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Simultaneous determination of neutral and amino sugars in biological materials

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ABSTRACT

A method is described for the simultaneous analysis of nine neutral and three amino sugars. Mixtures of standard sugars and biological samples were acid hydrolyzed with a two step Saeman procedure, neutralized with $BaCO_3$, reduced with sodium borohydride, acidified, evaporated and alditol acetates prepared. Baseline resolution was achieved on a glass-capillary SP-2340 column in *ca.* 52 min. Reproducibility, response factors and hydrolysis losses were determined. Quantitation was linear over the range of 10–20 μg/ml to 2000 μg/ml. Conditions were defined for the reproducible quantitation of muramic acid.

INTRODUCTION

Several gas (GC) and high-performance liquid chromatographic and colorimetric methods have been used to measure the neutral sugars in the complex mixture of plant polysaccharides termed dietary fiber[1–3]. Understanding the role of dietary fiber in the gastrointestinal tract requires additional analytical capabilities to distinguish exogenous from endogenous carbohydrate polymers, and bacteria. Plant polysaccharides are comprised largely of neutral sugars whereas both neutral and amino sugars are present in bacteria and mucin. One amino sugar, muramic acid, has been used as a marker for bacteria[4]. Our interest in the analysis of polysaccharides typically found in the lumen of the gastrointestinal tract instituted a search for an analytical method that would allow complete determination of both neutral and amino sugars and wich would permit analysis over a wide range of absolute amounts of sugars in any particular sample. Earlier GC methods for measurement of both neutral and amino sugars as alditol acetate derivatives exhibited long retention times, poor chromatography of the amino sugars[5] or inadequate separation of the neutral sugars[6]. Deamination of amino sugars produced shorter retention times for amino sugar derivatives. However, multiple products were produced from each amino sugar and some neutral sugars partially decomposed under the deamination conditions[7].

Methods which give satisfactory results for specific types of samples have been

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reported, for example, non-starch polysaccharides in foods[2,3,8], plant cell walls[9], bacterial cell walls[10,11], and glycoproteins[12,13]. Each of these kinds of samples often contain relatively high concentrations of carbohydrates or specific polysaccharides, that simplify their analysis. We report here a method, derived by combining and modifying a number of existing methods, for the simultaneous analysis and quantitation of neutral and amino sugars, including muramic acid, in the CHO mixtures typically found in the lumen of the gastrointestinal tract.

EXPERIMENTAL

Chemicals

All sugars, sodium borohydride, acetic anhydride, octanol and methylene chloride were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were reagent grade. Whatman GF/A glass micro fiber filter paper (4.25 cm) was purchased through VWR Scientific (Chicago, IL, U.S.A.). 12 *M* H_2SO_4 was made by adding 1226 g ultra pure sulfuric acid, purchased from VWR, to 408 g of distilled water. The specific gravity was checked and adjusted, if necessary, to 1.63 g/ml and the solution stored in a desiccator. The standard sugar mixture consisted of 1 mg/ml of each of nine neutral and three amino sugars in distilled water.

Biological samples

All samples that were analyzed were obtained from experiments conducted in this laboratory. Rat feces were collected from animals fed an AIN 76A purified diet[14,15] into which 40% oat bran (Quaker Oats, Barrington, IL, U.S.A.) had been incorporated to make a diet containing 8% dietary fiber[16]. Complete fecal collections were made daily for 14 days from 10 animals, blended with distilled water and lyophilized. A single composite consisting of 10% of the dry output from each rat was prepared for analysis. One composite of human feces was prepared from samples collected during 5 to 7 days of ingestion of a mixed solid food diet supplemented with 30 g/day of soft white wheat bran (American Association of Cereal Chemists, St. Paul, MN, U.S.A.); dietary fiber intake was 33–35 g/day[17]. Nine stools from five male subjects were combined to prepare the composite. The other human feces sample was collected during consumption of a fiber free diet during the last 10 days of a 60-days study of a nutritionally complete liquid diet (Ensure®, Ross Labs. Columbus, OH, U.S.A.)[18]. A single composite from the 10 days of excreta of six subjects was prepared for analysis by combining 2% of dry stool from each subject.

Acid hydrolysis

All biological samples were acid hydrolyzed by a modified Saeman hydrolysis procedure. Dry ground samples or standards were thoroughly mixed with 12 *M* H_2SO_4 (1.0 ml acid/50 mg of sample)[19] and allowed to incubate at ambient temperatures for 1 h with mixing every 15 min; 11 ml water/ml of 12 *M* H_2SO_4 was added to produce 1 *M* H_2SO_4. Samples were then autoclaved for 1 h at 121°C (15 p.s.i.). After cooling to ambient temperature, 0.2 ml of the internal standards mixture was added. The internal standards were allose at 10 mg/ml and N-methylglucamine (N-methyl-2-amino-2-deoxy-D-glucose) at 10 mg/ml. The hydrolysates were filtered through tared filter paper (Whatman GF/A) and neutralized with $BaCO_3$ (ref. 20).

Sugar derivatization

Reduction was accomplished by heating 1 ml of the acid hydrolysate from 25 mg of a biological sample or 1 ml from 24 mg of the standard mixture with 0.1 ml of a sodium borohydride solution (100 mg/ml in 3 *M* ammonium hydroxide, 1 h, 40°C). Glacial acetic acid (0.1 ml) and 3 ml methanol were added and sample evaporated with simultaneous vortexing under vacuum to remove excess borate as methylborate and to remove water. The temperature of the evaporator was started at 40°C and raised to 60°C when the pressure gauge indicated most of the methanol had evaporated. This step was repeated three more times with drying time increased to 3 h for the last time. Derivatization was accomplished essentially by the method of Whiton *et al.*[21] with 2.0 ml acetic anhydride at 100°C for 16 h. If a larger aliquot of the hydrolysate was used, the amounts of reduction and acetylation reagents were proportionately increased. The procedure was modified to remove contaminants by incorporating the following extractive steps. After acetylation, samples were extracted three times with 1 ml of methylene chloride. The pooled methylene chloride extracts were backwashed with 2 ml of water, followed by 2 ml of 30% sodium hydroxide and finally 2 ml of water. The washed extracts were dried under a steam of nitrogen, redissolved in 0.5–1.0 ml of methylene chloride and stored at −20°C until analyzed.

Gas chromatography

GC analyses were carried out on a Model HP-5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with a flame ionization detector, and containing a 30 m × 0.5 mm I.D. borosilicate glass column (SP-2340, Supelco, Bellefonte, PA, U.S.A.) fitted with 0.32 mm inert fused-silica tubing at both ends. Samples were injected (0.5–1.0 μl) using a split ratio of 10:1 and a column helium flow of 5.5 ml/min. The injection port temperature was 250°C, while the detector temperature was 300°C. The column oven program started at 185°C for 10 min then programmed at 3°C/min to 220°C, held at this temperature for 5 min, and then increased to a final temperature of 235°C at 15°C/min. The final temperature was held for 30 min. Data were collected on a Hewlett-Packard HP-3392A integrator.

Evaluation of method

Linearity of the method was determined by GC analysis of sets of sugar standards containing approximately 10, 20, 50, 500, 1000 and 2000 μg/ml of each sugar along with 0.2 ml of an internal standard mixture which contained 5.0 mg/ml of allose and 5.0 mg/ml of N-methylglucamine. Linearity was demonstrated by correlating the area ratio of each sugar, *i.e.*area of sugar/area of internal standard with the weighed amount of sugar. Response factors (*RF*) of standard sugar mixtures (24 mg sugar/ml) were determined by the equation: RF = (area of internal standard/mg of internal standard) × (mg of unknown/area of unknown). Hydrolysis losses were calculated as: [1.0 − (*RF* of sugars without acid treatment/*RF* of sugars with acid treatment)] × 100.

The effect of different concentrations of closely eluting pairs of neutral sugars, *i.e.* rhamnose and fucose, and ribose and arabinose, on the *RF* values for these sugars was evaluated by GC analysis of four mixtures of standard sugars without acid treatment: (1) the four sugars each at 2.0 mg/ml; (2) the four sugars each at 0.2 mg/ml; (3) rhamnose and ribose at 0.2 mg/ml, and fucose and arabinose at 2.0 mg/ml; and (4) rhamnose and ribose at 2.0 mg/ml and fucose and arabinose at 0.2 mg/ml.

Reproducibility of the method was evaluated by conducting all analyses in triplicate, determining the standard deviations and coefficients of variation. Reproducibility over time was determined by conducting triplicate analyses of four acid-treated standard sugar mixtures four times during two months.

RESULTS

Essentially baseline resolution was obtained for the nine neutral sugars, three amino sugars and two internal standards; the possible exceptions were the closely eluting pairs of rhamnose/fucose and ribose/arabinose (Fig. 1). The sugars eluted with these retention times: deoxyhexoses,at 8.02 and 8.60 min, pentoses, 12.06 to 16.39 min, hexoses 19.24 to 24.73 min, and amino sugars, 34.37 to 51.20 min (Table I).

Linearity for each neutral sugar was demonstrated over the range of 10 to 2000 μg/ml; the correlations between the area ratio of the sugar and the weighed amounts were $r \geqslant 0.999$. This linearity held throughout the entire range of concentrations tested; the coefficients of variation ($n=3$) for each neutral sugar at any concentration were all $\leqslant 4\%$. The mean coefficient of variation for all neutral sugars at 10 μg/ml was 1.9% (range: 1.0–3.6%), at 2000 μg/ml, 1.3% (range: 0.3–3.2%). None of the amino sugars were detectable at levels of 10 μg/ml; however, they were detected at 20 μg/ml. Linearity for each amino sugar was demonstrated over the range of 20 to 2000 μg/ml, with the correlations between the area ratio of the sugar and the weighed amounts of $r \geqslant 0.999$. At 20 μg/ml the mean coefficient of variation of triplicate analyses of two of the amino sugars, glucosamine and galactosamine, was 5.2%, and at 2000 μg/ml, 2.0%.

The individual sugars of the two closely eluting pairs, *i.e.* rhamnose/fucose and ribose/arabinose could be accurately measured whether concentrations of the two sugars in each pair varied by tenfold, 0.2 mg/ml and 2.0 mg/ml, or were at equal

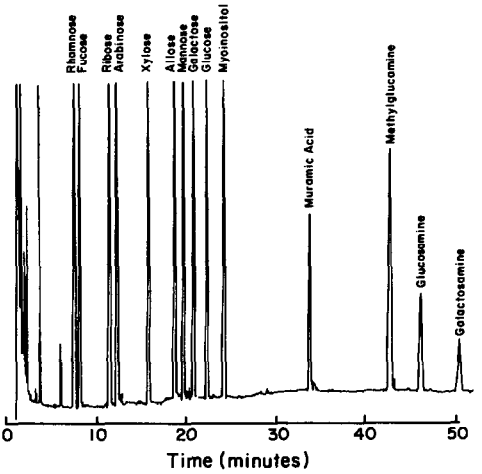

Fig. 1. Glass capillary column chromatogram of the alditol acetate derivatives of a mixture of hydrolyzed neutral and amino sugars each at a concentration of 2.0 mg/ml. Allose and methylglucamine were the internal standards.

TABLE I

RETENTION TIMES, RESPONSE FACTORS AND HYDROLYSIS LOSSES OF NEUTRAL AND AMINO SUGARS

I.S. = Internal standard.

Sugar	*Retention time (min)*	*Response factor (mean ± S.D., n=3)*		*Hydrolysis losses (%)*
		Without acid treatment	*With acid treatment*	
Rhamnose	8.02	0.94 ± 0.02	1.06 ± 0.03	11
Fucose	8.60	0.87 ± 0.01	0.98 ± 0.03	11
Ribose	12.06	0.89 ± 0.03	1.39 ± 0.04	36
Arabinose	12.85	0.88 ± 0.01	1.01 ± 0.03	13
Xylose	16.39	0.88 ± 0.01	1.18 ± 0.03	25
Allose (I.S.)	19.24	–	–	–
Mannose	20.19	0.95 ± 0.003	1.09 ± 0.03	13
Galatose	21.29	0.95 ± 0.004	1.09 ± 0.02	13
Glucose	22.80	0.94 ± 0.006	1.05 ± 0.03	11
Myoinositol	24.73	0.90 ± 0.01	0.97 ± 0.01	7
Muramic acid	34.37	–[a]	–[a]	–[a]
N-Methylglucamine (I.S.)	43.39	–	–	–
Glucosamine	46.02	1.90 ± 0.21	2.19 ± 0.05	13
Galactosamine	51.20	2.73 ± 0.06	3.26 ± 0.04	16

[a] See Table IV.

concentrations, 0.2 mg/ml or 2.0 mg/ml each. As shown in Table II, the *RF* for each individual sugar was essentially identical across the four different concentrations.

The extent of decomposition of monosaccharides during acid hydrolysis of carbohydrates was determined by subjecting a standard sugar mixture to the acid hydrolysis procedure and comparing the *RF* values of the same mixture without acid treatment. The *RF* values of standard sugar mixtures with and without acid treatment and the resulting hydrolysis losses are shown in Table I. The *RF* values of all of the neutral sugars without acid treatment were very similar, ranging from 0.87 for fucose

TABLE II

RESPONSE FACTORS OF TWO CLOSELY ELUTING PAIRS OF DERIVATIZED NEUTRAL SUGARS WITHOUT ACID TREATMENT

Concentrations (mg/ml)				R_F			
Rhamnose	*Fucose*	*Ribose*	*Arabinose*	*Rhamnose*	*Fucose*	*Ribose*	*Arabinose*
2.0	2.0	2.0	2.0	0.93[a]	0.86	0.85	0.85
0.2	0.2	0.2	0.2	0.87	0.80	0.80	0.80
0.2	2.0	0.2	2.0	0.85	0.82	0.80	0.83
2.0	0.2	2.0	0.2	0.93	0.86	0.86	0.81
Mean ± S.D.				0.90 ± 0.04	0.84 ± 0.03	0.83 ± 0.03	0.82 ± 0.02

[a] $n = 2$.

to 0.95 for both mannose and galactose. *RF* values for neutral sugars with acid treatment ranged from 0.97 for myoinositol to 1.39 for ribose; the *RF* values of the other neutral sugars were approximately 1.0 to 1.1. In contrast, the *RF* values of glucosamine and galactosamine without acid treatment were 1.90 and 2.73 and following acid treatment, 2.19 and 3.26, respectively (Table I). Seven of the nine neutral sugars showed about 10% to 15% decomposition during acid treatment while ribose and xylose exhibited 36 and 25% hydrolysis losses, respectively. Hydrolysis losses for glucosamine and galactosamine were 13 and 16%, respectively (Table I).

Reproducibility of the entire method, determined by analyzing four sets of triplicate aliquots of the standard sugar mixture, was very good, with coefficients of variation for the mean of means of the *RF* values of any sugar ranging from 1 to 4% (Table III). Precision within the sets of triplicates was also good, with coefficients of variation of 1 to 3%.

N-Acetylmuramic acid was included in the standard mixture and analyzed throughout these experiments. Data from four experiments in Table IV, part A, illustrate considerable variation within and between each set of triplicate analyses. Similar variability, with coefficients of variation, of 7 to 10% ($n=3$), was observed across the range of concentrations of 20 to 2000 mg/ml used to determine linearity of response. In addition, hydrolysis losses were variable and negative rather than positive numbers (Table IV, part A). The variability in the *RF* values suggested incomplete derivatization of N-acetylmuramic acid. The effects of the amounts of derivatizing reagents and total sugar content on the variability in N-acetylmuramic acid *RF* was evaluated by hydrolyzing and derivatizing N-acetylmuramic acid in the presence of constant amounts of derivatizing reagents and 8, 16 or 24 mg of total sugar. Indeed, the *RF* of N-acetylmuramic acid increased as the amount of total sugars increased in the mixture, indicating less complete derivatization of N-acetylmuramic acid in the presence of greater amounts of sugar (Table IV, part B). However, derivatizing

TABLE III

REPRODUCIBILITY OF RESPONSE FACTORS OF ALDITOL ACETATES OF MIXTURES OF NEUTRAL AND AMINO SUGARS WITH ACID TREATMENT

Sugar	*Response factor (mean ± S.D., n=3)*				*Mean of means ± S.D.*
	Day of Analysis				
	1	41	54	61	
Rhamnose	1.05 ± 0.03	1.06 ± 0.03	1.08 ± 0.01	1.08 ± 0.01	1.08 ± 0.02
Fucose	0.96 ± 0.03	0.97 ± 0.03	0.99 ± 0.01	0.98 ± 0.01	0.98 ± 0.01
Ribose	1.33 ± 0.03	1.39 ± 0.04	1.28 ± 0.01	1.36 ± 0.01	1.34 ± 0.05
Arabinose	0.99 ± 0.03	1.01 ± 0.03	1.03 ± 0.01	1.03 ± 0.01	1.02 ± 0.02
Xylose	1.14 ± 0.03	1.18 ± 0.03	1.14 ± 0.00	1.21 ± 0.01	1.17 ± 0.03
Mannose	1.03 ± 0.02	1.09 ± 0.02	1.10 ± 0.00	1.12 ± 0.01	1.09 ± 0.04
Galactose	1.04 ± 0.02	1.09 ± 0.02	1.11 ± 0.01	1.12 ± 0.01	1.09 ± 0.04
Glucose	1.00 ± 0.02	1.05 ± 0.03	1.04 ± 0.01	1.07 ± 0.01	1.04 ± 0.03
Myoinositol	1.03 ± 0.02	0.97 ± 0.02	1.00 ± 0.01	1.01 ± 0.01	1.00 ± 0.03
Glucosamine	2.10 ± 0.05	2.19 ± 0.05	2.16 ± 0.01	2.11 ± 0.03	2.14 ± 0.04
Galactosamine	3.09 ± 0.04	3.28 ± 0.04	3.27 ± 0.02	3.17 ± 0.03	3.20 ± 0.19

TABLE IV

RESPONSE FACTORS AND HYDROLYSIS LOSSES OF N-ACETYLMURAMIC AND MURAMIC ACID

	Response factor		*Hydrolysis loss (%)*
	Without acid treatment	*With acid treatment*	
(A) N-Acetylmuramic acid[a]			
Experiment 1	3.04 ± 0.30	2.29 ± 0.14	−33
Experiment 2	3.71 ± 0.29	2.48 ± 0.22	−50
Experiment 3	2.86 ± 0.80	2.90 ± 0.15	−16
Experiment 4	–	2.52 ± 0.10	–
(B) N-acetylmuramic acid[b]			
Sugar (mg) *Reagents*			
8 standard[a]		2.17 ± 0.08	
16 standard		2.50 ± 0.10	
24 standard		2.76 ± 0.20	
24 3 × standard		2.27 ± 0.04	
(C) N-Acetylmuramic and muramic acids			
N-Acetylmuramic acid	2.80 ± 0.08	2.17 ± 0.07	−29
Muramic acid	1.64 ± 0.07	2.16 ± 0.12	+24

[a] Experiments used amounts of reduction and derivatization reagents reported in methods for 1 ml of acid hydrolysate from 25 mg of biological sample.

[b] N-Acetylmuramic acid derivatized in presence of varying amounts of neutral and amino sugars and with two amounts of derivatization reagents.

N-acetylmuramic acid in the presence of 24 mg sugar with 3 times more reductive and acetylating reagents produced a *RF* of 2.27, almost identical to the *RF* of 2.17 determined with the lowest amount of sugars (Table IV, part B). The concentrations of the reducing and acetylating reagents had no effect on the *RF* values of all other sugars.

The problem of a negative hydrolysis loss for N-acetylmuramic acid was addressed by testing the hypothesis that the acetyl group interfered with derivatization[22]. First, N-acetylmuramic and muramic acids (1 mg) were individually derivatized in triplicate with the standard amounts of reagents, i.e. 0.1 ml of sodium borohydrate and 2 ml acetic anhydride. Comparison of the *RF* values for muramic and N-acetylmuramic acid without acid treatment indicated the acetyl group interfered with derivatization (Table IV, part C). Muramic acid was approximately 55% more efficiently acetylated than N-acetylmuramic acid, with corresponding *RF* values of 1.64 and 2.80. The experiment was repeated but with acid treatment of the samples before derivatization. Following acid treatment, both sugars yielded essentially identical *RF* values, 2.16 ± 0.12 and 2.17 ± 0.07, for muramic acid and N-acetylmuramic acid, respectively, and a positive hydrolysis loss of 24% for muramic acid (Table IV, part C).

The method described here was successfully used to determine sugar content of typical biological samples analyzed in this laboratory (Table V). Total neutral sugars

TABLE V

SIMULTANEOUS DETERMINATION OF NEUTRAL AND AMINO SUGARS IN BIOLOGICAL MATERIALS

n.d. = None detected.

Sugar	*Content (mg/100 mg dry feces) (mean ± S.D., n=3)*		
	Rat feces, oat bran diet	*Human feces*	
		Wheat bran diet	*Fiber-free diet*
Rhamnose	0.7 ± 0.0	0.5 ± 0.0	0.5 ± 0.1
Fucose	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
Ribose	0.5 ± 0.0	0.6 ± 0.0	0.2 ± 0.0
Arabinose	2.2 ± 0.1	4.8 ± 0.2	0.1 ± 0.0
Xylose	3.4 ± 0.1	5.2 ± 0.2	0.1 ± 0.0
Mannose	0.5 ± 0.0	0.5 ± 0.0	0.2 ± 0.0
Galactose	0.9 ± 0.0	1.3 ± 0.0	0.7 ± 0.1
Glucose	5.6 ± 0.1	8.1 ± 0.2	2.6 ± 0.2
Myoinositol	n.d.	n.d.	n.d.
Muramic acid	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
Glucosamine	0.6 ± 0.0	0.5 ± 0.0	0.7 ± 0.0
Galactosamine	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1

in the three samples ranged from 4.4 to 21.1 mg/100 mg of dry feces. In all samples glucose was the dominant sugar, accounting for about 40% of the neutral sugar content in the fiber-containing samples and 60% in the fiber-free sample. The two fiber-containing samples also had significant amounts of arabinose and xylose, whereas the feces from humans fed the fiber-free diet had only negligible amounts. All three amino sugars were detected in the three samples, and all at low levels. Muramic acid content was about 0.2% for each sample, and galactosamine concentrations were slightly higher, ranging from 0.2 to 0.3%. Glucosamine was the most abundant amino sugar in all three samples.

DISCUSSION

Simultaneous quantitation of nine neutral and three amino sugars typically found in biological samples was achieved with the method we developed and evaluated. GC analysis of the alditol acetate derivatives of the sugars was achieved in less than one hour. The borosilicate glass column that was used showed excellent resolution for all desired sugar derivatives, including the closely eluting pairs of rhamnose/fucose and ribose/arabinose. Although the retention times of amino sugars were relatively long, the column and conditions used are an acceptable compromise which allows quantitation of both neutral and amino sugars.

The limit of detection of neutral sugars we observed was at a concentration of about 10 μg/ml when 1.0 ml of methylene chloride was used as the final solvent. This translated to limits of 0.04 mg/100 mg of starting material which is similar to the limits of 0.1 mg/100 mg of starting material reported by Fox *et al.*[10]. Since detection of amino

sugars was less sensitive than that of neutral sugars, the limits of detection on a starting weight basis for the three amino sugars would be about two to three times higher. Theoretically lower limits of detection per starting weight could be attained by dissolving the derivatized sample in smaller volumes of methylene chloride.

Sawardeker *et al.*[23] reported *RF* values of 1.0 for a series of the alditol acetates of neutral sugars without acid treatment when compared to xylitol. Since xylose is routinely present in samples analyzed in this laboratory, allose was chosen as an internal standard for neutral sugars. The *RF* values for neutral sugars when compared to allose were all very similar, ranging from 0.87–0.95, but were slightly less than 1.0 reported by Sawardeker *et al.*[23]. We used N-methylglucamine as the internal standard for amino sugars since Fox *et al.*[10] reported improved precision for the analysis of amino sugars when N-methylglucamine was used as an internal standard *versus* the neutral sugar xylose.

Hydrolysis losses of sugars are sugar specific and highly dependent on the severity of hydrolytic conditions[9,24,25]. We found xylose and ribose to be the neutral sugars most sensitive to hydrolytic decomposition. Hough *et al.*[24] also observed the most decomposition of these sugars when samples were heated to 100°C in 2 *M* H_2SO_4 for 6 h. They recovered only 48% of the ribose, and 67% of the xylose, while glucose and fucose recoveries were $\geqslant 90\%$, and mannose and galactose recoveries were 84 and 87% respectively. The higher recoveries we observed for ribose and xylose, 64 and 75% respectively, agree with the conclusions of Selvendran *et al.*[25], that Saeman hydrolysis methods result in less degradation than hydrolysis methods using only dilute H_2SO_4 and longer heating times. The need for anaerobic conditions for amino sugar acid hydrolysis is unclear[26,27]. Under our experimental conditions, the losses of glucosamine and galactosmine, 13% and 16% respectively, were similar to those of the neutral sugars even though they were hydrolyzed under aerobic conditions. Muramic acid, with hydrolysis losses of 24%, was decomposed to a greater extent than the two other amino sugars.

Difficulties in the GC analysis of amino sugars are well documented, regardless of the derivative prepared[28]. It was thought that these problems were due primarily to chromatographic problems rather than derivatization problems[12]. Hudson *et al.*[29] found the behavior of the alditol acetate derivatives of amino sugars to be highly dependent on the pretreatment of a capillary GC column. For example after pretreatment with basic conditions the amino sugar derivatives were completely adsorbed to or decomposed on the column. However, if deactivated inlets and capillary columns were used, they found that the response of the amino sugar derivatives were about 90% of that of the neutral sugar derivatives. Fox *et al.*[10] also have suggested that the sensitivity of amino sugars may be lower than those for neutral sugars because they may adsorb to the column. This lower sensitivity would explain the higher *RF* values we observed for the amino sugars and suggests some adsorption onto the column. The *RF* values we determined for the amino sugars have been reproducible and suitable for the quantitation of small amounts of amino sugars in biological samples.

Many of the currently available methods were designed for the carbohydrate analysis of specific types of samples with high levels of carbohydrates or specific polysaccharides, characteristics which simplified their analysis. The samples analyzed for sugar composition in this report not only contained a complex mixture of

polysaccharides derived from a variety of sources, plant cell walls, bacterial cell walls, bacterial exopolysaccharides and mucins, but also contained only very small amounts of some of the sugars. To determine complete sugar composition of these biological samples, it was necessary to take either a large portion of or the entire acid hydrolysate from 25 mg of sample for GC analysis. This was achieved by neutralization of the hydrolysate with barium carbonate and removal of the sulfate anion as barium sulfate by centrifugation. Part or all of the supernatant was then taken for subsequent reduction and derivatization.

Since muramic acid determination was desired, we found that the thorough evaporative and derivatizing procedures reported by others[21] were required. If muramic acid determination is not desired, derivatization of the supernatant by a methylimadizole catalized method[9] could be utilized. Muramic acid (13-O-lactyl-glucosamine) is chemically unique among amino sugars in that under dehydrating conditions it forms a lactam ring[10,21]. The acetylated lactam is the derivative analyzed by GC. Lactam formation is essential for muramic acid analysis by the method we report, as the free carboxyl would bind to the SP2340 GC column[21].

Acetylation of the amine nitrogen interferes with the lactam ring formation, explaining the less efficient derivatization of N-acetylmuramic acid we observed. Such interference would lead to an underestimation of the actual amount of muramic acid when starting with N-acetylmuramic acid. This underestimation would explain the negative hydrolysis losses of N-acetylmuramic acid in the standard sugar mixture. Since the acetyl group of N-acetylmuramic acid was cleaved during hydrolysis, thereby yielding muramic acid[22], identical *RF* values for the two sugars were achieved.

Following acetylation and extraction, it would be possible to inject samples directly into the GC system. However, we have found that sample clean up and drying prior to GC injection prolongs the life of the column; it may also eliminate extraneous peaks from the chromatogram. The clean up procedure we described should remove both acidic and basic contaminants and provide an essentially water-free extract for GC analysis.

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