Journal of Chromatography, 303 (1984) 380–385
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,154

Note

Sensitive assay for amino sugars using capillary gas chromatography with nitrogen-selective detection

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(Received July 16th, 1984)

Several recent reports have described high-resolution gas[1–4] and liquid[5–7] chromatographic systems capable of separating complex mixtures of carbohydrates including amino sugars. Chromatographic assays for carbohydrates are limited by the lack of selectivity and sensitivity of the detectors most frequently used for gas chromatography (GC) and high-performance liquid chromatography (HPLC)[8]. The problem of low detector selectivity has been overcome by combining GC with mass spectrometry[9,10]. However the mass spectrometer is a high cost instrument not routinely available in most laboratories.

Commercially available nitrogen-selective detectors do not seem to have been widely exploited for amino sugar analysis. In this report the performance of the nitrogen–phosphorus-selective detector (NPD) is compared with that of the non-specific flame ionisation detector (FID) for the GC assay of amino sugars as their alditol peracetate derivatives. The enhanced selectivity and sensitivity of the nitrogen–phosphorus detector was exploited for analysis of the sugar composition of a fungal spore wall preparation.

EXPERIMENTAL

Materials

N-Acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-β-D-mannosamine · H_2O, D(+)-galactose and D(+)-mannose were purchased from Sigma (Poole, U.K.). D-Glucose was from Fisons Scientific apparatus, (Loughborough, U.K.). All chemicals were reagent grade and were used without further purification.

Derivatisation

Sugars were analysed by GC of their alditol peracetates[11,12]. A standard mixture containing 50 μmoles of each sugar was reduced by treatment with 50 ml of a freshly-prepared solution of sodium borohydride in 1 *M* ammonium hydroxide (2 mg ml^{-1}) for 1 h at room temperature. After neutralisation with glacial acetic acid, the mixture of alditols was evaporated from methanol–benzene (5:1, v/v) and dried *in vacuo* over phosphorus pentoxide. Dry samples were acetylated by treatment with 75 ml of acetic anhydride for 30 min at 100°C. Excess acetic anhydride was removed *in vacuo* and alditol acetates diluted in chloroform or ethyl acetate to produce a range

0021-9673/84/$03.00

of concentrations for GC analysis. Tris(hydroxymethyl)aminomethane (Tris) peracetate was added to samples before GC as an internal standard to allow correction for sample losses by injection and evaporation.

An acid hydrolysate (6 *M* hydrochloric acid) equivalent to 10 mg dry weight of the spore walls of the fungus *Plasmodiophora brassicae* was reduced with sodium borohydride and prepared for GC analysis as described above.

Gas chromatography

Alditol peracetates were separated on a 25 m × 0.2 mm I.D. BP-1 vitreous silica capillary column (bonded dimethylsilicone phase equivalent to OV-1 and SE-30, Scientific Glass Engineering, U.K.) in a Sigma 3B GC (Perkin-Elmer) and analysed both by nitrogen–phosphorus detection (NPD) and by flame ionisation detection (FID). The carrier gas was hydrogen at a flow-rate of 0.63 ml min^{-1} through the column. Injector and detector temperatures were maintained at 250 and 300°C respectively. Approximate gas flows for the detectors were: FID, air 500 and hydrogen 60 ml min^{-1}; NPD in the nitrogen–phosphorus mode, air 100 and hydrogen 3 ml min^{-1}. A make-up supply of 15 ml min^{-1} of oxygen-free nitrogen was supplied

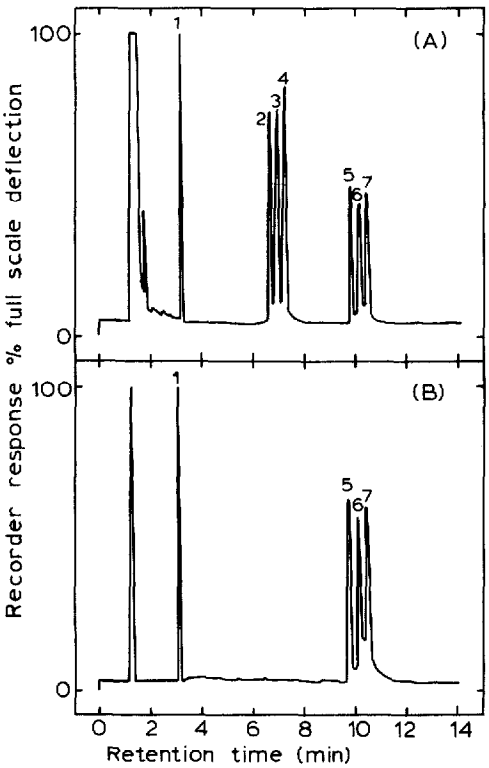

Fig. 1. Gas chromatography of alditol acetates of standard sugar mixture. (A) FID chromatogram of 250 pmol of 1 = Tris-acetate and 50 pmol (on-column) each of 2 = D-mannitol; 3 = D-glucitol; 4 = D-galactitol; 5 = D-glucosaminitol; 6 = D-mannosaminitol; 7 = D-galactosaminitol. Split ratio = 50:1. Attenuation 8 mV. (B) NPD chromatogram of 50 pmol Tris-acetate and 10 pmol (on-column) of each of the standards. Split ratio = 50:1. Attenuation 32 mV.

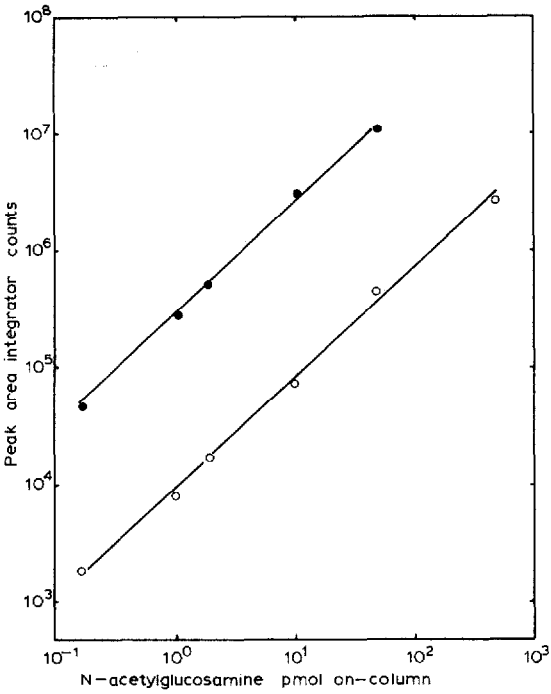

Fig. 2. Detector sensitivity to N-acetylglucosamine analysed as its alditol peracetate. Glucosaminitol peracetate was analysed by NPD (○—○) and FID (●—●).

to the detectors. Samples were analysed using a split ratio of 50:1 and a temperature programme of 200 to 215°C at 1°C min^{-1}. Peak areas were recorded with a Hewlett-Packard 3390A integrator.

RESULTS AND DISCUSSION

Chromatographic conditions for analysis of the standard sugar mixture were established using non-specific FID which responds to virtually all C–H-containing compounds. The six sugars as their alditol peracetates were well-separated on the BP-1 capillary column by temperature programming in 12 min (Fig. 1A). The separation obtained was similar to that of a conventional, OV-1 wall-coated capillary column[1] with the exception of glucitol and galactitol peracetates whose order of elution was reversed. This difference presumably reflects the slightly altered chromatographic behaviour of a bonded compared to a wall-coated stationary phase.

The standard sugar mixture was also analysed by NPD. Compared to FID, NPD gave a greatly enhanced response to each of the amino sugars tested but produced no detectable response to the non-nitrogen-containing hexoses (Fig. 1B). NPD therefore showed both increased sensitivity and selectivity for amino sugars compared to FID. The relative sensitivity of the detectors for N-acetylglucosamine is compared in Fig. 2. Based on a peak height of four times background the minimum detectable amounts of N-acetylglucosamine, measured as its peracetate, were 14 and

TABLE I

RELATIVE SENSITIVITY OF NPD AND FID TO AMINO AND NON-AMINO SUGARS

Sugar	*Molar response relative to glucose**	
	NPD	*FID*
N-Acetylglucosamine	3670	113
N-Acetylmannosamine**	3670	117
N-Acetylgalactosamine	4330	107
Glucose	10	170
Mannose	17	160
Galactose	10	157

* Assuming the molar response of NPD to glucose = 10.
** Corrected for 1 mole of water of crystallisation.

442 pg for NPD and FID respectively. Both detectors had a linear response range of at least 10^3; higher concentrations overloaded the capillary column and led to peak splitting. Quantitatively very similar results were obtained with the two other amino sugars tested. Overall, NPD increased sensitivity for amino sugar analysis approximately 30-fold compared to FID. The relative response of NPD and FID to non-nitrogen-containing sugars was tested by chromatographing increasing amounts of glucose, mannose and galactose (Table I). FID showed a comparable sensitivity both to hexoses and to hexosamines. In contrast, although it was possible with NPD to obtain distinct peaks corresponding to each of the hexoses, the response was very much less than with FID. NPD response exhibited a very high selectivity (approximately 300-fold) for hexosamines compared to the corresponding hexoses.

The performance of NPD was exploited for analysis of the amino sugar composition of spore walls of the fungus *Plasmodiophora brassicae*. An acid hydrolysate of the spore wall was analysed by NPD and FID (Fig. 3). Both chromatograms showed a peak which co-chromatographed with authentic glucosaminitol peracetate. NPD and FID techniques gave closely similar values of 323 and 319 μg glucosamine per mg dry weight respectively for the composition of the spore walls, in satisfactory agreement with previous results[13]. GC–NPD analysis, however, required injection of approximately 30 times less of the hydrolysate to produce a chromatogram quantitatively similar to FID. The sensitivity of NPD would theoretically allow reliable measurement of glucosamine concentration using as little as 50 ng of hydrolysed wall. The detection limit of the method might be lowered further by using a splitless injection technique[4] to allow a larger proportion of the sample onto the GC column.

NPD coupled to capillary column GC provides a sensitive and selective assay for amino sugars particularly useful where only limited amounts of material are available for analysis. The technique is more sensitive than existing GC[1–4,14] and HPLC[5–7] methods and could be applied directly, with the same degree of sensitivity, to the analysis of other nitrogen-containing sugar derivatives *e.g.* sialic and muramic acids. Preparation of nitrogen-containing derivatives such as O-methyloxime[15] or aldononitrile acetates[16,17] would extend the use of GC–NPD for the analysis of non-nitrogen-containing sugars.

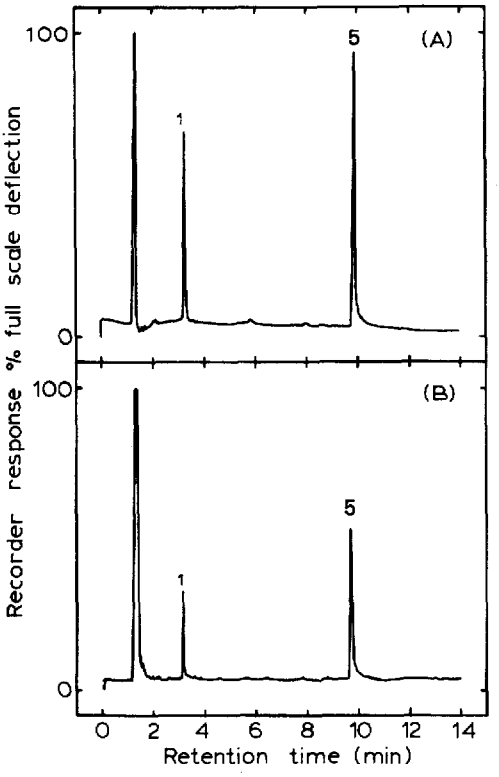

Fig. 3. Gas chromatography of sugars from hydrolysed spore walls of *Plasmodiophora brassicae* analysed as alditol acetates. (A) NPD chromatogram of 12.5 ng (on-column) hydrolysed wall fraction containing 3.125 pmol Tris-acetate. Split ratio = 50:1. Attenuation 64 mV. Peaks as in Fig. 1. (B) FID chromatogram of 200 ng (on-column) wall containing 50 pmol Tris. Split and attenuation as in A. Peaks as in Fig. 1.

ACKNOWLEDGEMENTS

I thank Dr. R. S. S. Fraser for helpful discussions and S. E. Moxham for samples of hydrolysed *Plasmodiophora* spore walls.

REFERENCES

1 R. Oshima, J. Kumanotani and C. Watanabe, *J. Chromatogr.*, 250 (1982) 90.
2 R. Oshima, A. Yoshikawa and J. Kumanotani, *J. Chromatogr.*, 213 (1981) 142.
3 A. G. W. Bradbury, D. J. Halliday and D. G. Medcalf, *J. Chromatogr.*, 213 (1981) 146.
4 R. E. Hicks and S. Y. Newell, *Anal. Biochem.*, 128 (1983) 438.
5 J. Golik, H-W. Liu, M. Dinovi, J. Furukawa and K. Nakanishi, *Carbohyd. Res.*, 118 (1983) 135.
6 A. Hjerpe, C. A. Antonopoulos, B. Classon and B. Engfeldt, *J. Chromatogr.*, 202 (1980) 453.
7 S. Honda, T. Konishi, S. Suzuki, M. Takahashi, K. Kakehi and S. Ganno, *Anal. Biochem.*, 134 (1983) 483.
8 G. D. McGinnis and P. Fang, *Methods Carbohyd. Chem.*, 8 (1980) 33.
9 I. Funakoshi and I. Yamashina, *Anal. Biochem.*, 107 (1980) 265.
10 B. Fournet, G. Strecker, Y. Leroy and J. Montreuil, *Anal. Biochem.*, 116 (1981) 489.
11 J. Sawardeker, J. Sloneker and A. Jeanes, *Anal. Chem.*, 37 (1965) 1602.

12 L. A. Torello, A. J. Yates and D. K. Thompson, *J. Chromatogr.*, 202 (1980) 195.
13 S. E. Moxham and S. T. Buczacki, *Trans. Br. Mycol. Soc.*, 80 (1983) 297.
14 M. Tomana, W. Niedermeier and C. Spivey, *Anal. Biochem.*, 89 (1978) 110.
15 T. P. MaWhinney, M. S. Feather, G. J. Barbero and J. R. Martinez, *Anal. Biochem.*, 101 (1980) 112.
16 B. A. Dmitriev, L. V. Buckinowsky, O. S. Chizhov, B. M. Zolotarev and N. K. Kochetkou, *Carbohyd. Res.*, 19 (1971) 432.
17 R. Varma, R. S. Varma, W. S. Allen and A. H. Wardi, *J. Chromatogr.*, 86 (1973) 205.