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GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF HYDROXYETHYL-STARCH

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SUMMARY

A gas–liquid chromatographic (GLC) method for the analysis of hydroxyethyl-starch (HES) is described. This method introduces a Schiff-base formation step to eliminate different anomeric forms of reducing sugars in HES, resulting in only one GLC peak for each monosaccharide constituent. Using this GLC method, the major constituents in HES can be separated and reproducibly quantitated using a conventional column under isothermal conditions in less than 20 min. The distribution of sugar constituents in HES at three different molar substitutions was determined and the major monosubstituted glucose was 2-O-hydroxyethylglucose, followed by 6-O-hydroxyethylglucose and 3-O-hydroxyethylglucose. The synthesis of 2-O-(2′-hydroxyethoxy)ethylglucose is described.

INTRODUCTION

Hydroxyethyl-starch (HES) is a macromolecular polysaccharide prepared by reacting naturally occurring amylopectin with ethylene oxide under alkaline conditions. HES has valuable medical applications as a plasma volume expander[1,2], leukapheresis agent[3,4] and cryopreservative[5].

Gas–liquid chromatography (GLC) of HES requires acid hydrolysis of the polysaccharide into its monosaccharide constituents, followed by a derivatization step forming volatile derivatives, and then chromatography.

Several GLC methods have been used to analyse reducing sugars[6–8]. These methods, however, suffer from poor quantitative capability as different anomeric forms of reducing sugars exist, which result in multiple peaks for each sugar. Chromatography of a complex mixture such as acid-hydrolysed HES, which contains many reducing sugars, results in many overlapping peaks, making accurate quantitation of each sugar extremely difficult[9,10]. In addition, many of the literature methods require capillary GLC columns and temperature programming with run times typically between 30 and 60 min per sample[11–13].

Formation of the oxime derivatives of reducing sugars as an approach to

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reduce the number of GLC peaks was first examined by Sweeley *et al.*[6] and subsequently applied successfully to the analysis of monosaccharides[14], disaccharides[14,15] and polysaccharides[16,17].

This paper describes a GLC method that incorporates the oxime formation step in the procedure. The method greatly simplifies the GLC analysis of HES using only a conventional glass column under isothermal conditions.

EXPERIMENTAL

Chemicals

Amberlite IR-45 CP, Amberlite IR-120, sulphuric acid and glucose were purchased from Mallinckrodt (St. Louis, MO, U.S.A.), pyridine and hydroxylamine hydrochloride from Eastman-Kodak (Rochester, NY, U.S.A.), dulcitol from Sigma (St. Louis, MO, U.S.A.) and bis(trimethylsilyl)trifluoroacetamide, acetyl chloride, dihydropyran, lithium aluminium hydride ($LiAlH_4$), *n*-butyllithium, lithium hydride and methyl bromoacetate from Aldrich (Milwaukee, WI, U.S.A.). Carbon tetrachloride, hexane, tetrahydrofuran (THF), silica gel, diethyl ether, magnesium sulphate and ethyl acetate were purchased from American Scientific Products (McGaw Park, IL, U.S.A.). Hydroxyethyl-starch (molar substitution = 0.26, 0.48, 0.70) and [^{14}C]hydroxyethyl starch (molar substitution = 0.70) were obtained from McGaw Laboratories (Irvine, CA, U.S.A.). 2-O-Hydroxyethylglucose[18,19], 3-O-hydroxyethylglucose[18] and 6-O-hydroxyethylglucose[18] were synthesized according to literature methods.

Synthesis of 2-O-(2′-hydroxyethoxy)ethylglucose

*Methyl 2-O-acetyl-4,6-O-benzylidene-α-*D*-glucopyranoside.* This compound was prepared by a modified literature method using a 1.5 molar excess of acetyl chloride[18]. Two recrystallizations from carbon tetrachloride–hexane (2:1) yielded 42% of the title compound, m.p. 132–133°C (lit.[18] m.p. 129–131°C).

*Methyl 3-O-(tetrahydropyran-2-yl)-4,6-O-benzylidene-α-*D*-glucopyranoside.* The compound prepared from the above experiment was reacted with excess of dihydropyran in dry THF for 2.5 h at 25°C. The reaction mixture was then added slowly to a slurry of $LiAlH_4$ in THF. After stirring for 30 min, the resulting mixture was treated with water and 15% sodium hydroxide solution. Filtration and evaporation of the filtrate and recrystallization of the residue from carbon tetrachloride–hexane (1:1) afforded the product (53%), m.p. 142–143°C (lit.[18], m.p. 135–142°C). A second crop (30%), which melted at 132–138°C, was obtained from the mother liquor.

*Methyl 2-O-(2′-hydroxyethyl)-3-O-(tetrahydropyran-2-yl)-4,6-O-benzylidene-α-*D*-glucopyranoside.* The synthesis was essentially the same as reported[18], except that excess of *n*-butyllithium (1.6 *M* in hexane) was used instead of sodium hydride. The crude alkoxyacetate was reduced with $LiAlH_4$ to the hydroxyethyl derivative and the product was chromatographed over silica gel/ether to give an oil (63.3%); thin-layer chromatography (TLC) (silica gel/ether), $R_F = 0.15$.

*Methyl 2-O-(carbomethoxymethoxy)ethyl-3-O-(tetrahydropyran-2-yl)-4,6-O-benzylidene-α-*D*-glucopyranoside.* To a cold solution of methyl 2-O-(2′-hydroxyethyl)-3-O-(tetrahydropyran-2-yl)-4,6-O-benzylidene-α-D-glucopyranoside (13.5 g, 32.9 mmol) in THF (130 ml) was slowly added dropwise *n*-butyllithium in hexane (1.6 *M*,

41 ml) at −50°C. After stirring for 10 min, a solution of methyl bromoacetate (910 g, 65.8 mmol) in THF (40 ml) was slowly added. Stirring was continued for another h at −50°C and the mixture was warmed to room temperature overnight. The solvent was evaporated and the residue was dissolved in ether, washed several times with water, dried over magnesium sulphate and evaporated to a dark oil. This oil was chromatographed on silica gel using ethyl acetate–hexane (2:1) as eluent to give 6.0 g (37.8%) of oil; TLC (silica gel/2:1 ethyl acetate–hexane), R_F = 0.4; NMR and IR data were consistent with the assigned structure. Elemental analysis; calculated, C 59.74, H 7.10%; found, C 59.78, H 7.19%.

*Methyl 2-O-(2'-hydroxyethoxy)ethyl-3-O-(tetrahydropyran-2-yl)-4,6-O-benzylidene-α-*D*-glucopyranoside.* To a stirred suspension of $LiAlH_4$ (0.45 g, 12 mmol) in THF (50 ml) was added dropwise a solution of the compound from the previous experiment (4.8 g, 10 mmol) in THF (50 ml). The resulting mixture was stirred for 30 min, treated with water (0.45 ml), sodium hydroxide solution (15%, 0.45 ml), and again with water (1.35 ml). After stirring for 10 min, the mixture was filtered and the filtrate was evaporated to 4.4 g of oil (97.6%). NMR and IR data were consistent with the assigned structure. Elemental analysis: calculated, C 60.78, H 7.54%; found, C 61.27, H 7.83%.

2-O-(2'-Hydroxyethoxy)ethylglucose. A mixture of the compound from the previous experiment (4.4 g, 9.7 mmol), pre-washed Amberlite IR-120 (15 g) and water (50 ml) was heated at 90°C for 18 h. After the reaction mixture had cooled, pre-washed Amberlite IR-45 was added until the pH was about 7. The mixture was filtered and the filtrate washed several times with ether and evaporated to an oil. The syrup was stored under vacuum for several days to remove traces of water until the weight remained constant. NMR and IR data were consistent with the assigned structure. Elemental analysis for the monohydrated product, $C_{10}H_{20}O_8 \cdot H_2O$: calculated, C 41.95, H 7.75%; found, C 41.50, H 7.53%.

Method of sample preparation

A 1-ml volume of a 1% solution of HES was transferred into a test-tube containing 10 μg of dulcitol (internal standard) and 0.5 ml of 2.0 *M* sulfuric acid. The sample was placed into a 90°C oil-bath for 3.0 h. A 1-ml volume of the acid hydrolyzate was added on top of a 3-ml disposable syringe filled with Amberlite IR-45 CP. The volume eluted after application of the sample was discarded. The sugars were eluted from the column by the addition of three 1-ml volumes of distilled water. The column eluates were pooled and placed in a Virtis Model 10-030 freeze-dryer (Gardiner, NY, U.S.A.) until the sample was completely dry. The sugar residues were dissolved in 1.0 ml of 1% hydroxylamine hydrochloride solution in dry pyridine and heated at 60°C for 30 min. Then 0.5 ml of bis(trimethylsilyl)trifluoroacetamide was added and the sample was heated at 60°C for 30 min.

Gas–liquid chromatography

The samples were analyzed using a Packard Model 428 gas chromatograph equipped with a flame-ionization detector. The 2 m × 4 mm I.D. glass column was packed with 3% SP-2250 on 80–100-mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The column, injector and detector temperatures were 220, 245 and 300°C, respectively. For analysis of trisubstituted hydroxyethylglucoses, the column temperature was increased to 230°C. The carrier gas flow-rate was 35 ml/min.

Data analyses

Identification of the individual peaks was accomplished by referring to the retention time (R_t) of authentic standards and/or mass spectral analyses[20]. Quantitation of the relative amounts of each sugar was accomplished using a Hewlett-Packard Model 3354 Automated Laboratory System (Rolling Meadows, IL, U.S.A.), which determined the ratio of the area of the peak of interest to that of the internal standard.

RESULTS

After reacting glucose, 2-O-hydroxyethylglucose (2-HEG), 3-O-hydroxyethylglucose (3-HEG), 6-O-hydroxyethylglucose (6-HEG) and 2-O-(2′-hydroxyethoxy)-ethylglucose (2,2-diHEG) with hydroxylamine, forming the oxime derivative prior to trimethylsilylation, all of the monosaccharides resulted in one distinct peak instead of multiple peaks which are the different possible anomeric forms of each sugar (data not shown). Oximes can exist in the *syn* and *anti* forms. As only one peak was detected for the oxime derivative, either the chromatographic conditions did not resolve the two conformations or under the conditions of preparation, one of the two forms of the oxime derivative is thermodynamically more stable and thus predominates in the final product.

A chromatogram of acid-hydrolyzed HES without and with the oxime formation step is shown in Figs. 1 and 2, respectively. It is apparent that the proposed GLC method results in a much simpler chromatogram. By reference to the R_t of authentic standards or mass spectral analyses[16], the GLC peaks in Fig. 2 were identified with increasing R_t as follows: dulcitol, glucose, pyranose and furanose forms of 1,2-O-ethyleneglucose, 3-HEG, 2-HEG, 6-HEG, 2,3-dihydroxyethylglucose (2,3-diHEG), 2,6-dihydroxyethylglucose (2,6-diHEG) and 2,2-diHEG. The trisubstituted hydroxyethylglucoses were analyzed at a higher column temperature. The amount of trisubstituted hydroxyethylglucoses was very low in the range of molar substitutions, *i.e.*,

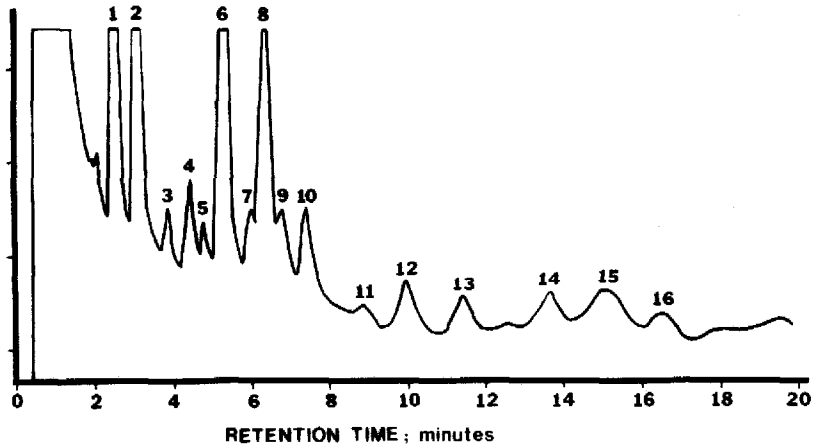

Fig. 1. Gas–liquid chromatogram of trimethylsilylated monosaccharides resulting from acid hydrolysis of hydroxyethyl-starch. Peaks: 1, 2 = glucose; 3, 5, 6, 8 = 2-hydroxyethylglucose; 4, 7 = 3-hydroxyethylglucose; 9, 10 = 6-hydroxyethylglucose; 11–16 = disubstituted glycosides.

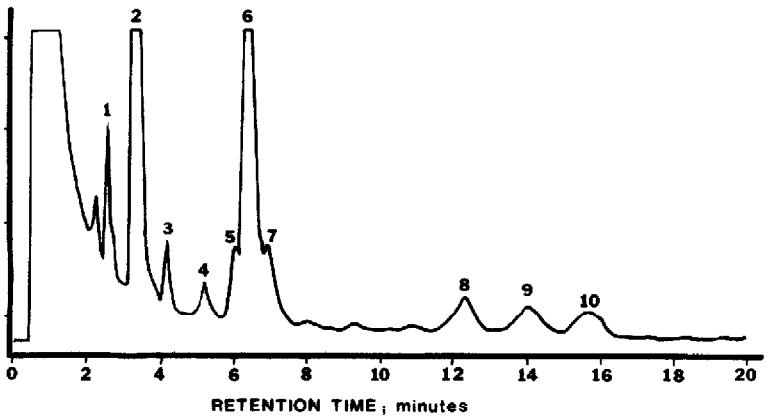

Fig. 2. Gas–liquid chromatogram of trimethylsilylated oxime derivatives of monosaccharides resulting from acid hydrolysis of hydroxyethyl-starch. Peaks: 1 = dulcitol (internal standard); 2 = glucose; 3, 4 = 1,2-O-ethyleneglucose; 5 = 3-hydroxyethylglucose; 6 = 2-hydroxyethylglucose; 7 = 6-hydroxyethylglucose; 8 = 2,3-dihydroxyethylglucose; 9 = 2,6-dihydroxyethylglucose; 10 = 2,2-dihydroxyethylglucose.

the ratio of moles of ethylene oxide that reacted per mole of glucose, that was being examined. Therefore, an unequivocal identification of the sugars was not possible.

Formation of the furanose and pyranose forms of 1,2-O-ethyleneglucose from 2-HEG has been reported in the literature[21,22] and the two compounds represent dehydration products of 2-HEG under acidic conditions at high temperature. The amount of 1,2-O-ethyleneglucose increased dramatically when the hydrolysis time for HES was increased to 4 h at 90°C.

As this method involves passing the acid hydrolyzate through an anion-exchange column, a possibility exists that preferential binding of specific sugars by the

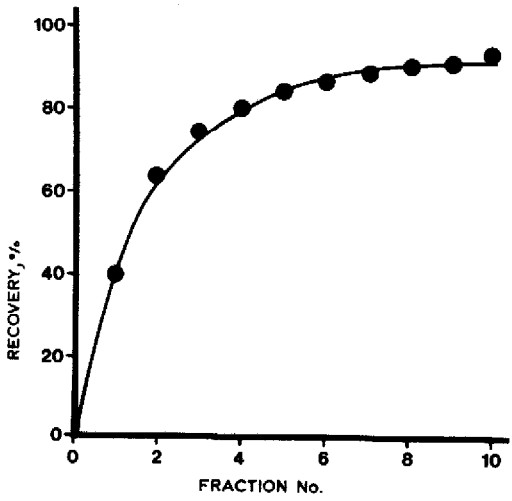

Fig. 3. Recovery of acid-hydrolyzed [^{14}C]HES from the anion-exchange column as a function of fractions collected.

TABLE I

SUGAR COMPOSITION OF FRACTIONS FROM ANION-EXCHANGE COLUMN

Results are total sugar (%) in each fraction.

Sugar	*Fraction No.*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Glucose	53	53	56	58	61	60	57	60	58
3-HEG	3	3	3	3	3	3	3	3	3
2-HEG*	30	29	28	27	30	32	35	32	33
6-HEG	5	5	5	5	5	5	5	5	5
2,3-DI	2	3	2	2	Below limit of detection				
2,6-DI	3	3	3	2	Below limit of detection				
2,2-DI	3	3	3	2	Below limit of detection				
TRI	Below limit of detection								

* 2-HEG represents the sum of 2-O-ethyleneglucose and 2-HEG.

resin may occur. This was examined in two ways. Recovery of sugars from the anion-exchange column was determined using acid-hydrolyzed [^{14}C]HES, and the results are shown in Fig. 3. More than 70% of the radioactivity eluted from the column in the first three 1-ml fractions. The remainder of the radioactivity eluted from the column at a much slower rate, so that an additional seven fractions were required in order to elute the remaining radioactivity.

TABLE II

EFFECT OF VARYING CONCENTRATIONS OF 2-HEG ON THE QUANTITATION OF 2-HEG, 3-HEG AND 6-HEG

The amounts of the internal standard, 3-HEG and 6-HEG were kept constant while the amounts of 2-HEG were varied.

*2-HEG in mixture of HEGs (%)**	*Peak-area ratio***		
	2-HEG	*3-HEG*	*6-HEG*
0	0	0.832	1.22
16.0	0.403	0.892	1.22
29.9	0.915	0.890	1.25
41.1	1.42	0.785	1.23
49.6	1.98	0.795	1.22
55.1	2.64	0.850	1.27
60.3	3.22	0.829	1.26
65.1	3.88	0.793	1.23
68.1	4.54	0.809	1.24
80.4	6.02	0.796	1.21
Mean ± S.D.	–	0.827 ± 0.039	1.24 ± 0.020

* Values are expressed in terms of the total area of monosubstituted HEGs.

** HEG to internal standard.

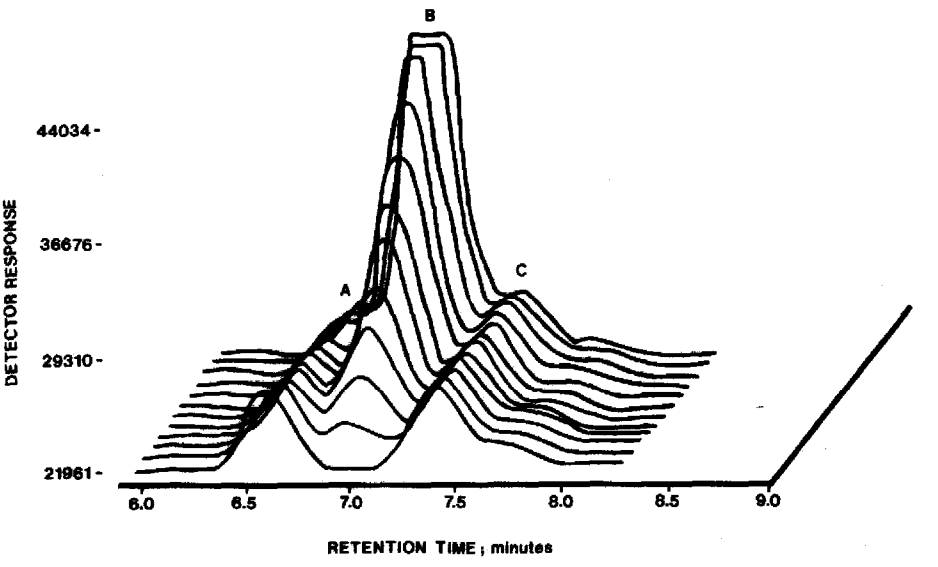

Fig. 4. Graphical representation of increasing the amount of 2-HEG (B) in a mixture of HEGs while maintaining a constant amount of 3-HEG (A) and 6-HEG (C).

The sugar composition of each 1-ml fraction from the column was also determined, and the results are shown in Table I. The relative amounts of different monosaccharides in the first four fractions were very similar and the last six fractions contained the same ratios of glucose to the three monosubstituted hydroxyethylglucoses (HEGs), indicating that an accurate monosaccharide distribution pattern was obtained by analyzing the first three fractions.

With incomplete chromatographic resolution of 2-HEG with 3-HEG and 6-HEG, quantitation of each monosubstituted glucose by the integrator may be concentration dependent. This possibility was examined by adding increasing amounts of 2-HEG to a mixture containing constant amounts of 3-HEG, 6-HEG and internal standard. Table II gives the results that are presented graphically in Fig. 4. The data clearly show that 2-HEG and 6-HEG can be reproducibly quantitated in the presence of different amounts of 2-HEG.

TABLE III

PATTERNS OF O-HYDROXYETHYL SUBSTITUTION OF GLUCOSE IN HES AT VARIOUS DEGREES OF SUBSTITUTION (D.S.) AND MOLAR SUBSTITUTION (M.S.)

D.S.	*M.S.*	*Percentage of total**							
		GLU	*2-HEG*	*3-HEG*	*6-HEG*	*2,3-Di*	*2,6-Di*	*2,2-Di*	*Tri*
0.24	0.26	75.7	16.9	2.38	2.92	0.86	0.69	0.55	N.D.**
0.39	0.48	60.7	27.1	2.51	2.60	2.37	2.03	1.49	1.20
0.54	0.70	46.4	32.2	2.94	4.65	3.95	4.27	3.50	2.20

* The values are the means of six determinations with a standard deviation of $\leqslant 3\%$ for each lot of HES.

** None detected.

The sugar distribution patterns of three lots of HES with different molar substitution values is shown in Table III. As the molar substitution values increased from 0.26 to 0.70, the glucose content of HES decreased with corresponding increases in substituted glucoses. The major substituted glucose in the range of molar substitution values examined was 2-HEG. The amount of 6-HEG was greater than that of 3-HEG. The three disubstituted glucoses were about equal in amount. At a molar substitution value of 0.26 there were no detectable amounts of trisubstituted HEGs but at a value of 0.70, the trisubstituted HEGs constituted about 2% of the total sugars found in HES.

DISCUSSION

Addition of the Schiff-base formation step greatly simplifies the GLC analysis of acid-hydrolyzed HES. The proposed method allows the separation and accurate quantitation of the major constituents of HES using a conventional GLC column and isothermal conditions instead of capillary columns and temperature programming[11–13]. More importantly, the data obtained using this simplified method are consistent with those from the more time-consuming procedures in the literature[11,13].

As the major substituent sugars in HES can be accurately quantitated, the molar substitution and the degree of substitution, *i.e.*, percentage of glucose in HES that is substituted with one or more hydroxyethyl groups, can be determined using the same method. Both of these parameters are important determinants not only of the hydroxyethyl substitution patterns of glucose in HES but also of the physiological properties of HES such as plasma volume expansion and duration of action[1,23].

REFERENCES

1 W. L. Thompson, in G. A. Jamieson, and T. J. Greenwalt (Editors), *Blood Substitites and Plasma Expanders*, Alan R. Liss, New York, 1978, p. 283.
2 A. Yacobi, R. G. Stoll, C. Y. Sum, C. M. Lai, S. D. Gupta and J. D. Hulse, *J. Clin. Pharmacol.*, 22 (1982) 206.
3 L. C. Maguire, R. G. Strauss, J. A. Koepke, R. J. Boromon, K. R. Zalenski, R. M. Lambert, J. D. Hulse and A. K. Atnip, *Transfusion*, 21 (1981) 347.
4 R. G. Strauss, *Vox Sang.*, 37 (1979) 268.
5 J. M. Mishler and E. S. Parry, *Vox Sang.*, 36 (1979) 337.
6 C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Amer. Chem. Soc.*, 85 (1963) 2497.
7 K. M. Brobst and C. E. Lott, *Cereal Chem.*, 43 (1966) 35.
8 D. C. DeJongh, T. Radford, J. H. Hribar, S. Hanession, M. Briban, G. Dawson and C. C. Sweeley, *J. Amer. Chem. Soc.*, 91 (1969) 1728.
9 C. E. Lott and K. M. Brobst, *Anal. Chem.*, 38 (1966) 1767.
10 O. Ramnas and O. Samuelson, *Svensk. Papperstidn.*, 71 (1968) 647.
11 J. W. Mourits, H. G. Merkus and L. de Galan, *Anal. Chem.*, 48 (1976) 1557.
12 H. G. Merkus, J. W. Mourits, L. de Galan and W. A. de Jong, *Starke*, 29 (1977) 406.
13 O. Larm, K. Larsson and O. Theander, *Starke*, 33 (1981) 240.
14 B. S. Mason and H. T. Slover, *J. Agr. Food Chem.*, 19 (1971) 551.
15 T. Toba and S. Adachi, *J. Chromatogr.*, 135 (1977) 411.
16 R. Varma, R. S. Varma, W. S. Allen and A. H. Wardi, *J. Chromatogr.*, 86 (1973) 205.
17 R. Varma and R. S. Varma, *J. Chromatogr.*, 139 (1977) 303.
18 A. N. Debelder and E. Wiren, *Carbohydr. Res.*, 24 (1972) 166.
19 W. P. Shyluk and T. E. Timell, *Can. J. Chem.*, 34 (1956) 571.
20 T. F. Kalhorn and C. Y. Sum, in preparation.
21 A. N. Debelder and B. Norman, *Carbohydr. Res.*, 10 (1969) 391.
22 H. E. Srivastava, K. V. Ramalingam, N. M. Doshi and A. S. Chaudhari, *Carbohydr. Res.*, 12 (1970) 23.
23 J. M. Mishler, *Int. J. Clin. Pharmacol. Ther. Toxicol.*, 18 (1980) 67.