temperature. At higher temperatures there is a preference for the formation of $[MH-MeOH]^+$ and $[MH-HOAc]^+$ ions whereas the MH^+ ions are enhanced at lower temperatures. In contrast to the mentioned insensitivity of EI to stereochemical differences, CI(isobutane) has shown that it is possible to differentiate between diastereomers having identical locations of *O*-methyl and *O*-acetyl substituents in the alditol chain (139). Additional data on CI(isobutane)-MS of deoxyhexitol, hexitol and pentitol derivatives have been reported in (60). For a study of alditols derived from fructose, see (140). It is evident that CI-MS is highly suited for the recording of mass chromatograms based on quasi-molecular ions.

Partially methylated alditol acetates of neutral and amino sugars have also been investigated by CI(methane)-MS (141). The mass spectra show intense $[MH-HOAc]^+$ ions for neutral sugar derivatives, and MH^+ ions for amino sugar derivatives. In addition some fragmentation is observed.

Finally, data on the use of CI(ammonia)-MS have been reported (105). Ammonia may be considered to be a more suitable reagent gas than isobutane or methane. At an ion-source temperature of about 120 °C partially methylated alditol acetates of neutral sugars form mainly $[M + NH_4]^+$ ions. In the case of amino sugar derivatives there is a preference for the formation of MH^+ ions.

Table 2. Primary fragment ions (EI-MS) characteristic for the substitution pattern of partially methylated alditol acetates

Position of OMe groups	*m/z* values	Position of OMe groups	*m/z* values
Pentitol		*6-Deoxyhexitol*	
2 (4)	261,117	2	275,117
3	189	3	203,189
5	45	4	261,131
2,3 (3,4)	189,161,117	2,3	203,161,117
2,4	233,117	2,4	247,233,131,117
2,5	233,117,45	3,4	189,131
3,5	189,161,45	2,3,4	175,161,131,117
2,3,4	161,117	2,3,5	175,161,117,59
2,3,5	161,117,45	*Heptitol*	
Hexitol		3	333,189
2 (5)	333,117	2,6	117
3 (4)	261,189	2,7	117,45
6	45	3,6	189,117
2,3	261,161,117	4,6	261,233,117
2,4 (3,5)	305,233,189,117	6,7	89,45
2,5	305,117	2,3,6	305,161,117
2,6	305,117,45	2,3,7	305,161,117,45
3,4	233,189	2,4,6	233,117
3,6	233,189,45	2,4,7	233,117,45
4,6	261,161,45	2,6,7	377,349,117,89,45
5,6	333,89,45	4,6,7	261,205,89,45
2,3,4	233,189,161,117	2,3,4,6	321,277,233,161,117
2,3,5	233,161,117	2,3,4,7	277,233,205,161,117,45
2,3,6	277,233,161,117,45	2,3,6,7	277,161,117,89,45
2,4,6	277,233,161,117,45	2,4,6,7	349,321,233,205,117,89,45
2,5,6	117,89,45	3,4,6,7	205,189,89,45
3,4,6	233,205,189,161,45	2,3,4,6,7	249,205,161,117,89,45
3,5,6	305,205,189,89,45		
1,3,4,6	205,161,45		
2,3,4,6	205,161,117,45		
2,3,5,6	277,205,161,117,89,45		
1,2,3,4,5	177,161,133,117,89,45		
1,2,3,5,6	249,205,133,89,45		
1,2,4,5,6	249,205,133,89,45		
1,3,4,5,6	249,205,161,133,89,45		

3.3 Methylation analysis procedures based on partially methylated alditol acetates

Several detailed practical procedures for the methylation analysis of oligosaccharide-alditols, polysaccharides, glycoproteins, glycopeptides and glycolipids have been reported (103–106, 109, 142). Some of them can be used for quantities below 1 μg. Here, procedures will be presented essentially as described in (105) and (106).

Table 3. Primary fragment ions (EI-MS) characteristic for the substitution pattern of partially methylated 2-deoxy-2-(*N*-methyl)acetamidohexitol acetates

Position of OMe groups	*m/z* values
2-deoxy-2-(N-methyl)acetamidohexitol	
3	261,202,158
4	274,189,158
6	158,45
3,4	246,233,202,189,158
3,6	233,202,158,45
4,6	274,161,158,45
1,3,5	318,290,233,174,130,117,45
1,4,5	318,290,246,161,130,117,45
3,4,6	246,205,202,161,158,45
1,3,5,6	290,205,174,130,89,45
1,3,4,5	290,218,174,161,130,117,45
1,4,5,6	290,246,133,130,89,45

An aqueous solution of 50 µg glycan is transferred to a 1 ml reacti-vial. After lyophilisation the residue is dried for 18 h *in vacuo* over P_2O_5. The screw cap, septum and reacti-vial magnetic stirrer are also kept in the vacuum desiccator. The vial is flushed with dry argon or nitrogen and subsequently sealed. Dry dimethylsulfoxide (250 µl) is added with a syringe through the septum of the vial. The mixture is stirred magnetically (or sonicated) for 2 h at room temperature. For polysaccharides a much longer time may be required to dissolve the material. Subsequently, 60 µl 2 $mol \cdot l^{-1}$ sodium methylsulfinylmethanide in dimethylsulfoxide is added, followed by 35 µl methyl iodide after 1 h. Before adding the freshly distilled methyl iodide, the solution is frozen. During the whole procedure the mixture is continuously stirred. After 2 h a clear solution is obtained.

For purification of the permethylated glycan use is made of reversed-phase chromatography on Sep-Pak C_{18} cartridges. Before use, the cartridge is washed with 40 ml 100% ethanol. Subsequently, 2 ml 100% acetonitrile (HPLC-grade) followed by 4 ml water (HPLC-grade) are passed through the column. The methylation-reaction mixture is diluted with water, resulting in a dimethylsulfoxide/water (1:1, v/v) solution. The latter solution is slowly pushed (1 – 2 drops/s) with a syringe plunger, through the cartridge bed until the syringe is just empty. Take care to avoid air bubbles! The reacti-vial is washed with 500 µl dimethylsulfoxide/water (1:1, v/v), which is then passed through the cartridge in the same way. To remove more-polar contaminants from the cartridge, four 2 ml portions of water are added. The first three portions are pushed through the cartridge with the syringe plunger until the syringe is just empty; the fourth one is pushed completely through the cartridge. Subsequently, less-polar contaminants can be eluted with acetonitrile/water (3:17, v/v, solvent A; 1:4, v/v, solvent B). In the case of disaccharide-alditols four 2 ml portions of solvent A are added. For intermediate-sized glycans (d. p. 3 – 10) the cartridge is washed with three 2 ml portions of solvent A followed by one 2 ml portion of solvent B. Larger glycans need washing with two 2 ml portions of solvent A followed by two 2 ml portions of

solvent B. Then, permethylated glycans are eluted from the cartridge with 2 ml 100% acetonitrile, followed by 4 ml 100% ethanol. The collected solution containing the permethylated material is evaporated to dryness with a stream of nitrogen. To transfer the glycan material to the bottom of the tube, the residue is dissolved in 1 ml methylene chloride and evaporated again. The product is transferred with methylene chloride (five times 0.2 ml portions) to a 0.3 ml reacti-vial (procedure 1) or to a small Pyrex glass tube (procedure 2). Methylene chloride is evaporated with a stream of nitrogen.

Procedure 1:

To the residue 100 µl 4 mol · l^{-1} trifluoroacetic acid is added. After thorough mixing, the solution is heated in an aluminum block for 4 h at 100 °C. Then the cooled trifluoroacetic acid solution is evaporated with a stream of nitrogen at room temperature until the sample is just dry. In order to concentrate the mixture of partially methylated monosaccharides at the bottom of the vial cone, methanol is added to the reacti-vial and evaporated with a stream of nitrogen. Also traces of residual trifluoroacetic acid are removed in this way.

The residue is mixed with 50 µl aqueous $NaBH_4$ or NaB^2H_4 (10 µg reducing agent/µl water) and kept for 2 h at room temperature. To convert the excess of reducing agent into boric acid, 5 µl acetic acid is added. After the addition of 50 µl methanol the solvent is evaporated with nitrogen.

Boric acid is removed as trimethylborate by co-evaporation with 50 µl 10% methanolic acetic acid (four times) using a stream of nitrogen. Finally, one evaporation to dryness with 75 µl methanol is carried out.

For the acetylation 75 µl acetic anhydride is added to the reacti-vial. After mixing, the vial is heated for 3 h at 120 °C in an aluminum block. The vial is then allowed to cool down to room temperature, and 50 µl toluene is added. The mixed solution is evaporated with a stream of nitrogen just to dryness. One additional evaporation just to dryness with 50 µl toluene is then performed. The residue is mixed with 25 µl methylene chloride and equilibrated for at least 3 h before GC-MS analysis.

Procedure 2:

To the residue 500 µl 0.25 mol · l^{-1} H_2SO_4 in 90% aqueous acetic acid is added. The tube is flushed with argon and heated in an aluminum block for 4–6 h at 80 °C. After cooling and neutralisation with 550 µl 0.5 mol · l^{-1} NaOH, the mixture is evaporated under reduced pressure.

Two additional evaporations are carried out with 200 µl water.

The residue is dissolved in 300 µl water containing 3 mg $NaBH_4$ or NaB^2H_4 and kept for 3 h at room temperature. The solution is mixed with 2 mol · l^{-1} acetic acid until pH $\approx$ 5 is reached and then evaporated under reduced pressure. Boric acid is removed as trimethylborate by co-evaporation with 1.5 ml 1% methanolic acetic acid (four times); the solvent is blown dry with nitrogen each time. The residue is dried *in vacuo* over P_2O_5 for 1 h.

For the acetylation 400 µl acetic anhydride is added. The mixture is heated for 4 h at 105 °C under argon in an aluminum block and excess reagent is removed by blowing with nitrogen just to dryness. The residue is taken up in 4 ml methylene chloride. This

solution is extracted three times with 2 ml water. Finally, the methylene chloride phase is transferred to a Pyrex glass tube with a conical bottom and evaporated with nitrogen just to dryness. For GC-MS analysis the residue is taken up in 50 µl methylene chloride.

Several stationary phases for GC analysis of partially methylated alditol acetates have been recommended in the literature, e. g. Silar 9CP (59, 105), Dexsil 410 (59), SE-30 (59), OV-101 (59), OV-275 (60), OV-225 (103, 143, 144), ECNSS-M (103), SP-1000 (103, 145), OV-17 (104, 143, 144), DB-1 (105, 106), OV-1 (115, 146), CP-Sil 5 (120), Silar 10C (147). In several investigations it has been advised to use the retention data from more than one column. It has to be noted that the more polar columns as SP-1000 and Silar 9CP are not suitable for the analysis of amino sugar derivatives.

Notes:

1. For the preparation of sodium methylsulfinylmethanide, see (103).
2. The excess of methylsulfinylmethanide in the permethylation reaction can be checked with triphenylmethane. The latter reagent gives a red colour in the presence of the carbanion (148).
3. The Sep-Pak C_{18} purification procedure for permethylated glycans has been reported recently (106). Other procedures include dialysis (only applicable for permethylated polymers) (103), filtration of the methylation-reaction mixture over Sephadex LH-20 (105), chloroform or methylene chloride extraction of the permethylated material (103), eventually followed by filtration over silica gel (104) or Sephadex LH-20.
4. The hydrolysis of amino sugar-containing permethylated glycan chains needs special attention. When *N*-deacetylation of the methylated 2-acetamido-2-deoxyhexose units occurs as the first step, the adjacent glycosidic linkages at C1 become resistant to acid hydrolysis. For this reason several hydrolysis conditions have been studied (113, 114). The frequently used formolysis followed by hydrolysis with H_2SO_4 (103) results in good yields of partially methylated alditol acetates of neutral sugars, but give low and more variable yields of the amino sugar derivatives (115). The acetic acid/H_2SO_4 procedure described above gives good recoveries of amino sugar derivatives and of most of the neutral sugar derivatives (113, 114). Low molar ratios have been reported for trisubstituted β-D-mannose residues (2-*O*-methyl-mannitol) in glycoprotein glycans (115). Furthermore, the hydrolysis with $4\ mol \cdot l^{-1}$ trifluoroacetic acid as presented above gives good results (see also (116)).
5. In the case of hydrolysis with acetic acid/H_2SO_4, NaOH has been used for neutralisation. It is also possible to neutralise via anion-exchange chromatography on Bio-Rad AG-3 (acetate form) (104, 113).
6. Hydrolysis of permethylated carbohydrate chains containing a 2-acetamido-2-deoxyhexitol unit can give rise to some demethylation of the alditol residue. Some authors state that an *O*-demethylation at C1 occurs (121), whereas others conclude to a *N*-demethylation at C2 (118, 119). The extent of demethylation is also influenced by the substitution pattern of the alditols. Recently, *O*-demethylation at C1 and C3 has been discussed (121a).
7. Generally, the reduction with $NaBH_4$ or NaB^2H_4 is carried out in water. However, procedures have been published wherein the reduction is performed in alkaline medium with or without ethanol (see for instance (106).

8. For the analysis of partially methylated alditol acetates obtained from permethylated glycoproteins a final purification step on a silica gel G column is necessary (142). After washing the column with petroleum ether/ethyl acetate (2:1, v/v), neutral sugar derivatives are eluted with petroleum ether/ethyl acetate (1:1, v/v), and, subsequently, amino sugar derivatives with methanol.
9. The quantitative aspects of methylation analysis procedures are poorly understood. Several factors influence the recovery of partially methylated alditol acetates, e. g., undermethylation, incomplete hydrolysis, degradation and demethylation during hydrolysis, incomplete reduction, incomplete acetylation and contaminants. Also the degradation on the GC-column phase is important, especially for amino sugar derivatives (122). In general molar ratios of the different sugar derivatives are calculated on the basis of FID responses, taking into account similar molar adjustment factors for the various partially methylated alditol acetates. A better approach is the use of molar response-factors based on the effective-carbon-response theory (134). It has to be noted that in several examples the FID responses of certain amino sugar derivatives have shown to be lower than those of the neutral ones. To overcome these problems it is suggested to estimate the ratio of the total amount of amino sugar residues to that of the neutral sugar residues by sugar analysis. Other quantification procedures are based on selected ion-monitoring, especially in CI-approaches, which seem to give very acceptable results (105).

3.4 Other methylation analysis procedures

Instead of preparing alditol acetate derivatives after the hydrolysis step, other working-up procedures can be followed, e. g. leading to trimethylsilylated *O*-methyl oximes (149), acetylated oxime derivatives (30), and acetylated aldononitriles (26, 30, 150–154). The acetylated aldononitriles have been studied extensively by EI- and CI-MS. They give rise to EI fragmentation patterns comparable to those presented for the partially methylated alditol acetates.

In some approaches hydrolysis has been replaced by methanolysis, resulting in the formation of partially methylated methyl glycosides. The monosaccharide derivatives can be analysed in their underivatised forms, or as the corresponding trimethylsilylated or acetylated compounds (1–7, 75, 76, 155–162). A detailed procedure has been worked out for the methylation analysis of glycoprotein glycans using acetylated, methylated methyl glycosides (160). The report includes GC-retention times of various α and/or β glycosides and a detailed EI-fragmentation scheme for the determination of the positions of the methyl and acetyl substituents in galactose, glucose, mannose and *N*-acetylglucosamine derivatives. Moreover, a series of mass spectra have been depicted. It has to be noted that methanolysis (0.5 $mol \cdot l^{-1}$ methanolic HCl, 24 h, 80 °C) does not split quantitatively the linkage between *N*-acetylglucosamine and asparagine.

For the linkage analysis of sialic acids in non-reducing or internal positions, methanolysis is the appropriate method of solvolysis. The resulting (partially) methylated methyl ester β-methyl glycosides show very characteristic EI mass spectra (163, 164), after treatment with reagents for acetylation or *N*-acetylation/trimethylsilylation,

Table 4. GC and MS data of trimethylsilylated/methylated *N,N*-acyl,methyl-neuraminic acid methyl ester β-methyl glycosides. The R_{Neu5Ac}-values on a packed column (2 m × 4 mm, i.d.) of 3.8% SE-30 at 220 °C (163) and on a capillary column (80 m × 0.35 mm, i.d.) wall-coated with OV-101 at 215 °C (164) are given relative to Neu5Ac4,5,7,8,9Me_5 methyl ester β-methyl glycoside.

Sialic acid* (as methyl ester β-methyl glycoside)	R_{Neu5Ac} 3.8% SE-30	R_{Neu5Ac} OV-101	*m/z* values							
			A	B	C	D	E	F	G	H
Neu5Ac4,5,7,8,9Me_5	1.00	1.00	392	348	318	254	201	89	129	298
Neu5Ac4,5,7,8Me_4	1.30	1.31	450	406	318	254	201	147	129	298
Neu5Ac4,5,7,9Me_4	1.14	1.14	450	406	318	254	201	147	129	356
Neu5Ac4,5,8,9Me_4	1.07	1.06	450	406	376	312	201	89	129	298
Neu5Ac5,7,8,9Me_4		1.20	450	406	376	254	259	89	187	298
Neu5Ac4,5,7Me_3	1.55	1.52	508	464	318	254	201	205	129	356
Neu5Ac4,5,9Me_3	1.27	1.27	508	464	376	312	201	147	129	356
Neu5Ac5,7,8Me_3		1.60	508	464	376	254	259	147	187	298
Neu5Ac5,7,9Me_3		1.39	508	464	376	254	259	147	187	356
Neu5Ac5,8,9Me_3		1.23	508	464	434	312	259	89	187	298
Neu5Ac4,5Me_2	1.70	1.78	566	522	376	312	201	205	129	356
Neu5Ac5,7Me_2		1.91	566	522	376	254	259	205	187	356
Neu5Ac5,9Me_2	1.43	1.50	566	522	434	312	259	147	187	356
Neu5Ac5Me	1.89	2.05	624	580	434	312	259	205	187	356
Neu5MeGc4,5,7,8,9Me_5#			422	378	348	284	201	89	159	328
Neu5MeGc4,5,7,9Me_4			480	436	348	284	201	147	159	386

* Neu5Ac4,5,7,8,9Me_5 = 4,7,8,9-tetra-*O*-methyl-*N,N*-acetyl,methyl-neuraminic acid; etc.
\# MeGc = methylated glycolyl group.

(for a comprehensive review, see (36)). Tables 4 and 5 summarize the GC and MS data of a series of trimethylsilylated/methylated *N,N*-acyl,methyl-neuraminic acid methyl ester β-methyl glycosides and of a series of acetylated/methylated *N,N*-acyl,methyl-neuraminic acid methyl ester β-methyl glycosides, respectively. The presented fragment ions A-H have already been discussed in relation to Scheme 3. Typical mass spectra of some sialic acid derivatives are depicted in Figures 29–33. The choice of the applied methanolysis conditions in relation to the possible release of the *N*-acyl group seems to be very important. It has been shown that the *N*-acyl (*N*-acetyl or methylated *N*-glycolyl) groups of sialic acids in terminal positions of the permethylated carbohydrate chain are resistant to cleavage in 0.5 $mol \cdot l^{-1}$ methanolic HCl (18 h, 80 °C). However, internal sialic acids are *N*-deacylated to a large extent. Therefore, after methanolysis *N*-(re)acetylation is necessary in the working-up procedure for trimethylsilylated derivatives. The use of 0.05 $mol \cdot l^{-1}$ methanolic HCl (1 h, 80 °C) does not give *N*-deacylation, but these milder conditions do not liberate sialic acid quantitatively from the sialo-carbohydrate chain (36).

Table 5. GC and MS data of acetylated/methylated *N,N*.acyl,methyl-neuraminic acid methyl ester β-methyl glycosides. The R_{Neu5Ac}-values on a packed column (2 m × 4 mm, i.d.) of 3.8% SE-30 at 220 °C (163) are given relative to Neu5Ac4,5,7,8,9Me_5 methyl ester β-methyl glycoside. For an explanation of the '-' signs, see the discussion in relation to Scheme 3. For high-resolution exact mass measurements, see (36) and (163).

Sialic acid* (as methyl ester β-methyl glycoside)	R_{Neu5Ac}	*m/z* values						
		A	B	C	D	E	F	G
Neu5Ac4,5,7,8Me_4	1.47	420	376	318	254	201	117	129
Neu5Ac4,5,7,9Me_4	1.25	420	376	318	254	201	–	129
Neu5Ac4,5,8,9Me_4	1.08	420	376	–	–	201	89	129
Neu5Ac5,7,8,9Me_4		420	376	346	254	–	89	157
Neu5Ac4,5,7Me_3	1.75	448	404	318	254	201	–	129
Neu5Ac4,5,9Me_3	1.26	448	404	–	–	201	–	129
Neu5Ac4,5Me_2	1.70	476	432	–	–	201	–	129
Neu5Ac5,9Me_2	1.63	476	432	–	–	–	–	157
Neu5Ac5Me	2.17	504	460	–	–	–	–	157
Neu5MeGc4,5,7,9Me_4		450	406	348	284	201	–	159

* For an explanation of the abbreviations, see Table 4.

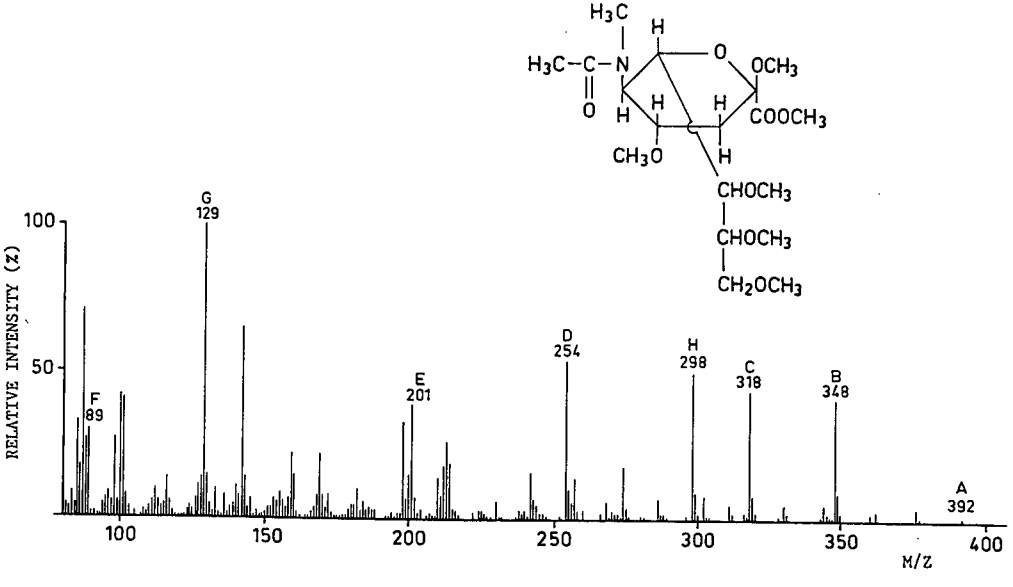


Figure 29. 70 eV EI mass spectrum of 4,7,8,9-tetra-*O*-methyl-*N,N*-acetyl,methyl-neuraminic acid methyl ester β-methyl glycoside.

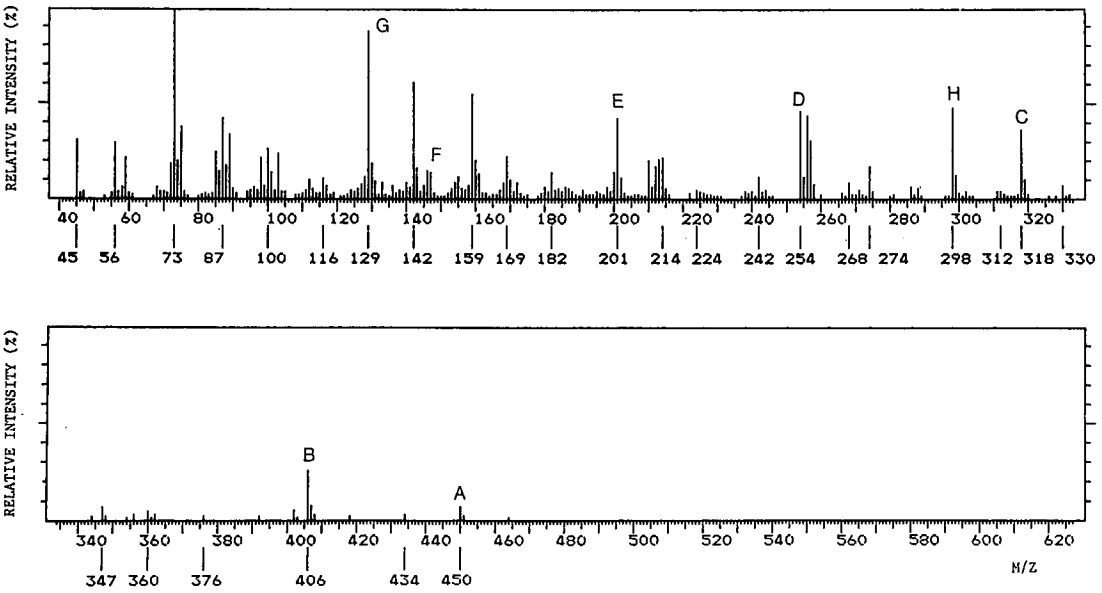


Figure 30. 70 eV EI mass spectrum of 4,7,8-tri-*O*-methyl-9-mono-*O*-trimethylsilyl-*N*,*N*-acetyl,methyl-neuraminic acid methyl ester β-methyl glycoside.

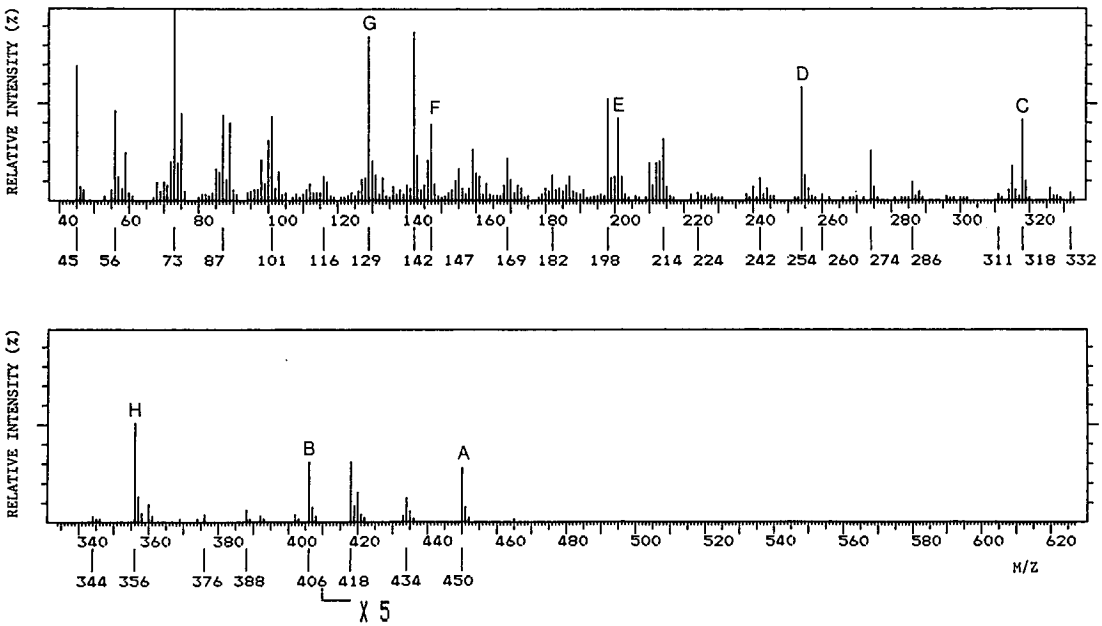


Figure 31. 70 eV EI mass spectrum of 4,7,9-tri-*O*-methyl-8-mono-*O*-trimethylsilyl-*N*,*N*-acetyl, methyl-neuraminic acid methyl ester β-methyl glycoside.

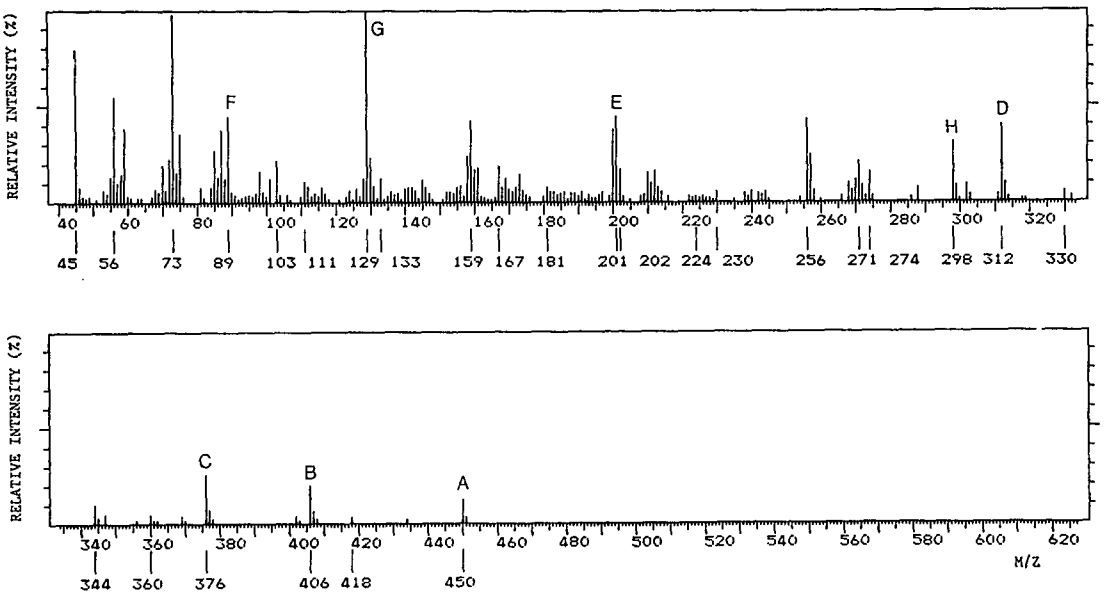


Figure 32. 70 eV EI mass spectrum of 4,8,9-tri-*O*-methyl-7-mono-*O*-trimethylsilyl-*N*,*N*-acetyl,methyl-neuraminic acid methyl ester β-methyl glycoside.

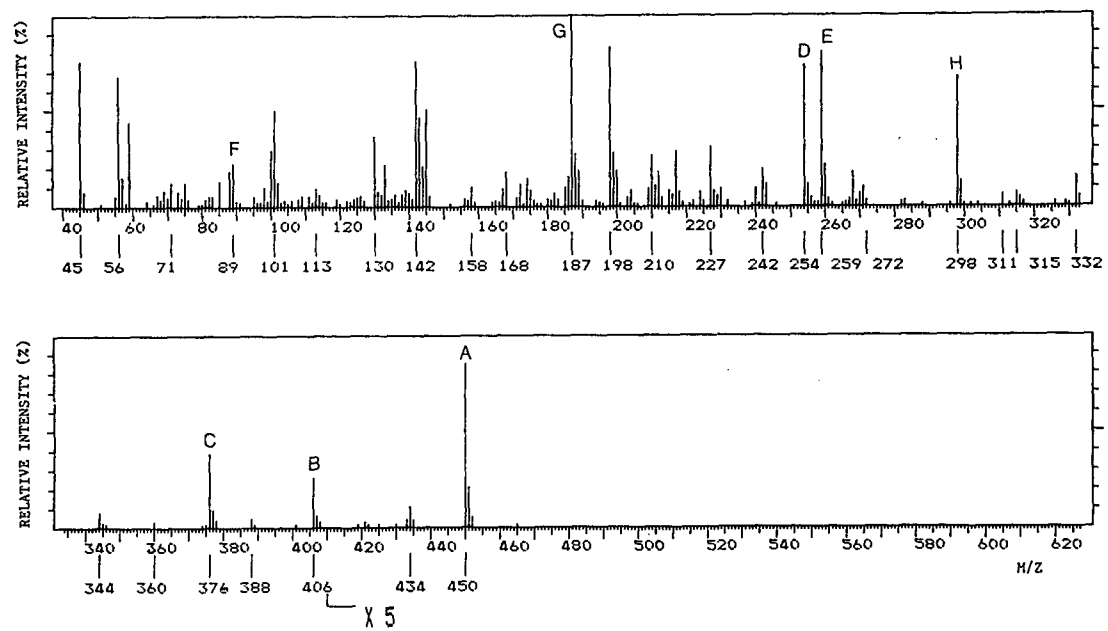


Figure 33. 70 eV EI mass spectrum of 7,8,9-tri-*O*-methyl-4-mono-*O*-trimethylsilyl-*N*,*N*-acetyl,methyl-neuraminic acid methyl ester β-methyl glycoside.

3.5 Methylation analysis of oligosaccharides isolated from physiological fluids

Methylation analysis is one of the methods incorporated in studies concerning the structure elucidation of oligosaccharides isolated from physiological fluids like milk and urine. In the case of patients with inborn errors of metabolism related to the carbohydrate catabolism of polysaccharides, glycoproteins and glycolipids relatively large amounts of carbohydrate material is available from the urine. For applications in the field of clinical biochemistry, a series of methylation analysis examples can be found in (165–199, 199a, 199b, 199c).

4 Sequence Analysis

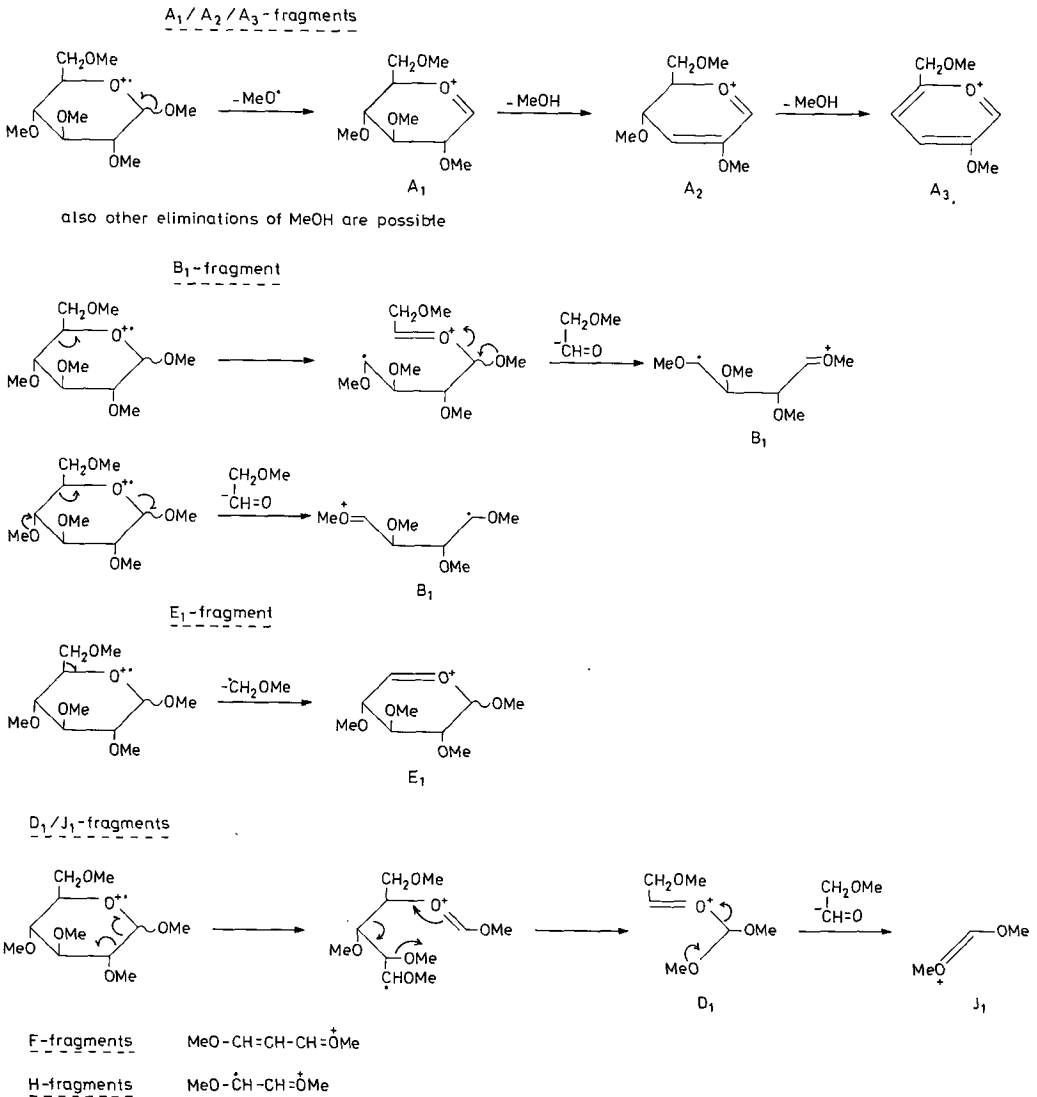
MS of oligosaccharides, oligosaccharide-alditols, glycopeptides and glycolipids have been reviewed regularly (1–3, 6–8, 200–202). Permethylated derivatives are among the most commonly used derivatives. However, trimethylsilylated and acetylated derivatives have also shown their suitability for structural studies. Most of the published MS data entail EI-MS, but CI-MS, DCI-MS, FI-MS, FD-MS, SI-MS and FAB-MS have been employed. For EI, CI and FI conversion of polar saccharides into thermally more stable, volatile derivatives is important (203–276) and when possible GC is used. DCI, FD, SI and FAB are ionization methods which are applicable for the analysis of derivatised and underivatised saccharides (277–310).

Fundamental studies have been carried out with the aim of developing general MS methods for the determination of the sequence of monosaccharide units in terms of hexoses, pentoses, heptoses, deoxyhexoses, uronic acids, aminohexoses, sialic acids, etc. Furthermore for disaccharide derivatives discrimination rules to establish the type of glycosidic linkage have been developed (see below). Higher saccharides were sometimes incorporated in the latter studies.

4.1 Characterisation of permethylated oligosaccharide-alditols

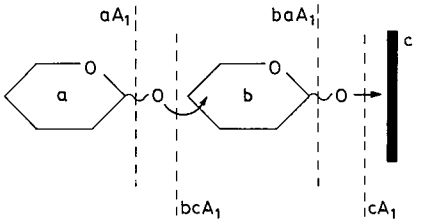
Analysis of reducing oligosaccharides is most conveniently made after conversion of these compounds into the corresponding oligosaccharide-alditols. The reduction can be carried out with $NaBH_4$ or NaB^2H_4. Then the oligosaccharide-alditols are permethylated as already described in the section on methylation analysis.

Permethylated oligosaccharide-alditols rarely give rise to molecular ions in EI-MS. Fortunately, molecular weights can often be deduced from specific fragment ions. EI mass spectra contain a great deal of structural information due to extensive fragmentation and, for the interpretation of these spectra, the general principles developed for methylated ring forms and alditol chains are applicable (1, 6). In Scheme 5 a number of mass spectral fragmentation pathways in permethylated methyl glucopyranoside

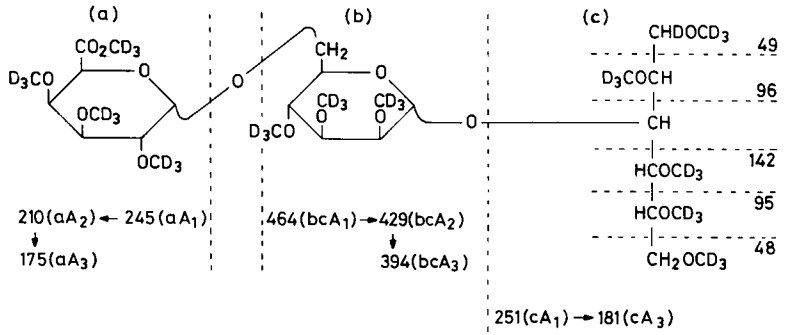


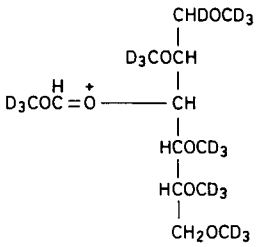
Scheme 5. Some important examples of fragmentation in permethylated methyl glucopyranoside. For detailed information, see (1).

are shown. Comparable breakdown pathways are followed in deoxy sugars, amino sugars and uronic acids. In furanose ring forms similar fragmentations are observed. The introduced nomenclature system with upper-case letters A-K to describe the various fragment ions (1), has also been used for the development of a fragmentation scheme for unbranched oligosaccharides (6, 203, 204) and oligosaccharide-alditols (6, 18). As has been depicted in Scheme 6 for the sequence fragment ions, the upper-case letters are supplied with lower-case letters a, b, c, etc., to denote the various sugar

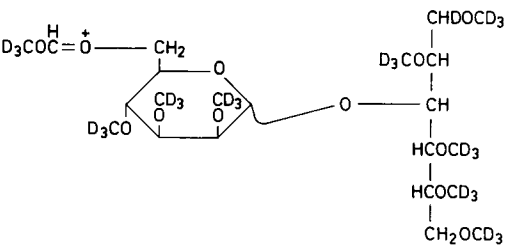


Scheme 6
Sequence fragment ions of the A-series in a linear trisaccharide-alditol a-b-c.





314 (bc J_1)



527 (abc J_1)

Figure 34. Important fragmentation pathways in the pertrideuteromethylated trisaccharide-alditol-[1-^{2}H] prepared from α-D-Gal*p*A-(1 → 6)-α-D-Man*p*-(1 → 3)-D-Man.

units starting from the non-reducing monosaccharide. When more than one lower-case letter has to be used, the first lower-case letter characterises the part of the molecule from which the fragment originates, whereas the second designates the unchanged part of the molecule, etc. For instance, baA_1 denotes an ion that has arisen from cleavage of ring b following pathway A and is substituted by ring a (Scheme 6). It may be evident that the A-series is highly valuable for the sequence analysis of oligosaccharide-alditols. In Figures 34 and 35 two examples are shown which include this type of fragmentation: the pertrideuteromethylated trisaccharide-alditol-[1-^{2}H] prepared from α-D-Gal*p*A-(1 → 6)-α-D-Man*p*-(1 → 3)-D-Man, which has been isolated from a partial hydrolysate of the polysaccharide associated with the coccoliths of the alga *Emiliania huxleyi* (Lohmann) Kamptner (205), and the permethylated trisaccharide-alditol-[1-^{2}H] prepared from α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 → 4)-D-GlcNAc,

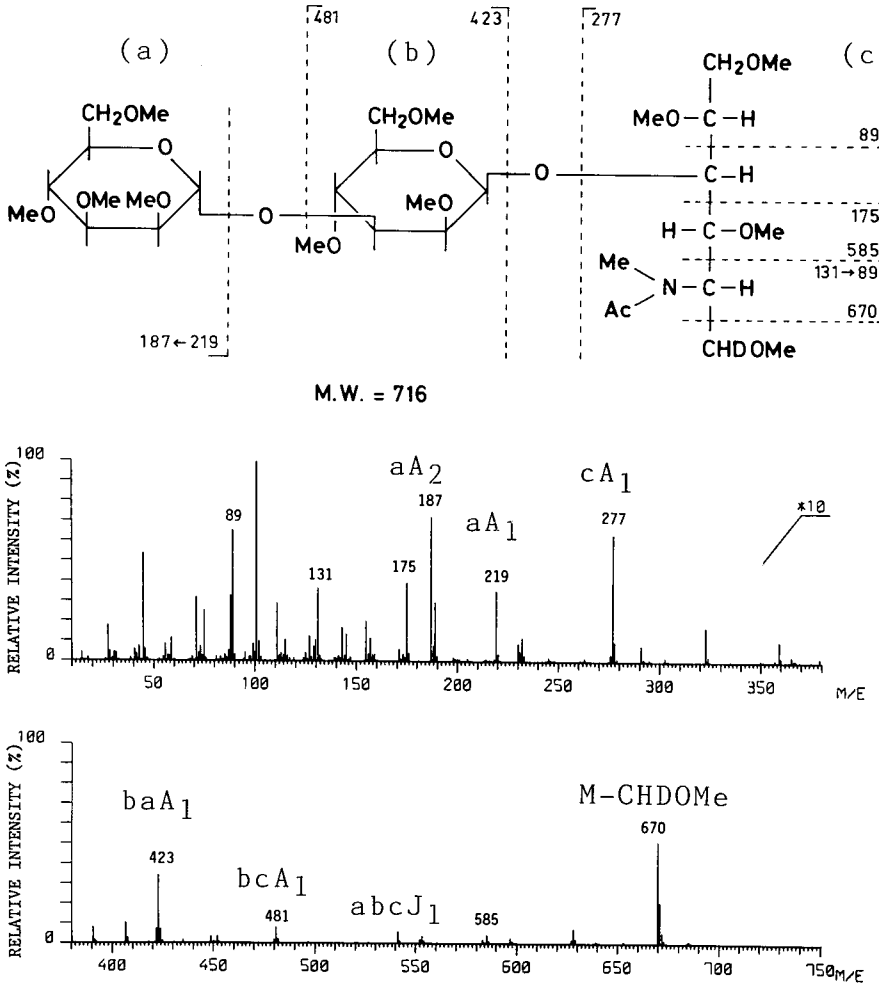


Figure 35. 70 eV EI mass spectrum of the permethylated trisaccharide-alditol-[1-^{2}H] prepared from urinary α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc.

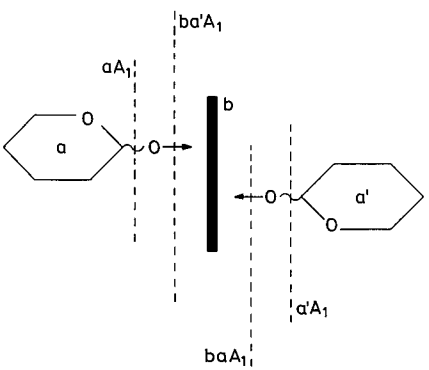
which has been isolated from the urine of a a-mannosidosis patient (206). Table 6 presents the *m/z* values of the A_1 fragments and the mass increments for internal sugar residues of different monosaccharide classes. It has to be noted that the expected theoretical A_1 fragments are not always observed.

Although model studies of di- and trisaccharides have shown that the EI mass spectra do contain information with regard to the type of glycosidic linkages, in most cases only the J_1 fragment is useful for this purpose in unknown saccharides. The reason is that discrimination rules are mainly based on intensity differences between selected peaks in the spectrum. The J_1 fragment ion is indicative for the presence or absence of 1→3 linkages (Scheme 5). For instance the absence of *m/z* 337 (bcJ_1 fragment ≡ CH_3OCHO-HexNAc-ol-[1-^{2}H] in the mass spectrum of Figure 35 suggests the occurrence of a →3)-Hex-HexNAc-ol-[1-^{2}H] unit.

Table 6. Survey of m/z-values of A_1 fragments and mass increments, corresponding to internal sugar residues, of a series of different monosaccharide classes

Non-reducing units m/z A_1 fragments		Internal units mass increments		Alditol-[1-^{2}H] units m/z A_1 fragments	
Hex →	219	→ Hex →	204	→ Hexol	236
Deoxyhex →	189	→ Deoxyhex →	174	→ Deoxyhexol	206
Pent →	175	→ Pent →	160	→ Pentol	192
HexA →	233	→ HexA →	218	→ HexAol	250
HexNAc →	260	→ HexNAc →	245	→ HexNAcol	277

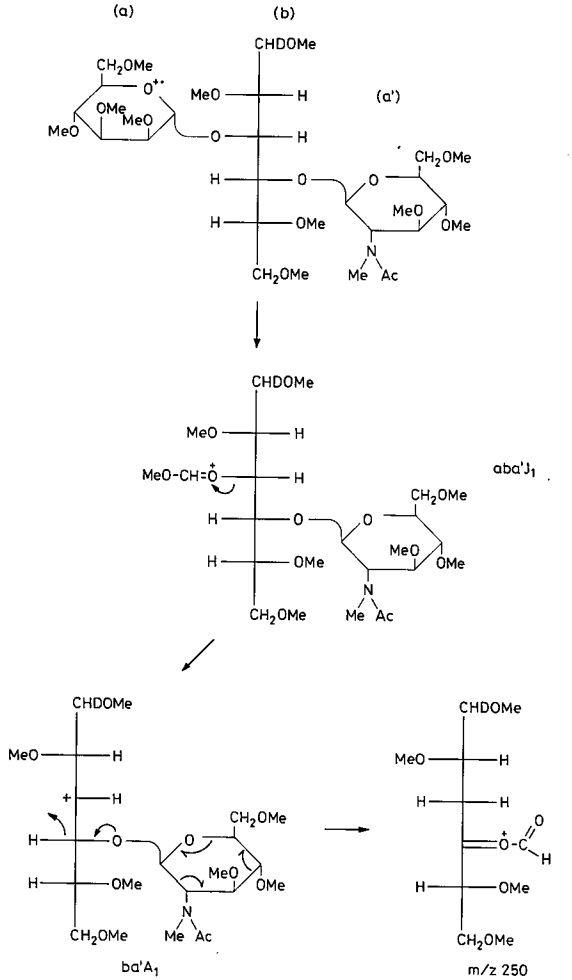
Hex = hexose; Deoxyhex = deoxyhexose; Pent = pentose; HexA = hexuronic acid; HexNAc = *N*-acetylhexosamine.



Scheme 7
Sequence fragment ions of the A-series in a branched trisaccharide-alditol a-(a'-)b.

The primary cleavages of the C-C bonds in the alditol unit give information about the type of glycosidic linkage next to this residue (207 – 214), although sometimes the intensity of characteristic fragments is not sufficiently high to serve as reliable criteria for the identification of the type of linkage. Comparison of the MS data of non-labeled and ^{2}H-labeled hexitols is useful. Discrimination rules have been reported for a series of (1 → 2)-, (1 → 3)-, (1 → 4)- and (1 → 6)-linked hexosyl-hexitols-[1-^{2}H] (207, 208), a series of (1 → 3)-, (1 → 4)- and (1 → 6)-linked hexosyl-2-acetamido-2-deoxy-hexitols (211), and a series of (1 → 3)-, (1 → 4)- and (1 → 6)-linked *N*-acetylglucosaminyl-*N*-acetylglucosaminitols (212). The presence of *N*,*N*-methyl,acetyl functions in the alditol chains strongly influences the peak patterns. See also Figures 34 and 35 for primary cleavages of the alditol C-C bonds.

The sequence analysis of branched oligosaccharide-alditols with a branching point at the alditol unit takes place in a similar way (Scheme 7). The m/z values of the A_1 fragments being indicative for the alditol units in linear oligosaccharide-alditols are absent (see cA_1 in Scheme 6 and Table 6). Instead, new fragment ions at higher m/z values corresponding with a substituted alditol unit appear in the mass spectra (e. g. baA_1 and $ba'A_1$ in Scheme 7). Furthermore, an additional fragmentation pathway has been observed, as indicated in Scheme 8. For a hexitol-[1-^{2}H] this fragmentation leads to the formation of m/z 250 (214). In the case of 2-acetamido-2-deoxy-hexitols this peak is observed at m/z 291 (215).



Scheme 8. Formation of the characteristic peak at *m/z* 250 in a branched oligosaccharide-alditol-[1-^{2}H].

Also CI-MS is currently used for the characterisation of permethylated oligosaccharide-alditols (214, 216–211). CI(isobutane)-MS of (1→2)-, (1→3)-, (1→4)- and (1→6)-linked aldosyl-alditols give rise to intense quasi-molecular MH^+ ions and characteristic sequence ions (216). Close inspection of the spectra of the various disaccharide-alditols showed that the intensity ratios of the alditol A_1^+ ions and the alditolhydrate $A_1OH_2^+$ ions could be correlated with the linkage positions between glucose and glucitol. Furthermore, the values found for the (1→4)-linked Glc*p*-Glc-ol and the trisaccharide-alditol Glc*p*(1→4)Glc*p*(1→4)Glc-ol were in the same range. It is not evident if these rules are generally applicable. Figure 36 shows the fragmentation in the mentioned trisaccharide-alditol. The formation of the cA_1^+ ion can be explained by protonation of the glycosidic oxygen followed by assisted cleavage of the bond at the alditol site of the molecule. The $cA_1OH_2^+$ ions probably arise by

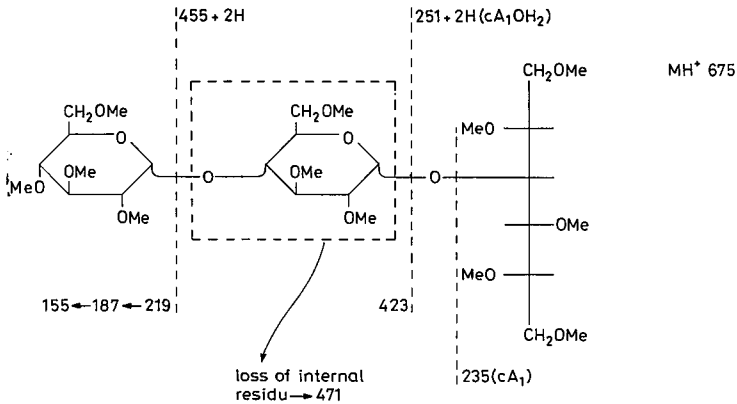


Figure 36. CI(isobutane) mass fragmentation pathways in a trisaccharide-alditol.

protonation at a site remote from the glycosidic oxygen followed by elimination of an unsaturated sugar molecule (H-rearrangement), but direct formation of cA_1^+ from $cA_1OH_2^+$ can not be excluded. The interpretation of the CI(isobutane) mass spectra of the tri- and higher oligosaccharide-alditols is complicated by the occurrence of an unusual elimination reaction (Fig. 36). The MH^+ ion can eliminate the glycosyl residue directly attached to the alditol. This results in a glycosyl-residue-elimination fragment ion corresponding with the MH^+ ion of an oligosaccharide-alditol with one sugar residue less (220). In this way a tetrasaccharide-alditol can eliminate successively two internal glycose residues. The elimination reaction is influenced by the substitution pattern of the sugar unit linked to the alditol. In the case of a $(1 \rightarrow 3)$-linked residue the relative abundance of the glycosyl-residue-elimination fragment ion decreases dramatically. The loss of an internal residue has not been detected in the ammonia/isobutane mass spectra (216). A detailed study on the CI(ammonia)- and CI(isobutane)-MS of sialo- and corresponding asialo-oligosaccharide-alditols derived from gangliosides has been published (221). The CI(ammonia) mass spectra show both protonated and ammonia adduct molecular ions. In addition several major fragment (sequence) ions are observed, formed by direct cleavage of the glycosidic bond as well as by cleavage of this bond involving H-rearrangement. They can occur as such in the protonated form, or as ammonia adduct ion. Also other more complicated pathways have been detected and detailed information can be obtained from the spectra with regard to the branching structures, CI(ammonia) and CI(isobutane) being complementary in the structural eludication of these oligosaccharides.

Hydrazinolysis-nitrous acid deamination of oligosaccharide chains containing 2-acetamido-2-deoxy-glucose residues leads to specific cleavage at C1 of the latter residues with formation of oligosaccharides ending on 2,5-anhydromannose. The permethylated oligosaccharide-2,5-anhydromannitols have been extensively studied by EI-MS, giving structural information comparable to that obtained from normal oligosaccharide-alditols (222 – 224).

An enormous number of papers in the literature report on the structural analysis of oligosaccharides and their corresponding alditols using MS of the permethylated

derivatives. In the field of clinical biochemistry structural studies have been carried out on a great variety of carbohydrate chains. Physiological fluids like milk and urine (especially in the case of lysosomal storage diseases) are main sources of oligosaccharide material. A series of interesting examples containing many reports of mass spectra can be found in (215, 222, 225–253, 199a, 253a, 253b).

4.2 Characterisation of permethylated glycopeptides

Only a few studies on the mass spectrometric analysis of glycopeptides are known. Permethylated sialo- and asialo-biantennary glycopeptides derived from *N*-glycosidic glycoproteins give rise to complicated EI-mass spectra with several sequence ions (254). Also the methylated-reduced and methylated-reduced-trimethylsilylated derivatives are incorporated in this study (see below, characterisation of permethylated glycosphingolipids). Furthermore, MS data of smaller glycopeptides are available (255, 256). *O*-Glycosidic glycopeptides will degrade via β-elimination under the conditions of permethylation, leading to peptides with one or more unsaturated amino acids and one or more oligosaccharide chains (257), (for an EI- and CI(methane)-MS study of permethylated glycopeptides containing *N*-acetylmuramyl residues, see (258)). Finally, one of the main urinary compounds excreted by patients with aspartylglycosaminuria is the glycopeptide 4-*N*-acetamido-2-deoxy-β-D-glucopyranosyl-L-asparagine. GC-MS data of the permethylated compound are available from (255, 259).

4.3 Characterisation of permethylated glycosphingolipids

The structural analysis of glycosphingolipids can conveniently be performed by EI-MS on permethylated derivatives. Besides the use of these derivatives permethylated-reduced and permethylated-reduced-trimethylsilylated compounds have been incorporated in several studies. The reduction of permethylated glycosphingolipids is carried out with $LiAlH_4$. In this way amide groups of ceramide and amino sugars are converted into amines, whereas esterified carboxyl groups of sialic acids are transformed into primary hydroxyl functions. The latter groups can be trimethylsilylated. In Figure 37 the different derivatives have been depicted for GM_1 ganglioside (260) containing C_{20}-sphingosine and stearic acid.

Detailed fragmentations of the glycosphingolipid derivatives have been discussed extensively (200–202, 260–265). In principle four series of characteristic primary fragments can be distinguished: 1. fragment ions derived from the molecular ion; 2. fragment ions related to the ceramide moiety; 3. fragment ions originating from the carbohydrate chain and 4. fragment ions consisting of parts from both units.

The molecular ion of derivatised glycosphingolipids is rarely observed in EI-mass spectra. However, in several cases, depending on the size of the structure and of the type of derivative, $[M-H]^+$ ions can be detected. Furthermore, loss of MeOH, $COOCH_3$ (in permethylated gangliosides) or CH_2OTMS (in permethylated-reduced-trimethylsilylated gangliosides) can take place. Several series of primary and rear-

rangement ions allow a conclusive interpretation of the monosaccharide sequence, and of the branching points in the carbohydrate chain. These fragments originate predominantly from cleavages around the glycosidic bonds but they can also contain parts of the ceramide residue (Fig. 37). Although the expected sequence ions are not always detectable for one of the derivatives, the application of three types of derivatives gives rise to reliable overlapping sequence data. Sometimes, additional information can be obtained about certain types of glycosidic linkages in individual glycosphingolipids.

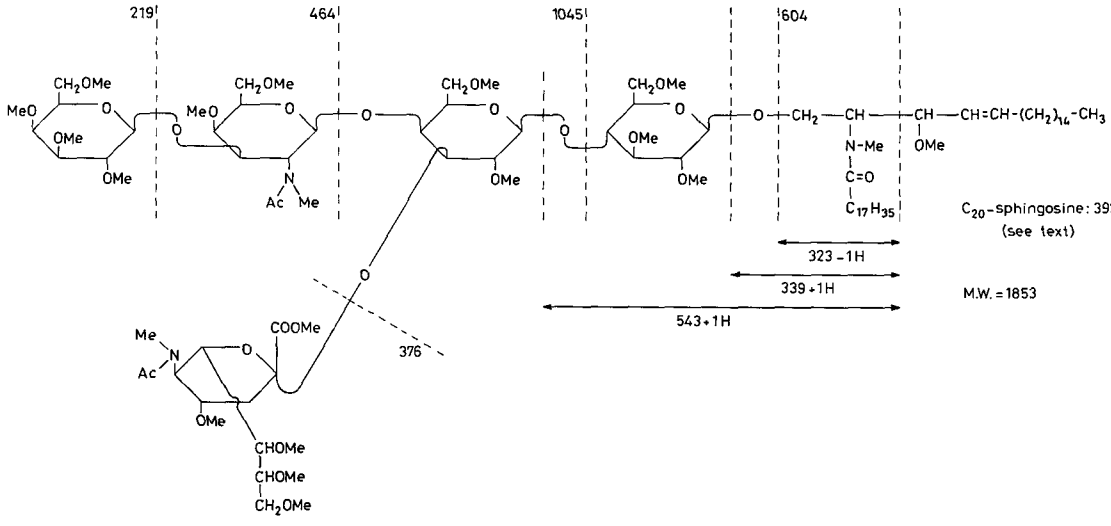
Because of the frequently occurring heterogeneity of the constituent fatty acids and long-chain-bases, the EI mass spectra of glycosphingolipids can be very complicated. Fragments characteristic of the ceramide components, elaborated for sphingosine and 4D-hydroxysphinganine, are presented schematically in Figures 38 and 39. Type A fragments are generally observed for permethylated glycosphingolipids and represent the entire ceramide moiety. In the permethylated-reduced derivatives fragment A' prevails. The type of sphingosine base is characterised by a fragment ion formed from A after cleavage between C3 and C4 of the fatty acid chain in the permethylated glycosphingolipid with rearrangement of one hydrogen atom, yielding *m/z* 364 for sphingosine and *m/z* 396 for 4D-hydroxysphinganine. Fragments of the B-type (minus 1 H) or B'-type (plus 1 H) (Fig. 38) furnish information concerning the fatty acid composition of the glycolipids with sphingosine as long-chain-base. Because of its low intensity, fragment C is not very useful for identification purposes. In the case of glycolipids with 4D-hydroxysphinganine (Fig. 39) the fragments D and D' are significant. In the non-reduced form there is a preferential fission leading to D' fragments, whereas the reduced form gives predominantly rise to D fragments. In the case of 2-hydroxy fatty acids as *N*-acyl substituents more complicated peak patterns have been observed.

Mixtures of permethylated glycosphingolipids and corresponding derivatives have been analysed by selected ion monitoring in the EI-mode. Samples were evaporated slowly from the direct inlet probe using a temperature program. A successive partial separation was obtained for glycosphingolipids containing one up to seven sugars (264, 265).

The various glycosphingolipid derivatives have also been studied with CI(ammonia)-MS. The neutral permethylated glycosphingolipids show intense $MH^+ \cdot MeOH$ ions. The quasi-molecular MH^+ and $(M + NH_4)^+$ ions have a low abundance. Fragment ions, produced by the successive loss of sugar moieties from the non-reducing end are prominent (266). Mass spectra of permethylated gangliosides furnish also peak patterns with information about molecular weight, ceramide structure and sugar sequences (267, 268). In Figure 40 the peak patterns of the permethylated, the permethylated-reduced and the permethylated-reduced-trimethylsilylated GM_1 ganglioside (stearic acid; C_{20}-sphingosine) are compared. It may be evident that overlapping sequence data are obtained for the three types of derivatives. An impression of the differences between the EI and CI(ammonia) mass spectra of the GM_1 ganglioside derivatives is evident from a comparison of Figures 37 and 40.

The mass spectrometric data obtained in the field of glycolipid research is vast and has to remain outwith the scope of this chapter. Some typical recent data can be found in (269–276). For results on oligosaccharides released from glycolipids via ozonolysis or trifluoroacetolysis, see (217, 221, 247, 252).

(a)



(b)

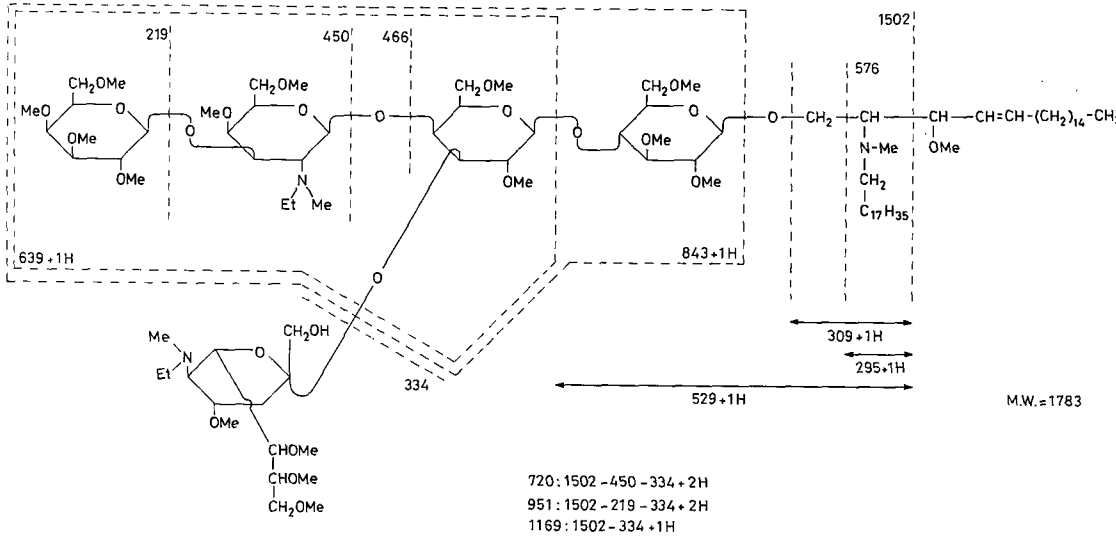
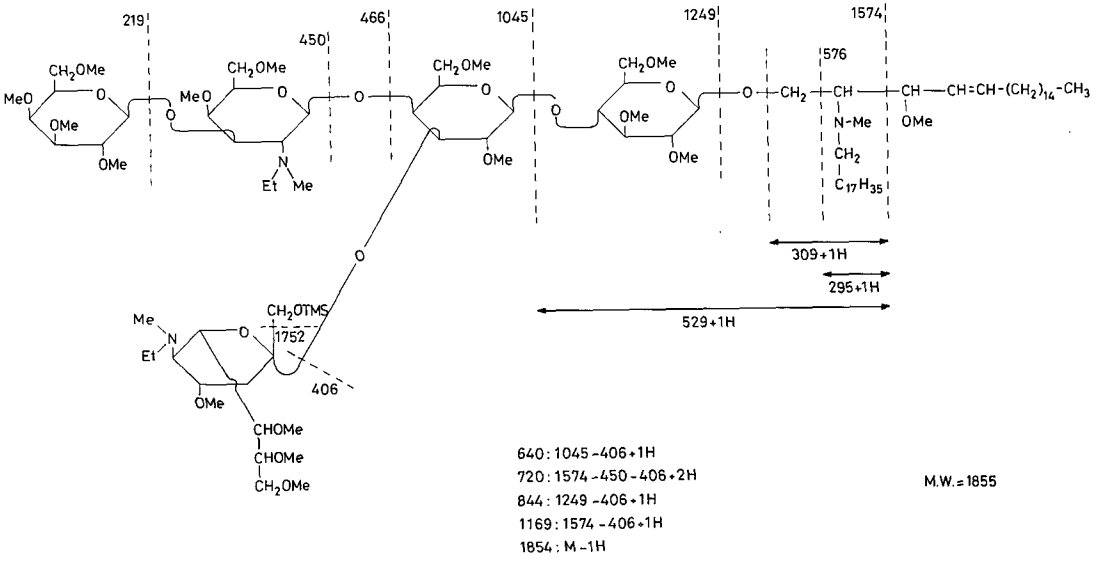


Figure 37. Fragmentation diagrams of GM_1 ganglioside (stearic acid; C_{20}-sphingosine): (a) permethylated; (b) permethylated-reduced; (c) permethylated-reduced-trimethylsilylated. The mass spectra were recorded in the EI-mode.

(c)



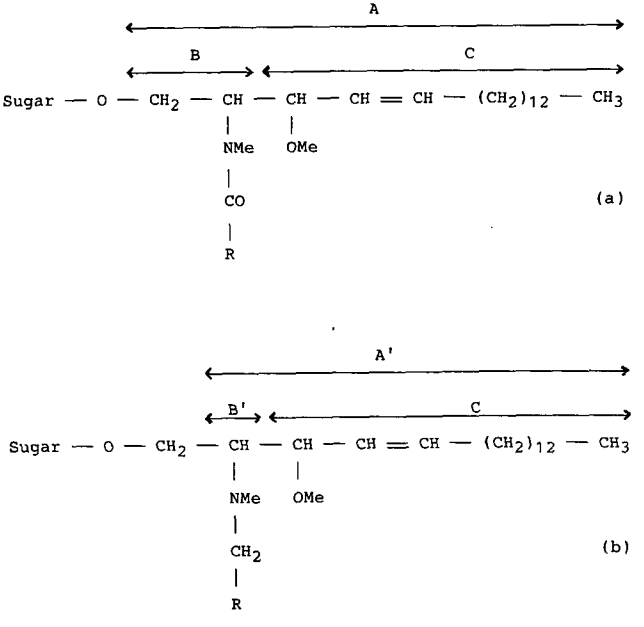


Figure 38. Schematic fragmentation patterns of permethylated (a) and permethylated and reduced (b) glycosphingolipids whose ceramide residue is composed of sphingosine and *n*-fatty acids (R = acyl group).

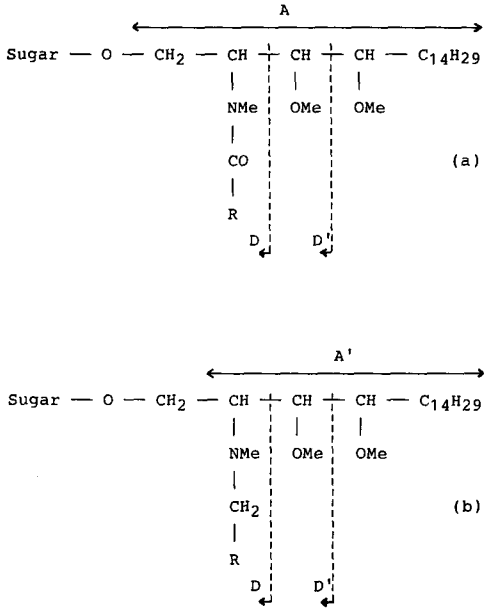
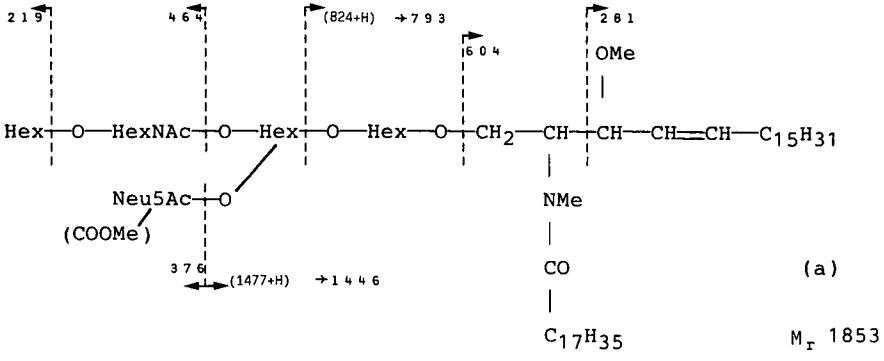


Figure 39. Schematic fragmentation patterns of permethylated (a) and permethylated and reduced (b) glycosphingolipids whose ceramide residue is composed of 4D-hydroxysphinganine and *n*-fatty acids (R = acyl group).

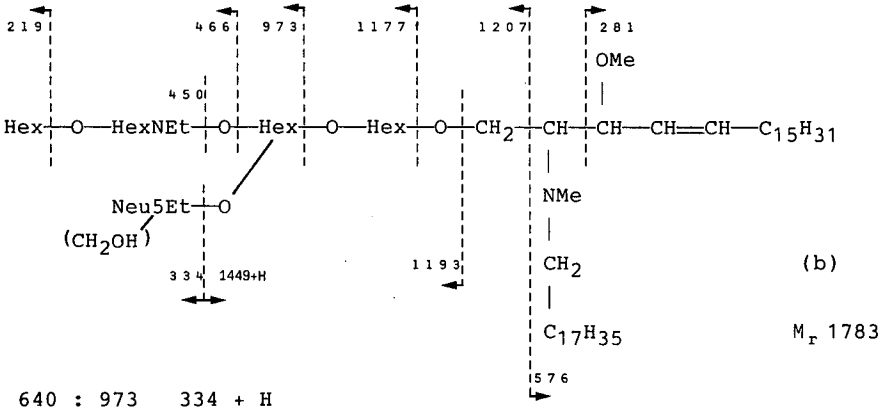
4.4 Fast atom bombardment mass spectrometry

The recent introduction of FAB-MS in carbohydrate chemistry has proven to be a valuable additional technique for the structural analysis of sugar chains, especially with respect to molecular weight determination and sequence analysis. Its combination with high field and high resolution magnetic sector mass spectrometers makes it possible to investigate polar non-volatile compounds or derivatives with relatively high molecular weights. Positive as well as negative ion FAB-MS data have been published for all categories of carbohydrate chains discussed in this review: oligosaccharides, oligosaccharide-alditols, glycopeptides and glycosphingolipids (277 – 304). Mainly underivatised, permethylated and peracetylated derivatives have been studied.

In the positive ion FAB mass spectra the molecular weight can be deduced from the relatively abundant protonated molecular ion $[M + H]^+$, and from the frequently present cationized molecular ions $[M + Na]^+$, $[M + K]^+$. The amounts of the latter species can be enhanced by the addition of suitable salts to the matrix. The negative ion FAB mass spectra are generally characterized by relatively abundant $[M - H]^-$ ions. The intense quasi-molecular ions in FAB-MS mass spectra may be used with great advantage to ascertain the homogeneity of the compounds under investigation (282), or just to study mixtures of oligosaccharide chains (281). Several solvents have been employed as matrix i. a. glycerol, 1-mercapto-2,3-propanediol (thioglycerol), polyethylene glycol and triethanolamine. Frequently use is made of additives like sodium acetate in methanol, acetic acid and 1,1,3,3,-tetramethylurea.



982 : 1478 - 464 - MeOH
1227 : 1478 - 219 - MeOH
1822 : MH - MeOH
1854 : MH



640 : 973 334 + H
844 : 1177 - 334 + H
1000 : 1450 - 450
1784 : MH

Figure 40. Fragmentation diagrams of GM_1 ganglioside (stearic acid; C_{20}-sphingosine); (a) permethylated; (b) permethylated-reduced: (c) permethylated-reduced-trimethylsilylated. The mass spectra were recorded in the CI(ammonia)-mode. For complete structures, see Figure 37.

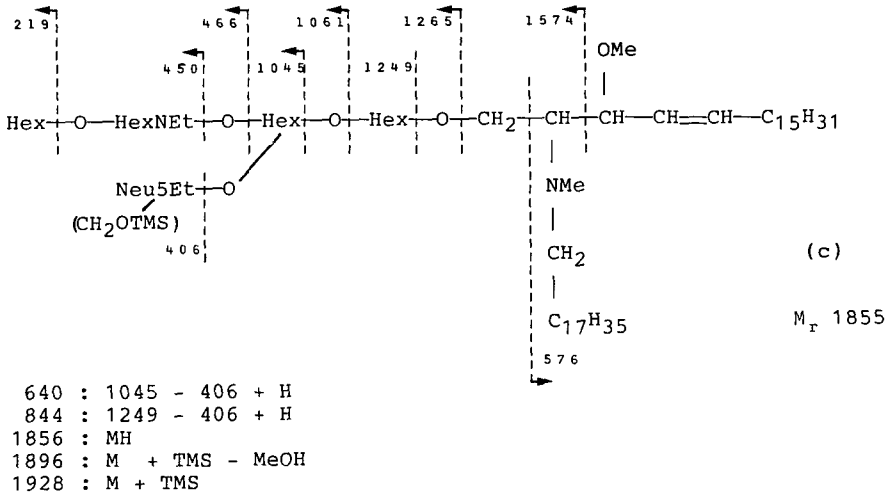
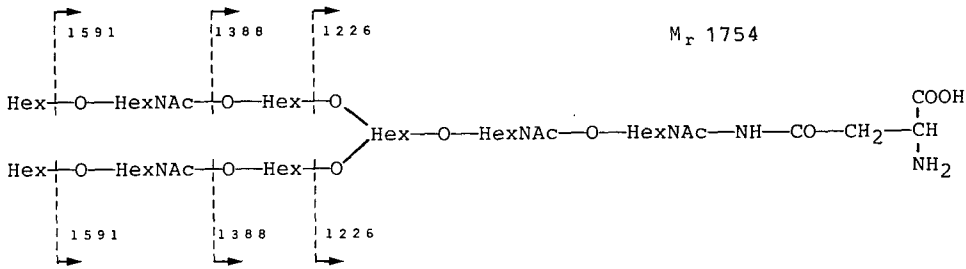


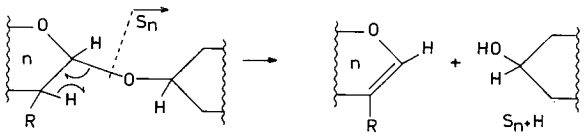
Figure 40 (Continued)



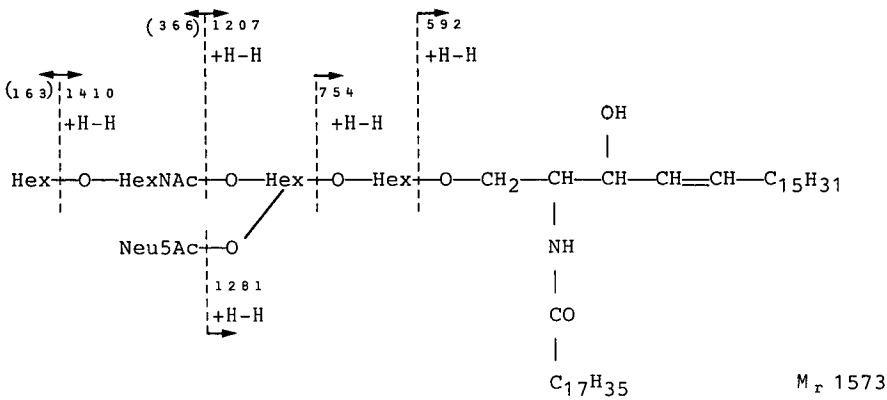
1753 : M - H
1735 : (M - H_2O) - H
1619 : (1591 + HCO) - H
1591 : (1591 + H) - H
1573 : (1591 + H - H_2O) - H
1416 : (1388 + HCO) - H
1388 : (1388 + H) - H
1370 : (1388 + H - H_2O) - H
1254 : (1226 + HCO) - H
1226 : (1226 + H) - H

Figure 41. Molecular weight and sequence information from the negative ion FAB mass spectrum of Gal*p*-β(1 → 4)-GlcNAc*p*-β-(1 → 2)-Man*p*-α(1 → 3)-[Gal*p*-β(1 → 4)-GlcNAc*p*-β(1 → 2)-Man*p*-α(1 → 6)]Man*p*-β(1 → 4)-GlcNAc*p*-β(1 → 4)-GlcNAc*p*-β(1 → N)Asn (glycerol as matrix).

In Figure 41 the schematic presentation of a biantennary asialoglycopeptide together with the molecular weight and sequence information deduced from the negative ion FAB mass spectrum is shown (280). The most important fragmentation reaction involves cleavage of the glycosidic bond between the anomeric carbon atom and the interglycosidic oxygen atom. This reaction is accompanied by a hydrogen migration to the interglycosidic oxygen, resulting in negative fragment ions ($[S_n + H]-H)^-$; see Scheme 9. In the case of analysis of underivatized glycosphingolipids, the matrix



Scheme 9. Sequence ion formation by glycosidic bond cleavage. The index n of Sn refers to the cleaved glycosidic bond at the anomeric carbon atom of monosaccharide n.



1572 : M - H
1119 : (1281 + H) - 163
916 : (1281 + H) - 366

Figure 42. Fragmentation diagram of underivatized GM_1 ganglioside (stearic acid; C_{20}-sphingosine) using negative ion FAB-MS with triethanolamine/1,1,3,3-tetramethylurea as matrix.

triethanolamine/1,1,3,3-tetramethylurea has shown to give excellent negative ion FAB mass spectra (283, 284). In Figure 42 the fragmentation for GM_1 ganglioside (with stearic acid and C_{20}-sphingosine) is presented (284). Structural isomers having different binding positions of sialic acid can be discriminated (293). Natural and synthetic neutral glycosphingolipids are discussed in (304). It has to be noted that examples have been reported showing no sequence information (292).

Positive ion FAB-MS has been successfully used for the analysis of permethylated and peracetylated derivatives. Besides the occurrence of quasi-molecular ions sequence ions are observed. Cleavages have been detected on either site of the glycosidic oxygen. Charged fragments can result from the non-reducing end of the chain as well as from the reducing end of the molecule. In the case of hexosamine-containing compounds, it has been found that fragment ions are predominantly formed by cleavage of the glycosidic bonds at C1 of the amino sugar residues, starting from the non-reducing terminal of the carbohydrate chain (278, 279, 294–299). This makes the technique highly effective for sequence analysis of permethylated polylactosamine carbohydrate

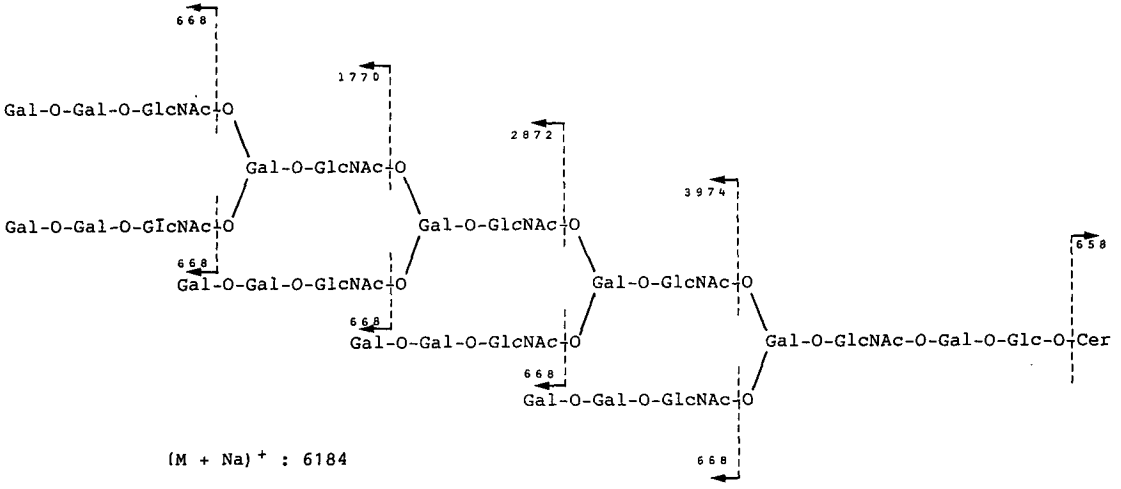


Figure 43. Fragmentation diagram of a permethylated glycolipid with molecular weight 6161 using positive ion FAB-MS with thioglycerol as matrix.

chains (296–299). An example of a permethylated glycolipid with a molecular weight of 6161 is given in Figure 43 (294, 295). Besides the cleavages in the carbohydrate chain, a specific cleavage occurs at the glycosidic linkage between the chain and the ceramide moiety. Intense ions are observed representing the whole ceramide residue. Analysis of peracetylated oligosaccharides using positive ion FAB-MS gives better results with thioglycerol as matrix than with glycerol (278, 279, 296).

FAB-MS has become a well established method for the structural analysis of carbohydrate chains and many papers have been published on its application. Space does not permit a detailed discussion of these, but data are available on glycoproteins (313–336), proteoglycans (337, 338), polysaccharides (339) and glycolipids (340–356). Recently, a review has apeared (357).

5 A General Strategy for Oligosaccharide-Analysis

To give an impression of the general strategy followed in the structural analysis of oligosaccharides isolated from biological fluids, tissues and cell systems, the characterisation of the simple mannosidosis trisaccharide α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc will be discussed in more detail.

Sugar analysis (Section 2). The mannosidosis-saccharide was subjected to the methanolysis procedure, leading to a mixture of trimethylsilylated methyl glycosides. Quantitative GC(-MS) analysis demonstrated the occurrence of Man and GlcNAc residues in a molar ratio of 2:1. In the case where sugar analysis was carried out on the reduced

oligosaccharide, Man and GlcNAc-ol were also found in the molar ratio 2:1. These data indicate the oligosaccharide to be a trisaccharide having GlcNAc as reducing unit. Determination of the absolute configuration by GC-MS of the trimethylsilylated (−)-2-butyl glycosides (Section 2.7) showed D-configuration for both monosaccharides.

Permethylation analysis (Section 3). In order to get information about the substitution patterns of the three monosaccharides, the trisaccharide-alditol-[1-^{2}H] was permethylated with CH_3I in the presence of sodium methylsulfinylmethanide. GC-MS analysis of the partially methylated alditol-[1-^{2}H] acetates showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-mannitol-[1-^{2}H], 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-mannitol-[1-^{2}H] and 4-mono-*O*-acetyl-1,3,5,6-tetra-*O*-methyl-2-*N*-methyl-acetamido-2-deoxy-glucitol-[1-^{2}H] (for EI mass spectra Figs. 26–28). The finding of these alditols points to the occurrence of a terminal Man*p*-residue, a 3-substituted Man*p*-residue and a 4-substituted GlcNAc-ol residue. Therefore the structure of the trisaccharide-alditol is: Man*p*-(1 → 3)-Man*p*-(1 → 4)-GlcNAc-ol[1-^{2}H].

Sequence analysis (Section 4). The permethylated trisaccharide-alditol-[1-^{2}H] was also studied directly by EI-MS (Figure 35). Although the spectrum does not show a molecular ion peak, the molecular weight can be deduced easily from the abundant primary fragment ion *m/z* 670 (M minus CHDOMe). The monosaccharide sequence Hex → Hex → HexNAc-ol-[1-^{2}H] is proved by the presence of the sequence fragment ions *m/z* 481, 423, 277, 219 and 187. Information concerning the (1 → 4)-linkage between Hex and HexNAc-ol-[1-^{2}H] is indicated by the *m/z* values 89 and 175. The absence of *m/z* 337 (bcJ_1) suggests the occurrence of a 3-linked Hex → HexNAc-ol-[1-^{2}H] unit. Although not discussed here, in addition other ionization techniques such as CI and FAB can be applied. For FAB-studies also the underivatized trisaccharide can be used.

Finally, the determination of the configuration of the glycosidic linkages has to be performed along other routes. Nowadays, mainly NMR techniques and enzymic approaches are incorporated for this purpose. It should be stressed that besides MS these methods are also of more general importance in carbohydrate analysis. For ^{1}H-NMR and ^{13}C-NMR data of the trisaccharide, see (311, 312). From these data it can be concluded that the terminal Man*p*-residue is coupled α-glycosidically to the internal Man*p*-residue, which in turn is coupled β-glycosidically to the reducing GlcNAc unit.

In conclusion, the oligosaccharide isolated from urine of a patient with α-mannosidosis has the structure α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 → 4)-D-GlcNAc.

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