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SILYLALDONITRILE DERIVATIVES FOR THE DETERMINATION OF SUGARS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A novel procedure for the derivatization of aldoses prior to gas chromatography–mass spectrometry is proposed. Reaction of the sugars with hydroxylamino-O-sulphonic acid and silylation of the hydroxyl groups yields silylaldonitrile derivatives, which give single chromatographic peaks and favourable mass spectrometric properties. The silylaldonitrile derivatives are easily separated by capillary gas chromatography and are suitable for both identification and quantification purposes.

INTRODUCTION

The determination of sugars by gas chromatography (GC) and GC coupled with mass spectrometry (GC–MS) has been accomplished with a number of derivatives, each possessing distinct advantages and drawbacks. In principle, an ideal derivative for GC–MS should yield a product eluting as a single peak, with intense and diagnostic high-mass ions in its mass spectrum. An additional useful feature in the spectra of polyhydroxy compounds is the occurrence of even-electron ion series, arising from α-cleavage next to each heteroatom-bearing carbon, which facilitates the recognition of functional groups on the carbon atoms. Among the commonly employed derivatives of simple sugars are methyl[1,2], silyl-[3] or acyl-protected[4,5] closed-ring acetals, which yield anomeric mixtures, their proportion depending largely on the reaction conditions. Some of these derivatives have been widely applied in the past for both structural and quantitative analysis.

Conversion of the oxo function to a suitable oxime derivative and silylation of the hydroxyl groups yields a mixture of *syn* and *anti* products[6,7], which can be separated by GC and give rise to intense C_6-containing ions in their mass spectra. A serious drawback of the methoxime-silyl (MO-TMS) derivatives is the excessive length of the GC run if the two isomers of each of glucose, mannose, galactose and fructose are to be separated[8].

Acetylated aldonitriles are obtained through dehydration of the corresponding aldoximes by means of acetic anhydride[9,10]. A single derivative is obtained for each aldose, and several can be separated in a chromatographic run, but the electron-

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impact (EI) mass spectra contain only two low-intensity, high-mass ions, resulting from the loss of the C_6 and C_6–C_5 fragments. This is of particular disadvantage if [6,6-$^2H_2$]glucose is to be employed in isotope dilution mass spectrometry (IDMS) or as a tracer in biochemical studies[11], as neither of these ions retains the carbon atom carrying the isotopic label.

Boronate cyclic esters[12] of many sugars are useful derivatives for GC–MS work, characterized by intense high-mass ions retaining the cyclic acetal moiety. The number of derivatives formed in the boroacetylation reaction is, however, strongly related to the stereochemistry and solution conformation of the molecule, and therefore the method does not appear to be suitable for general use in sugar derivatization.

Silylated aldonitriles were first described in the course of an investigation on cyanide addition to pentoses[13]. Such derivatives were reported to be not very stable, and no attempt was made to employ them for analytical purposes.

The aim of this work was to combine the advantages of two of the aforementioned derivatives, namely the single-peak sugar aldonitriles and the methyloxime-trimethylsilyl ethers, with C_6-containing fragments in their mass spectra. The silylated aldonitriles of various simple sugars were therefore synthetized and subjected to GC and GC–MS analyses.

EXPERIMENTAL

All solvents and reagents were of analytical-reagent or reagent grade and were used as received.

The sugars 2-deoxy-D-ribose (1), D-arabinose (2), D-xylose (3), D-lixose (4), D-ribose (5), 2-deoxy-D-glucose (6), D-galactose (7), D-mannose (8), D-gulose (9), D-allose (10), D-idose (11), D-altrose (12), D-talose (13), D-glucose (14) and D-glucuronic acid (15) were purchased from Sigma or obtained as pure standards.

Lyophilization and evaporation under vacuum were performed in a SpeedVac concentrator (Savant). All heating was accomplished with a Blok heater (Supelco).

GC analyses were performed on a Carlo Erba 4160 capillary gas chromatograph equipped with a split–splitless injector and flame ionization detector. A Waters 740 integrator was employed for recording the data. GC–MS analyses were performed on a Finnigan 4021 instrument with a Super-INCOS data system.

The capillary columns employed were of the bonded-phase fused-silica type: a Mega 25 m × 0.25 mm I.D. column coated with (5% phenyl–methyl)polysiloxane (SE-54; film thickness 0.4 μm) and a Chrompack 25 m × 0.32 mm I.D. column (50% phenyl–methyl)polysiloxane (CP-Sil-19CB; film thickness 0.1 μm). More polar columns were not tested, as the compromise between resolution, analysis time and column lifetime was judged to be sufficient with the above columns.

Synthesis of derivatives

An appropriate amount (50 μg–1 mg) of individual sugars or sugar mixture from water or methanol stock solutions was lyophilized in cone-bottomed glass tubes with PTFE-lined screw-caps. A 50–100-μl volume of the derivatizing reagent (a 1 *M* solution of hydroxylamine-O-sulphonic acid in methanol containing an equimolecular amount of triethylamine) was added and the solution reacted at room temperature for 30 min. The solvent was evaporated, 50 μl of bistrimethylsilyltrifluoroacet-

amide (BSTFA) and 50 μl of pyridine or acetonitrile were added and the suspension was heated at 80°C for 30 min.

When an internal standard was needed, as in the measurement of conversion efficiencies or retention time measurements, hexachlorobenzene (5 mg/ml in pyridine) was added in the silylation step.

RESULTS AND DISCUSSION

Derivatization

The silylated aldonitrile derivatives of several aldoses were prepared by a modification of the procedure of Fizet and co-workers[14,15], employing hydroxylamine-O-sulphonic acid, which they had applied to a variety of aliphatic, aromatic and heterocyclic aldehydes. The reaction scheme, exemplified for glucose, is depicted in Fig. 1. An earlier attempt to reproduce Fizet and co-workers' conditions, *i.e.*, carrying out the reaction in water at room temperature to 50°C, produced the silylated epimeric mixture as the only peaks in the GC run. Moreover, the reagent is scarcely, if at all, soluble in pyridine, and when a suspension was added to the sugars and reacted at 80°C for 30 min, as under the usual oxime formation conditions, a mixture containing the silylated anomers, in addition to the *syn–anti* isomers, was obtained. A very minor peak, eluting immediately before the oxime isomers, was also detected, and was also present when the silylation step was carried at temperatures higher than 100°C. It was therefore assumed that such a peak could be the expected product, formed through a thermally induced loss of silanol from the silyloxime moiety.

When an equimolar amount of a tertiary amine (pyridine or triethylamine) was added to a 1 *M* solution of hydroxylamine-O-sulphonic acid in methanol or acetonitrile, the pH of the solution increased to 5, in comparison with the value of 1 measured in water or methanol. This reagent was therefore employed for the derivatization,under a variety of conditions, which all led to the expected aldonitrile compounds, although in greatly different yields and with different rates.

Conversion efficiencies were measured for glucose in methanol and in acetonitrile and showed the conversion to be quantitative within 15 min in the former

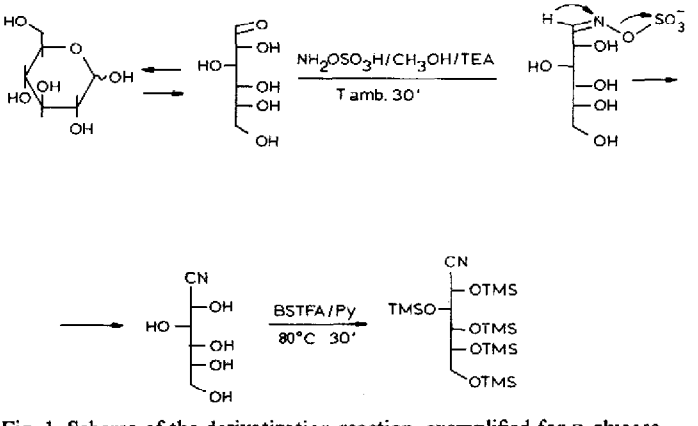

Fig. 1. Scheme of the derivatization reaction, exemplified for D-glucose.

solvent, whereas 16–24 h were necessary when the reaction was carried in acetonitrile. However, an advantage of employing acetonitrile as the reaction solvent is that lyophilization prior to silylation is unnecessary, and much less tanning of the reaction mixture occurs on storage of the samples, both at room temperature and at 4°C, in tightly closed vessels.

The reagent and the oxime-O-sulphonic acid intermediate are very prone to methanolysis when a molar excess of triethylamine is present, the measured pH being higher than 5, or when the reaction is carried out at temperatures over 40°C. Under these conditions, the silyloxime couple is formed instead of the silylaldonitrile. Further evidence of this explanation is that with aged solutions a sticky precipitate separates and the desired reaction does not occur. The derivatizing solution should therefore be used within a few hours after preparation.

The derivatized samples are stable (and can be employed for quantitative measurements) for several weeks even at room temperature, provided that the screw-capped vial is not opened.

The stability of glycosidic bonds to the reaction conditions was tested by subjecting β-methyl-D-mannopyranoside to the derivatization steps and analysing the products by gas chromatography. No mannose silylaldonitrile peak was detected, and the silyl derivative of the substrate compound was detected as the only product.

Gas chromatography

GC separation of several different aldohexoses and pentoses was accomplished on a moderately polar capillary column operated isothermally at 170–180°C (Fig. 2). At a 50:1 splitting ratio even the earliest eluting 2-deoxyribose derivative was well

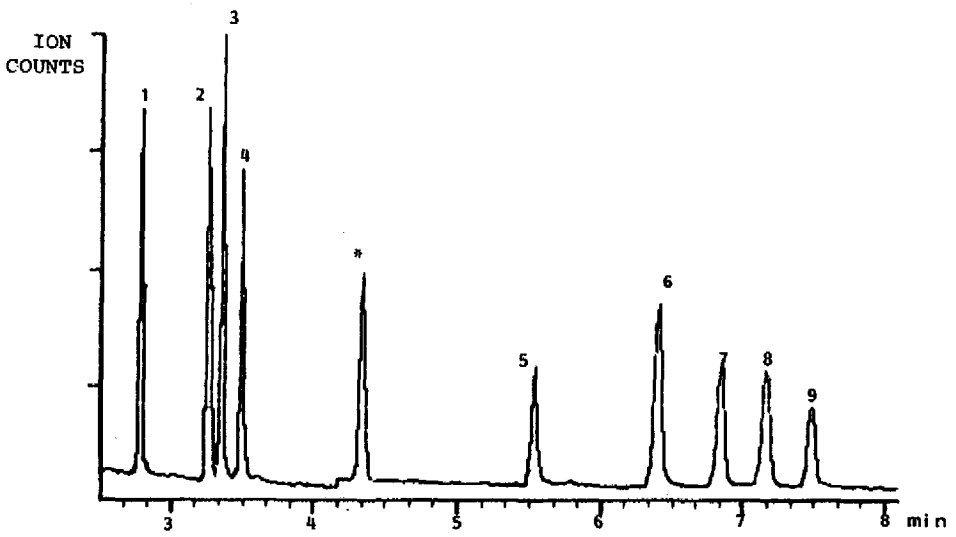

Fig. 2. Capillary GC separation of silylaldonitriles: 1, deoxyribose; 2, arabinose; 3, xylose; 4, ribose; 5, 2-deoxyglucose; 6, galactose; 7, mannose; 8, idose; 9, glucose. The peak marked with an asterisk is the internal standard (hexachlorobenzene). CP-Sil-19CB capillary column (25 m × 0.32 mm I.D., film thickness 0.1–0.15 μm); helium, 8 p.s.i.; injection temperature, 280°C; oven temperature, 180°C; coupled to a Finnigan 4021 quadrupole mass spectrometer continuously scanned between 33 and 650 u in 0.95 + 0.05 s; electron energy, 70 eV; emission current, 250 μA; multiplier voltage, 950 V.

TABLE I

RELATIVE RETENTION TIMES OF THE SILYLALDONITRILE DERIVATIVES OF VARIOUS SUGARS ON TWO COLUMNS

Isothermal operation at 180°C.

Compound	*SE-54*[a]	*CP-Sil-19CB*[b]
1	0.537	0.644
2	0.755	0.751
3	0.756	0.774
4	0.769	0.769
5	0.790	0.804
6	1.158	1.276
7	1.700	1.475
8	1.706	1.575
9	1.752	1.631
10	1.759	1.609
11	1.792	1.647
12	1.838	1.610
13	1.862	1.631
14	1.876	1.724
15	2.098	2.109

[a] Retention time of internal standard 4.525 min.
[b] Retention time of internal standard 4.537 min.

separated from the solvent peak. Differences in the elution order of the eight aldohexose steroisomers were noticed between the two phases, whereas all sugars were more retarded on the SE-54 column (Table I). Mannose and galactose could not be separated on the SE-54 column, whereas the three physiologically occurring aldohexoses

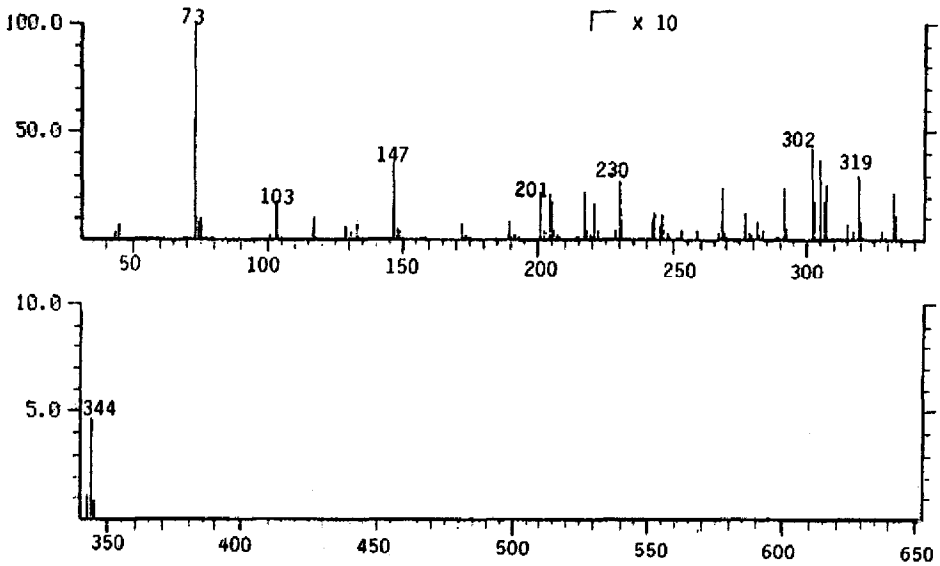

Fig. 3. 70-eV mass spectrum of D-glucose. GC–MS conditions as in Fig. 2.

(galactose, mannose and glucose), together with a diastereoisomeric internal standard (idose or gulose), were separately eluted from the CP-Sil-19CB column within 8 min. Temperature-programmed runs did not improve the separation of too closely eluting peaks.

Under the conditions used, less than 10 ng of glucose could be detected with flame ionization detection.

Mass spectrometry

The 70-eV EI mass spectrum of the glucose derivative is shown as an example in Fig. 3. The main features of the EI fragmentation parallel that of the corresponding MO-TMS derivatives, as reported by Laine and Sweeley[6,7], and their nomenclature for the fragment ions is employed here (Fig. 4 and Table II). Cleavages of the carbon skeleton α to each silyl-ether oxygen atom generate two series of even-electron oxonium ions, which can further eliminate trimethylsilanol, yielding the corresponding "prime"(') ions.

The difference in the behaviour of the two derivatives lies principally in the lower abundance of A' and B' fragments in the aldonitrile compounds. The smaller number of degrees of freedom (a cyano group *vs.* a methoxime) at C-1 can, of course, play a role in the more extensive fragmentation of these compounds in comparison with the MO-TMS derivatives. The derivatives of uronic acids yield ions that retain the silyl ester moiety, in addition to those related to the common part of the molecule. Other fragments, related to more complex cleavages of the silylated polyol chain, are also present (Fig. 5 and Table III).

In the spectrum of [6,6-$^2H_2$]glucose the 217/219 and 103/105 ion ratios are very close to that observed by Laine and Sweeley for the MO-TMS derivatives, thus confirming that a substantial non-specific hydrogen rearrangement takes place. This is also confirmed by the occurrence of the ion at *m/z* 103 in the derivative of the uronic acids. Spectra obtained at low electron energy (30–40 eV) show a prominent ion at *m/z* 201, which is probably generated from a weak precursor at *m/z* 291, by loss of

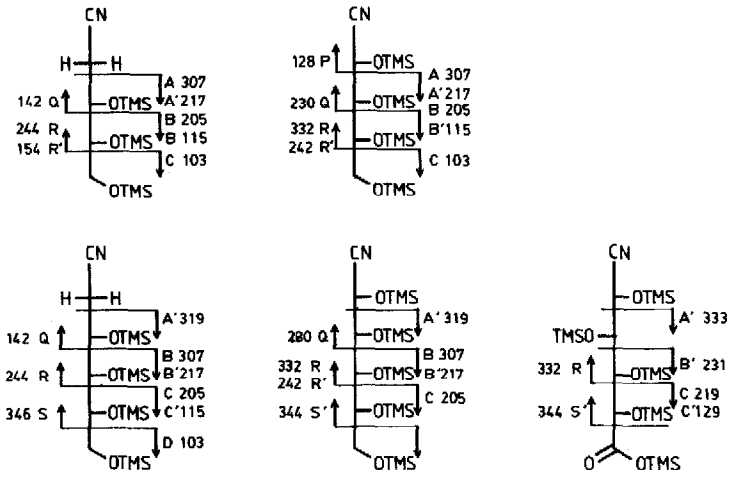

Fig. 4. Simple cleavage and silanol loss (primes) fragments in the spectra of silylaldonitrile derivatives.

TABLE II

FRAGMENT IONS FROM SIMPLE CHAIN FISSION AND SILANOL LOSS (PRIMES)

For the mass values of the fragments, see Fig. 4; the reported intensities are relative to *m/z* 73 = 100%.

Compound	*A*	*A'*	*B*	*B'*	*C*	*C'*	*D*	*Q*	*R*	*R'*	*S*	*S'*
1	–	5.72	13.30	–	6.91	–	–	6.11	4.20	–	–	–
2	1.62	11.57	17.62	–	15.76	–	–	0.67	1.20	0.76	–	–
3	1.70	12.88	14.64	–	16.11	–	–	0.84	1.22	0.66	–	–
4	2.40	11.60	10.82	–	19.51	–	–	0.65	0.93	–	–	–
5	3.42	15.11	13.23	1.01	19.63	–	–	0.78	0.55	1.01	–	–
6	–	–	2.16	13.91	17.26	–	15.29	5.75	4.18	–	2.70	–
7	–	3.98	2.53	24.54	16.29	–	16.82	3.12	1.05	–	–	7.29
8	–	5.71	2.59	21.16	18.66	–	16.83	3.12	1.61	–	–	6.61
9	–	–	1.23	10.50	11.24	–	16.14	1.79	–	0.42	–	4.26
10	–	6.84	1.42	14.34	16.47	–	13.87	3.18	0.56	0.69	–	1.63
11	–	3.84	3.55	23.32	15.98	–	18.04	2.83	3.07	–	–	6.00
12	–	4.14	1.48	13.31	15.62	–	15.34	2.61	0.61	0.67	–	4.43
13	–	2.99	1.71	11.50	11.45	–	14.23	2.76	0.51	0.46	–	4.67
14	–	2.99	2.63	21.31	17.52	–	17.23	2.70	2.19	–	–	4.67
15	–	7.15	–	0.69	2.47	2.94	–	–	2.65	–	–	0.88

trimethylsilanol. Such fragmentation is not shared by 2-deoxyaldoses, uronic acids and silylated polyols.

Applications

In order to explore the suitability of these derivatives to assist in the characterization of polysaccharides, lactose was hydrolysed under acidic conditions and the resulting sugar mixture was derivatized and subjected to GC analysis. Two peaks appeared in the chromatogram, corresponding to the expected galactose and glucose derivatives. A calibration graph ($r = 0.9989$) in the physiological range 50–350 mg per 100 ml was obtained for serum glucose, employing D-idose as the internal standard and flame ionization detection. This method could be suitable as a reference method

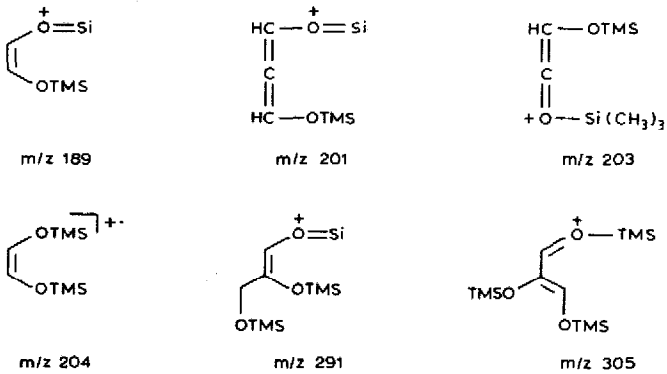

Fig. 5. Other important fragments in the spectra of silylaldonitriles.

TABLE III

OTHER IMPORTANT FRAGMENTS

For structures, see Fig. 5; intensity is relative to m/z 73 = 100%.

Compound	*Fragment ions (m/z)*					
	189	*201*	*203*	*204*	*291*	*305*
1	1.12	–	2.43	3.31	–	–
2	8.71	7.20	1.17	38.34	5.28	0.58
3	9.03	6.72	2.06	30.78	2.52	0.47
4	8.07	9.18	2.04	22.14	2.43	0.37
5	11.86	11.08	2.57	25.28	2.84	0.55
6	6.19	–	1.23	31.76	3.44	1.23
7	8.18	22.86	2.79	21.65	2.76	4.07
8	8.75	30.36	3.48	18.79	2.32	3.57
9	3.85	14.09	2.27	13.29	1.51	0.90
10	7.37	21.88	2.62	13.04	1.27	1.93
11	9.36	18.33	2.59	22.36	2.78	4.17
12	8.26	25.88	2.82	15.51	1.51	2.11
13	4.94	15.12	1.85	13.39	0.92	1.69
14	8.61	22.04	2.99	20.44	2.41	3.72
15	7.97	1.12	1.13	15.90	1.58	3.42

to assess the precision of glucose measurements in clinical chemistry. The use of an isomer rather than an isotopically labelled analogue and of simple capillary GC in the place of GC–MS would be highly cost effective, as pointed out by Kinter *et al.*[16] in a recent paper on serum cholesterol measurement.

CONCLUSIONS

Trimethylsilyl aldonitriles can be synthesized under mild conditions from aldoses carrying a variety of functional groups, and the derivatives of several sugars can be separated as single peaks in a short isothermal GC run. The mass spectra allow identification of the substituents on the carbon chain and contain ions suitable for IDMS quantification.

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