F. Gas-Liquid Chromatography and Mass Spectrometry of Sialic Acids

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I. Introduction

For the structural analysis of the carbohydrate chains of glycoconjugates, oligosaccharides and polysaccharides, the introduction of gas-liquid chromatography (g.l.c.) and later on of gas-liquid chromatography combined with mass spectrometry (g.l.c./m.s.) has proved to be of great importance. As has been summarized in several comprehensive reviews, many protecting groups are in use to prepare volatile sugar derivatives (Bishop 1964, Clamp *et al.* 1971, Dutton 1973, 1974).

Among the monosaccharides which occur as constituents of glycoconjugates and polysaccharides, sialic acid is a complicated compound. From a chemical point of view it is a C_9-monosaccharide with several different functional groups, which have each to be taken into account when developing derivatization techniques. One can discern a carboxyl group, a keto-acetal function, an acetylated or glycolylated amino function, hydroxyl groups with different reactivities, and a deoxy-group adjacent to the anomeric centre. One or more of the hydroxyl groups can be acetylated, lactylated, methylated or sulfated. In water, sialic acids exist predominantly in the β-anomeric form (equatorial carboxyl group in the stable $^2C_5$-conformation) (Haverkamp *et al.* 1982). Methyl ester methyl glycosides of sialic acids obtained by treatment with methanolic HCl have also mainly the β-configuration.

In glycoproteins and glycolipids, sialic acids occupy generally terminal positions

in the carbohydrate chain; they are coupled via α-glycosidic linkages (MACHER and SWEELEY 1978, MONTREUIL 1980). Sometimes oligomeric chains of sialic acid residues are attached to the carbohydrate backbone of these molecules (FINNE *et al.* 1977, MACHER and SWEELEY 1978, INOUE and MATSUMURA 1980). Also internal sialic acid residues have been detected, even as branching points (HOTTA *et al.* 1973, SUGITA 1979a, b, SMIRNOVA and KOCHETKOV 1980, VAN DER MEER *et al.* 1982). Furthermore, sialic acids have been found as constituents of homo- and hetero-polysaccharides (BHATTACHARJEE and JENNINGS 1976).

II. Gas-Liquid Chromatography

Protection of sialic acids by trimethylsilylation is widely applied in g.l.c. analysis. Several variants have been described: trimethylsilylation of sialic acid methyl esters (SWEELEY *et al.* 1963, SCHAUER *et al.* 1976), of sialic acid methyl ester methyl glycosides (SWEELEY and WALKER 1964, YU and LEDEEN 1970, HAVERKAMP *et al.* 1975, KAMERLING *et al.* 1975a, MONONEN and KÄRKKÄINEN 1975), of free sialic acids (CRAVEN and GEHRKE 1968, CASALS-STENZEL *et al.* 1975, ROBOZ *et al.* 1978), and of deaminated sialic acid methyl ester methyl glycoside (MONONEN 1981). Furthermore, the analyses of trifluoroacetylated (ZANETTA *et al.* 1972, YOHE and YU 1981), permethylated and partially O-methylated (BHATTACHARJEE and JENNINGS 1976, RAUVALA and KÄRKKÄINEN 1977, VAN HALBEEK *et al.* 1978, INOUE and MATSUMURA 1979, BRUVIER *et al.* 1981) sialic acid methyl ester methyl glycosides have been reported. The latter derivatives are analyzed after subsequent trimethylsilylation or acetylation of free hydroxyl groups.

The release of monosaccharides from glycoconjugates and polysaccharides can be carried out via hydrolysis or methanolysis. The stabilities of free monosaccharides to aqueous acid vary greatly, necessitating different hydrolysis conditions optimal for each type of sugar. On the other hand, it has been reported that methanolysis is as efficient as hydrolysis in cleaving glycosidic bonds, and causes less destruction of sugars than does aqueous acid. This observation made it possible to develop single step methods for the qualitative and quantitative analyses of commonly occurring monosaccharides as (methyl ester) methyl glycoside derivatives.

Generally used methanolysis conditions are 0.5-1.0 N methanolic HCl (16-24 h, 80-85 °C) (CHAMBERS and CLAMP 1971, CLAMP *et al.* 1971, ZANETTA *et al.* 1972, KAMERLING *et al.* 1975a). Although the glycosidic bonds of the sialic acids are cleaved in a good yield and destruction is minimized, these conditions lead to complete N,O-deacylation. Therefore, most authors apply a (re-)N-acetylation step in the derivatization procedure, resulting in the formation of Neu5Ac methyl ester methyl glycoside (CHAMBERS and CLAMP 1971, CLAMP *et al.* 1971, KAMERLING *et al.* 1975a). After trimethylsilylation this approach makes it possible to determine the sialic acids in the form of Neu5Ac. Details including g.l.c. are given in section II.1. When trifluoroacetylation is applied, the (re-)N-acetylation step can be omitted; sialic acids are analyzed in the form of N-trifluoroacetyl-neuraminic acid (ZANETTA *et al.* 1972).

Special attention has been paid to the protection of N- and O-acyl substituents against methanolic HCl. YU and LEDEEN (1970) proposed a mild methanolysis

procedure (0.05 N methanolic HCl; 1 h, 80 °C), which would supposedly lead to only a small degree of N-deacylation. Based on this study, they developed a quantitative g.l.c. method for the combined determination of Neu5Ac and Neu5Gc. It should be noted that O-deacylation occurs even when 0.02 N methanolic HCl is employed (CASALS-STENZEL *et al.* 1975). Application of the latter conditions has the disadvantage of incomplete release of sialic acid as methyl ester methyl glycoside.

Terminal sialic acids are released in a good yield under mild acidic conditions like formic acid, pH 2 (1 h, 70 °C), 0.1 N H_2SO_4 or 0.1 N HCl (1 h, 80 °C) (SCHAUER 1978). These conditions do not lead to N-deacylation; however, O-deacylation occurs to an extent of about 50%. Mild acid hydrolysis has frequently been chosen to release N,O-acylneuraminic acids from biological materials for identification by g.l.c./m.s. Derivatization procedures are presented in sections II.3 and II.4. For g.l.c./m.s., see section III.2. Quantitative g.l.c. procedures after pertrimethylsilylation (section II.4) have been worked out by CASALS-STENZEL *et al.* (1975). Because of partial O-deacylation during hydrolysis and subsequent isolation, quantitative analysis of the various N,O-acylneuraminic acids present in *native* biological material is still a serious problem. The nonavailability of pure N,O-acylneuraminic acids as standard compounds makes it also difficult to determine reliable molar adjustment factors for g.l.c. analysis.

SUGITA (1979 a) developed a different approach for the combined determination of Neu5Ac and Neu5Gc, by applying solvolysis with 1.0 N *n*-butanolic HCl. The formed *n*-butyl acetate and *n*-butyl glycolate are analyzed by g.l.c. (section II.5).

1. Quantitative Sugar Analysis, Including Sialic Acid
(CHAMBERS and CLAMP 1971, CLAMP *et al.* 1971, KAMERLING *et al.* 1975 a)

In an ampoule the sialoglycoconjugate or polysaccharide (0.5–2 mg) is mixed with a mannitol solution (internal standard; 25–100 nmol). After lyophilization and drying over P_2O_5 in a vacuum desiccator, the residue is dissolved in 1.0 N methanolic HCl (0.5 ml). Nitrogen is bubbled through the solution for 30 sec, and then the ampoule is sealed. The solution is heated for 24 h at 85 °C; subsequently, neutralization is carried out by the addition of solid silver carbonate (pH-paper). (Re-)N-acetylation is performed by the addition of acetic anhydride (10–50 μl). The obtained mixture 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. The final residue is dried for 12 h in a vacuum desiccator over P_2O_5. Before g.l.c. 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 CPsil5 WCOT fused silica capillary column (25 m × 0.32 mm, i.d.) using flame-ionization detection. The carrier gas nitrogen flow-rate is 1.5 ml/min and the make-up gas nitrogen flow-rate 35 ml/min. The split-ratio amounts 1 : 10. The injection-port temperature is 210 °C, and the detector temperature 230 °C. The oven temperature is

programmed from 130 to 220 °C at 2 °C/min and kept isothermally at 220 °C for 1 min. A typical gas chromatogram of a standard mixture is presented in Fig. 1.

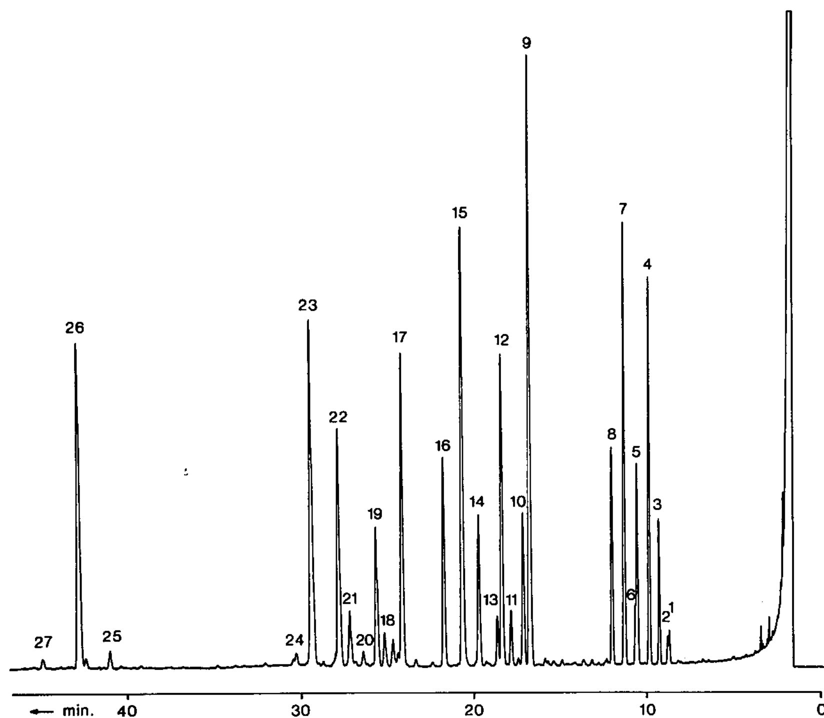

Fig. 1. Gas chromatogram of trimethylsilylated (methyl ester) methyl glycosides on a CPsil5 WCOT fused silica capillary column (25 m × 0.32 mm). Oven temperature program: 130 to 220 °C at 2 °C/min; 1 min at 220 °C. The peaks are numbered in their order of elution and are assigned as follows: *1* Xylose (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-acetylmannitol; *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* Neu5Ac (α); *26* Neu5Ac (β); *27* Neu5,9Ac$_2$. *f* furanoside; *p* pyranoside.

Remarks: 1. The amount of added internal standard depends on the carbohydrate-content in the sialoglycoconjugate; 2. When too much acetic anhydride is added, the primary hydroxyl functions of mannitol are O-acetylated giving rise to an additional small peak in the gas chromatogram. The same holds for the primary hydroxyl function of Neu5Ac methyl ester methyl glycoside; 3. Molar adjustment factors of monosaccharides except Neu5Ac are determined by application of the methanolysis procedure on standard mixtures of sugars and internal standard. For Neu5Ac the molar adjustment factor is determined using a

methanolyzed known sialooligosaccharide; 4. Under the conditions used, the GlcNAc—Asn linkage is hardly cleaved. This has to be taken into account when calculating ratios.

2. *Hydrolytic Release of Sialic Acids from Sialobiopolymers*

These data are discussed in detail in chapter C.

3. *Preparation and Analysis of Trimethylsilylated N,O-Acylneuraminic Acid Methyl Esters*

(KAMERLING *et al.* 1975c, SCHAUER *et al.* 1976)

Sialic acid dried over P_2O_5 (50-100 μg) dissolved in dry methanol (0.5 ml) is treated with diazomethane in ether until a faint-yellow colour is obtained. The solution is immediately evaporated under reduced pressure, and the residue dissolved in pyridine (1 ml). Subsequently, hexamethyldisilazane (0.2 ml) and chlorotrimethylsilane (0.1 ml) are added. After 2 h at room temperature, chloroform (2 ml) and water (2 ml) are added to the turbid mixture. The chloroform layer is dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The g.l.c. analysis is carried out on a glass column (2 m × 4 mm, i.d.) packed with 3.8% SE-30 on Chromosorb W HP, 80-100 mesh, using flame-ionization detection. The oven temperature is 215 °C and the carrier gas nitrogen flow-rate 40 ml/min. Retention times R_{Neu5Ac} are given relative to the trimethylsilyl derivative of Neu5Ac methyl ester (see Table 2 on page 104).

4. *Preparation and Analysis of Pertrimethylsilylated N,O-Acylneuraminic Acids*

(CASALS-STENZEL *et al.* 1975)

Sialic acid dried over P_2O_5 (10-100 μg) is mixed with N-trimethylsilylimidazole (25 μl) in a test tube. The tube is flushed with nitrogen and whirled for either

Table 1. *Gas-liquid chromatography of pertrimethylsilylated N,O-acylneuraminic acids. The R_{Neu5Ac}-values on 3.5% OV-17 (temperature program: 200 to 280 °C at 2 °C/min) are given relative to pertrimethylsilylated Neu5Ac*

Sialic acid	R_{Neu5Ac} (β-anomers)
Neu5Ac	1.00
Neu4,5Ac_2	1.37
Neu5,7Ac_2	1.20
Neu5,9Ac_2	1.28
Neu4,5,9Ac_3	1.60
Neu5,7,9Ac_3	1.53
Neu5,8,9Ac_3	1.64
Neu5,7,8,9Ac_4	1.87
Neu5Gc	1.43
Neu9Ac5Gc	1.75
Neu7,9$Ac_2$5Gc	2.06
Neu2en5Ac	1.32

15 min at room temperature or 5 min at 60 °C in a heating block to complete the silylation reaction. For the trimethylsilylation, a mixture of pyridine-hexamethyldisilazane-chlorotrimethylsilane, 5 : 1 : 1, can also be used (reaction conditions: 3 h at room temperature). The g.l.c. analysis is carried out on a glass column (2.2 m × 2 mm, i.d.) packed with 3.5% OV-17 on Chrom GAW-DMCS, 80–100 mesh, using flame-ionization detection. The oven temperature is programmed from 200 to 280 °C at 2 °C/min. The carrier gas nitrogen flow-rate is 30 ml/min. The derivatives are stable in the refrigerator. Table 1 gives a survey of the relative retention times of a series of pertrimethylsilylated N,O-acylneuraminic acids.

5. Analysis of Acetyl and/or Glycolyl Residues of Sialic Acid (SUGITA 1979 a)

Glycoconjugate dried over P_2O_5 (1 mg) is treated with 1.0 N *n*-butanolic HCl for 3 h at 100 °C. After cooling, the acidic *n*-butanol solution is neutralized with silver carbonate (pH-paper). An aliquot of the *n*-butanol solution is analyzed for *n*-butyl acetate and/or *n*-butyl glycolate by g.l.c. on 3% diethylene glycol succinate on Shimalite, 80–100 mesh, at an oven temperature of 40 °C (column: 2 m × 3 mm, i.d.).

III. Gas-Liquid Chromatography/Mass Spectrometry

In structure analyses of sialic acids, the combination of g.l.c. and m.s. is almost indispensable. By careful studies of the mass spectra of the trimethylsilylated methyl esters of Neu5Ac and Neu5Gc and of some related derivatives, a general electron impact (e.i.) mass spectrometric micromethod has been developed for the identification of N,O-acylneuraminic acids isolated from biological material (KAMERLING *et al.* 1974, 1975 c, 1978). The method has also proved to be useful for the analysis of other isolated sialic acids, of (partially) O-methylated sialic acid methyl ester methyl glycosides as obtained in methylation analyses, and of synthetic sialic acid(s) (derivatives).

1. Mass Spectrometric Identification Procedure

To obtain volatile sialic acid derivatives, free carboxyl groups are converted into methyl esters and free hydroxyl groups into methyl ethers, trimethylsilyl ethers or acetyl esters. In methylation analysis studies, the acetylated or glycolylated amino function is also methylated. Figs. 2 and 3 present the e.i. mass spectra of the trimethylsilylated methyl ester of Neu5Ac, and of the permethylated methyl ester methyl glycoside of Neu5Ac, respectively. In Fig. 4, a schematic survey is depicted showing the selected fragment ions *A–H*, which furnish the information (abundances and *m/e* values of the ions) necessary to deduce the complete structure of the sialic acids. Although it has not been checked in detail, it is highly probable that the use of trimethylsilyl esters instead of methyl esters will not change the identification procedure. Fragments *A* and *B* indicate the molecular weight of the sialic acid derivatives and thereby the number and type of substituents. Fragments *C–H* contain the information concerning the position of the different substituents.

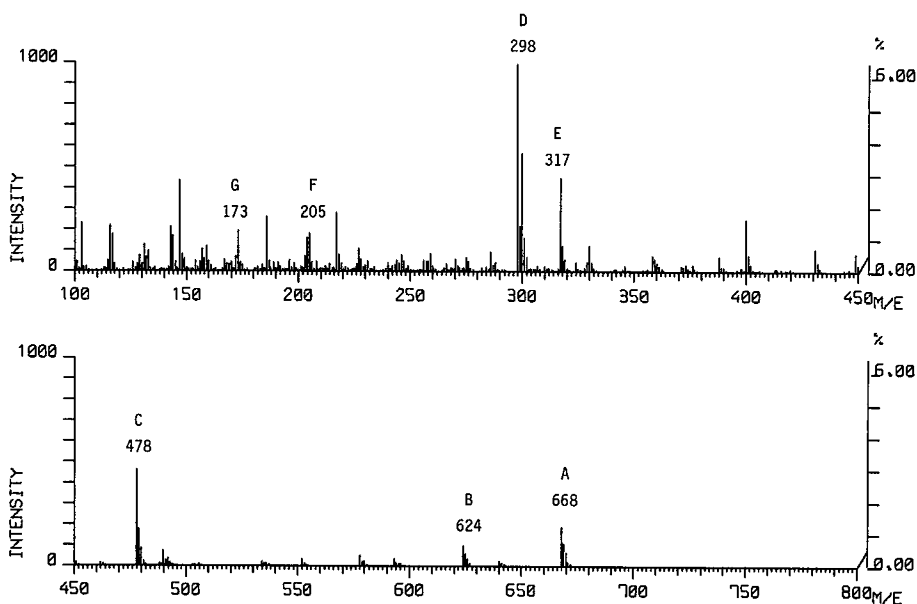

Fig. 2. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu5Ac (β-form).

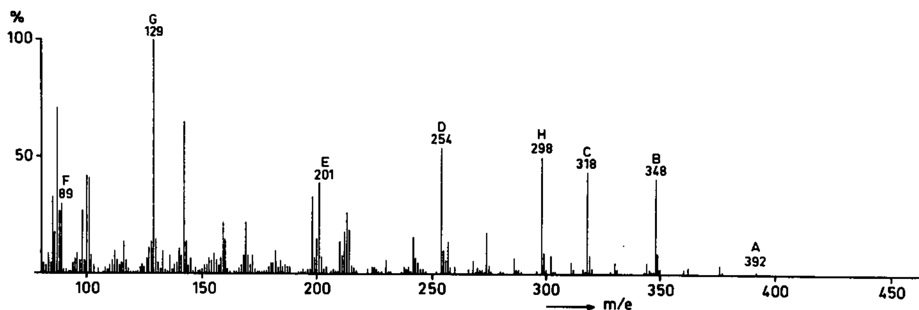

Fig. 3. E.i.-mass spectrum (70 eV) of the permethylated methyl ester β-methyl glycoside of Neu5Ac.

Fragment *A* is formed by elimination of a methyl group from the molecular ion *M*. In trimethylsilylated (O-acylated) N-acylneuraminic acid derivatives, the methyl group originates from a trimethylsilyl substituent, whereas in acetylated partially O-methylated N,N-acetyl,methyl-neuraminic acid methyl ester methyl glycosides the N,N-acetyl,methyl group is responsible. Trimethylsilylated partially O-methylated N,N-acetyl,methyl-neuraminic acid methyl ester methyl glycosides give rise to both possibilities, but the elimination from a trimethylsilyl group dominates (Van Halbeek *et al.* 1978).

Fragment *B* is obtained by release of the C-1 part of the sialic acid molecule. Eliminations of $OCOCH_3$ in O-acetylated neuraminic acid derivatives and of NH_2COCH_3 in Neu5Ac derivatives, which in principle give rise to the same *m/e* value as fragment *B* in the case of $R_1 = CH_3$, contribute little to the abundancy of this ion.

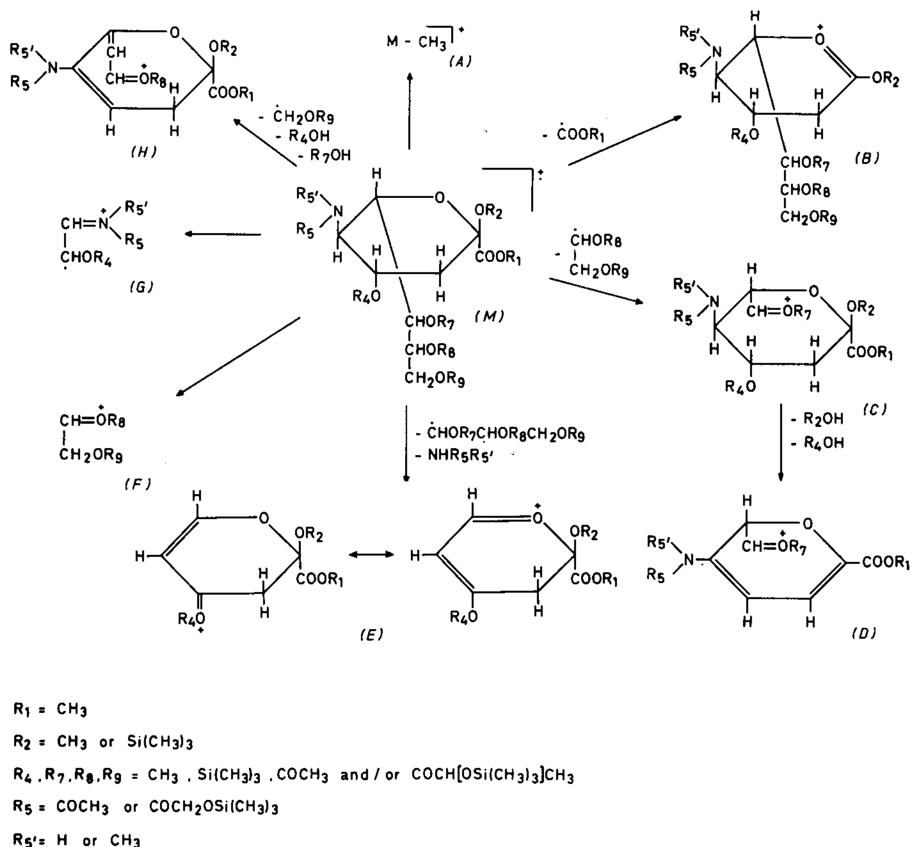

Fig. 4. Survey of the selected fragment ions *A-H* worked out for trimethylsilylated (O-acylated) N-acylneuraminic acid methyl ester(s) (methyl glycosides) and for (partially) O-methylated N,N-acetyl,methyl-neuraminic acid methyl ester methyl glycosides. Partially O-methylated compounds are trimethylsilylated or acetylated.

Fragment *C* is formed by elimination of the C-8,9 part, with localization of the charge on position 7. For cleavage of partially methylated alditol acetates, it has been demonstrated that the charge is preferentially located on an ether oxygen instead of on an ester oxygen (BJÖRNDAL *et al.* 1970, LÖNNGREN and SVENSSON 1974). Therefore, in general, cleavage occurs between two alkoxylated (methoxylated or trimethylsiloxylated) carbon atoms, or between an acetoxylated and an alkoxylated carbon atom, rather than between two acetoxylated carbon atoms. Fragment *C* has only significant abundance if C-7 bears an ether group. In

case when at C-7 an ester group is present, 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 *C*.

Fragment *E* is formed by elimination of the side-chain C-7,8,9 and the substituent at C-5. This fragment ion is not observed if an O-acetyl group is attached to C-4, illustrating that the transition state in the McLafferty rearrangement is disfavored when the substituent at C-4 is an ester group rather than an ether group.

Fragment *F* contains the C-8,9 part. 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 connected at C-8.

Fragment *G* consists of the C-4,5 part of the sialic acid molecule.

Fragment *H* is formed by elimination of the C-9 part of the molecule, followed by elimination of R_4OH and R_7OH. This fragment ion is useful for discriminating between an O-trimethylsilyl group at C-8 or C-9 in trimethylsilylated partially methylated N-acylneuraminic acid methyl ester(s) (methyl glycosides). For the identification of trimethylsilylated O-acylated N-acylneuraminic acid derivatives and acetylated partially methylated N-acylneuraminic acid derivatives, it is not necessary to take fragment *H* into consideration.

2. *Analysis of (O-Acylated) N-Acylneuraminic Acids Isolated from Biological Material*

In Table 2 a series of (O-acylated) N-acylneuraminic acids isolated from various biological sources is presented (see also chapter B and C). These sialic acids have been analyzed by g.l.c./m.s. after esterification with diazomethane and trimethylsilylation (section II.3): the Table includes relative retention times of the derivatives on g.l.c. and *m/e* values of the fragment ions *A–G*. As was mentioned earlier, sialic acids predominantly occur in the β-anomeric form. However, on 3.8% SE-30 the α-anomer could sometimes be detected separately from the β-anomer (in most cases as a small shoulder). Mass spectra are depicted in Figs. 2 and 5–17.

From Table 2, it is evident that the replacement of one O-trimethylsilyl group by one O-acetyl group causes in involved fragments a negative shift of 30 a.m.u. For two O-acetyl groups a shift of 60 a.m.u. is observed, etc. In the same way, the presence of a trimethylsilylated O-lactyl group instead of an O-trimethylsilyl group causes a positive shift of 72 a.m.u. Replacement of an N-acetyl group by a trimethylsilylated N-glycolyl group shows a positive shift of 88 a.m.u. In conclusion, each sialic acid derivative gives rise to a unique series of fragment ions *A–G*.

Fragment *G* has to be discussed in more detail (KAMERLING *et al.* 1975c, 1978). The occurrence of an O-acetyl group instead of an O-trimethylsilyl group at C-4 as in the derivatives of $Neu4,5Ac_2$, $Neu4,5,9Ac_3$, $Neu4,5Ac_29Lt$, and Neu4Ac5Gc leads to a negative shift of 30 a.m.u. for this fragment ion. Therefore, in the mass spectra of 4-O-acetylated N-acetylneuraminic acids the peak at *m/e* 173 is absent:

Table 2. *G.l.c. and m.s. 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 the esterification and trimethylsilylation procedure and additional analysis conditions, see section II.3. For an explanation of the — signs, see sections III.1 and III.2*

Sialic acid	R_{Neu5Ac} β-anomer	R_{Neu5Ac} α-anomer	*m/e* values							References
			A	*B*	*C*	*D*	*E*	*F*	*G*	
Neu5Ac	1.00	1.05	668	624	478	298	317	205	173	KAMERLING *et al.* 1974, 1975c, SCHAUER *et al.* 1976
Neu4,5Ac$_2$	1.18	1.21	638	594	448	298	—	205	143	KAMERLING *et al.* 1975c, 1982a, REUTER *et al.* 1980a
Neu5,7Ac$_2$	1.04	1.00	638	594	—	—	317	205	173	REUTER *et al.* 1982, SCHAUER *et al.* 1976
Neu5,8Ac$_2$	1.05		638	594	478	298	317	—	173	REUTER *et al.* 1982
Neu5,9Ac$_2$	1.13		638	594	478	298	317	175	173	GHIDONI *et al.* 1980, HAVERKAMP *et al.* 1976, 1977b, KAMERLING *et al.* 1975c, 1982b, REUTER *et al.* 1980b, SCHAUER *et al.* 1976
Neu4,5,9Ac$_3$	1.31		608	564	448	298	—	175	143	KAMERLING *et al.* 1975c, REUTER *et al.* 1980a
Neu5,7,9Ac$_3$	1.14	1.07	608	564	—	—	317	175	173	KAMERLING *et al.* 1975c, REUTER *et al.* 1982, SCHAUER *et al.* 1976
Neu5,8,9Ac$_3$	1.19		608	564	478	298	317	—‡	173	REUTER *et al.* 1982
Neu5,7,8,9Ac$_4$	1.15		578	534	—	—	317	—	173	REUTER *et al.* 1982
Neu5Ac9Lt†	2.55		740	696	478	298	317	277	173	HAVERKAMP *et al.* 1976, SCHAUER *et al.* 1976
Neu4,5Ac$_2$9Lt	3.01		710	666	448	298	—	277	143	REUTER *et al.* 1980a
Neu5Gc	1.81	1.90	756	712	566	386	317	205	261	KAMERLING *et al.* 1974, 1975c, SCHAUER *et al.* 1976
Neu4Ac5Gc	2.02		726	682	536	386	—	205	231	KAMERLING *et al.* 1975c
Neu7Ac5Gc	1.83		726	682	—	—	317	205	261	REUTER *et al.* 1982
Neu9Ac5Gc	2.04		726	682	566	386	317	175	261	KAMERLING *et al.* 1975c, 1980, SCHAUER *et al.* 1976
Neu7,9Ac$_2$5Gc	2.01		696	652	—	—	317	175	261	REUTER *et al.* 1982
Neu8,9Ac$_2$5Gc	1.99		696	652	566	386	317	—‡	261	REUTER *et al.* 1982
Neu7,8,9Ac$_3$5Gc	1.93		666	622	—	—	317	—	261	REUTER *et al.* 1982

† The absolute configuration of the lactyl (Lt) substituent was found to be **L** (SCHAUER *et al.* 1976).

‡ The small peak at *m/e* 145, also present in the mass spectra of other sialic acids, has not been checked by exact mass measurements (see also footnote in Table 5).

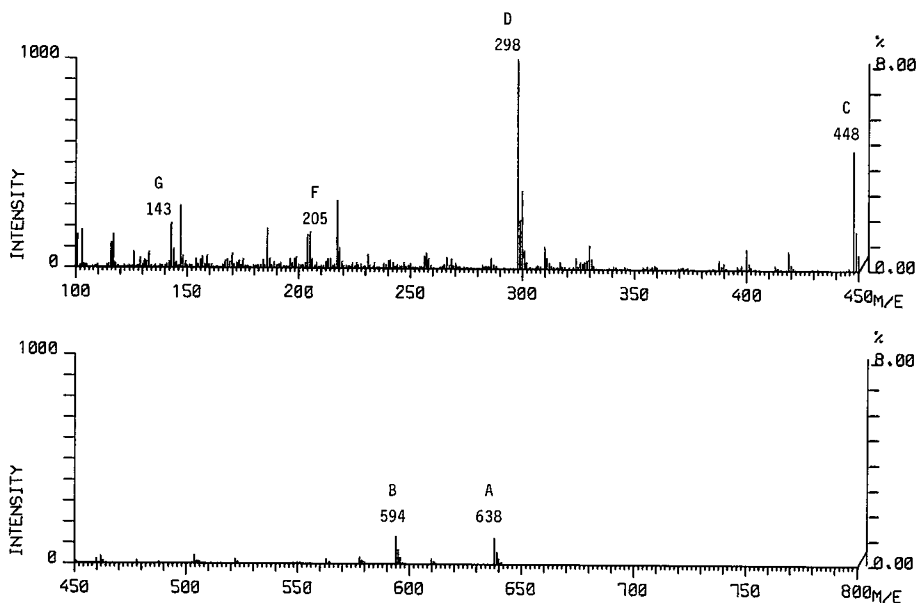

Fig. 5. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu4,5Ac$_2$ (β-form).

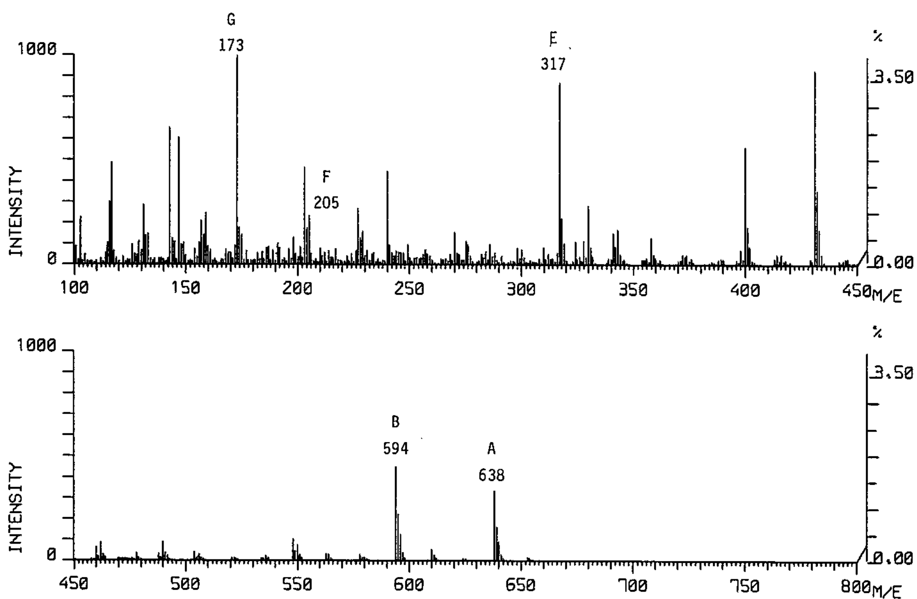

Fig. 6. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu5,7Ac$_2$ (β-form).

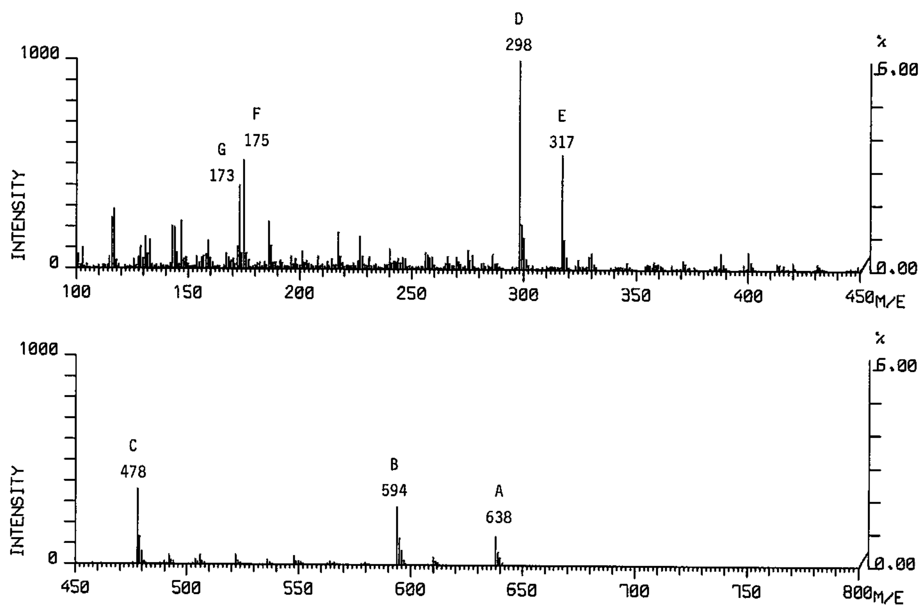

Fig. 7. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu5,9Ac$_2$.

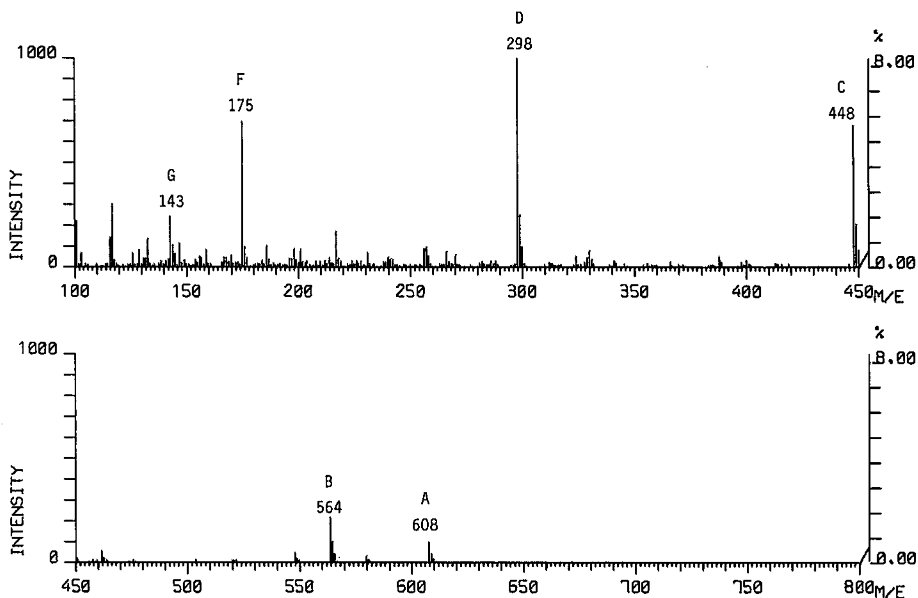

Fig. 8. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu4,5,9Ac$_3$.

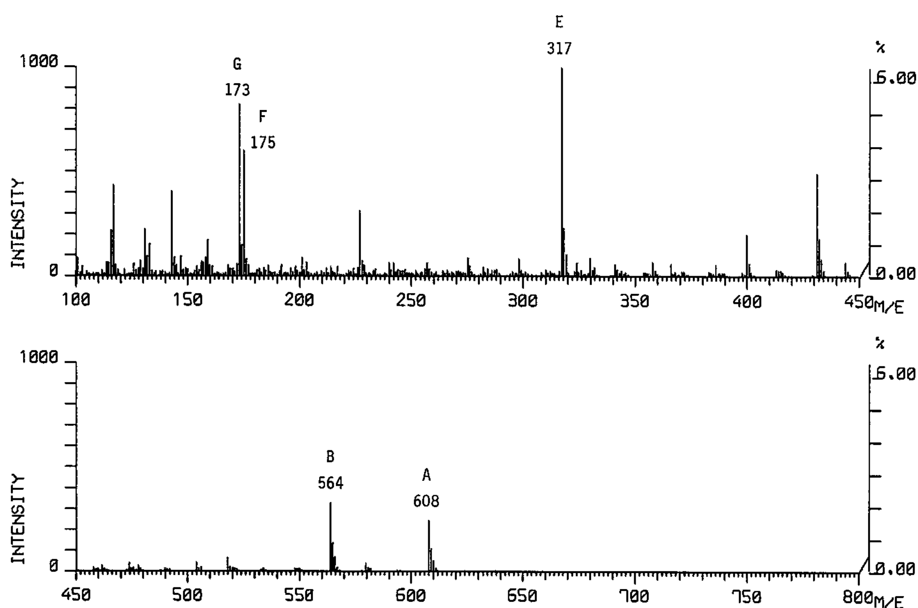

Fig. 9. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu5,7,9Ac$_3$ (β-form).

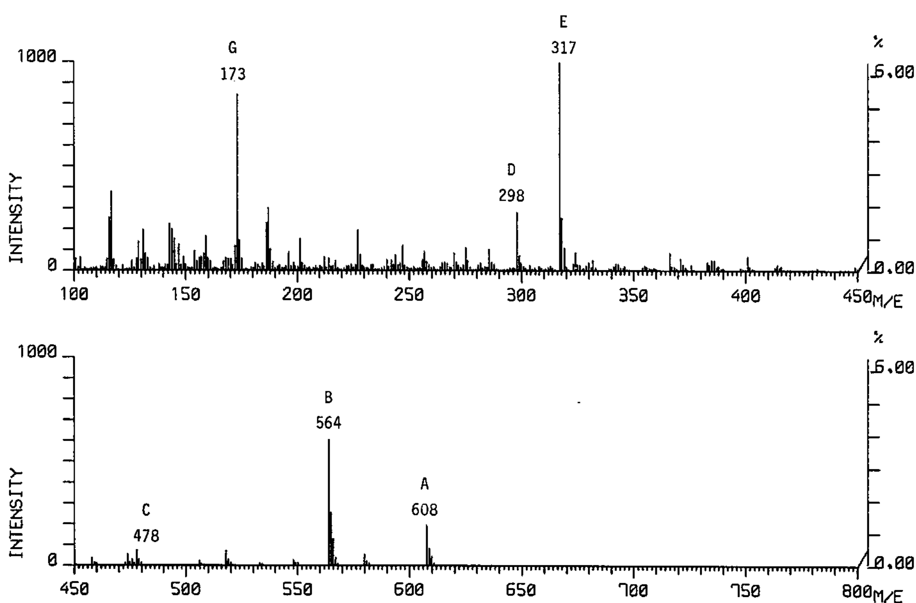

Fig. 10. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu5,8,9Ac$_3$.

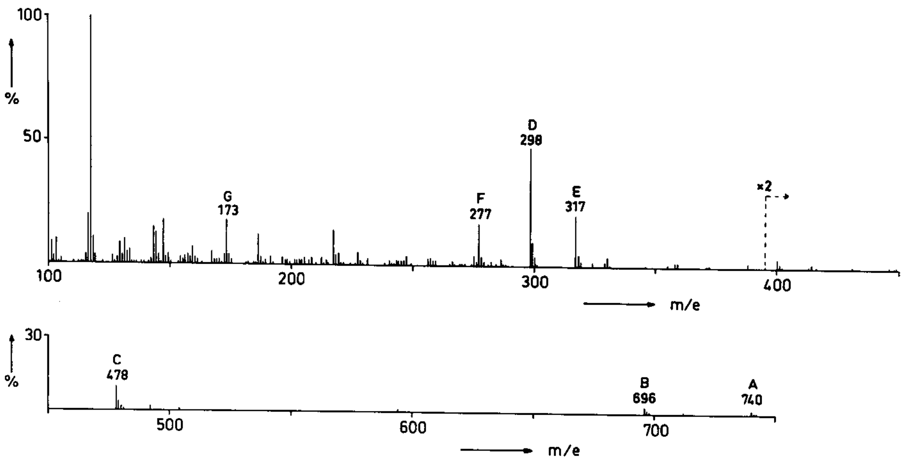

Fig. 11. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu5Ac9Lt (Lt, lactyl).

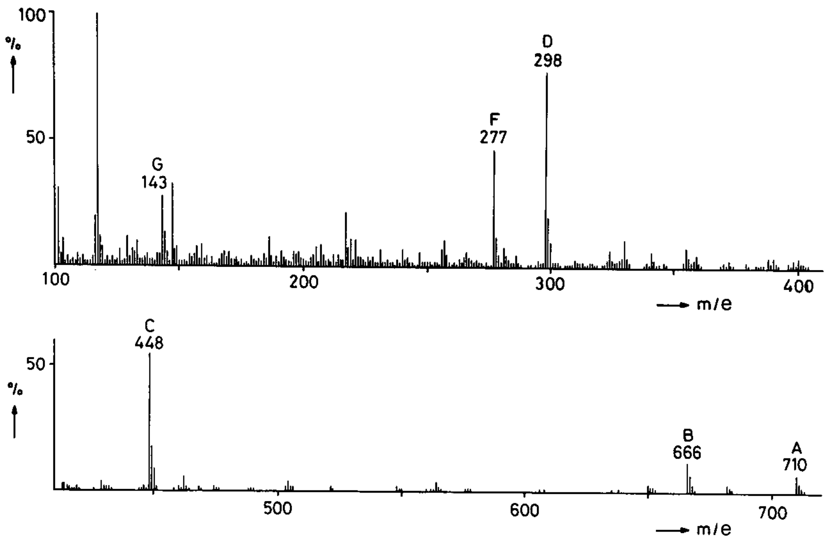

Fig. 12. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu4,5Ac$_2$9Lt.

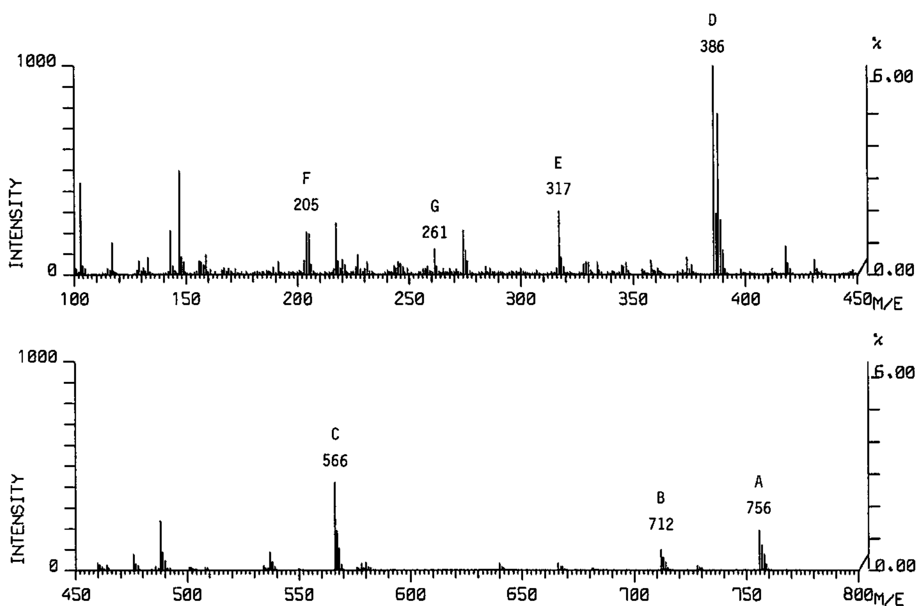

Fig. 13. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu5Gc (β-form).

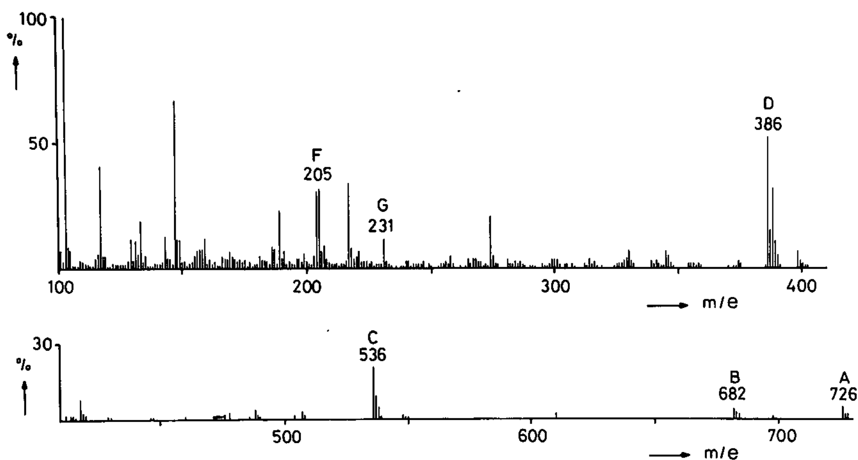

Fig. 14. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu4Ac5Gc.

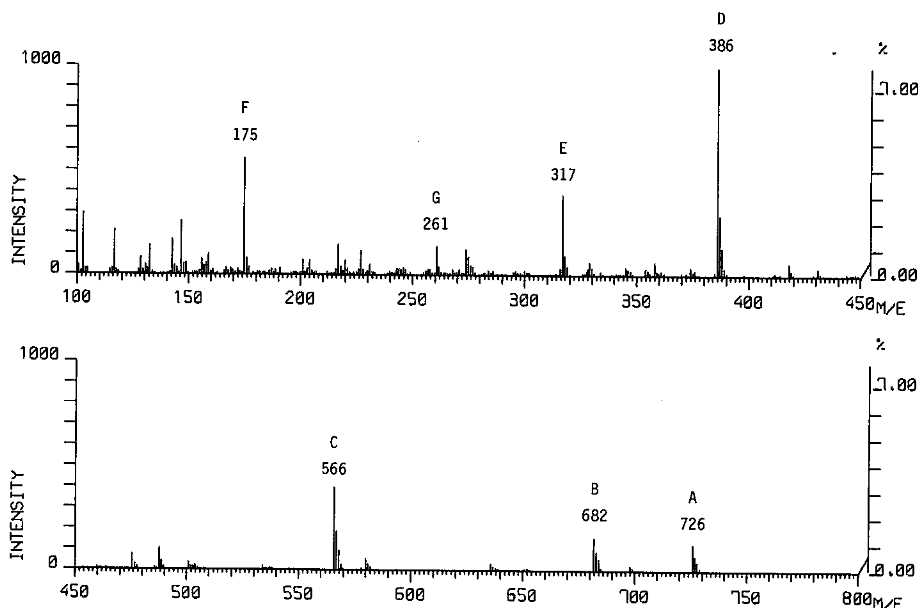

Fig. 15. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu9Ac5Gc.

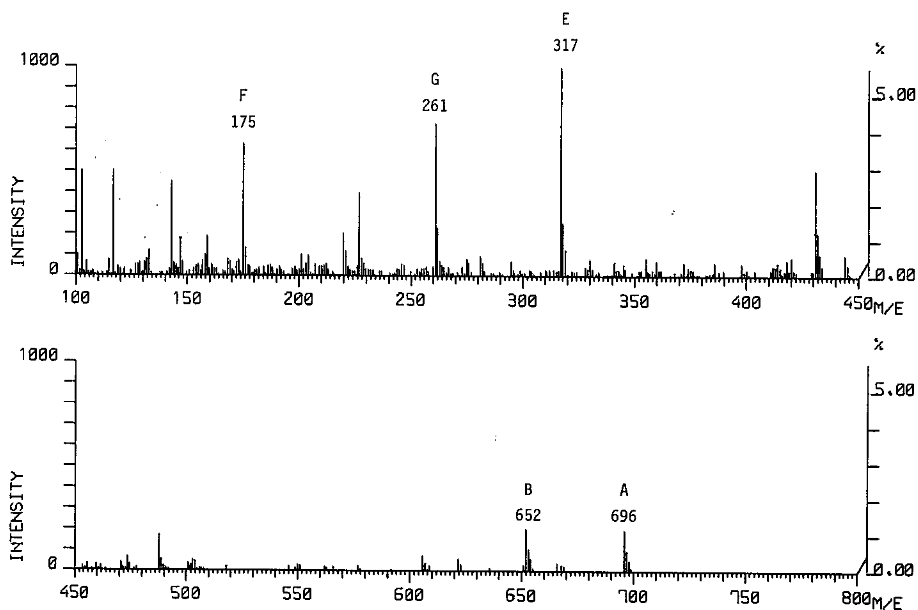

Fig. 16. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu7,9Ac$_2$5Gc.

m/*e* 173 → *m*/*e* 143. However, in all mass spectra a peak at *m*/*e* 143 with a general formula $C_6H_{11}O_2Si$ (143.0528) is observed. But in the mass spectra of Neu4,5Ac_2, Neu4,5,9Ac_3, and Neu4,5$Ac_2$9Lt the main contribution to the abundancy of *m*/*e* 143 originates from fragment *G* ($C_6H_9NO_3$; 143.0582). In the mass spectrum of Neu4Ac5Gc the peak at *m*/*e* 261 is not observed. For this compound fragment *G* shifts to *m*/*e* 231. By high-resolution mass spectrometry, this fragment ion could not be distinguished from other generally occurring fragment ions in sialic acid, which contribute also to the intensity of the peak at *m*/*e* 231.

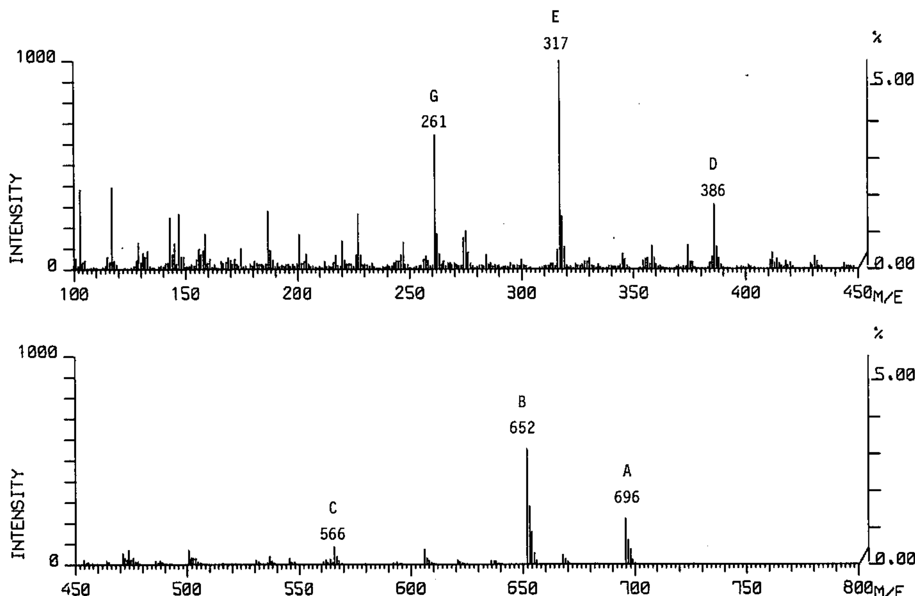

Fig. 17. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu8,9$Ac_2$5Gc.

Using the fragment ions *A*-*G*, Neu5,8Ac_2 and Neu5,9Ac_2 are only distinguished on the basis of the intensity of the peak at *m*/*e* 175 (fragment *F*). Of course, the mass spectra of these compounds differ also in other aspects. For instance, the side-chain CH_2OCOCH_3—$CHOSi(CH_3)_3$—$CH=\overset{+}{O}Si(CH_3)_3$ in Neu5,9Ac_2 clearly eliminates CH_3COOH, giving rise to the fragment ion *m*/*e* 217. In Neu5,8Ac_2 the side chain $CH_2OSi(CH_3)_3$—$CHOCOCH_3$—$CH=\overset{+}{O}Si(CH_3)_3$ eliminates CH_3COOH (*m*/*e* 217) as well as $HOSi(CH_3)_3$ (*m*/*e* 187). See also Kamerling *et al.* (1975 c) and Lönngren and Svensson (1974).

It has to be noted that the fragment ion at *m*/*e* 103 ($CH_2=\overset{+}{O}Si(CH_3)_3$) is not characteristic for a primary trimethylsiloxyl group in the sialic acid derivatives, but can also be formed along other routes (Kamerling *et al.* 1978). Furthermore, the fragment ions at *m*/*e* 186 ($CH_3CO\overset{+}{N}H=CH$—$CH=CHOSi(CH_3)_3$ and $CH_3CO\overset{+}{N}H=CH$—$C(OSi(CH_3)_3)=CH_2$) in N-acetylneuraminic acids and at *m*/*e* 274 in N-glycolylneuraminic acids only give information about the type of substitution at C-5 (amino group).

Table 3. *G.l.c. and m.s. 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 on Chromosorb W-AW DMCS HP, 80-100 mesh at 220 °C* (VAN HALBEEK *et al.* 1978) *and on a capillary column (80 m × 0.35 mm, i.d.) wall-coated with OV-101 at 215 °C* (BRUVIER *et al.* 1981) *are given relative to Neu5Ac4,5,7,8,9Me₅ methyl ester β-methyl glycoside. For the trimethylsilylation procedure, see section II.3*

Sialic acid (as methyl ester β-methyl glycoside)	R_{Neu5Ac} 3.8% SE-30	R_{Neu5Ac} OV-101	*m/e* values								References
			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	BHATTACHARJEE and JENNINGS 1976, BRUVIER *et al.* 1981, RAUVALA and KÄRKKÄINEN 1977, VAN HALBEEK *et al.* 1978
Neu5Ac4,5,7,8Me_4	1.30	1.31	450	406	318	254	201	147	129	298	BHATTACHARJEE and JENNINGS 1976, BRUVIER *et al.* 1981, VAN HALBEEK *et al.* 1978
Neu5Ac4,5,7,9Me_4	1.14	1.14	450	406	318	254	201	147	129	356	BHATTACHARJEE and JENNINGS 1976, BRUVIER *et al.* 1981, HAVERKAMP *et al.* 1977a, VAN HALBEEK *et al.* 1978
Neu5Ac4,5,8,9Me_4	1.07	1.06	450	406	376	312	201	89	129	298	BRUVIER *et al.* 1981, VAN HALBEEK *et al.* 1978
Neu5Ac5,7,8,9Me_4		1.20	450	406	376	254	259	89	187	298	BHATTACHARJEE and JENNINGS 1976, BRUVIER *et al.* 1981
Neu5Ac4,5,7Me_3	1.55	1.52	508	464	318	254	201	205	129	356	BRUVIER *et al.* 1981, KAMERLING *et al.* 1978
Neu5Ac4,5,9Me_3	1.27	1.27	508	464	376	312	201	147	129	356	BRUVIER *et al.* 1981, VAN HALBEEK *et al.* 1978
Neu5Ac5,7,8Me_3		1.60	508	464	376	254	259	147	187	298	BRUVIER *et al.* 1981
Neu5Ac5,7,9Me_3		1.39	508	464	376	254	259	147	187	356	BRUVIER *et al.* 1981
Neu5Ac5,8,9Me_3		1.23	508	464	434	312	259	89	187	298	BRUVIER *et al.* 1981
Neu5Ac4,5Me_2	1.70	1.78	566	522	376	312	201	205	129	356	BRUVIER *et al.* 1981, VAN HALBEEK *et al.* 1978
Neu5Ac5,7Me_2		1.91	566	522	376	254	259	205	187	356	BRUVIER *et al.* 1981
Neu5Ac5,9Me_2	1.43	1.50	566	522	434	312	259	147	187	356	BRUVIER *et al.* 1981, VAN HALBEEK *et al.* 1978

Neu5Ac5Me	1.89	2.05	624	580	434	312	259	205	187	356	BRUVIER *et al.* 1981, VAN HALBEEK *et al.* 1978
Neu5MeGc4,5,7,8,9Me$_5$ [†]			422	378	348	284	201	89	159	328	INOUE and MATSUMURA 1979, RAUVALA and KÄRKKÄINEN 1977
Neu5MeGc4,5,7,9Me$_4$ [†]			480	436	348	284	201	147	159	386	SEKINE *et al.* 1981

[†] MeGc = methylated glycolyl group.

Table 4. *G.l.c. and m.s. 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 on Chromosorb W-AW DMCS HP, 80–100 mesh at 220 °C* (VAN HALBEEK *et al.* 1978) *are given relative to Neu5Ac4,5,7,8,9Me$_5$ methyl ester β-methyl glycoside. The acetylation is carried out at 100 °C for 30 min with acetic anhydride-pyridine (1 : 1). For an explanation of the — signs, see section III.1*

Sialic acid [†] (as methyl ester β-methyl glycoside)	R_{Neu5Ac}	*m/e* values							References
		A	*B*	*C*	*D*	*E*	*F*	*G*	
Neu5Ac4,5,7,8Me$_4$	1.47	420	376	318	254	201	117	129	BHATTACHARJEE and JENNINGS 1976, VAN HALBEEK *et al.* 1978
Neu5Ac4,5,7,9Me$_4$	1.25	420	376	318	254	201	—	129	BHATTACHARJEE and JENNINGS 1976, HAVERKAMP *et al.* 1977 a, RAUVALA and KÄRKKÄINEN 1977, VAN HALBEEK *et al.* 1978
Neu5Ac4,5,8,9Me$_4$	1.08	420	376	—	—	201	89	129	VAN HALBEEK *et al.* 1978
Neu5Ac5,7,8,9Me$_4$		420	376	346	254	—	89	157	BHATTACHARJEE and JENNINGS 1976, SMIRNOVA and KOCHETKOV 1980
Neu5Ac4,5,7Me$_3$	1.75	448	404	318	254	201	—	129	KAMERLING *et al.* 1978
Neu5Ac4,5,9Me$_3$	1.26	448	404	—	—	201	—	129	VAN HALBEEK *et al.* 1978
Neu5Ac4,5Me$_2$	1.70	476	432	—	—	201	—	129	VAN HALBEEK *et al.* 1978
Neu5Ac5,9Me$_2$	1.63	476	432	—	—	—	—	157	VAN HALBEEK *et al.* 1978
Neu5Ac5Me	2.17	504	460	—	—	—	—	157	VAN HALBEEK *et al.* 1978
Neu5MeGc4,5,7,9Me$_4$ [††]		450	406	348	284	201	—	159	INOUE and MATSUMURA 1979

[†] For data of Neu5Ac4,5,7,8,9Me$_5$ and Neu5MeGc4,5,7,8,9Me$_5$, see Table 3.
[††] MeGc = methylated glycolyl group.

3. *Sialic Acids and Methylation Analysis*

Methylation analysis is generally applied for the determination of the position of glycosidic linkages in glycoconjugates, oligosaccharides, and polysaccharides. After permethylation the biopolymer is solvolyzed (e.g., hydrolyzed or methanolyzed) and the obtained mixture of partially methylated monomers is analyzed (BJÖRNDAL *et al.* 1970, STELLNER *et al.* 1973, LÖNNGREN and SVENSSON 1974, RAUVALA *et al.* 1981). For the linkage analysis of sialic acids in non-reducing and internal positions, methanolysis is the appropriate method of solvolysis. From the analytical data available so far, it can be concluded that after methanolysis the (partially) methylated sialic acids occur predominantly as their methyl ester β-methyl glycosides; only a small percentage of the corresponding α-anomers have been detected (HAVERKAMP *et al.* 1977 a). Partially O-methylated N,N-acyl,methyl-neuraminic acid methyl ester methyl glycosides are analyzed by g.l.c./m.s. after trimethylsilylation or acetylation of free hydroxyl groups.

In literature, the preparation and g.l.c./m.s. data of a large series of partially methylated sialic acid methyl ester methyl glycosides as reference compounds have been reported. For this purpose, specific procedures (VAN HALBEEK *et al.* 1978) as well as methanolysis of permethylated sialobiopolymers (BHATTACHARJEE and JENNINGS 1976, RAUVALA and KÄRKKÄINEN 1977, VAN HALBEEK *et al.* 1978) and non-specific partial methylations (undermethylation) (BRUVIER *et al.* 1981) have been employed. Table 3 summarizes the relative retention times on g.l.c. and the *m/e* values of the fragment ions *A-H* of (partially) O-methylated N,N-acyl,methyl-neuraminic acid methyl ester β-methyl glycosides after trimethylsilylation. Table 4 contains similar information about several sialic acid derivatives after acetylation (fragments *A-G*). In Fig. 3 the mass spectrum of permethylated N,N-acetyl,methyl-neuraminic acid methyl ester β-methyl glycoside is presented. Figs. 18-21 show the mass spectra of the various tri-O-methyl-N,N-acetyl,methyl-neuraminic acid methyl ester β-methyl glycosides after trimethylsilylation. In principle, the latter derivatives are obtained from internal non-branching N-acylneuraminic acids. For additional mass spectra, see BHATTACHARJEE and JENNINGS (1976), RAUVALA and KÄRKKÄINEN (1977), HAVERKAMP *et al.* (1977 a), VAN HALBEEK *et al.* (1978), INOUE and MATSUMURA (1979), and BRUVIER *et al.* (1981).

The choice of the applied methanolysis conditions in relation to the possible release of the N-acyl group is very important for the methylated compounds too. Using 0.5 N methanolic HCl (18 h, 80 °C) the N-acyl (N-acetyl or methylated N-glycolyl) groups of sialic acids in terminal positions of the carbohydrate chain are resistant to methanolic cleavage. However, internal sialic acids are N-deacylated to a large extent. For this reason, after methanolysis (re-)N-acetylation is necessary in the working-up procedure (also perhaps with deuterated acetic anhydride). The use of 0.05 N methanolic HCl (1 h, 80 °C) seems to give no N-deacylation (INOUE and MATSUMURA 1979, 1980). However, these milder conditions do not liberate sialic acid quantitatively from the sialobiopolymer.

In order to verify the general formula of the selected fragment ions, the sialic acid methyl ester β-methyl glycosides of Neu5Ac4,5,7,8,9Me_5 and of Neu5Ac4,5,7,8Me_4, Neu5Ac4,5,7,9Me_4, Neu5Ac4,5,8,9Me_4, Neu5Ac4,5,7Me_3,

Neu5Ac4,5,9Me$_3$, Neu5Ac4,5Me$_2$, Neu5Ac5,9Me$_2$, and Neu5Ac5Me after trimethylsilylation or acetylation, have been studied by high-resolution mass spectrometry (VAN HALBEEK *et al.* 1978). These investigations have indicated that especially the fragment ions *F* and *G* have to be considered in more detail.

Fragment *F*: i. Two different ions contribute to the intensity of the peak at *m/e* 89 in the trimethylsilyl derivative of Neu5Ac4,5,8,9Me$_4$ methyl ester β-methyl glycoside (Table 3), namely $CH_2OCH_3—CH=\overset{+}{O}CH_3$ (*F*; $C_4H_9O_2$) and $\overset{+}{O}Si(CH_3)_3$ (C_3H_9OSi). The fragment C_3H_9OSi can always be detected in the mass spectra of trimethylsilylated carbohydrates. One can assume that for the trimethylsilyl derivatives of Neu5Ac5,7,8,9Me$_4$ and Neu5Ac5,8,9Me$_3$ methyl ester β-methyl glycoside (Table 3) the same reasoning with respect to *m/e* 89 holds. ii. Two different fragment ions contribute to the intensity of the peak at *m/e* 147 in the trimethylsilyl derivatives of Neu5Ac4,5,9Me$_3$ and Neu5Ac5,9Me$_2$ methyl ester β-methyl glycoside (Table 3), namely $CH_2OCH_3—CH=\overset{+}{O}Si(CH_3)_3$ (*F*; $C_6H_{15}O_2Si$) and $(CH_3)_3SiO\overset{+}{S}i(CH_3)_2$ ($C_5H_{15}OSi_2$). The fragment with formula $C_5H_{15}OSi_2$ is generally present in the mass spectra of trimethylsilylated sugars with more than one O-trimethylsilyl group. In this case it can be assumed that for the trimethylsilyl derivatives of Neu5Ac5,7,8Me$_3$ and Neu5Ac5,7,9Me$_3$ methyl ester β-methyl glycoside (Table 3) the peak at *m/e* 147 is also composed of two fragments. iii. In the trimethylsilyl derivatives of Neu5Ac4,5,7Me$_3$, Neu5Ac4,5Me$_2$, and Neu5Ac5Me methyl ester β-methyl glycoside the peak at *m/e* 147 originates only from $C_5H_{15}OSi_2$. The same can be expected for the trimethylsilyl derivatives of Neu5Ac5,8,9Me$_3$ and Neu5Ac5,7Me$_2$ methyl ester β-methyl glycoside (Table 3).

Fragment *G*: i. Mass spectra of trimethylsilylated sugars always contain a peak at *m/e* 129 with low intensity, originating from the fragments $C_5H_9O_2Si$ and $C_6H_{13}OSi$. In the trimethylsilyl derivatives of Neu5Ac5,9Me$_2$ and Neu5Ac5Me methyl ester β-methyl glycoside the peak at *m/e* 129 ($C_5H_9O_2Si$ and $C_6H_{13}OSi$) is of low intensity ($R_4=Si(CH_3)_3$). A similar composition can be expected for the trimethylsilyl derivatives of Neu5Ac5,7,8,9Me$_4$, Neu5Ac5,7,8Me$_3$, Neu5Ac5,7,9Me$_3$, Neu5Ac5,8,9Me$_3$, and Neu5Ac5,7Me$_2$ methyl ester β-methyl glycoside (Table 3). ii. The main contribution to the intense peak at *m/e* 129 in the trimethylsilyl derivatives of Neu5Ac4,5,7,8Me$_4$, Neu5Ac4,5,7,9Me$_4$, Neu5Ac4,5,8,9Me$_4$, Neu5Ac4,5,7Me$_3$, Neu5Ac4,5,9Me$_3$, and Neu5Ac4,5Me$_2$ methyl ester β-methyl glycoside (Table 3) represents fragment *G* ($C_6H_{11}NO_2$; $R_4=CH_3$). iii. In the acetyl derivatives of Neu5Ac4,5,7,8Me$_4$, Neu5Ac4,5,7,9Me$_4$, Neu5Ac4,5,8,9Me$_4$, Neu5Ac4,5,7Me$_3$, and Neu5Ac4,5,9Me$_3$ methyl ester β-methyl glycoside (Table 4) the peak at *m/e* 129 consists mainly of fragment *G*; a small contribution of $C_6H_9O_3$ has been detected.

The foregoing demonstrates that if exact mass measurements are carried out, each fragment as such can provide essential structural information. However, it should be emphasized that, if working under low-resolution conditions only, the whole series of selected fragment ions form a self-consisted system, in which the interpretation of the fragment ions should support each other. It is obvious that to arrive at an unambiguous conclusion about the substitution pattern, the whole mass spectrum should also be considered.

Finally, it has to be mentioned that methylation analysis data have also been

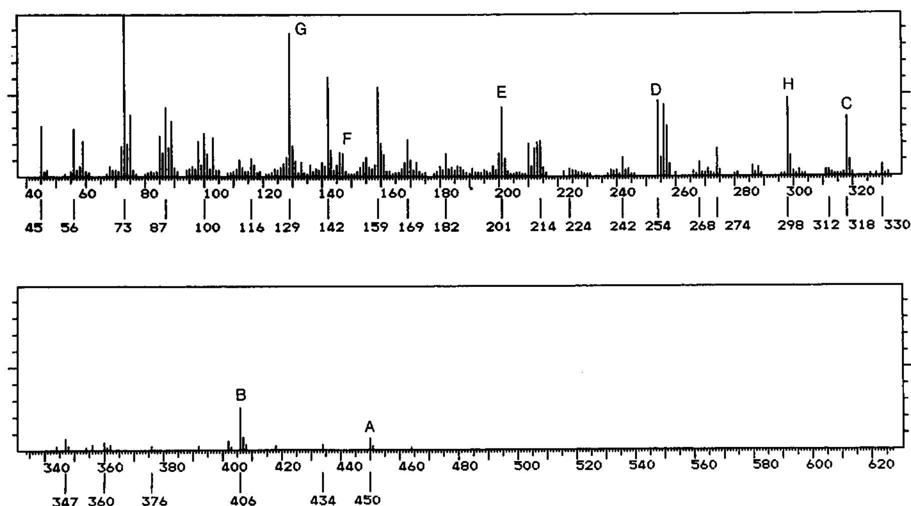

Fig. 18. E.i.-mass spectrum (70 eV) of Neu5Ac4,5,7,8Me$_4$ methyl ester β-methyl glycoside.

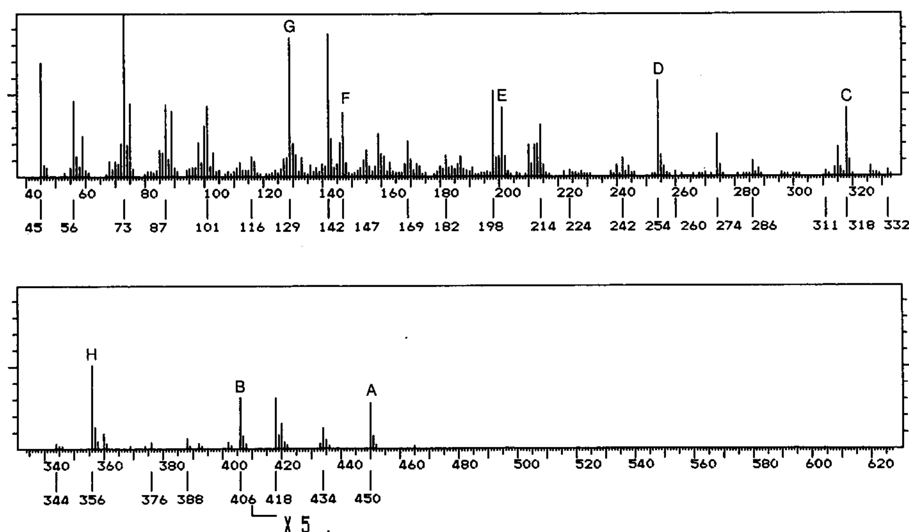

Fig. 19. E.i.-mass spectrum (70 eV) of Neu5Ac4,5,7,9Me$_4$ methyl ester β-methyl glycoside.

published for neuraminitols (SUGITA 1979 a, b). These derivatives are especially important for oligosaccharides having sialic acid in a reducing position. Before permethylation and methanolysis the oligosaccharides are converted into their corresponding oligosaccharide-alditols. Mass spectra have been reported of two partially methylated N,N-acetyl,methyl-neuraminitol acetate methyl esters, namely, the 2,6,7,8,9-penta-O-methyl and 2,6,7,9-tetra-O-methyl derivatives (SUGITA 1979 a). For mass spectral data of peracetylated N-acetyl- and N-glycolylneuraminitol methyl esters, see SMIRNOVA *et al.* (1977).

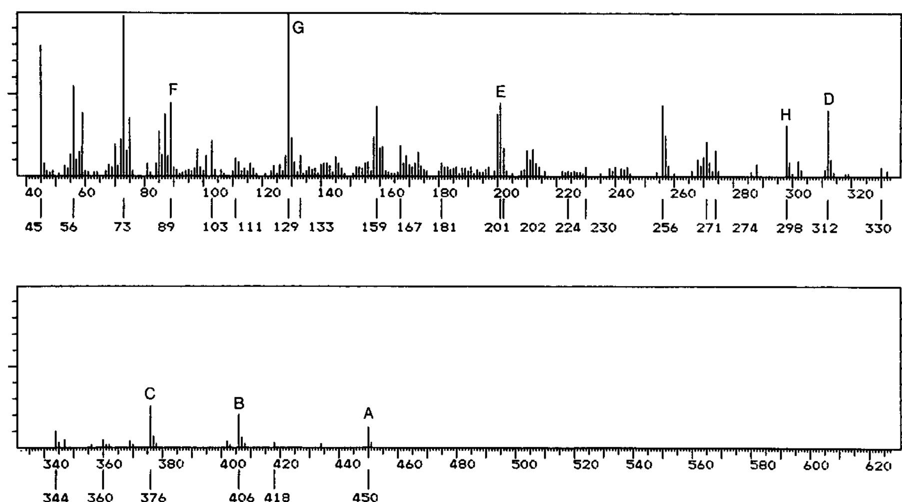

Fig. 20. E.i.-mass spectrum (70 eV) of Neu5Ac4,5,8,9Me_4 methyl ester β-methyl glycoside.

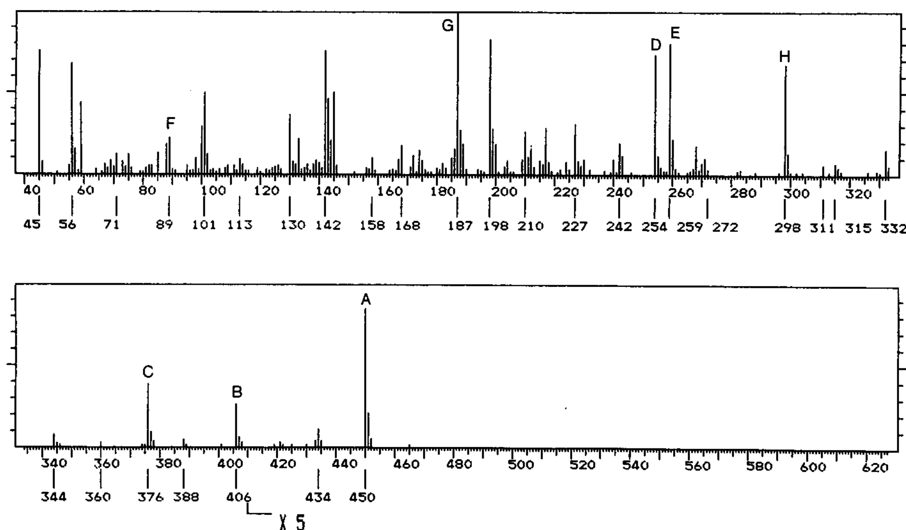

Fig. 21. E.i.-mass spectrum (70 eV) of Neu5Ac5,7,8,9Me_4 methyl ester β-methyl glycoside.

4. Miscellaneous Sialic Acids and Sialic Acid Derivatives

Partial O-acetylation of Neu5Ac methyl ester β-methyl glycoside led to the formation of several O-acetylated compounds (Haverkamp *et al.* 1975, Van Halbeek *et al.* 1978). In Table 5 the relative retention times on g.l.c. and the m.s. data in terms of the fragment ions *A*-*G* of the various trimethylsilyl derivatives are summarized. Compared with the intensity of the peak at *m/e* 175 (fragment *F*) in the mass spectra of the 9-O-acetylated compounds, this peak is observed only in a

Table 5. *G.l.c. and m.s. data of trimethylsilylated (O-acetylated) Neu5Ac methyl ester β-methyl glycosides. The R_{Neu5Ac}-values on 3.8% SE-30 at 210 °C are given relative to the trimethylsilylated Neu5Ac methyl ester β-methyl glycoside. For the trimethylsilylation procedure and additional analysis conditions, see section II.3. For an explanation of the — signs, see sections III.1 and III.4*

Sialic acid (as methyl ester β-methyl glycoside)	R_{Neu5Ac}	*m/e* values						
		A	*B*	*C*	*D*	*E*	*F*	*G*
Neu5Ac	1.00	610	566	420	298	259	205	173
Neu5,8Ac_2	1.12	580	536	420	298	259	—	173
Neu5,9Ac_2	1.20	580	536	420	298	259	175	173
Neu4,5,8Ac_3	1.28	550	506	390	298	—	—	143
Neu4,5,9Ac_3	1.46	550	506	390	298	—	175	143
Neu4,5,8,9Ac_4	1.42	520	476	390	298	—	—†	143

† The small peak at *m/e* 145 corresponds with $C_6H_{13}O_2Si$.

Table 6. *G.l.c. and m.s. data of trimethylsilylated methyl ester derivatives of degradation products obtained by periodate oxidation of bound Neu5Ac and Neu5Gc* (VEH *et al.* 1977, PFANNSCHMIDT and SCHAUER 1980). *The R_{Neu5Ac}-values on 3.8% SE-30 at 215 °C are given relative to the trimethylsilylated Neu5Ac methyl ester. For the esterification and trimethylsilylation procedure, see section II.3*

Sialic acid derivative	R_{Neu5Ac}	*m/e* values						
		A	*B*	*C*	*D*	*E*	*F*	*G*
C_7-Neu5Ac	0.29	464	420			317		173
C_8-Neu5Ac	0.50	566	522	478	298	317	"103"	173
C_7-Neu5Gc	0.60	552	508			317		261
C_8-Neu5Gc	0.95	654	610	566	386	317	"103"	261

low intensity in the spectra of the 8-O-acetylated ones. For a discussion of fragment *G* (*m/e* 173 or *m/e* 143) and of an additional criterion for the discrimination between 8-O-acetylated and 9-O-acetylated compounds, see section III.2. Some of the mass spectra have been published by HAVERKAMP *et al.* (1975), MONONEN and KÄRKKÄINEN (1975), and SUGITA (1979 b).

Neu5Ac8Me and Neu5Gc8Me have been shown to be constituents of some glycolipids (KOCHETKOV *et al.* 1973, SUGITA 1979 b). Mass spectra of the acetylated and trimethylsilylated methyl ester methyl glycosides of Neu5Ac8Me have been reported by KOCHETKOV *et al.* (1973) and SUGITA (1979 b). In one of these studies (KOCHETKOV *et al.* 1973), the mass spectra of the peracetylated methyl ester methyl

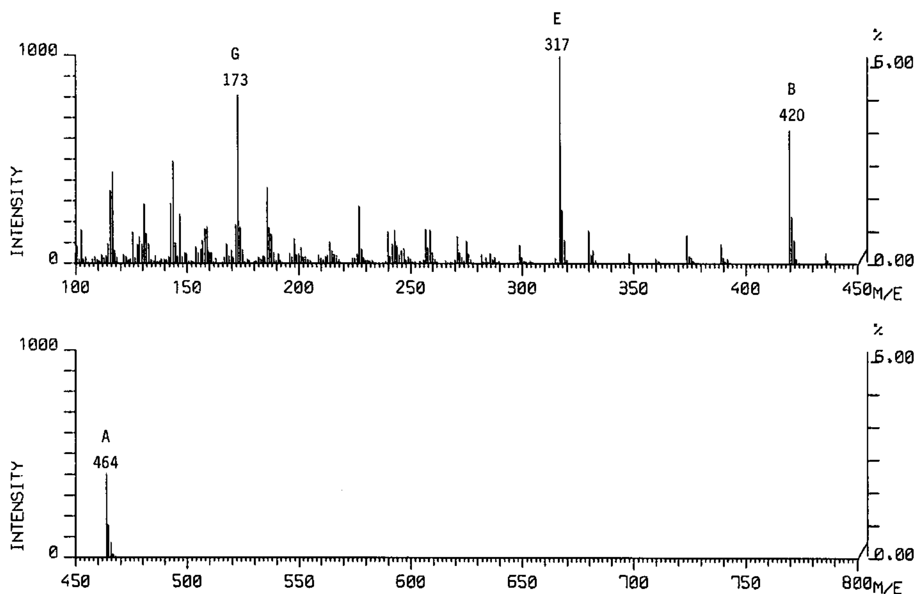

Fig. 22. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of C_7-Neu5Ac.

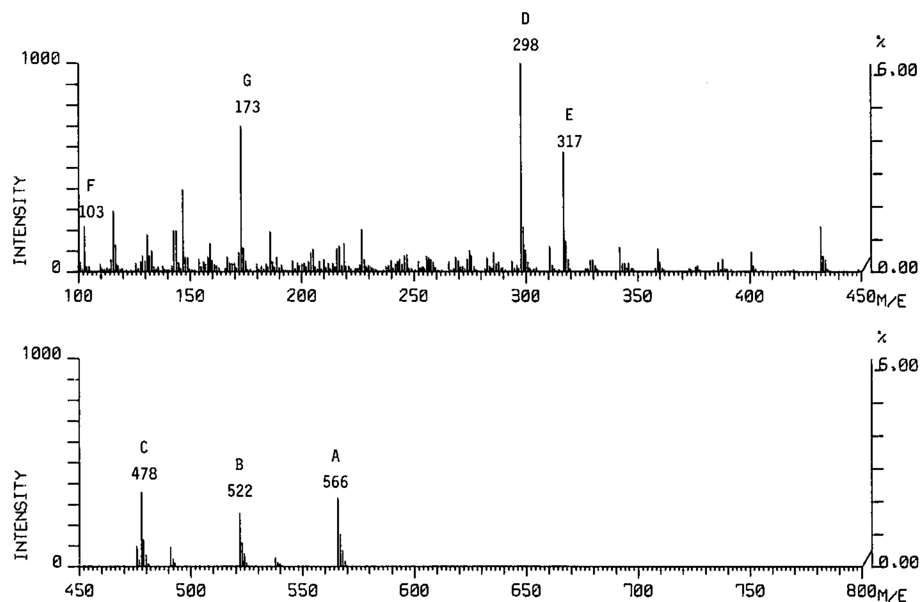

Fig. 23. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of C_8-Neu5Ac.

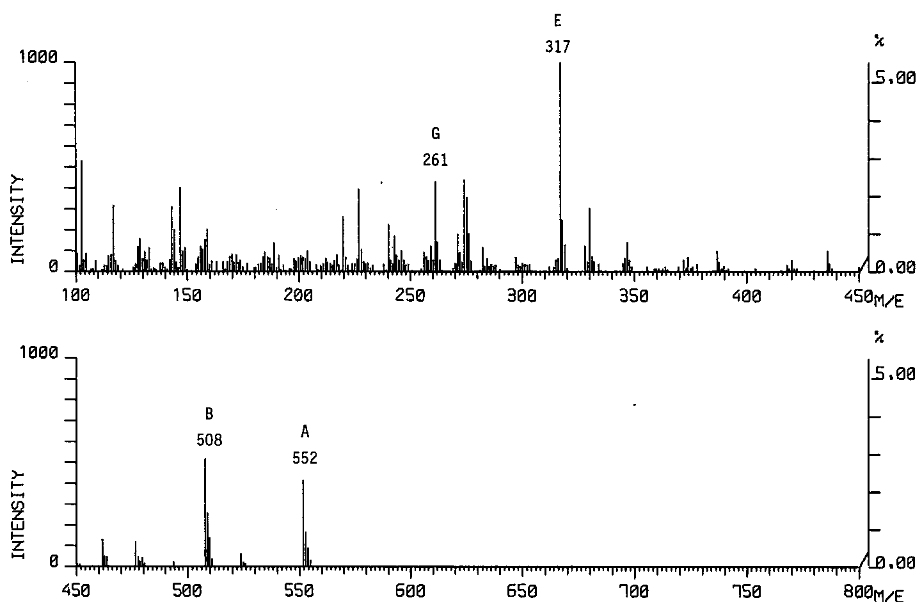

Fig. 24. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of C_7-Neu5Gc.

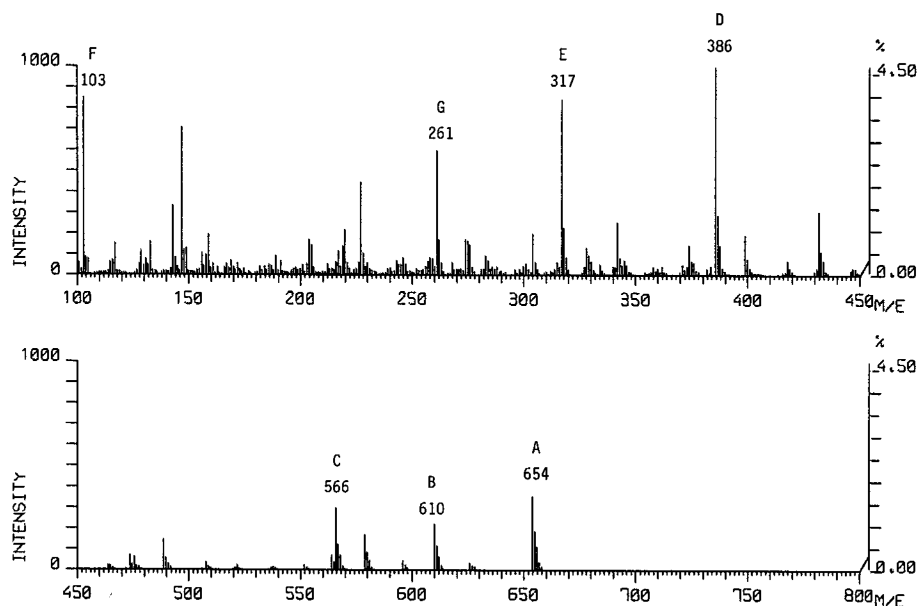

Fig. 25. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of C_8-Neu5Gc.

glycosides of Neu5Ac and Neu5Gc have also been included. Furthermore, mass spectral data have been published for the trimethylsilylated methyl ester of Neu5Ac4Me (BEAU *et al.* 1978) and for the trimethylsilylated methyl ester methyl glycoside of Neu (SWEELEY and VANCE 1967). For mass spectra of pertri-

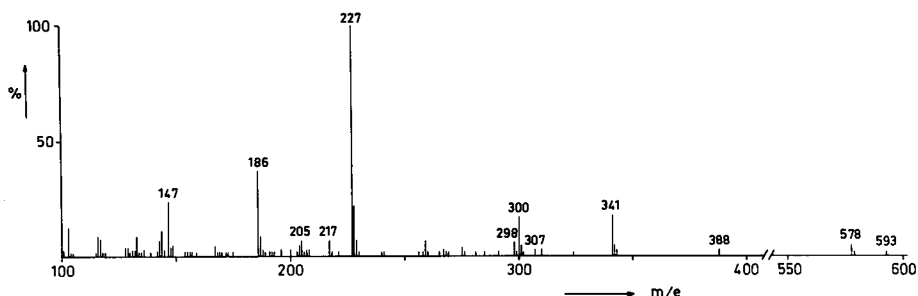

Fig. 26. E.i.-mass spectrum (70 eV) of the trimethylsilylated methyl ester of Neu2en5Ac.

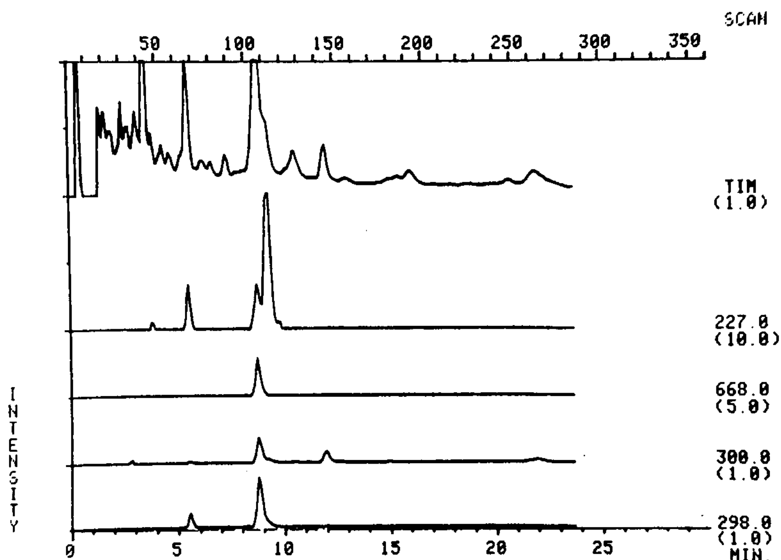

Fig. 27. Mass chromatography of the trimethylsilylated methyl esters of Neu5Ac (*m/e* 668, 300, 298) and Neu2en5Ac (*m/e* 227).

fluoroacetylated Neu and C_7-Neu methyl ester methyl glycosides, see YOHE and YU (1981).

In Table 6 the relative retention times on g.l.c. and the m.s. data of C_7-Neu5Ac, C_8-Neu5Ac, C_7-Neu5Gc, and C_8-Neu5Gc after esterification and trimethylsilylation have been summarized (VEH *et al.* 1977, PFANNSCHMIDT and SCHAUER 1980). In Figs. 22–25 the various mass spectra are presented. G.l.c. data of the trimethylsilyl methyl ester methyl glycosides and of the pertrimethylsilylated derivatives of C_7-Neu5Ac and C_8-Neu5Ac have been reported by McLEAN *et al.* (1971) and SUTTAJIT and WINZLER (1971), respectively.

Finally, on guidance of the developed fragmentation scheme in Fig. 4, an unsaturated sialic acid, namely Neu2en5Ac, was also found to be present in different biological sources (KAMERLING *et al.* 1975b, HAVERKAMP *et al.* 1976). In Fig. 26 the mass spectrum of the trimethylsilylated methyl ester of Neu2en5Ac (R_{Neu5Ac}-value 1.09 on 3.8% SE-30 at 215 °C) is depicted. Usually in biological samples, Neu2en5Ac is present in relatively small amounts. To detect Neu2en5Ac as a contaminant in Neu5Ac (R_{Neu5Ac} 1.00) preparations, mass chromatography using the base peak at *m/e* 227 (M-$CHOSi(CH_3)_3CHOSi(CH_3)_3CH_2OSi(CH_3)_3$-$NH_2COCH_3$) is an excellent method. Fig. 27 shows an example of such an analysis.

5. *Quantitative Analysis of Sialic Acids by g.l.c./m.s.*

MONONEN and KÄRKKÄINEN (1975) have reported a quantitative determination method for sialic acid with g.l.c./e.i.-m.s. using the multiple-ion-detection technique. Sialic acids in biological samples were liberated by methanolysis. Subsequently, the formed methyl ester methyl glycoside of Neu was N-acetylated and trimethylsilylated (see section II.1). Mass spectral data of the formed trimethylsilylated Neu5Ac methyl ester methyl glycoside are included in Table 5. The internal standard Neu5Ac trideuteromethyl ester trideuteromethyl glycoside was added after the N-acetylation. For quantification, the intensities of the *C* fragment ions *m/e* 426 ↔ *m/e* 420 and the *D* fragment ions *m/e* 301 ↔ *m/e* 298 were used. It has been stated by the authors that the accurate determination of 1 ng is possible. The method has been applied for the analysis of the sialic acid content in a crude protein fraction from rat brain.

Another approach has been published by ROBOZ *et al.* (1978). Neu5Ac was released from glycoproteins by sialidase or by acid hydrolysis and subsequently trimethylsilylated with N,O-bis(trimethylsilyl)-trifluoroacetamide plus 1% chlorotrimethylsilane and pyridine (3 : 1) for 1 h at 100 °C, also leading to trimethylsilylation of the N-acetyl function. As the internal standard trimethylsilylated Neu β-methyl glycoside was used. The quantification was carried out with g.l.c./c.i.-m.s. (isobutane) using the intense peak at *m/e* 814 $[(M+H)^+]$ for Neu5Ac and the intense peak at *m/e* 714 $[(M'+H)^+]$ for the internal standard. The detection limit for pure Neu5Ac is 200 pg. The method has been applied in the study of leukemic myeloblasts.

A method to determine sialic acid in erythrocyte ghosts has been developed by ASHRAF *et al.* (1980). For the release of sialic acid, mild methanolysis conditions were chosen (0.1 N methanolic HCl; 1 h, 90 °C). After trimethylsilylation the obtained derivative was analyzed using g.l.c./c.i.-m.s. (methane) with the trimethylsilylated derivative of N-acetylglucosamine α-phenyl glycoside as the internal standard. For quantification, the abundancies of the $[MH\text{-}16]^+$-ions were used: *m/e* 610 for the trimethylsilylated methyl ester methyl glycoside of Neu5Ac and *m/e* 498 for the internal standard. The limit of detection was found to be below 0.4 ng (useful range 10 ng–1 μg).

MIYATAKE *et al.* (1979) reported the use of mass fragmentography in sialidase activity studies. For the analysis of released Neu5Ac, the carboxyl group as well as the various hydroxyl functions were trimethylsilylated. As the internal standard the trimethylsilylated derivative of N-acetylgalactosamine α-phenyl glycoside was

employed. The quantification was carried out using fragment *D* of the sialic acid derivative (*m/e* 356) and *m/e* 330 of the internal standard (g.l.c./e.i.-m.s.).

6. *Sialooligosaccharides, Sialoglycolipids, and Sialoglycopeptides*

Mass spectrometric investigations of derivatized sialooligosaccharide-alditols, sialoglycolipids, and sialoglycopeptides have been carried out by several authors. Because of the relatively low volatility of these substances, g.l.c. cannot in general be used as m.s. inlet system. In the framework of this chapter, only some examples will be mentioned.

M.s. data of the pertrimethylsilylated derivatives of the methyl esters of Neu5Ac-α(2→3)- and Neu5Ac-α(2→6)-lactose have been reported by KAMERLING *et al.* (1974). The same type of derivatives were discussed for Neu5Ac-α(2→6)- and β(2→6)-galactose (VAN DER VLEUGEL *et al.* 1982 a) and for Neu5Ac-β(2→6)-N-acetylglucosamine (VAN DER VLEUGEL *et al.* 1982 b). For Neu4,5Ac_2-α(2→3)-lactose, see KAMERLING *et al.* (1982 a). Trimethylsilylated gangliosides as G_{M1}, G_{M2}, G_{M3}, G_{D1a}, G_{D1b}, and G_{D3} have been analyzed by SWEELEY and DAWSON (1969) and DAWSON and SWEELEY (1971).

Data on the mass spectral analysis of permethylated sialooligosaccharide-alditols have been reported, for instance, by VAN HALBEEK *et al.* (1981) (oligosaccharide-alditols from hog-submaxillary-gland mucin glycoproteins), RAUVALA *et al.* (1981), SAITO *et al.* (1981) (oligosaccharide-alditols from bovine colostrum ϰ-casein), and KAMERLING *et al.* (1982 a) (sialyl-lactitols). Comprehensive data on permethylated sialoglycolipids from various biological sources have been published; see for instance KARLSSON (1978). A permethylated sialoglycopeptide (biantennary structure) from human transferrin has been analyzed by KARLSSON *et al.* (1978).

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