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DETECTION OF NEUTRAL AND AMINOSUGARS FROM GLYCOPROTEINS AND POLYSACCHARIDES AS THEIR ALDITOL ACETATES

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SUMMARY

A new method for the preparation and separation of alditol acetates from neutral sugars has been applied to aminosugars. Reduced aminosugars were rapidly acetylated using 1-methylimidazole as the catalyst without removal of borate formed during reduction. The alditol acetates were separated by glass capillary gas chromatography on Silar 10C. The alditol acetates of aminosugars had retention times much longer than those of neutral sugars. However, the alditol acetates of the deamination products of aminosugars had shorter retention times and were resolved from those of neutral sugars. This method was used for the simultaneous detection of neutral and aminosugars in acid hydrolysates of chitin and the glycoproteins, ovalbumin and peroxidase.

INTRODUCTION

Aminosugars occur widely in nature. For example, they are found in chitin, many plant and animal glycoproteins, proteoglycans and antibiotics[1]. Monosaccharides released by acid or enzymic hydrolysis of these compounds are often determined by gas chromatography (GC) of their alditol acetates[2]. However, alditol acetates of aminosugars have long retention times and have been reported to be thermally unstable[3]. The alditol acetates of deaminated aminosugars[4,5] have shorter retention times and are thus more suitable for chromatography with alditol acetates derived from neutral sugars[6].

Methods for preparing alditol acetates have involved long acetylation times at high temperatures following the tedious removal of borate formed during reduction with sodium borohydride[2]. Recently, Blakeney *et al.*[7] reported a simple and rapid procedure for the formation of alditol acetates in which removal of borate is unnecessary. The alditol acetates are separated by glass capillary GC on Silar 10C. This polar phase was found to resolve completely complex mixtures of alditol acetates[7]

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and has been reported to have good thermal stability[8]. We now report the application of this method to the analysis of aminosugars.

EXPERIMENTAL

Gas chromatography

Alditol acetates were separated on a 28.5 m × 0.5 mm I.D. Silar 10C support-coated open tubular (SCOT) glass capillary column (SGE, Melbourne, Australia) in a Hewlett-Packard 5710A chromatograph equipped with a flame ionization detector and a SGE Unijector capillary injection system, used in the split mode. High-purity hydrogen was used as the carrier gas at a flow-rate of 81 cm/sec (determined using dichloromethane). Two temperature programmes were used: (a) 230°C for 4 min, followed by a 4°C/min rise to 250°C, for the analysis of the alditol acetates of aminosugars and (b) 190°C for 4 min, followed by a 4°C/min rise to 230°C, where high resolution of alditol acetates of neutral sugars and deaminated aminosugars was required. The injection port and detector were heated to 250°C and 300°C respectively. Peak areas and retention times were recorded using a Hewlett-Packard Model 3380A reporting integrator.

Reagents

Dichloromethane (Cat. No. 6049), dimethyl sulphoxide (DMSO) (Cat. No. 802912) (stored over molecular sieve type 4A) and sodium borohydride (Cat. No. 806373) were obtained from E. Merck (Darmstadt, G.F.R.). 1-Methylimidazole was obtained from Fluka (Buchs, Switzerland) and stored over molecular sieve type 4A.

Sugars were obtained from the following sources: L-arabinose, erythritol and myo-inositol (BDH, Poole, Great Britain); 2-deoxy-D-ribose (Koch-Light, Colnbrook, Great Britain); D-mannose (Pfanstiehl Labs., Waukegan, IL, U.S.A.); D-allose, 2-deoxy-D-glucose, L-fucose, D-galactose, D-glucose, L-rhamnose, D-ribose, D-xylose, D-galactosamine, D-glucosamine, D-mannosamine, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and N-acetyl-D-mannosamine (Sigma, St. Louis, MO, U.S.A.). Sugars were stored over silica gel.

Inositol hexaacetate (m.p. 217°C) was prepared from inositol as follows. Inositol (5 g) was suspended in 1-methylimidazole (5 ml) and acetic anhydride (50 ml) added slowly with mixing. After 15 min, water (200 ml) was added to destroy the excess acetic anhydride. The acetylated inositol was extracted into dichloromethane (200 ml), and the extract decolourised with carbon (5 g), filtered and evaporated to dryness (80°C). The residue was dissolved in acetone (200 ml) and precipitated by the addition of cold water (800 ml). The reprecipitated acetate was dried, dissolved in dichloromethane and crystallized under vacuum. Xylitol pentaacetate (m.p. 62°C) was prepared by the method of Abdel-Akher *et al.*[9].

Ovalbumin was obtained from BDH and horseradish peroxidase (Type II) from Sigma.

Chitin, isolated from *Sepia* sp. using EDTA and proteolytic enzymes[10], was the kind gift of Mr. M. V. Tracey, C.S.I.R.O., Institute of Biological Resources, Canberra, A.C.T., Australia.

All other reagents were of analytical grade.

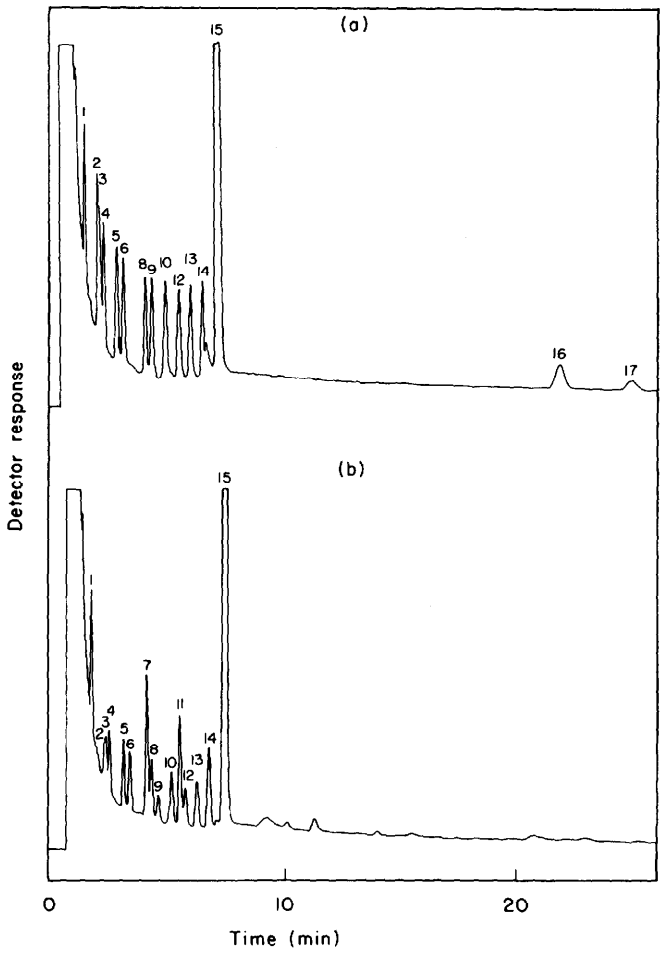

Fig. 1. Gas chromatography of alditol acetates of aminosugars and neutral sugars on Silar 10C glass capillary column using temperature program a: 230°C for 4 min followed by 4°C/min rise to 250°C. (a) Before deamination; (b) following nitrous acid deamination of aminosugars. Peaks: 1 = erythrose; 2 = 2-deoxyribose; 3 = rhamnose; 4 = fucose; 5 = ribose; 6 = arabinose; 7 = 2,5-anhydromannose (produced by deamination of glucosamine); 8 = xylose; 9 = 2-deoxyglucose; 10 = allose; 11 = 2,5-anhydrotalose (produced by deamination of galactosamine); 12 = mannose; 13 = galactose; 14 = glucose; 15 = inositol; 16 = glucosamine; 17 = galactosamine.

Hydrolysis of glycoproteins and chitin

Ovalbumin and peroxidase were hydrolysed with 4 *M* hydrochloric acid at 100°C for 4 h under argon[11] and chitin with 6 *M* hydrochloric acid at 100°C for 14 h under argon[12].

Deamination of aminosugars

Aminosugars were deaminated with nitrous acid as follows: an aqueous sample (0.1 ml) containing up to 2 mg of aminosugar was added to solid sodium nitrite (100 mg), the solution placed on ice and 9 *M* sulphuric acid (0.1 ml) added.

After 5 min the reaction mixture was allowed to warm to room temperature. After a further 25 min the reaction mixture was made alkaline with 15 *M* ammonia solution (0.2 ml) and then reduced and acetylated.

Reduction and acetylation of sugars

Sugars were reduced and acetylated by the method of Blakeney *et al.*[7]. Sodium borohydride (2 g) was dissolved in anhydrous DMSO (100 ml) at 100°C. Routinely, sugars were reduced at 40°C for 90 min by adding 1 ml of sodium borohydride solution to 0.1 ml of the sample in 1 *M* ammonia. After reduction, excess sodium borohydride was destroyed by the addition of glacial acetic acid (0.1 ml). 1-Methylimidazole (0.2 ml), followed by acetic anhydride (2 ml) were then added and the solution mixed. After 10 min at room temperature, water (5 ml) was added to destroy excess acetic anhydride. When cool, dichloromethane (1 ml) was added and mixed on a vortex mixer. After the phases had separated, the lower phase was removed with a Pasteur pipette and 2 μl of this phase analysed by GC.

TABLE I

RETENTION TIMES OF THE ALDITOL ACETATES OF NEUTRAL AND AMINOSUGARS ON A SILAR 10C GLASS CAPILLARY COLUMN

Temperature programmes: (a) 230°C for 4 min, 4°C/min to 250°C; (b) 190°C for 4 min, 4°C/min to 230°C.

Sugar	*Temperature program a*		*Temperature program b*	
	Retention time (min)	*Relative retention time (inositol = 1.00)*	*Retention time (min)*	*Relative retention time (inositol = 1.00)*
Erythritol	1.63	0.22	4.67	0.28
2-Deoxyribose	2.18	0.29	6.60	0.39
Rhamnose	2.20	0.29	6.86	0.41
Fucose	2.42	0.32	7.22	0.43
Ribose	3.00	0.40	8.87	0.53
Arabinose	3.28	0.44	9.30	0.56
2,5-Anhydromannose*	4.06	0.54	10.58	0.63
Xylose	4.23	0.57	11.07	0.66
2-Deoxyglucose	4.52	0.61	11.45	0.68
Allose	5.11	0.68	12.50	0.75
2,5-Anhydrotalose**	5.50	0.74	12.69	0.76
Mannose	5.70	0.76	13.23	0.79
Galactose	6.20	0.83	14.03	0.84
Glucose***	6.73	0.90	15.11	0.90
Inositol	7.46	1.00	16.74	1.00
Glucosamine§	22.20	2.98	–§§	
Mannosamine§	24.63	3.30	–§§	
Galactosamine§	25.28	3.39	–§§	

* Major product of nitrous acid deamination of glucosamine.

** Major product of nitrous acid deamination of galactosamine.

*** Major product of nitrous acid deamination of mannosamine.

§ N-Acetylaminohexoses gave identical derivatives.

§§ Retention times of the alditol acetates of aminohexoses were very long with this temperature program and could not be determined.

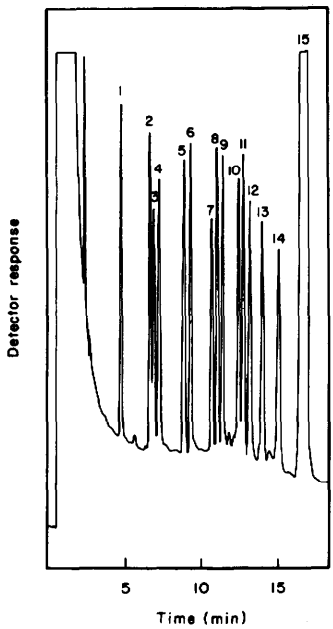

Fig. 2. Gas chromatography of alditol acetates of deaminated aminosugars and neutral sugars on Silar 10C glass capillary column. Temperature program b: 190°C for 4 min followed by a 4°C/min rise to 230°C. Peaks as in Fig. 1.

Reduction and acetylation of aminosugars and the corresponding N-acetyl-aminosugars resulted in identical derivatives.

RESULTS AND DISCUSSION

Gas chromatography of aminosugars

The alditol acetates of glucosamine, galactosamine and mannosamine had long retention times relative to those of neutral sugars when chromatographed on a Silar 10C glass capillary column, even when high column temperatures were used (program a) (Table I and Fig. 1). These temperatures did not allow optimum resolution of the alditol acetates of neutral sugars. On a less polar phase, SP 2100, the retention times of the alditol acetates of aminosugars were shorter, but resolution of the alditol acetates of aminosugars and neutral sugars was poor. The alditol acetates of galactosamine and mannosamine were incompletely resolved on Silar 10C. Niedermeier and Tomana[13] have successfully resolved the alditol acetates of glucosamine, galactosamine and mannosamine on Poly A-103 and Green *et al.*[14] separated them on a glass capillary column coated with a chiral polysiloxane phase. However, separation of the alditol acetates of neutral sugars on these phases was inferior to that obtained on Silar 10C.

Recently, Oshima *et al.*[15] used fused-silica wall-coated open tubular (WCOT) capillary columns coated with OV-1, SE-54 and Carbowax 20M to separate the alditol acetates of neutral and aminosugars. The best resolution was obtained on Carbowax 20M, which gave baseline separation of the alditol acetates of aminosugars

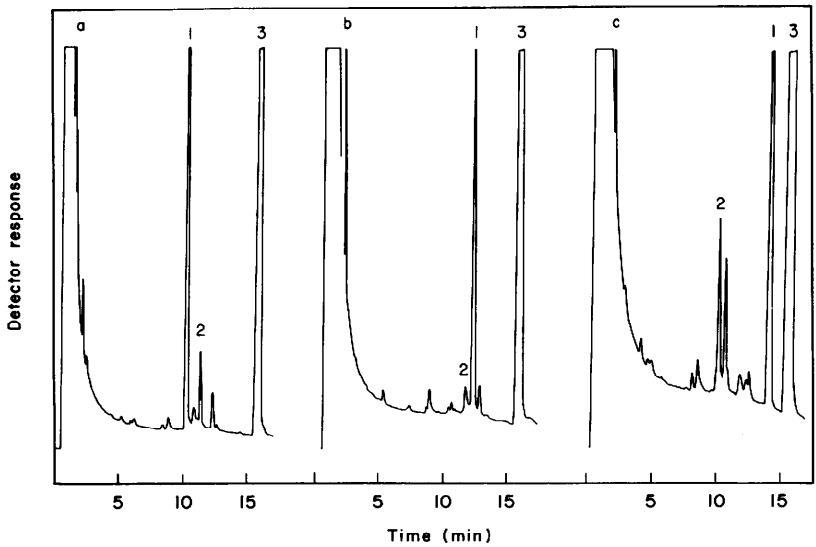

Fig. 3. Gas chromatography of alditol acetates of deaminated aminosugars on a Silar 10C glass capillary column. Temperature program b. (a) Glucosamine; (b) galactosamine; (c) mannosamine. Peaks: 1 = major product of deamination; 2 = second greatest product of deamination; 3 = inositol hexaacetate (internal standard).

and all neutral sugars tested except glucose and galactose. Carbowax 20M is less polar than Silar 10C which gives excellent separation of the alditol acetates of neutral sugars, but comparatively poor separation of the alditol acetates of aminosugars. The retention times of the alditol acetates of aminosugars appear to increase with increasing column polarity[15].

Determination of aminosugars

Nitrous acid deamination of aminosugars produced products, the alditol acetates of which were resolved on Silar 10C using a temperature program that also gave good resolution of the alditol acetates of neutral sugars (Fig. 2). Deamination of glucosamine, galactosamine and mannosamine gave 2,5-anhydromannose, 2,5-anhydrotalose and glucose, respectively, as the major products, although a number of other minor products were formed (Fig. 3)[5,16]. The alditol acetates of 2,5-anhydrotalose and 2,5-anhydromannose were resolved from those of the neutral sugars on Silar 10C (Fig. 2). Their retention times are given in Table I. 2,5-Anhydromannose, 2,5-anhydrotalose and glucose represented 83%, 77% and 57% of the total deamination products from glucosamine, galactosamine and mannosamine respectively (Fig. 3). Glucosamine was 96% deaminated by this procedure as determined by comparison of peak areas. However, as previously reported[16], N-acetylhexosamines were not deaminated.

The effects of deamination on sugar mixtures are shown in Fig. 1. The use of deamination in the quantitative analysis of aminosugars would require careful control of deamination conditions because of the formation of more than one deamination product for each aminosugar. Although deamination causes some selective losses of neutral sugars, with adequate controls deamination could provide a simple

method for the detection and identification of aminosugars.

The alditol acetates of xylose and 2,5-anhydromannose have not been resolved in other chromatographic systems[6,17], and because of this other sugar derivatives, such as aldononitriles, have been investigated[2]. However, the aldononitriles have relatively long retention times and the derivatives of xylose and 2,5-anhydromannose are still poorly resolved. Furthermore, the solubility of these derivatives in water makes their preparation for chromatography difficult[18,19]. The use of other derivatives, such as trimethylsilyl ethers, in which the anomeric centre has not been eliminated, leads to multiple peaks for each sugar[20]. The chromatographic system used in the present study allows the simultaneous separation of the alditol acetates of neutral sugars and deaminated aminosugars.

Analysis of chitin

On GC of a reduced acetylated hydrolysate of chitin, no peaks with retention times less than the internal standard, inositol hexaacetate, were observed (Fig. 4a). After deamination, one major peak with a retention time coincident with the alditol acetate of 2,5-anhydromannose was found (Fig. 4b). Since the rate constants for de-acetylation of N-acetylaminosugars are of a similar order of magnitude to those for the hydrolysis of glucosidic linkages[6], the N-acetylglucosaminyl residues of chitin would be de-acetylated and glucosamine would be expected as the final product in an acid hydrolysate of chitin. However, enzymic hydrolysis could release N-acetylglucosamine. The alditol acetate of N-acetylglucosamine can be detected directly using a high temperature program (program a). Alternatively, the N-acetylglucosamine can be de-acetylated to allow deamination.

Analysis of neutral and aminosugars from glycoproteins

The method was applied to the analysis of neutral sugars and aminosugars in acid hydrolysates of two glycoproteins, ovalbumin and horseradish peroxidase. The hydrolysate of ovalbumin contained mannose, galactose and glucosamine (Fig. 4c and d). Mannose, N-acetylglucosamine and galactose have been reported as constituents of ovalbumin[11]. Horseradish peroxidase contained fucose, arabinose, xylose, mannose, galactose, glucose and glucosamine (Fig. 4e and f) as previously reported[21]. We found only small amounts of xylose in peroxidase, but detected an unidentified component with a retention time greater than that of xylitol pentaacetate. Cochromatography with authentic xylitol pentaacetate confirmed that this unidentified component was not xylose and was probably an artifact. This component was found in the ovalbumin hydrolysate (Fig. 4d).

The carbohydrate components represent only about 3% of ovalbumin[11]. Despite the presence of a large excess of amino acids in the ovalbumin hydrolysate that would be rapidly acetylated in the procedure used[22], acetylation of alditols apparently was not affected, nor was the chromatography. The method of Blakeney *et al.*[7] involves extraction of the alditol acetates from an aqueous solution with dichloromethane. N-acetylated amino acids would remain in the water phase when using this procedure. The substantial partitioning of acetylated amino acids into the water phase was confirmed experimentally using casein hydrolysate, glycine, hydroxyproline, lysine and tryptophan.

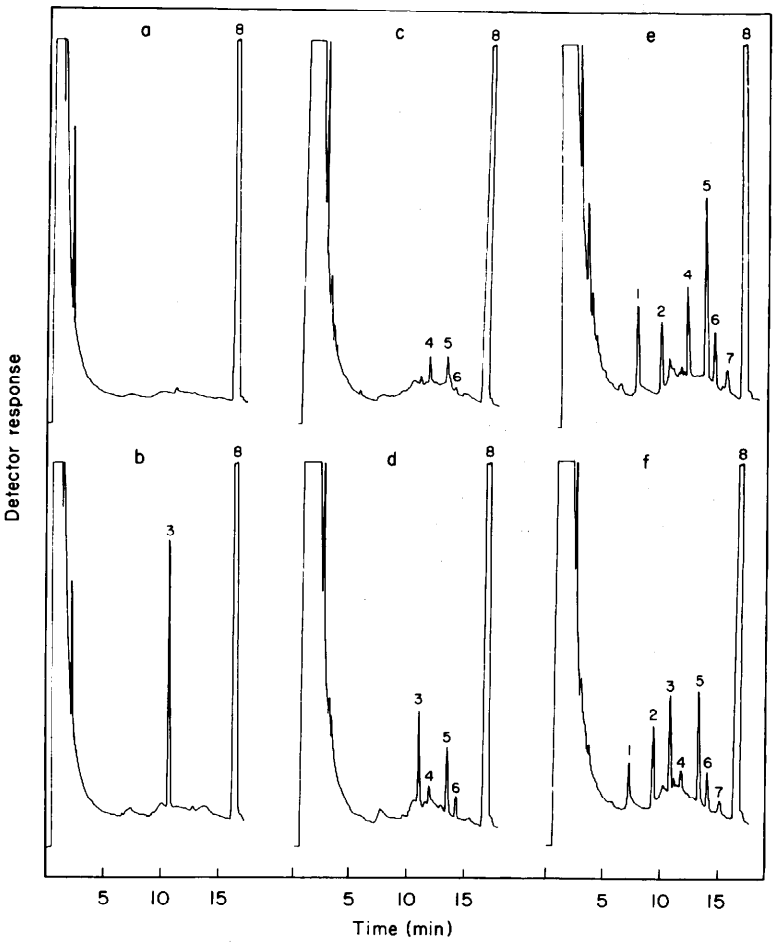

Fig. 4. Gas chromatography of alditol acetates produced from (a) hydrolysate of chitin, (b) deaminated hydrolysate of chitin, (c) hydrolysate of ovalbumin, (d) deaminated hydrolysate of ovalbumin, (e) hydrolysate of peroxidase, (f) deaminated hydrolysate of peroxidase on a Silar 10C glass capillary column. Temperature program b. (a), (c) and (e) Before deamination; (b), (d) and (f) following nitrous acid deamination of aminosugars. Peaks: 1 = fucose; 2 = arabinose; 3 = 2,5-anhydromannose (produced by deamination of glucosamine); 4 = unknown; 5 = mannose; 6 = galactose; 7 = glucose; 8 = inositol (internal standard).

CONCLUSION

The rapid method for the reduction and acetylation of neutral sugars described by Blakeney *et al.*[7] has been successfully applied in the separation of aminosugars and their deamination products. Major advantages of this method are that it can be performed in a single tube without transfers or evaporations, acetylation is complete in 10 min at room temperature and the removal of borate, formed during reduction with sodium borohydride, is unnecessary. GC on a Silar 10C glass capillary column separates the alditol acetates of neutral sugars and deaminated aminosugars. Amino acids did not interfere and thus the method is suitable for the separation of the

carbohydrate components of glycoproteins without the requirement of a deionisation step after hydrolysis.

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REFERENCES

1 D. Horton and J. D. Wander, in W. Pigman and D. Horton (Editors), *The Carbohydrates*, Vol. 1B, Academic Press, New York, 2nd ed., 1980, p. 643.
2 G. G. S. Dutton, *Carbohydr. Chem. Biochem.*, 28 (1973) 11.
3 L. J. Griggs, A. Post, E. R. White, J. A. Finkelstein, W. E. Moeckel, K. G. Holden, J. A. Zarembo and J. A. Weisback, *Anal. Biochem.*, 43 (1971) 369.
4 F. Shafizadeh, in M. L. Wolfrom (Editor), *Advances in Carbohydrate Research*, Vol. 13, Academie Press, New York, 1958, p. 9.
5 J. Defaye, *Advan. Carbohydr. Chem. Biochem.*, 25 (1970) 181.
6 W. H. Porter, *Anal. Biochem.*, 63 (1975) 27.
7 A. B. Blakeney, P. J. Harris, R. J. Henry and B. A. Stone, *Carbohydr. Res.*, 113 (1983) in press.
8 S. Hirase, K. Watanabe and R. Takano, *Agr. Biol. Chem.*, 42 (1978) 1065.
9 M. Abdel-Akher, J. K. Hamilton and F. Smith, *J. Amer. Chem. Soc.*, 73 (1951) 4691.
10 M. V. Tracey, *Rev. Pure. Appl. Chem.*, 7 (1957) 1.
11 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins* (*B.B.A. Library*, Vol. 5), Elsevier, Amsterdam, 1972, p. 732.
12 S. Bartnicki-Garcia and W. J. Nickerson, *Biochim. Biophys. Acta*, 58 (1962) 102.
13 W. Niedermeier and M. Tomana, *Anal. Biochem.*, 57 (1974) 363.
14 C. Green, V. M. Doctor, G. Holzer and J. Oró, *J. Chromatogr.*, 207 (1981) 268.
15 R. Oshima, J. Kumanotani and C. Watanabe, *J. Chromatogr.*, 250 (1982) 90.
16 J. E. Shively and H. E. Conrad, *Biochemistry*, 9 (1970) 33.
17 T. Anastassiades, R. Puzic and O. Puzic, *J. Chromatogr.*, 225 (1981) 309.
18 R. Varma and R. S. Varma, *J. Chromatogr.*, 128 (1976) 45.
19 T. P. Mawhimney, M. S. Feather, G. J. Barbero and J. R. Martinez, *Anal. Biochem.*, 101 (1980) 112.
20 W. S. Rickert and P. A. McBride-Warren, *Biochem. Biophys. Acta*, 336 (1974) 437.
21 J. Clarke and L. M. Shannon, *Biochim. Biophys. Acta*, 427 (1976) 428.
22 K. A. Connors and N. K. Pandit, *Anal. Chem.*, 50 (1978) 1542.