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# GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF THE MONOSACCHARIDE COMPOSITION OF ACID GLYCOSAMINOGLYCANS (MUCOPOLYSACCHARIDES) DERIVED FROM ANIMAL TISSUES

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## ABSTRACT

Quantitative, gas–liquid chromatography was investigated for analysis of the monosaccharide composition of acid mucopolysaccharides from animal tissues. The method entailed the analysis of the trimethylsilyl ($Me_3Si$) derivatives of methyl glycosides on two liquid phases. Good resolution of monosaccharides was achieved by use of columns of SE-30 and Apiezon-M. The procedure was tested with chondroitin 4-sulfate, and the results were slightly different from those of Mathews *et al.* When the analysis is performed according to this method, important points are: (*1*) absolutely anhydrous, methanolic hydrogen chloride is necessary, to ensure detection of hexosamines and sialic acid; and (*2*) high moisture in the air obstructs high recovery of methyl glycosides and their $Me_3Si$ derivatives, except in the case of neutral sugars.

## INTRODUCTION

Most of the acid mucopolysaccharides from animal tissues are simpler in monosaccharide composition than other protein– or lipid–polysaccharide complexes. but some of the monosaccharides composing acid mucopolysaccharides are liable to decompose during hydrolysis[1–6]. This makes it difficult to obtain high recovery of hydrolyzates and to analyze the monosaccharide composition correctly. In the analysis of monosaccharides composing acid mucopolysaccharide by gas–liquid chromatography (g.l.c.), high recovery of hydrolyzates and their derivatives is also necessary. One method, acid hydrolysis followed by acetylation, trimethylsilylation, or trifluoroacetylation, has not succeeded because of low recovery of products, whereas another method, methanolysis followed by trimethylsilylation or trifluoroacetylation, has been successful[7–11].

Zanetta *et al.*[10] described a method using methanolysis–trifluoroacetylation that offers good recovery of methanolyzates and their derivatives, and good separation of the derivatives in g.l.c., and that is adaptable to monosaccharide analysis of polysaccharides in general. However, when this method is used, it is necessary to

perform methanolysis and trifluoroacetylation under certain severe conditions, and when such conditions are met, the trifluoroacetyl (TFA) derivatives are not satisfactorily separated, as shown by our gas–liquid chromatographic results (see Discussion). These results led us to examine the adaptability of some liquid phases and trimethylsilylation to g.l.c. analysis of acid glycosaminoglycans (mucopolysaccharides) derived from animal tissues.

## EXPERIMENTAL

*Reagents.* — Standard carbohydrates were purchased from Wako Pure Chemical Industries, Ltd., Seikagaku Kogyo Co., Ltd., and Koso Chemical Co., Ltd., all of Tokyo, Japan. Dimethyl-*N*-(trimethylsilyl)amine (TMSDMA), chlorotrimethylsilane (TMCS), and acetone dimethyl acetal were obtained from Tokyo Kasei Co., Ltd., Tokyo, Japan. Chondroitin 4-sulfate and dermatan sulfate were supplied by Dr. M. B. Mathews, Department of Pediatrics, The University of Chicago.

*Chromatographic materials and apparatus.* — Apiezon-M (Api-M), SE-30, OV-1, SE-52, and Chromosorb WAW DMCS (100–120 mesh) were purchased from Nihon Chromat Works, Ltd., Tokyo, Japan. All analyses were conducted with a Shimazu GC-4BM gas chromatograph.

*Methanolysis.* — An aqueous solution of a monosaccharide or mucopolysaccharide (10–1,000 μg of carbohydrate) was placed in a 5-ml ampoule, together with *myo*-inositol (20–40 μg) as an internal standard. The sample was lyophilized, and kept overnight over $P_2O_5$ in a desiccator. To the material was added 0.5M methanolic hydrogen chloride (1–2 ml), and the ampoule was sealed with a flame, shaken vigorously, and kept for 20 h at 80°. The contents of the ampoule were poured into a 5-ml, conical, Pyrex tube to which acetone dimethyl acetal (5 drops from a capillary pipet) and $Ag_2CO_3$ (200–400 mg) were added. The tube was kept for 20 min at room temperature, with occasional, vigorous shaking. The supernatant liquor, isolated by centrifugation, was removed to an ampoule (5 ml) with a capillary pipet. The methanol was removed under a stream of nitrogen, or over $P_2O_5$ in a desiccator at room temperature. Methanol that was not absolutely anhydrous was usually used for the preparation of MeOH–HCl. Absolutely anhydrous MeOH–HCl was prepared according to the method of Zanetta *et al.*[10].

*Trimethylsilylation.* — The dried methanolyzate was dissolved in a mixture of pyridine (0.6 ml), TMSDMA (0.3 ml), and TMCS (0.1 ml). The ampoule was sealed with a flame, and kept for 24 h at 120°. After cooling, the contents were poured into a 2–5-ml, conical, Pyrex tube. To eliminate $NH_4Cl$, the procedure of Yamakawa *et al.*[11] was used. An equal volume of $CHCl_3$ was added, and mixed well by shaking. Then, a volume of water equal to that of the $CHCl_3$ was added, and the contents were mixed vigorously (with a Vortex mixer) and centrifuged. The aqueous layer was removed with an aspirator. Washing with water (to eliminate $NH_4Cl$) was repeated three times. The $CHCl_3$ layer was then concentrated under a stream of nitrogen at room temperature, for injection into a gas–liquid chromatograph.

*Conditions of gas–liquid chromatography.* — Api-M, SE-30, OV-1, and SE-52 (3% of any one) on Chromosorb WAW DMCS (100–120 mesh) were used. Chromosorb and the liquid phase were placed in a 1-liter suction flask and covered with $CHCl_3$. After mixing, the solvent was evaporated. To the residue, the same volume of $CHCl_3$ was added, and the material was treated three times in the same way. After the solvent had been removed, the Chromosorb particles coated by the liquid phase were packed, with gentle tapping, into a spiral, Pyrex colunm (2 m × 3 mm i.d.), and the column was conditioned for 24 h. The operating conditions for g.l.c. are given in Table I.

*Chromatographic resolution of trimethylsilylated methyl glycosides.* — The resolution of monosaccharides on columns packed with Api-M and SE-30, respectively, is shown in Figs. 1 and 2. The relative retention-time and peak-height ratio (the ratio of a peak to the main peak of a trimethylsilylated methyl glycoside) are given in Table II. Monosaccharides examined were those which compose acid mucopolysaccharides derived from animal tissues, together with some common ones. The overlapping, or incompletely separated, isomers of trimethylsilylated methyl glycosides on each column were as follows. On Api-M: (*1*) Man–1st peak ($P_1$) of Gal ($P_1$[Gal]) and (*2*) $P_1$[GlcNAc]–$P_1$[Glc]. They were incompletely separated, depending on their molar ratio. $P_2$[GlcNAc] could be used for determination of GlcNAc, when they were not separated. (*3*) $P_2$[Glc]–$P_2$[GlcN]. $P_1$[GalNAc] could be better separated from Man on Api-M than on SE-30, and was detected between $P_2$ and $P_3$ of Gal. On SE-30: (*1*) $P_1$[GalNAc]–Man; incomplete separation of these derivatives was sometimes detected, depending on their molar ratio. (*2*) $P_1$[Glc]–$P_2$[GlcN].

*Determination of the relative, molar response of monosaccharides.* — A mixture of different molar amounts of each carbohydrate and *myo*-inositol was treated by trimethylsilylation after methanolysis. Aliquots of the product were injected into the gas–liquid chromatograph. The area of each peak was determined by weight, and the areas of all peaks of a monosaccharide were summed (total peak-area). The ratio of total peak-area to its molar amount (total peak-area/mole) was calculated. The ratio between total peak-area/mole of a monosaccharide and that of *myo*-inositol was defined as the relative, molar ratio (RMR). RMR values of each monosaccharide are shown in Table III. RMR values of neutral sugars were good, but those of *N*-acetylhexosamines, hexosamines, and sialic acid were not satisfactory. Values indicated in parentheses were obtained when absolutely anhydrous MeOH–HCl was employed. Under our laboratory conditions, it was difficult to maintain the absolutely anhydrous state of MeOH–HCl. This kind of MeOH–HCl, therefore, was used for the methanolysis of several monosaccharides. Use of non-anhydrous MeOH–HCl made the RMR values poor.

*Application to chondroitin 4-sulfate.* — Chondroitin 4-sulfate (Ch-4s) was subjected to methanolysis for 8, 20, 30, and 40 h in the presence of *myo*-inositol as the internal standard. The liberation of monosaccharides was monitored by g.l.c. analysis of $Me_3Si$ derivatives of the products (see Fig. 3). The maximum liberation of monosaccharides was achieved after methanolysis for 20 h, but there was no degrada-

TABLE I

CONDITIONS FOR GAS–LIQUID CHROMATOGRAPHY

| | | |
|---|---|---|
| Liquid phase | 3% of Api-M | 3% of SE-30 |
| Column size | 2 m × 3 mm i.d. | 2 m × 3 mm i.d. |
| Column temperature (°C) | 110 → 280 | 110 → 200 |
| Temperature-programming rate (°C/min) | 8 | 2 |
| Detector-cell temperature (°C) | 290 | 290 |
| Injector temperature (°C) | 280 | 280 |
| Gas flow-rate | | |
| nitrogen carrier gas (ml/min) | 60 | 20 |
| hydrogen (kg/cm²) | 0.8 | 0.8 |
| air (kg/cm²) | 1.3 | 1.3 |

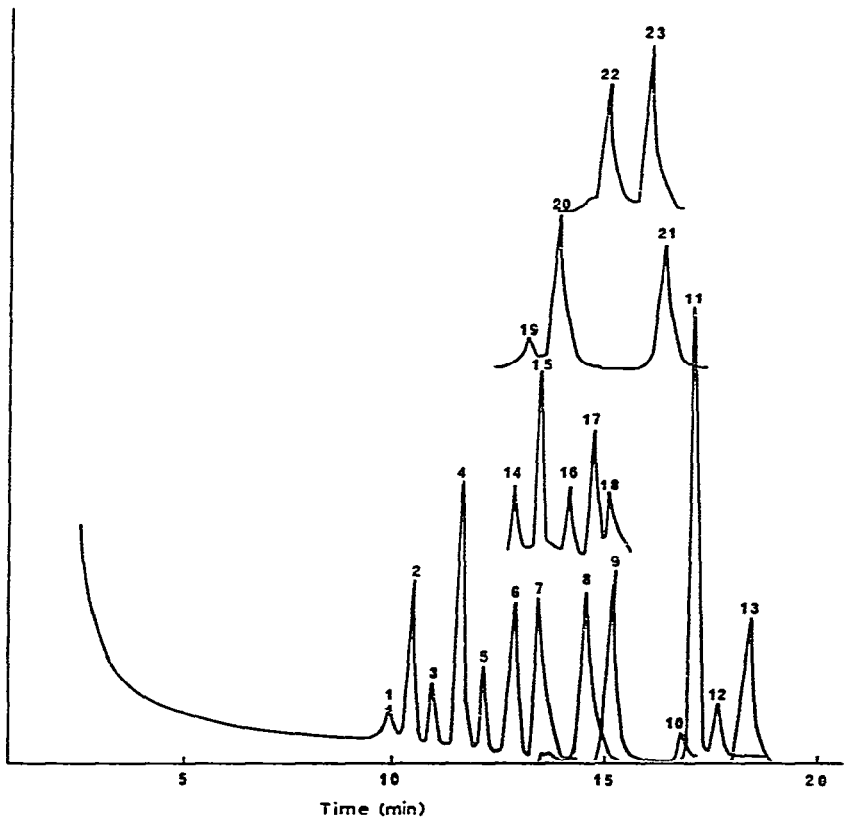


Fig. 1. G.l.c. resolution of standard, trimethylsilylated methyl glycosides on Apiezon-M. (Fuc, 1,2,3; Xyl, 4,5; Man, 6; GalNAc, 7,10; GlcNAc, 8,12; GlcA. 9; *myo*-inositol, 11; AcNeu, 13; Gal, 14,15,16; Glc, 17,18; GalN, 19,20,21; and GlcN, 22,23).

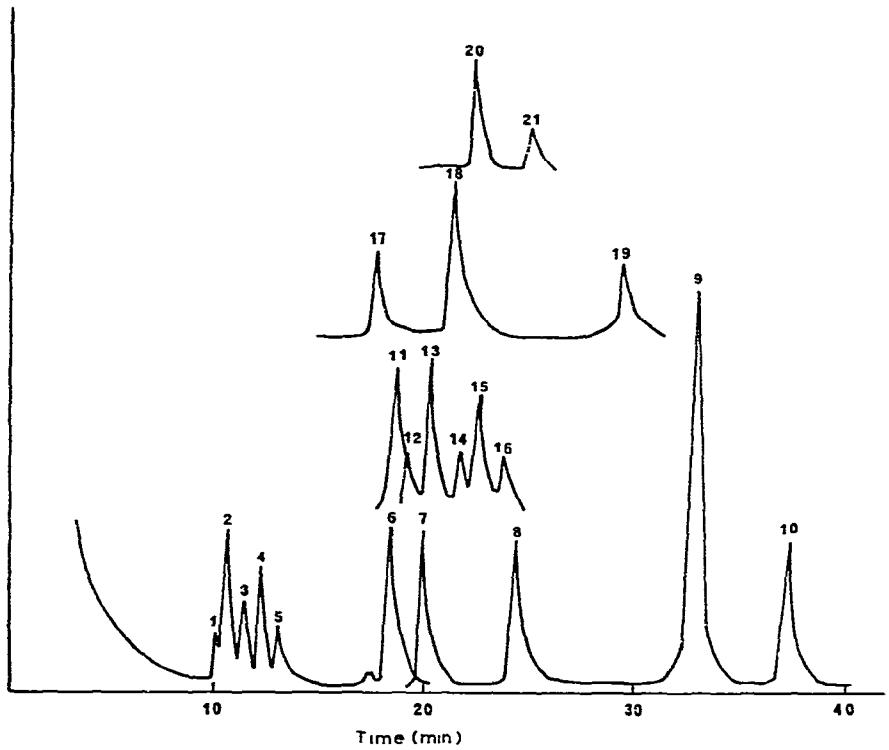


Fig. 2. G.l.c. resolution of standard, trimethylsilylated methyl glycosides on SE-30. (Fuc, 1,2,3; Xyl, 4,5; GalNAc, 6; GlcNAc, 7; GlcA, 8; *myo*-inositol, 9; AcNeu, 10; Man, 11; Gal, 12,13,14; Glc, 15,16; GalN, 17,18,19; and GlcN, 20,21).

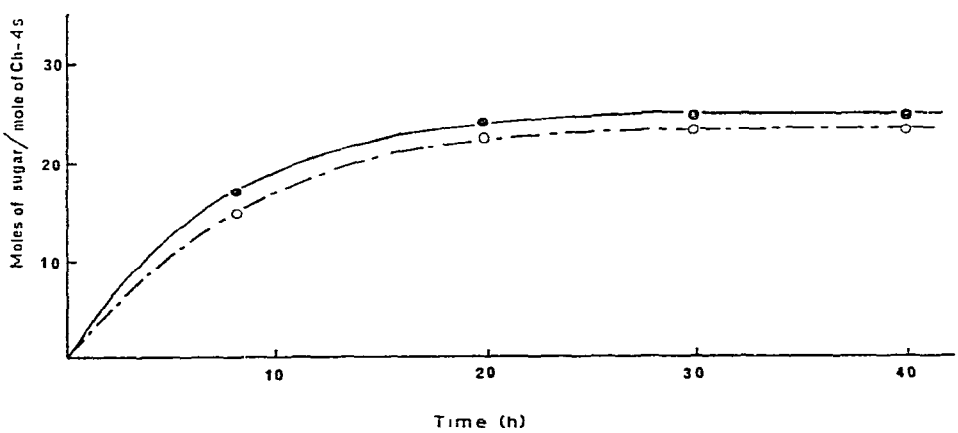


Fig. 3. Kinetics of the liberation of monosaccharides from chondroitin 4-sulfate by methanolysis. [The products were analyzed by g.l.c. of the trimethylsilylated methyl glycosides in the presence of *myo*-inositol as the internal standard. Values are expressed in moles of sugar/mole of chondroitin 4-sulfate (mol. wt. 12,000*). ———, GlcA; — — —, GalNAc. * According to Mathews, Cifonelli, and Rodén[15].]

TABLE II

RELATIVE RETENTION-TIMES AND RELATIVE PEAK-HEIGHTS OF TRIMETHYLSILYLATED METHYL GLYCOSIDES OF MONOSACCHARIDES

| *Compound* | *Peak* | *On Api-M* | | *On SE-30* | |
|---|---|---|---|---|---|
| | | *R.p.h.*[a] | *R.r.t.*[b] | *R.p.h.*[a] | *R.r.t.*[b] |
| Fuc | 1 | 0.20 | 0.56 | 0.30 | 0.295 |
| | 2 | 1.00 | 0.59 | 1.00 | 0.301 |
| | 3 | 0.43 | 0.62 | 0.55 | 0.34 |
| Xyl | 1 | 1.00 | 0.66 | 1.00 | 0.36 |
| | 2 | 0.53 | 0.69 | 0.50 | 0.39 |
| GalNAc | 1 | 1.00 | 0.785 | 1.00 | 0.55 |
| | 2 | 0.16 | 0.99 | 0.11 | 0.958 |
| GlcNAc | 1 | 1.00 | 0.835 | 1.00 | 0.585 |
| | 2 | 0.34 | 1.04 | 0.16 | 0.96 |
| GlcA | | 1.00 | 0.88 | 1.00 | 0.73 |
| *m*-I[c] | | *1.00* | *1.00* | *1.00* | *1.00* |
| Man | | 1.00 | 0.74 | 1.00 | 0.535 |
| Gal | 1 | 0.37 | 0.73 | 0.36 | 0.57 |
| | 2 | 1.00 | 0.77 | 1.00 | 0.60 |
| | 3 | 0.37 | 0.81 | 0.43 | 0.65 |
| Glc | 1 | 1.00 | 0.85 | 1.00 | 0.67 |
| | 2 | 0.45 | 0.87 | 0.42 | 0.71 |
| GalN | 1 | 0.21 | 0.76 | 0.47 | 0.52 |
| | 2 | 1.00 | 0.80 | 1.00 | 0.64 |
| | 3 | 0.83 | 0.95 | 0.46 | 0.88 |
| GlcN | 1 | 1.00 | 0.87 | 1.00 | 0.67 |
| | 2 | 0.88 | 0.93 | 0.43 | 0.75 |
| AcNeu | | 1.00 | 1.09 | 1.00 | 1.14 |

[a]R.p.h., relative peak-height. [b]R.r.t., relative retention-time. [c]*m*-I,*myo*-Inositol.

TABLE III

RELATIVE MOLAR RESPONSE OF TRIMETHYLSILYLATED METHYL GLYCOSIDES ON AN SE-30 COLUMN

| *Parent monosaccharide* | *Relative molar response of trimethylsilylated methyl glycoside* |
|---|---|
| D-Xylose | 0.760 (0.760) |
| D-Galactose | 0.714 (0.830) |
| D-Glucuronic acid | 0.533 (0.803) |
| 2-Acetamido-2-deoxy-D-galactose | 0.344 (0.795) |
| 2-Acetamido-2-deoxy-D-glucose | 0.321 (0.780) |
| 2-Amino-2-deoxy-D-glucose | 0.111 (0.765) |
| *N*-acetylneuramic acid | 0.065 (0.658) |
| *myo*-Inositol | *1.000* (*1.000*) |

TABLE IV

MONOSACCHARIDE COMPOSITION OF CHONDROITIN 4-SULFATE

| | *Weight* (%) | |
|---|---|---|
| | *Found* | *Reported*[a] |
| Hexuronic acid (HexA) | 39.4 | 34.1 |
| *N*-Acetylhexosamine (HexNAc) | 42.8 | 27.2 |
| Molar ratio (HexA/HexNAc) | 1.06 | 1.4 |

[a]By Mathews, Cifonelli, and Rodén[15].

tion of the products, even after methanolysis for 40 h. The carbohydrate composition of Ch-4s is presented in Table IV. The molar ratio of GlcA to GalNAc was 1.06:1 when methanolysis was performed for 20 or more hours. The molar amounts of GlcA and GalNAc from 1 mole of Ch-4s calculated from the molar ratio (1.06) and molecular weight (M.W.) of Ch-4s (12,000) were 24.5 (GlcA) and 32.2 moles (GalNAc), respectively. These amounts correspond to 82.4% of 1 mole of Ch-4s. The molar ratio of hexuronic acid to hexosamine found by Mathews *et al.*[15] was 1.4:1. It is difficult to judge which value, 1.06 or 1.4, is the more accurate, because the theoretical value for the molar ratio of Ch-4s is obscure.

## DISCUSSION

Simultaneous separation of monosaccharide mixtures and estimation of each component in one step by g.l.c. has been developed. Among the reports dealing with one-step analysis of monosaccharide mixtures by g.l.c., the method of Zanetta *et al.*[10] is said to have several advantages: (*1*) methanolysis with 0.5M MeOH–HCl does not cause deacetylation of *N*-acetylhexosamines; (*2*) uronic acid and sialic acid are not easily decomposed by 0.5M MeOH–HCl; (*3*) TFA derivatives of methyl glycosides of monosaccharides are eluted at relatively low temperatures, so that this method is not time-consuming; and (*4*) isomers of monosaccharides are separated from each other without overlapping.

However, on using a gas–liquid chromatograph and spiral-shaped, glass columns, we did not achieve the results just described. Even under the conditions of low flow-rate of $N_2$: or the use of columns longer than 2 m (4 or 6 m), good separation of pentoses from hexoses was not achieved: arabinose, fucose, rhamnose, ribose, and xylose were not separated; and, also, separation of *N*-acetylhexosamines from other monosaccharides was not satisfactory.

The following conditions were necessary in order to achieve RMR values of uronic acid and sialic acid as good as those of Zanetta *et al.*[10]: (*1*) complete elimination of water or moisture from the MeOH, (*2*) complete elimination of trifluoroacetic acid (TFA) from trifluoroacetic acid anhydride (TFAA), (*3*) less than 40% of moisture in the air during methanolysis and trifluoroacetylation. More than 50% of moisture

gave RMR values for these monosaccharides (especially sialic acid) that were unsatisfactory.

The separation of mixtures of $Me_3Si$ derivatives of methyl glycosides of monosaccharides on the liquid phases Api-M, SE-30, OV-1, and SE-52 were investigated, because complete elimination of TFA and establishment of less than 40% of moisture in the air were difficult under our conditions. The gas–liquid chromatograph and spiral columns we employed gave better resolution of isomers of $Me_3Si$ derivatives of methyl glycosides of pentoses, hexoses, and *N*-acetylhexosamines than did those of TFA derivatives of them. The liquid phases Api-M and SE-30 provided good separation of the isomers of $Me_3Si$ derivatives of methyl glycosides, as compared with OV-1 and SE-52. The results obtained with Api-M and SE-30 are presented.

Trimethylsilylated methyl glycosides of monosaccharides are identified by determining the percentage distributions and retention times of isomers of trimethylsilylated methyl glycosides. If, in g.l.c., isomers of $Me_3Si$ derivatives of monosaccharides always give a certain percentage distribution under certain conditions of trimethylsilylation, determination of retention times of isomers and of peak-height ratios of isomers to the highest (main isomer) peak among them can identify a trimethylsilylated methyl glycoside of a monosaccharide. In this report, therefore, the peaks of the $Me_3Si$ derivative of the methyl glycosides of each monosaccharide were numbered in the order of their retention times, and the peak-height ratio of each was indicated.

In our experiments, RMR values of $Me_3Si$ derivatives prepared after methanolysis of monosaccharides were satisfactory for neutral sugars, but poor for hexosamines, *N*-acetylhexosamines, sialic acid, and D-galacturonic acid (GalA). The RMR values of hexamines and sialic acid directly trimethylsilylated, or trimethylsilylated after methanolysis with absolutely anhydrous MeOH–HCl, were 0.65–0.80 (compared with *myo*-inositol as unity). The low RMR values of these monosaccharides was due to their decomposition during methanolysis. In agreement with Zanetta *et al.*[10], we found that satisfactory results for the methanolysis were obtained only by use of absolutely anhydrous samples, MeOH, and hydrogen chloride in MeOH. Sodium metal should be used for the preparation of absolutely anhydrous MeOH, as Zanetta *et al.*[10] mentioned. High moisture in the air leads to unsatisfactory results for RMR values in all stages of methanolysis, trimethylsilylation, and gas–liquid chromatographic operation. Moisture is more destructive in trifluoroacetylation, because the TFA liberated from TFAA by the moisture decomposes monosaccharides.

Washing with water–chloroform had no deleterious effect on $Me_3Si$ derivatives. This procedure removes the pyridine and $NH_4Cl$ produced during trimethylsilylation. Elimination of $NH_4Cl$ is especially necessary after trimethylsilylation of micro-weight samples, as, when samples containing it are concentrated after trimethylsilylation, $NH_4Cl$ fills the needle of the microsyringe, and the $Me_3Si$ derivatives will not pass through the needle at sampling. For trimethylsilylation, TMSDMA was used instead

of HMDS, based on the report that it trimethylsilylates amino groups in amino acids more effectively than HMDS[12–14]. We did not find that TMSDMA was more effective than HMDS in the case of amino groups in hexosamines.

Generally, the monosaccharides composing acid mucopolysaccharides are GalNAc, GlcNAc, 2-deoxy-2-(sulfoamino)-D-glucose (GlcNS), GlcA, iduronic acid (IdA), and Gal. The relative retention time of IdA compared with that of *myo*-inositol as unity was 0.90–0.91 on Api-M, and 0.74–0.75 on SE-30 (analytic result for dermatan sulfate). GlcNS was detected as the $Me_3Si$ derivative of GlcN resulting from liberation of the sulfate group from *N*-sulfated hexosamine during methanolysis. Resolution of the $Me_3Si$ derivatives of the methyl glycosides of these monosaccharides was satisfactory on Api-M or SE-30, but unsatisfactory on OV-1 and SE-52. Monosaccharide components of heparitin sulfate, having a complex composition of sugars (GlcNS, GlcNAc, GlcA, and IdA) are best determined with Api-M or SE-30, and those of keratan sulfate (GlcNAc and Gal) with Api-M. Even if GalNAc is one of the components of keratan sulfate, $P_1$[GalNAc] is detected between $P_2$[Gal] and $P_3$[Gal] on Api-M. The use of anhydrous MeOH–HCl ensures detection of *N*-acetylhexosamines, hexosamines, and sialic acid, and is especially necessary for the detection of hexosamines and sialic acid.

On applying the method reported here to analysis of the monosaccharide composition of as-yet-unstudied mucopolysaccharides, the following should be noted. (*1*) Absolutely anhydrous MeOH–HCl and a low degree of moisture in the air are required for positive determination of hexosamines and sialic acid, as, otherwise, they cannot easily be detected. (*2*) Trimethylsilyl derivatives of GlcNAc and GlcA are sometimes not separated from $Me_3Si$ derivatives of Glc on Api-M, depending on their molar ratio. $P_2$[GlcNAc], when overlapping, is useful for the detection of GlcNAc. Trimethylsilyl derivatives of GlcA and Glc can be separated on SE-30. (*3*) Separation of the $Me_3Si$ derivative of Man and $P_1$ of the $Me_3Si$ derivative of Gal is not achieved on Api-M, but they are sometimes separated on SE-30, depending on their molar ratio. Analysis of the monosaccharide composition is often made difficult by Man in the components of polysaccharides.

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