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STUDY OF NEUTRAL AND AMINOMONOSACCHARIDES BY GAS-LIQUID DIFFERENTIAL CHROMATOGRAPHY: APPLICATION TO THREE REFERENCE GLYCOPROTEINS

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SUMMARY

A method is described for the determination of protein sugars by gas–liquid differential chromatography. After methanolysis and trimethylsilylation with trimethylsilylimidazole, the neutral monosaccharides are determined by flame ionization detection and the aminomonosaccharides by thermionic detection (nitrogen–phosphorus selective detection).

This technique was applied to three reference glycoproteins (ovalbumin, ovomucoid and uromucoid); the results obtained in 48 h, using 0.2–0.6 mg of protein, were satisfactory.

INTRODUCTION

The systematic analysis of the molecular composition of glycoprotein sugars demands the use of a simple and rapid technique. Gas–liquid chromatography (GLC)[1–3] is often preferred to the classical colorimetric methods applied to different preparative techniques[4].

There are two essential steps in the determination of protein sugars by GLC. Firstly, the O-glycosidic bonds must be chemically broken by an acid; aqueous acid hydrolysis requires different conditions for each type of monosaccharide (neutral monosaccharides, aminomonosaccharides and sialic acids)[4], whereas methanolysis liberates all sugars in a single step without either inducing their breakdown or attacking the peptide chain. In a second step, the sugars are converted into volatile derivatives[5] by methylation, acetylation, trimethylsilylation[6,7] or trifluoroacetylation[8,9], all of which substitute a more stable radical in place of the active hydrogen atoms of the hydroxyl and amino groups.

This paper describes a differential technique for the study of protein sugars (amino and neutral) by trimethylsilylation of the O-methylglycosides. The aminomonosaccharides are determined with the use of a thermionic detector (nitrogen–phosphorus selective detector, NPSD)[10–13], which functions as a flame-ionization detector (FID) except that the flame is reinforced by the combustion of an alkali

metal salt (potassium chloride). The ionization current or chromatographic signal that results is selectively modified by the presence of alkaline complexes formed with the nitrogen- and phosphorus-containing compounds.

EXPERIMENTAL

Methanolysis

The methanolysis conditions adopted were those described by Clamp and co-workers[7,3,14] in which anhydrous methanolic hydrochloric acid (1.5 *M*) reacts with the dry glucidic residue for 22 h at 85° in a sealed ampoule. After neutralization of the excess of acid with silver carbonate, re-acetylation of the hexosamines with acetic anhydride and washing with methanol of the precipitate formed, the pooled supernatants are evaporated to dryness.

Trimethylsilylation

The reagent (trimethylsilylimidazole, TSIM) was provided by Regis Co. (Morton Grove, Ill., U.S.A.). All other reagents were obtained from Merck (Darmstadt, G.F.R.), except the reference glucides, which were products of Sigma (St Louis, Mo., U.S.A.).

The trimethylsilylation reaction is performed according to a previously described procedure[15] in tubes kept overnight in a desiccator. A 20-μl volume of reagent is added to the dry residue, which is dissolved by grinding and then taken up in 80 μl of doubly distilled water and 50 μl of carbon disulphide. After mixing the solution, it is left to stand and two phases separate; the excess of TSIM remains in the aqueous phase, leaving the derivatives in the organic phase. A 0.5–1 μl volume of the latter phase is injected directly into the apparatus.

Gas–liquid chromatography

Apparatus and materials. The stationary phases and supports were purchased from Touzart and Matignon (Paris, France) and Carlo Erba (Milan, Italy). Columns of stainless steel, with an internal diameter of 2 mm and rolled into a spiral, were used. All analyses were performed with a Carlo Erba Fractovap Linea 1300 chromatograph equipped with an electrometer, a two-column oven, both an FID and an NPSD and a temperature programmer.

Analytical conditions. The neutral monosaccharides were studied in a 2-m column filled with 3% (w/w) of OV-17 as stationary phase on Gas-Chrom Q (100–120 mesh). The operating conditions for the FID detector were as follows: column temperature, 130°; temperature of the injector and detector, 250°; gas flow-rates, nitrogen (carrier gas) 1.65 kg/cm^2 (13 ml/min), hydrogen 0.55 kg/cm^2 (26 ml/min) and air 1.10 kg/cm^2 (350 ml/min). It should be noted that in studies of oligosaccharides, these conditions must be modified either by shortening the column or by increasing the temperature.

The aminomonosaccharides were analysed in a column of the same dimensions as above (2 m × 2 mm), but containing 4% (w/w) of SE-30 on Chromosorb W HP (80–100 mesh). The operating conditions were as follows for the NPSD: column temperature, 175° for 30 min, then increased at 1°/min to 200°, which was maintained for 10 min; temperature of injector and detector, 250°; gas flow-rates (which must be

carefully maintained as they determine the constancy of the ionizing flux of molten potassium chloride in the flame), nitrogen (carrier gas) 3 kg/cm^2 (30 ml/min), hydrogen 0.70 kg/cm^2 (30 ml/min) and air 0.85 kg/cm^2 (300 ml/min). The adjustment of the height of the potassium salt and the polarization electrode above the flame is performed so that the salt is at its maximal ionization and the electrode is sufficiently high to reduce the standing current and to select the nitrogen-containing substances.

Choice of internal standard. For the OV-17 phase as an exception, the two anomeric forms of methylglucose are mixed. Because of this α-methyl-D-glucose is used for the analysis of neutral monosaccharides in known samples. On the other hand, for unknown test samples the standard is inositol. This choice avoids errors due to the presence (although very rare) of glucose in the carbohydrate moiety or as a contaminant in complex preparations (saccharose, ficoll).

For the aminomonosaccharides, it is essential to use a nitrogenous standard as a thermionic detector gives a very poor response to compounds that lack a nitrogen atom. To achieve this, we used racephedrine (the optical isomers are not separated by GLC).

Interpretation of chromatograms. Each sugar is characterized relative to the internal standard, by the retention time of each of its isomers and by the coefficient of the relative molar response (RMR).

The areas of the peaks are calculated manually by triangulation. The reproducibility of the measurements and the significance of the difference between the values obtained and those in the literature are obtained by statistical analysis.

RESULTS

GLC of standard monosaccharide mixtures

Neutral monosaccharides. In the GLC of a mixture of free monosaccharides after trimethylsilylation (Fig. 1a and Table I), when all of the sugars are present together overlapping of certain peaks is observed (for instance, the first xylose with the second fucose peak and the first glucose with the third galactose peak), but their areas can nevertheless be calculated by taking into account the constancy of the relative proportions of the isomers at equilibrium in aqueous solution[6].

In the GLC of a mixture of trimethylsilylated (TMS) monosaccharides after methanolysis (Fig. 1b and Table I), all monosaccharides are completely resolved. Only one peak is observed for methylxyloside, although this may be due to the polarity of OV-17, as two isomeric forms are found on SE-30.

Aminomonosaccharides. In the GLC of a mixture of free monosaccharides after trimethylsilylation (Fig. 2a and Table II), inositol was used as an internal standard as racephedrine is not detected directly by trimethylsilylation but only after methanolysis. The sialic acids were studied by direct injection of the reaction mixture, including the TSIM as solvent[16], otherwise they are soluble in the aqueous phase owing to their very polar carboxyl radical, which is blocked with difficulty by the TSIM.

The GLC of a mixture of trimethylsilylated monosaccharides after methanolysis was carried out with both an FID (Fig. 2b and Table II) and an NPSD (Fig. 2c and Table II). Carbon disulphide is an ideal solvent for flame ionization as it gives a very feeble response for the injection peak, but on the other hand it alters the stability

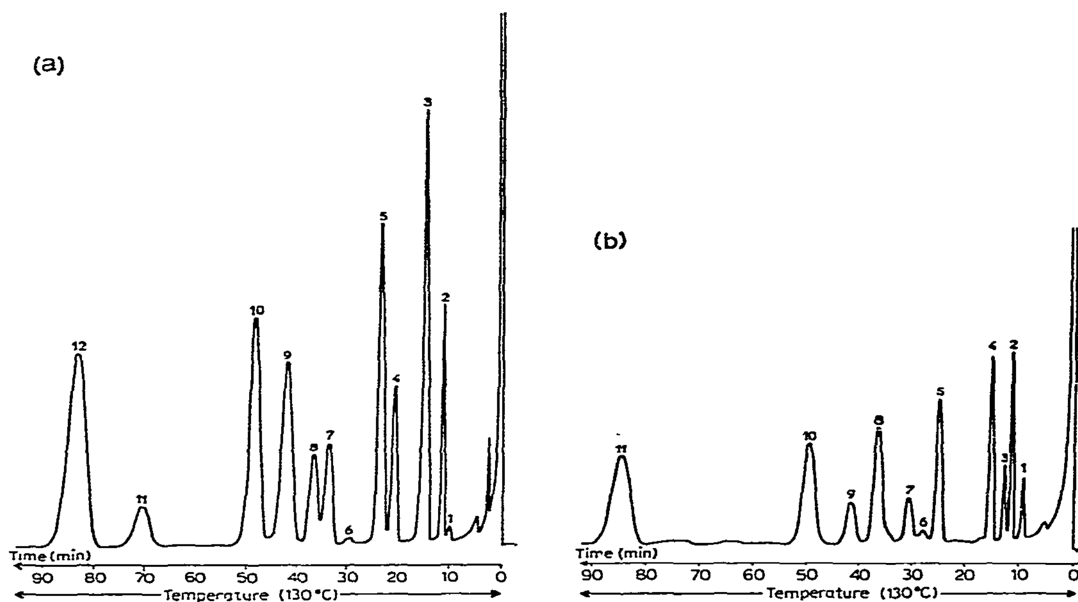

Fig. 1. Separation by GLC of neutral monosaccharides commonly occurring in glycoproteins. GLC conditions as defined in the text. (a) GLC resolution of the TMS derivatives of standard sugars. The peaks are numbered in their order of emergence: 1, 2, 3 = fucose; 3, 4 = xylose; 5, 8 = mannose; 6, 7, 9 = galactose; 9, 11 = glucose; 10 = α-methyl-D-glucoside; 12 = inositol. (b) GLC resolution of the TMS derivatives of O-methylglycosides. Peaks: 1, 2, 3 = fucose; 4 = xylose; 5, 6 = mannose; 7, 8, 9 = galactose; 10 = α-methyl-D-glucoside; 11 = inositol.

of the NPSD flame and *n*-hexane is to be preferred. Consequently, the compounds studied in a carbon disulphide medium are evaporated nearly to dryness under a stream of nitrogen and the residue is dissolved in the necessary amount of *n*-hexane.

After methanolysis, N-acetyl- and N-glycolylneuraminic acids have the same retention times. Contrary to the usual method[13,14], two peaks are observed, which are common in the two instances but with a different anomerization ratio (see Table II). A control experiment using mass spectrometry is under investigation in order to verify that these two peaks belong to sialic acids.

Determination of the coefficient of the relative molar response (RMR) for each sugar

This coefficient is a measure of the particular behaviour of the detector for each sugar with respect to the internal standard under defined operating conditions. It is represented by the slope of the graph of the total peak area ratio against the molar ratio.

The RMRs are given in Table III. There is a slight variation of the correlation coefficient (r) according to the osides studied; $r = 0.99$ for the hexoses, $r = 0.98$ for the hexosamines and $r = 0.96$ for the sialic acids. Also, the RMRs for the amino-monosaccharides with respect to methylracephedrine differ according to their mode of detection. This can be explained by the fact that the sensitivity of the NPSD for amino compounds depends on the nitrogen content of their molecule[10].

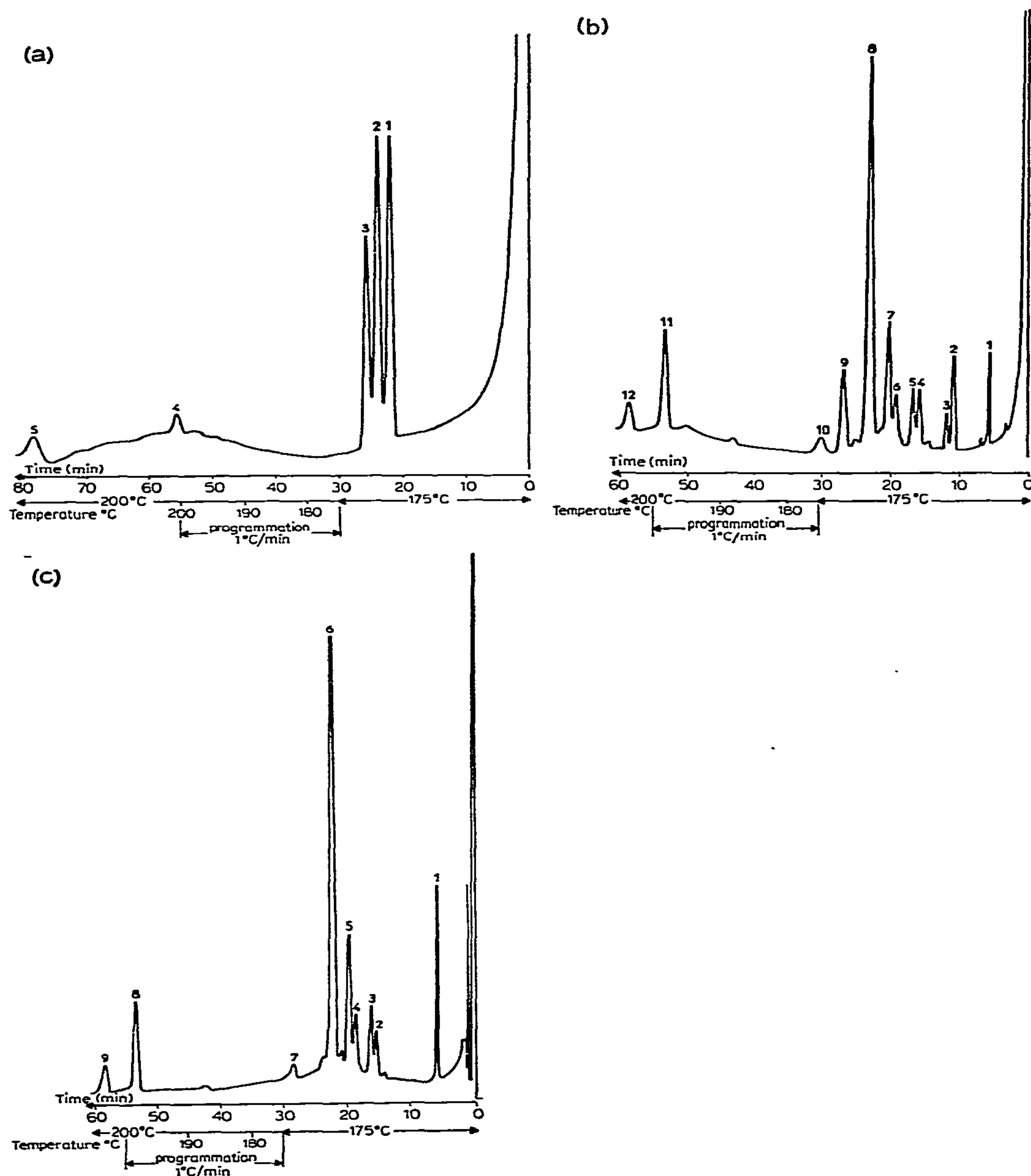

Fig. 2. Separation by GLC of aminomonosaccharides commonly occurring in glycoproteins. GLC conditions as defined in the text. (a) GLC resolution of the TMS derivatives of standard sugars. The peaks are numbered in their order of emergence: 1 = N-acetylgalactosamine; 2 = N-acetylglucosamine; 3 = inositol; 4 = N-acetylneuraminic acid; 5 = N-glycolylneuraminic acid. (b) GLC resolution of the TMS derivatives of O-methylglycosides with FID. Peaks: 1 = racephedrine; 2, 3 = α-methyl-D-glucoside; 4, 6, 8, 10 = N-acetylglucosamine; 5, 7 = N-acetylgalactosamine; 9 = inositol; 11, 12 = N-acetylneuraminic acid; 11, 12 = N-glycolylneuraminic acid. (c) GLC resolution of the TMS derivatives of O-methylglycosides with NPSD. Peaks: 1 = racephedrine; 2, 4, 6, 7 = N-acetylglucosamine; 3, 5 = N-acetylgalactosamine; 8, 9 = N-acetylneuraminic acid; 8, 9 = N-glycolylneuraminic acid.

TABLE I

GAS CHROMATOGRAPHIC PROPERTIES OF SOME NEUTRAL MONOSACCHARIDES (COMMONLY OCCURRING IN GLYCOPROTEINS AND GLYCOPEPTIDES) AND THEIR DERIVATIVES

Parent monosaccharide	*Free monosaccharides**			*O-Methylglycosides*		
	*Retention time of isomer*** relative to α-methyl-D-glucoside (R_t = 50 min)*	*Retention time of isomer relative to inositol (R_t = 85 min)*	*Percentage distribution of isomer****	*Retention time of isomer relative to α-methyl-D-glucoside (R_t = 50 min)*	*Retention time of isomer relative to inositol (R_t = 85 min)*	*Percentage distribution of isomer*
Xylose	0.33	0.20	43 ± 0.02	0.31	0.18	100
	0.44	0.26	57 ± 0.02			
Fucose	0.21	0.12	3.1 ± 0.10	0.19	0.11	11.1 ± 0.05
	0.24	0.15	39.3 ± 0.02	0.23	0.14	61.8 ± 0.03
	0.31	0.18	57.6 ± 0.02	0.26	0.16	27.1 ± 0.02
Mannose	0.49	0.29	66.3 ± 0.03	0.51	0.30	88 ± 0.02
	0.76	0.45	33.7 ± 0.05	0.57	0.33	12 ± 0.11
Galactose	0.61	0.35	4.2 ± 0.10	0.62	0.37	14 ± 0.04
	0.70	0.41	38.2 ± 0.03	0.74	0.44	59 ± 0.02
	0.87	0.51	57.6 ± 0.02	0.84	0.50	27 ± 0.04
Glucose	0.87	0.51	40 ± 0.03	1.00	0.59	100
	1.40	0.84	60 ± 0.02			
α-Methyl-D-glucoside	1.00	0.59	100	1.00	0.59	100
Inositol	1.70	1.00	100	1.70	1.00	100

* Free monosaccharides trimethylsilylated directly.
** Configuration of isomers not defined.
*** Peak area of each isomer expressed as a percentage of the total peak area.

TABLE II

GAS CHROMATOGRAPHIC PROPERTIES OF SOME AMINOMONOSACCHARIDES (COMMONLY OCCURRING IN GLYCOPROTEINS AND GLYCOPEPTIDES) AND THEIR DERIVATIVES

Parent monosaccharide	*Free monosaccharides**			*O-Methylglycosides*					
	*FID***			*FID*				*NPSD****	
	Retention time of isomer§ *relative to inositol* (R_t = *26 min)*	*Retention time of isomer relative to perseitol* (R_t = *42 min)*	*Percentage distribution of isomer*§§	*Retention time of isomer relative to inositol* (R_t = *26 min)*	*Retention of isomer relative to perseitol* (R_t = *42 min)*	*Retention time of isomer relative to methylracephedrine* (R_t = *6 min)*	*Percentage distribution of isomer*	*Retention time of isomer relative to methylracephedrine* (R_t = *6 min)*	*Percentage distribution of isomer*
N-Acetylglucosamine	0.93	0.57	100	0.59	0.37	2.76	7.5 ± 0.15	2.76	5.2 ± 0.18
				0.73	0.46	3.38	7.3 ± 0.16	3.38	7.5 ± 0.11
				0.86	0.54	4.00	80.2 ± 0.03	4.00	84.3 ± 0.02
				1.13	0.71	5.28	3 ± 0.20	5.28	3 ± 0.16
N-Acetylgalactosamine	0.82	0.50	100	0.63	0.39	2.92	32.5 ± 0.06	2.92	32.5 ± 0.06
				0.76	0.48	3.55	67.5 ± 0.03	3.55	67.5 ± 0.03
N-Acetylneuraminic acid	2.20	1.38	100	2.00	1.23	9.15	74.2 ± 0.05	9.15	74.2 ± 0.05
				2.20	1.35	10.10	25.8 ± 0.15	10.10	25.8 ± 0.15
N-Glycolylneuraminic acid	3.08	1.90	100	2.00	1.23	9.15	62.3 ± 0.06	9.15	62.3 ± 0.06
				2.20	1.35	10.10	37.7 ± 0.18	10.10	37.7 ± 0.18
Racephedrine	—	—	—	0.22	0.13	1.00	100	1.00	100
Inositol	1.00	0.61	100	1.00	0.63	4.67	100	4.67	100
Perseitol	1.64	1.00	100	1.60	1.00	7.42	100	7.42	100

* Free monosaccharides trimethylsilylated directly.
** Flame-ionization detection.
*** Nitrogen-phosphorus selective detection.
§ Configuration of isomers not defined.
§§ Peak area of each isomer expressed as a percentage of the total peak area.

TABLE III

RELATIVE MOLAR RESPONSE FACTORS OF MONOSACCHARIDES COMMONLY OCCURRING IN GLYCOPROTEINS AND GLYCOPEPTIDES

Parent monosaccharide	Neutral monosaccharides*		Aminomonosaccharides**					Nitrogen (%)***
	FID§		FID				NPSD§§	
	Relative molar response factor§§§ (relative to α-methyl-D-glucoside)	Relative molar response factor§§§ (relative to inositol)	Relative molar response factor† (relative to α-methyl-D-glucoside)	Relative molar response factor (relative to inositol): Free sugar	Relative molar response factor (relative to inositol): O-Methylglycoside	Relative molar response factor† (relative to methylracephedrine)	Relative molar response factor† (relative to methylracephedrine)	
Xylose	0.94	0.59						
Fucose	0.95	0.57						
Mannose	1.00	0.60						
Galactose	0.98	0.59						
Glucose	1.00	0.60						
Racephedrine	—	—	0.73	—	0.50	1.00	1.00	
α-Methyl-D-glucoside	1.00	0.60	1.00	0.71	0.72	1.35	—†††	
Inositol	1.55	1.00	1.48	1.00	1.00	2.00	—†††	
N-Acetyl-glucosamine			0.65	0.42	0.45	0.85	0.62	6.34
N-Acetyl-galactosamine			0.66	0.42	0.43	0.82	0.60	6.34
N-Acetyl-neuraminic acid			0.45	0.25	0.31	0.57	0.40	4.53
N-Glycol-neuraminic acid			—††	—††	0.30	0.58	0.37	4.31

* GLC was performed isothermally at 130°.
** GLC conditions as defined in the text.
*** Percentage of nitrogen in the molecule.
§ FID = flame-ionization detection.
§§ NPSD = nitrogen–phosphorus selective detection.
§§§ RMR defined for trimethylsilyl derivatives of free monosaccharides and O-methylglycosides.
† RMR defined for trimethylsilyl derivatives of O-methylglycosides.
†† Not determined.
††† Near to zero.

Application to three glycoproteins of known composition

The GLC analysis of standard mixtures of monosaccharides has contributed to the elaboration of the method, but in order to test the value of this technique we

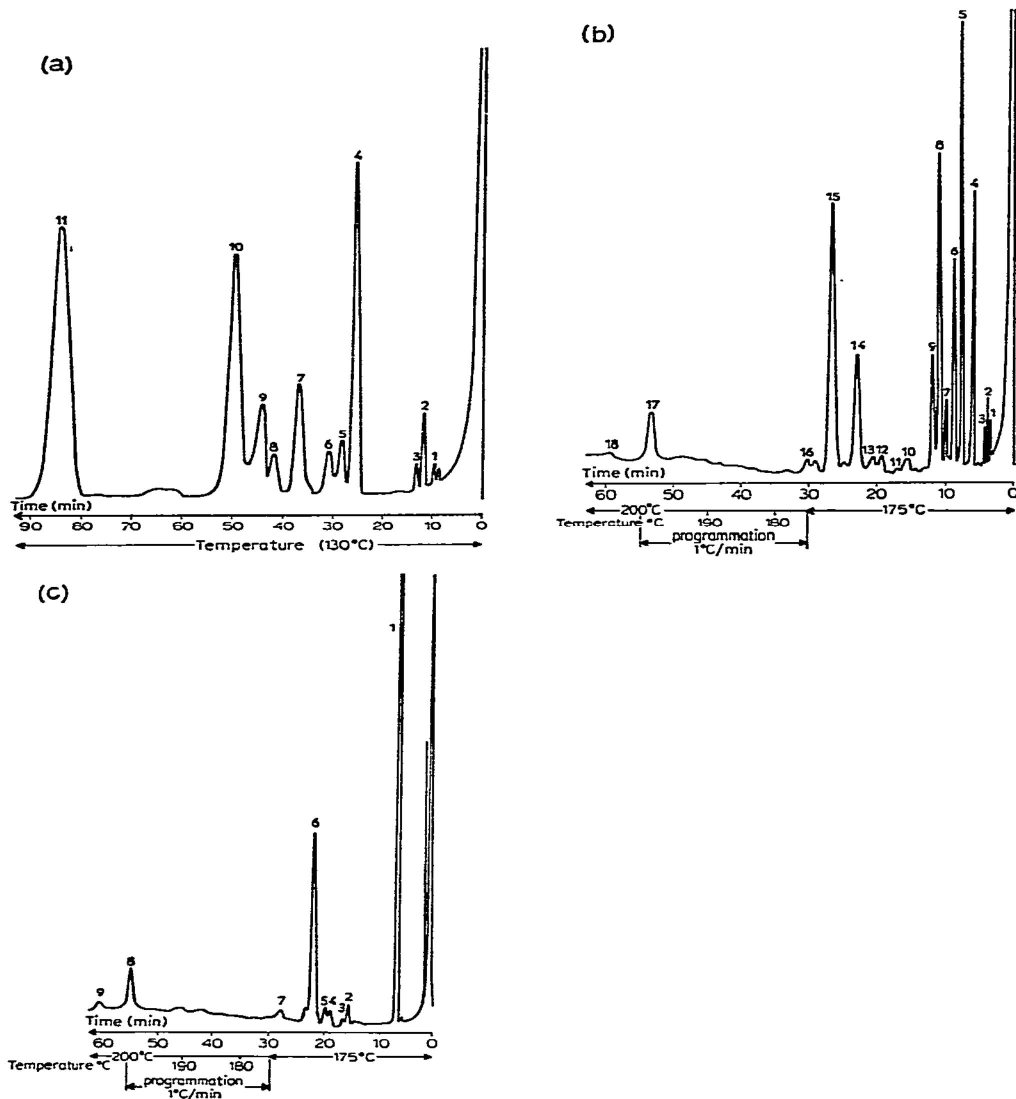

Fig. 3. GLC after trimethylsilylation of the methanolysis residue from Tamm and Horsfall glycoprotein. Conditions of chromatography as defined in the text. (a) GLC of neutral monosaccharides. The peaks numbered in their order of emergence: 1, 2, 3 = fucose; 4, 5 = mannose; 6, 7, 8 = galactose; 9 = methylracephedrine; 10 = α-methyl-D-glucoside; 11 = inositol. (b) GLC of amino-monosaccharides with FID. Peaks: 1, 2, 3 = fucose; 4 = methylracephedrine; 5, 6 = mannose; 6, 7 = galactose; 8, 9 = α-methyl-D-glucoside; 10, 12, 14, 16 = N-acetylglucosamine; 11, 13 = N-acetylgalactosamine; 15 = inositol; 17, 18 = N-acetylneuraminic acid. (c) GLC resolution of amino-monosaccharides with NPSD. Peaks: 1 = methylracephedrine; 2, 4, 6, 7 = N-acetylglucosamine; 3, 5 = N-acetylgalactosamine; 8, 9 = N-acetylneuraminic acid.

TABLE IV

CARBOHYDRATE COMPOSITION OF GLYCOPROTEINS

Monosaccharide	*Ovalbumin*[a]				*Ovomucoid*[b]			*T and H*[b]			
	Reported		*Found*		*Reported*	*Found*		*Reported*		*Found*	
	Colorimetry[c]	*GLC*[d]	*Colorimetry*	*GLC*	*Colorimetry*[e]	*Colorimetry*	*GLC*	*Colorimetry*[f]	*GLC*[g]	*Colorimetry*	*GLC*
Neutral monosaccharides	(5.0)	(5.10)	(5.8)	(5.4)	(8.5)	(8.5)	(8.1)	(12.34)	(14.1)	(9.94)	(11.08)
Fucose	—	—	—	—	—	—	—	0.82	1.1	—	0.73
Hexoses											
Mannose	5.0	5.10	5.8	5.4	} 8.5	} 8.5	6.9	5.76	6.5	} 9.94	5.8
Galactose	—	—	—	—			1.2	5.76	6.5		4.6
Ratio Man/Gal[k]					(5/1)		(5.7/1)	(1/1)	(1/1)		(1.3/1)
Hexosamines								(11.04)	(10.6)	(11.26)[h]	(10.1)
N-Acetylglucosamine	3.0	3.85	3.1[h]–3.7[i]	3.7	14	12.4[h]–15.2[i]	13	9.94	9.6	8.12[i]	8.9
N-Acetylgalactosamine	—	—	—	—	—	—	—	1.10	1.0	0.98[i]	1.2
Ratio GlcNAc/GalNAc[l]								(9/1)	(9.6/1)	(8.3/1)	(7.4/1)
Sialic acid											
N-Acetylneuramicic acid	—	—	—	—	0.9	0.5	0.5	4.63	6.1	—[j]	5.9
Total	8.0	8.95	8.9	9.1	23.4	21.4	21.6	28.01	30.8		27.1

[a] Values are given in moles per mole of protein.
[b] Values are given as a percentage of dried protein.
[c] According to Johansen *et al.*[18].
[d] According to Zanetta *et al.*[5].
[e] According to Adam-Chosson and Montreuil[19].
[f] According to Fletcher *et al.*[20].
[g] According to Stevenson and Kent[21].
[h] Determined as free hexosamines by classical colorimetry according to Montreuil and Spik[4].
[i] Determined as free hexosamines with a Beckman 120C amino acid Autoanalyser after hydrolysis for 4 h in 4 *N* hydrochloric acid at 100°.
[j] Not determined.
[k] Ratio mannose/galactose.
[l] Ratio N-acetylglucosamine/N-acetylgalactosamine.

applied it to the analysis of the glycans of three well known glycoproteins: ovalbumin, ovomucoid and uromucoid or Tamm and Horsfall (T and H) protein. The first two proteins were Sigma products (ovalbumin, grade V, and ovomucoid, trypsin inhibitor type II-O); the T and H was prepared in the laboratory from normal urines by precipitation with 0.58 *M* sodium chloride solution[17] and its purity was controlled by immunochemistry and electron microscopy.

The measurements were carried out at least six times on each batch of protein using 0.2–0.6 mg of material. All of the monosaccharides studied were from the same sample: firstly, 1–2 μl of each carbon disulphide extract was injected to give a chromatogram of the neutral monosaccharides (Fig. 3a), then secondly, the aminomonosaccharides were analysed by injection of 1–2 μl of the extract in n-hexane (Figs. 3b and 3c).

The values found by GLC were compared with those in the literature (Table IV); colorimetric titrations carried out in parallel served as a control. All of the results, which are accurate to within 5%, are close to the literature values[8,18–21] and the few differences observed, which are hardly significant, are due to technical factors.

DISCUSSION

The utilisation of TSIM as a silylating agent has several advantages over the classical reaction mixture[6] (pyridine–hexamethyldisilazane–trimethylchlorosilane, 5:1:1). It is easy to use as it reacts instantaneously, it causes no precipitation and elimination of an excess of it suppresses a long tail at the level of the injection peak. It can be used to give highly reproducible determinations and a better conservation of the TMS derivatives than in pyridine medium, where their hydrolysis is evident after 24 h.

The advantages of methanolysis, discussed in many papers[7–9,13,14], were confirmed by this work.

Of particular interest is the use of the NPSD in the study of the aminomonosaccharides. The selectivity of this detector for these compounds is greater (by a factor of 100 in the present case) and the sensitivity of their detection is therefore enhanced (about 5-fold) in comparison with the FID analysis. All of the peaks of non-nitrogenous compounds are virtually eliminated from the chromatograms, facilitating their interpretation, and the baseline is nearly horizontal.

The NPSD is operated in the same manner as the FID, but some precautions must be taken in order to maintain its performances. We have established, for instance, that the carrier gas flow-rate must be 30 ml/min in order to obtain good results, as it conditions the ionization current of substances that burn in the flame. Also, the adjustment of the height of the salt and the polarization electrode over the flame is very important as it allows, depending on the type of analysis required, a preferential modification of either the selectivity or the sensitivity of response. In our study, the selectivity is of greater importance than the sensitivity because the amino sugars and the neutral monosaccharides are found in the same extract. Before each series of analyses a check of the NPSD adjustment is necessary.

The preparation of the samples can be simplified in some instances because non-nitrogenous compounds are not detected. We have shown that all of the usual buffers (veronal, Tris, citrate), in addition to sodium chloride and EDTA, have no influence under the operating conditions chosen.

When performing a differential study of the neutral monosaccharides and aminomonosaccharides, the NPSD allows a considerable saving of time and avoids the loss of material which occurs in the technique involving the separation of the hexosamines and neutral monosaccharides after running a hydrolysate through a cationic resin.

The use of the NPSD, which is already employed extensively in the screening of nooanaleptics and pesticides, could be extended to the analysis of sugars, among which there is a wide variety of nitrogen- and phosphorus-containing compounds. This work was limited to the sugars of glycoproteins. Nevertheless, from our first results we can conclude that the NPSD gives better performances with phosphorus- than with nitrogen-containing compounds. Hence it can be recommended for the determination of phosphorus-containing sugars.

Finally very small amounts of proteins are necessary for a complete analysis, for the results are adequate for quantitative purposes with 10 ng of each sugar. The number of successive steps is limited, favouring good reproducibility, and an accuracy of 2–3% is obtained for ten replicate experiments using the same standard solution. The standard deviation is therefore small in comparison with errors due to the calculation of the areas, which are about 2%. These results confirm the validity of the proposed method.

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