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METHYLATION ANALYSIS IN GLYCOPROTEIN CHEMISTRY: LOW-BLEEDING COLUMNS FOR THE GAS CHROMATOGRAPHIC ANALYSIS OF METHYLATED SUGAR DERIVATIVES

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SUMMARY

Columns packed with 0.3–0.4% OV-225 on Chromosorb surface modified with high-molecular-weight polyethylene glycol have been developed for the gas chromatographic analysis of methylated sugar derivatives. These columns provide a better separation of O-acetyl-O-methylmannitols and -galactitols than those usually employed (packed with 3% ECNSS-M or 3% OV-225) and an exceptionally good resolution of O-trimethylsilyl(TMS)-O-methylalditols. Methylated derivatives of glucosamine could be chromatographed well both in the form of N,O-acetylglucosaminitols and as the corresponding N-acetyl-O-TMS derivatives. Short retention times make it possible to perform the analyses rapidly and at temperatures 40–50° lower than the upper operation limit of the packing (*ca.* 250°). Column bleeding is negligible, thus ensuring that the retention parameters are constant for long-term functioning of the column.

INTRODUCTION

Methylation analysis is one of the most powerful tools used for elucidating the oligosaccharide structure of glycoproteins[1]. The analysis of the methylated sugar derivatives is a very difficult task, which involves separation of a number of closely related compounds. The great separating power of gas–liquid chromatography (GLC) prompted some investigators[2,3] to use it for this purpose. Recent advances in structural studies of glycoprotein carbohydrate moieties and the development of GLC itself have been intimately connected with the gas chromatographic analysis of methylated sugars (for reviews see refs. 1, 4 and 5).

Methylated sugars are usually analysed as the corresponding alditol acetates on columns with the polar stationary phases ECNSS-M[6] or OV-225[7]. Recently, OV-275–XF-1150[8] and Silar 10C[9] have been proposed. However, an inadequate resolution of the mixtures being analysed often calls for the use of mass spectrometry for identification of the components and complicates their quantification.

In laboratory practice, it would be desirable to have a gas chromatographic

technique that would ensure the complete separation, identification and quantification of the products of solvolysis of permethylated glycopeptides or glycoproteins on a single chromatographic column without the need for additional analytical methods. To achieve this it seems necessary to attempt a comprehensive study of the gas chromatographic behaviour of various volatile derivatives of methylated sugars and to develop columns that combine high selectivity with regard to methylated carbohydrates with high efficiency. In this respect, we have investigated the high-performance packings described by Aue *et al.*[10,11], which involve Chromosorb surface modified with high-molecular-weight polyethylene glycol.

EXPERIMENTAL

Materials

Mixtures of tetra-, tri-, di- and monomethyl ethers of methyl-α-D-mannopyranoside and methyl-α-D-galactopyranoside (from Serva, Heidelberg, G.F.R.) were prepared by methylation according to Handa and Montgomery[12] as described by Fournet and co-workers[13,14]. The individual methyl ethers were isolated from the mixtures by liquid chromatography (the technique will be published separately) and identified by mass spectrometry after being converted into the corresponding alditol acetates[15]. The methylated derivatives of methyl-N-acetyl-α-D-glucosaminide[16] were synthesized according to conventional techniques[17,18].

Methods

Methyl glycosides were hydrolysed in sealed glass ampoules at 100°. Heating with 2 *N* trifluoroacetic acid for 6 h was employed with methyl glycosides of neutral sugars and heating with 4 *N* hydrochloric acid for 3 h with methyl glucosaminides. The methylated sugars were reduced with excess of sodium borohydride at ambient temperature and pH 10–10.5 for 3 h. The acetylation and O-trimethylsilylation were performed by conventional procedures[19,20]. For the preparation of N-acetyl-O-TMS derivatives, the technique of Hara and Matsushima[21] was used.

A detailed description of the whole procedure for the quantitative conversion of methylated methyl glycosides into the corresponding O-TMS-O-methylalditols will be published elsewhere.

Gas chromatography

Analyses were performed with a Chrom 41 apparatus (Laboratorní Přistroje, Prague, Czechoslovakia) equipped with a linear temperature programmer, dual flame-ionization detectors and IT-1 integrator. The stainless-steel injection-port liners were replaced with liners made of silanized glass. Coiled glass columns (*ca.* 2.5 mm I.D. and 1.5, 2.4 and 3.0 m long) were silanized. Different temperature programmes were employed (see legends to the figures and tables). Helium, hydrogen and air flow-rates were optimized for each column used.

Chromosorb W AW (80–100 mesh) (Johns-Manville, Denver, Colo., U.S.A.), polyethylene glycol 20,000, for gas chromatography (Merck, Darmstadt, G.F.R.), OV-225 (Varian Aerograph, Walnut Creek, Calif., U.S.A.) and 3% OV-1 on Gas-Chrom Q (100–120 mesh) and 3% OV-225 on the same support (Serva) were employed.

The surface-modified Chromosorb was prepared according to Aue *et al.*[10] and then coated with OV-225 by the solution-coating technique. Packed columns were conditioned overnight at 240–250° with a slight flow of helium.

RESULTS

Characteristics of the columns

Preliminary experiments showed that the surface-modified Chromosorb (SMC) was a rather selective stationary phase with respect to various methylated sugar derivatives. However, in spite of the extraordinarily high efficiency inherent in the SMC columns, their separating capability, specifically with regard to O-acetyl-O-methylalditols, was lower than that of columns packed with 3% OV-225 on Gas-Chrom Q. Also, the low capacity of the SMC columns led to some inconvenience in operation.

We found that the coating of SMC with OV-225 at concentrations lower than 1% (w/w) contributed greatly to the selectivity and capacity of the columns, whereas their efficiency remained very high. A 0.3–0.4% loading was found to be optimal for preparing columns with high separating capabilities. The interaction of the compounds

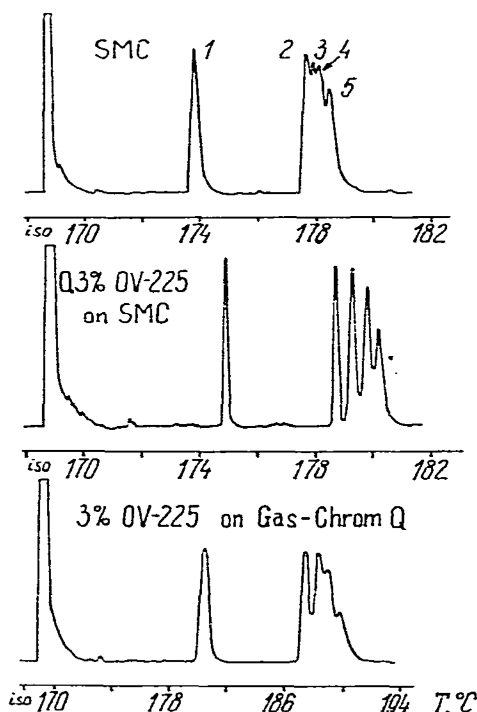

Fig. 1. Comparison of separating capabilities of columns with various packings with respect to tetra- and tri-O-methyl derivatives of mannitol acetates. After the injection of the samples, the columns (240 × 0.25 cm) were kept for 2 min at 170°, then the temperature was increased at the rate of 1°/min. The order of the appearance of the substances was the same with all columns: 1 = 2,3,4,6-tetra-O-methyl-; 2 = 3,4,6-tri-O-methyl-; 3 = 2,4,6-tri-O-methyl-; 4 = 2,3,6-tri-O-methyl-; 5 = 2,3,4-tri-O-methylmannitol.

being analysed both with the liquid phase and with the modified surface of the support seems likely to be significant in achieving good separations. A further increase in the liquid phase concentration resulted in an appreciable decrease in column efficiency. Fig. 1 illustrates the separation of several O-acetyl-O-methylmannitols on columns with various packings.

Evaluation of the theoretical plates number with respect to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol gave a value of *ca.* 18,000 for the 3-m column packed with 0.3% OV-225 on SMC (at the optimal carrier gas flow-rate). A slight slope on the right-hand branch of the Van Deemter plot (Fig. 2) affords an opportunity for increasing the carrier gas flow-rate and shortening the analysis time without a substantial decrease in column efficiency.

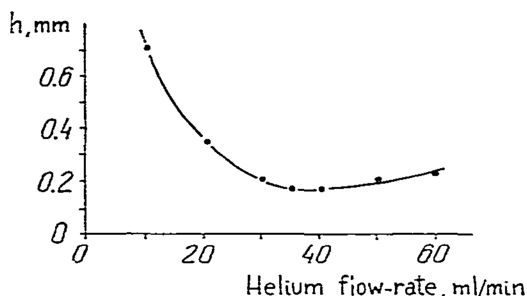

Fig. 2. Dependence of the efficiency of the column (300 × 0.25 cm) packed with 0.3% OV-225 on SMC on the carrier gas flow-rate. Compound: 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol. Temperature: 170°. h = height equivalent to a theoretical plate.

The upper temperature limit of the OV-225–SMC packing operation is *ca.* 250°. Below this temperature, the extent of column bleeding is negligible. Even prolonged intensive use of the columns (for at least 6 months) did not cause any alteration in the separating capability of the columns or in the retention parameters of the compounds being analysed. The low bleeding ensures a high stability of the baseline on the chromatograms and enables one to operate in the single-column mode and to utilize the most sensitive amplifier ranges without any compensation. The methylated methyl glycosides, monosaccharides and alditols (as both their acetyl and TMS derivatives) give symmetrical peaks on the chromatograms. Within the limits of experimental error, the peak areas remained constant under the different temperature regimes studied, indicating the absence of the irreversible adsorption and decomposition in the columns.

Analysis of O-methylmannitols and galactitols

Table I gives the relative retention times of O-acetyl-O-methylmannitols and -galactitols on columns with different packings. Comparison of these parameters shows that the 0.3% OV-225–SMC column gives better separations than the columns usually employed packed with 3% ECNSS-M or 3% OV-225 (it is noteworthy that the peaks are much narrower when using the OV-225–SMC column). The retention temperatures of the alditol acetates on the OV-225–SMC column are comparatively low, and the last peak (1,3,4,5,6-penta-O-acetyl-2-O-methylmannitol) emerges at a temperature *ca.* 40° lower than the upper operating limit of the packing.

TABLE I

RELATIVE RETENTION TIMES OF PARTIALLY METHYLATED ALDITOL ACETATES ON COLUMNS WITH VARIOUS PACKINGS

Column with 0.3% OV-225 on SMC (240 × 0.25 cm) was kept for 2 min at 170° and then heated at a rate of 1°/min to 210°.

Parent monosaccharide	*Position of methoxy groups*	*Relative retention time*		
		3% ECNSS-M (ref. 15)	*3% OV-225 (ref. 7)*[*]	*0.3% OV-225 on SMC*
Mannose	2, 3, 4, 6	1.00	1.00	1.00
	3, 4, 6	1.95	1.84	1.62
	2, 4, 6	2.09	1.92	1.71
	2, 3, 6	2.20	—	1.79
	2, 3, 4	2.48	2.21	1.86
	4, 6	3.29	2.95	2.44
	3, 6	—	3.71	2.76
	3, 4	5.37	4.40	3.03
	2, 4	5.44	4.56	3.11
	2, 6	3.35	—	2.87
	6	—	—	3.32
	2	—	—	3.75
Galactose	2, 3, 4, 6	1.25	1.20	1.15
	2, 4, 6	2.28	2.05	1.77
	2, 3, 6	2.42	—	1.84
	3, 4, 6	2.50	—	1.89
	2, 3, 4	3.41	2.92	1.99

[*] Data by Lönngren and Pilotti[7] were re-calculated by us based on the assumption that the relative retention time of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol is 1.00.

Trimethylsilylation of free hydroxyl groups has hardly ever been used in the GLC of methylated alditols[22]. We have succeeded, however, in achieving far better separations of O-TMS-O-methylalditols than of their acetylated analogues. Figs. 3

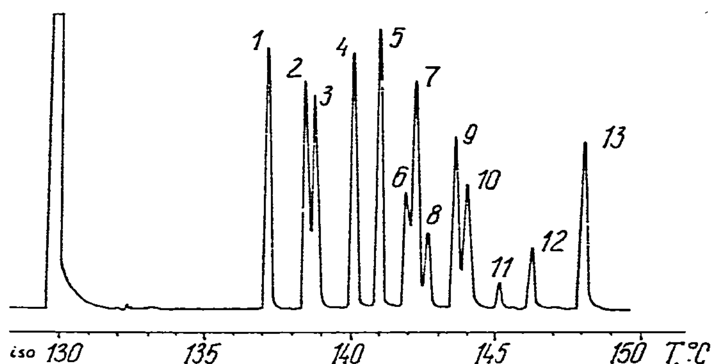

Fig. 3. Separation of the mixture of O-TMS-O-methylmannitols (*ca.* 0.3 μmole total) on the OV-225–SMC column. Chromatographic conditions as in Table II. Peaks: 1 = 2,3,4,6-tetra-O-methyl-; 2 = 2,4,6-tri-O-methyl-; 3 = 2,3,6-tri-O-methyl-; 4 = 3,4,6-tri-O-methyl-; 6 = 2,3,4-tri-O-methyl-; 7 = 3,6-di-O-methyl-; 8 = 4,6-di-O-methyl-; 9 = 3,4-di-O-methyl-; 10 = 2,4-di-O-methyl-; 11 = 2,6-di-O-methyl-; 12 = 6-mono-O-methyl-; 13 = 2-mono-O-methylmannitol; 5 = penta-O-TMS-arabitol (internal standard).

and 4 show that the OV-225–SMC columns ensure an excellent separation of silylated O-methylmannitols and O-methylgalactitols. Hence the problem of the separation of the most important methylated derivatives of mannose and galactose, when employing the methylation analysis in structural studies of glycoproteins, is practically solved. The relative retention times of a number of O-TMS-O-methylalditols are given in Table II.

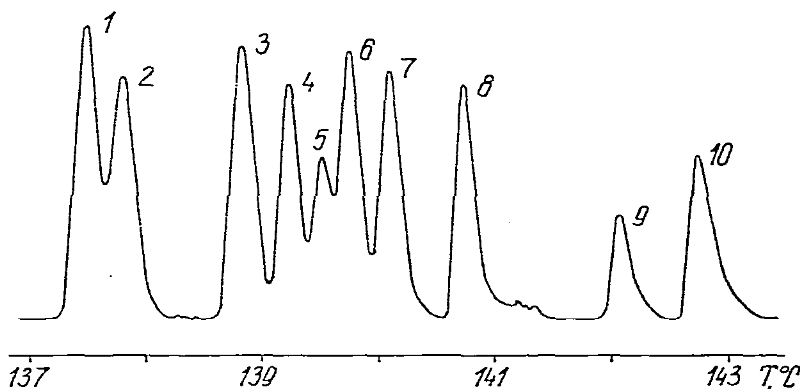

Fig. 4. Separation of a mixture of O-TMS-O-methylmannitols and O-TMS-O-methylgalactitols (part of chromatogram). Total amount of substances: *ca.* 0.1 μmole. Chromatographic conditions as in Fig. 3. Peaks: 1 = 2,3,4,6-tetra-O-methyl-; 3 = 2,4,6-tri-O-methyl-; 4 = 2,3,6-tri-O-methyl-; 8 = 3,4,6-tri-O-methyl-; 10 = 2,3,4-tri-O-methylmannitol; 2 = 2,3,4,6-tetra-O-methyl-; 5 = 2,4,6-tri-O-methyl-; 6 = 3,4,6-tri-O-methyl-; 7 = 2,3,6-tri-O-methyl-; 9 = 2,3,4-tri-O-methylgalactitol.

TABLE II

RELATIVE RETENTION TIMES OF O-TMS-O-METHYLALDITOLS ON THE 0.4% OV-225–SMC COLUMN

Column: 300 × 0.25 cm. Temperature programme: 130° for 2 min, then heated at 1°/min to 150°. The retention times were determined with respect to penta-O-TMS-arabitol.

Parent monosaccharide	*Position of methoxy groups*	*Relative retention time*
Mannose	2, 3, 4, 6	0.66
	2, 4, 6	0.77
	2, 3, 6	0.80
	3, 4, 6	0.92
	2, 3, 4	1.08
	3, 6	1.11
	4, 6	1.15
	3, 4	1.23
	2, 4	1.27
	2, 6	1.36
	6	1.47
	2	1.63
Galactose	2, 3, 4, 6	0.69
	2, 4, 6	0.82
	3, 4, 6	0.84
	2, 3, 6	0.87
	2, 3, 4	0.98

The high selectivity of the polar stationary phase with respect to TMS derivatives is rather surprising. Columns packed with 3% OV-1 or 3% OV-225 on Gas-Chrom Q gave the worst results.

Analysis of methylated glucosaminitols

Solvolysis of oligosaccharides is known[23,24] to be attended by the deacetylation of acetamidohexose residues. The GLC of monosaccharides and methyl glycosides usually requires preliminary re-N-acetylation. We found that the re-N-acetylation step is also necessary in the analysis of methylated glucosaminitols. Otherwise, these compounds tended to be adsorbed irreversibly on the packing.

On the OV-225–SMC columns, both N-acetyl-N-methyl-O-acetyl-O-methylglucosaminitols and the corresponding N-acetyl-N-methyl-O-TMS-O-methyl derivatives were resolved well. Even the mono-O-methyl derivatives were eluted from the column at temperatures substantially lower than the maximal operating temperature of the packing: *ca.* 210° for O-TMS derivatives and *ca.* 230° for O-acetyl derivatives.

The relative retention times of some N-acetyl-N-methyl-O-TMS-O-methylglucosaminitols are given in Table III and their separation is illustrated in Fig. 5.

TABLE III

RELATIVE RETENTION TIMES OF 2-DEOXY-2-N-METHYLACETAMIDO-O-TMS-O-METHYLGLUCITOLS ON THE 0.4% OV-225–SMC COLUMN

Column: 240 × 0.25 cm. Temperature programme: 130° for 2 min, then heated at 4°/min to 205°. The retention times were determined with respect to penta-O-TMS-arabitol.

Position of methoxy groups	*Relative retention time*
3, 4, 6	1.67
3, 6	1.77
3, 4	1.88
3	2.03

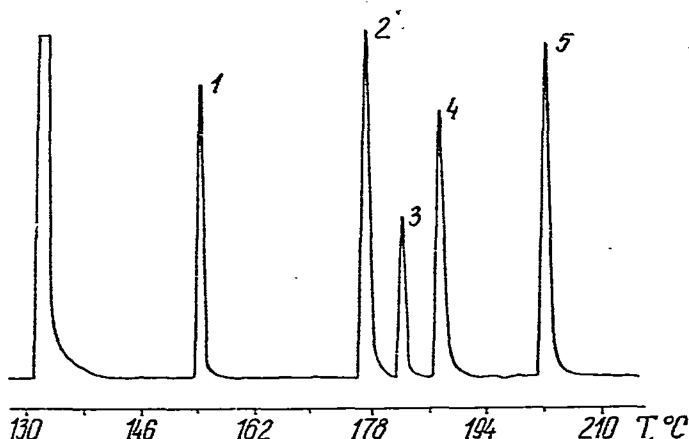

Fig. 5. Separation of a mixture of 2-deoxy-2-N-methylacetamido-O-TMS-O-methylglucitols (*ca.* 0.05 μmole total). Chromatographic conditions as in Table III. Peaks: 1 = penta-O-TMS-arabitol; 2 = 3,4,6-tri-O-methyl; 3 = 3,6-di-O-methyl; 4 = 3,4-di-O-methyl; 5 = 3-mono-O-methyl derivatives.

Analysis of methylated monosaccharides and methyl glycosides

The immediate products of solvolysis of a permethylated oligosaccharide are either methylated monosaccharides (in the case of acid hydrolysis) or methylated methyl glycosides (in the case of proton-catalysed methanolysis). For the GLC analysis of these compounds, it is sufficient to carry out a one-step reaction to protect free hydroxyl groups, and most methylated methyl glycosides are volatile enough to be analysed directly[13]. However, the solvolysis gives rise to two or, possibly, four anomers of every methylated monosaccharide (methyl glycoside). In general, the separation of a large number of such closely related substances can hardly be achieved when employing packed columns, even high-efficiency columns.

However, the GLC of methylated monosaccharides and methyl glycosides is of interest as a useful technique for controlling the steps of the hydrolysis of methyl glycosides and the reduction of monosacchraides when developing a procedure for the quantitative analysis of O-methylalditols. For this purpose, a reliable group separation of methylated methyl glycosides, monosaccharides and alditols is desirable. As can be seen from Fig. 6, the OV-225–SMC column permits compounds of the specified types with equal numbers of methoxyl groups to be clearly separated.

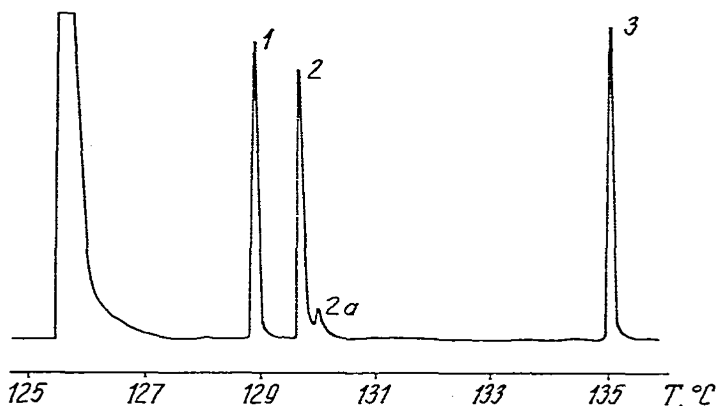

Fig. 6. Simultaneous analysis of methyl tetra-O-methyl-α-D-mannopyranoside (peak 1), 2,3,4,6-tetra-O-methylmannose (peaks 2 and 2a) and 1,5-di-O-TMS-2,3,4,6-tetra-O-methylmannitol (peak 3); *ca.* 0.01 μmole of each. Column: 240 × 0.25 cm, 0.3% OV-225 on SMC. Sample was injected at 125°, then the temperature was increased at the rate of 1°/min.

DISCUSSION

Following the investigations of Chizhov *et al.*[25] and Björndal and coworkers[6,15,26], the techniques for the analysis of methylated sugars as the corresponding alditol acetates by GLC–mass spectrometry become conventional. This approach was the basis of many structural studies of glycoprotein carbohydrate moieties. The oligosaccharide structure of a number of glycoproteins has now been determined and the general principles of the organization of the iso-glycanic structures, "high-mannose" and O-glycosidically linked oligosaccharides have been established.

More complicated problems are presented with glycoproteins that occur in organisms at a very low level and microheterogeneity, which is common with many glycoproteins[27]. These problems require a critical examination of quantitative aspects

of the analytical procedures used. Unfortunately, since a review published by Lindberg[4] in 1972 there have been no systematic studies of the reproducibility of the quantitative transformation of methylated sugars into volatile derivatives and of the accuracy and the reproducibility of their analysis. Investigators have developed and used various analytical procedures[28–32] proceeding from the specific features of a glycoprotein under investigation. The absence of a generally accepted procedure complicates investigations of new substances and sometimes makes it difficult to compare the data reported by different workers. In addition, we consider GLC to be powerful enough to solve the problem of the analysis of methylated sugars without the simultaneous use of mass spectrometry or other additional methods.

When attempting to develop a simple and reliable procedure for the analysis of methylated sugars, which would be useful for investigating glycoproteins, we examined a scheme involving methanolysis of a permethylated glycopeptide (glycoprotein), separation of methylated derivatives of neutral sugars and those of hexosamines, transformation into the corresponding O-methylalditols, and quantitative analysis on a single gas chromatographic column. We started by developing a technique of GLC separation that would enable us to use GLC not only to identify and quantify methylate dalditols, but also to check the completeness of the stages of the scheme.

The application of O-TMS-O-methylalditols in combination with columns packed with 0.3–0.4% OV-225 on SMC provides a very good resolution of methylated derivatives of mannose, galactose and glucosamine. These monosaccharides are the main constituents of the iso-glycanic and "high-mannose" oligosaccharide chains. Structures of such types occur widely[5] and their elucidation seems to be the most complex and important task in structural glycoprotein chemistry. The feasibility of the simultaneous analysis of methylated methyl glycosides, monosaccharides and alditols allows us to use methyl ethers of methyl glycosides as model substances in developing a reproducible procedure for the quantitative preparation of O-TMS-O-methylalditols.

An important feature of the OV-225–SMC columns developed is the very low column bleeding. Even long-term operation of the columns at temperatures up to 240° did not lead to any appreciable decrease in the retention temperatures of the compounds being analysed or in the resolving power of the columns, whereas an almost two-fold decrease in retention times after about 1 month's operation was reported[9] for the ECNSS-M column. The low column bleeding also provides for the use of the most sensitive ranges of amplification in a single-column mode of operation and, therefore, for the analysis of ultramicro amounts of methylated sugars (as low as *ca.* 100 pmole in the injected sample).

The GLC conditions described have been used in our laboratory for developing a reliable and convenient procedure for the analysis of the solvolysis products of permethylated glycopeptides and glycoproteins. The results will be published shortly.

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