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CAPILLARY GAS CHROMATOGRAPHIC ANALYSIS OF ALDITOL ACETATES OF NEUTRAL AND AMINO SUGARS IN BACTERIAL CELL WALLS

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SUMMARY

Several improvements in the preparation of alditol acetates of neutral and amino sugars and in the preparation of glass capillary columns for their separation are described. Modifications in sample preparation permitted the simultaneous processing of multiple samples and eliminated extraneous background peaks. Efficient and inert columns were tailor-made for the separation of alditol acetates of neutral and amino sugars by leaching glass capillaries with aqueous hydrochloric acid and dynamically coating with SP-2330.

INTRODUCTION

The prepation of volatile alditol acetate derivatives of carbohydrates and their separation by gas chromatography (GC) is a popular analytical method for determining the sugar composition of biomolecules[1–3]. The principal advantage of the alditol acetate method over other procedures, such as trimethylsilylation[4], is that single derivatives are produced for each component sugar. In the analysis of a mixture of sugars from a complex biological matrix, the production of multiple peaks will lead to a more complicated chromatogram which might confound both qualitative identification and quantitative measurement, particularly if these peaks are poorly resolved from one another or the background. Furthermore, the production of multiple peaks from a single sugar might adversely affect the limit of detection.

The application of a derivatization GC procedure to the determination of individual sugars in a complex polysaccharide involves several steps. The carbohy-

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drate polymer must first be treated to release component monosaccharides. Next, these non-volatile monomers must be isolated from contaminating materials and then derivatized prior to GC analysis. Extraneous, and possibly interfering, chromatographic peaks can result from both contamination of the original sample and contamination introduced by the reagents or reactions employed. Two additional difficulties are encountered in the GC separation of mixtures of neutral and amino sugars: lengthy analysis times are required for adequate separation, and the sensitivity for amino sugars is often significantly lower than for neutral sugars[5–7]. Several recent articles have described separations of the alditol acetates of neutral and amino sugars by capillary GC[8–12]. In the present article, we report improvements in the alditol acetate method for mixtures of neutral and amino sugars. Modifications in the hydrolysis, neutralization, isolation and derivatization steps have led to a considerable decrease in extraneous peaks. We have described the development of improved high resolution glass capillary columns for the separation of neutral and amino sugars alditol acetates in a separate article[13]. Glass capillary column technology enables the rapid and efficient separation of neutral and amino sugars, while increasing the sensitivity for amino sugars. The utility of this derivatization and capillary GC method for carbohydrate profiling is demonstrated by the analysis of bacterial cell wall preparations isolated from *Streptococcus pyogenes.*

EXPERIMENTAL

Materials

Samples to be analyzed consisted of either mixtures of pure sugar standards or bacterial cell walls. L-Rhamnose, L-fucose, D-xylose, D-mannose, D-galactose and D-glucose were obtained from Supelco (Bellefonte, PA, U.S.A.). 2-Deoxy-D-ribose, D-ribose, muramic acid, D-glucosamine hydrochloride and D-galactosamine hydrochloride and methylglucamine were obtained from Sigma (St. Louis, MO, U.S.A.). *Streptococcus pyogenes* cell walls (ATCC 10389) were prepared as described previously[14]. The following derivatization reagents and chromatographic solvents were glass distilled: acetic anhydride (Applied Science Labs, State College, PA, U.S.A.), Ultrex grade acetic acid (J. T. Baker, Phillipsburgh, NJ, U.S.A.), chloroform and methanol Burdick & Jackson Labs, Muskegon, MI, U.S.A.). N,N-Dioctylmethylamine was purchased from ICN (Plainview, NY, U.S.A.). Reagent grade sulfuric acid and sodium borohydride were obtained from Fisher (Fairlawn, NJ, U.S.A.). Clin Elut hydrophilic extraction columns and Bond Elut hydrophobic extraction columns were purchased from Analytichem International (Lawndale, CA, U.S.A.). All glassware was acid washed prior to use.

Sample preparation

Hydrolysis of bacterial cell walls started with 1 mg of lyophilized sample in 0.5 ml sulfuric acid in a reaction tube with an adjustable PTFE plunger (Pierce, Rockford, IL, U.S.A.). After evacuation and flushing with nitrogen, the sample was heated at 100°C in a heating module (Pierce) for several hours and then cooled in an ice-bath. The hydrolysis time and concentration of acid employed were varied depending on the sample, with typical conditions of 2 *N* sulfuric acid for 3 h. The acid solution was neutralized with 2.5 ml 20% N,N-dioctylmethylamine in chloroform

and 24 μg of the xylose and methylglucamine internal standards in 50 μl water were added. The aqueous phase was placed on a C_{18} Bond elut column that had been prewetted with 2 ml methanol followed by 2 ml distilled water. Sodium borohydride solution (50 μl containing approximately 5 mg) was added, the reaction mixture was pulled through the column by vacuum and the column was rinsed with 1 ml water. The sample was then allowed to react overnight at 4°C. The Vac Elut system (Analytichem) permitted up to ten samples to be run simultaneously on a single module.

Sodium borohydride was removed from the samples by adding 3 ml of acetic acid–methanol (1:200 v/v) and by evaporating to dryness under vacuum at 60°C in a Vortex Evaporator (Buchler, Fort Lee, NJ, U.S.A.); this step was repeated four additional times. As many as 36 samples could be evaporated simultaneously. The samples were further dried for 3 h under vacuum. After the addition of 300 μl of acetic anhydride, the samples were heated at 100°C in a heating module for 13–16 h in reaction vials with screw caps and PTFE silicone discs (Pierce). The samples were then evaporated to dryness. One ml water and one ml chloroform were added and the mixture was poured onto a Clin Elut column (Analytichem). The sample was eluted with chloroform and evaporated to dryness. Approximately 25 μl of chloroform was added and the sample was ready for GC analysis.

Standard mixtures of sugars were included for calibration with each batch of analytical samples. These standard samples were placed in sulfuric acid solution and carried through the entire procedure with the exception of the initial heating step.

Gas chromatography

Packed columns were prepared by cleaning a 6 ft. × 2 mm I.D. glass column (Supelco) with distilled water, 1 *M* HCl, distilled water, acetone and glass distilled methanol in that order. After silanizing with Sylon CT (Supelco), the columns were rinsed with toluene followed by glass distilled methanol and then packed with Supelcoport (100–200 mesh) coated with 3% SP-2330 (Supelco). Packed column GC was performed on a Packard Model 417 gas chromatograph (Packard, Downers Grove, IL, U.S.A.) equipped with a flame ionization detector. Chromatographic conditions were: injector temperature 245°C, detector temperature 295°C, initial oven temperature 200°C, no initial temperature hold, temperature programmed at 4°C/min to a final temperature of 245°C. The helium carrier gas flow-rate was 45 ml/min. Injections were on-column to eliminate possible contact with the hot injector port.

Glass capillary columns were prepared in our own laboratory. All were drawn from 4 ft. × 9 mm O.D. × 2.8 mm I.D. Pyrex tubing (A. H. Thomas, Philadelphia, PA, U.S.A.) on a GDM-1B glass drawing machine (Shimadzu Seisakusho, Kyoto, Japan). Metal ions were removed from the column by filling the capillary with 20% HCl solution, sealing the ends and heating at 180°C for 14 h. The columns were rinsed with 0.5 *M* HCl and heated at 150°C for 30 min to remove residual acid and coated with 20% (w/v) solution of SP-2330 in chloroform by the dynamic mercury plug method[15]. Capillary GC was performed on a Hewlett-Packard 5831A gas chromatograph fitted with a Hewlett-Packard 18835B capillary inlet system and a flame ionization detector. Chromatographic conditions were: injector temperature 245°C, detector temperature 295°C, initial oven temperature 100°C, initial hold 0.5 min, temperature programmed at 30°C/min to a final temperature of 245°C. The column helium flow-rate was approximately 2 ml/min.

RESULTS AND DISCUSSION

Improvements in sample preparation

The classical alditol acetate derivatization method for the GC determination of carbohydrates introduced by Gunner *et al.*[1] and modified by others[2,16] is time-consuming and involves a considerable number of manual operations. Furthermore, the final chromatogram can be easily contaminated with extraneous peaks by uncontrolled side reactions or by the use of impure reagents. The modifications adopted in our procedure answer many of these criticisms of the alditol acetate method. One of the first considerations faced was the desire to run multiple samples through the procedure simultaneously. The use of hydrolysis reaction tubes instead of flame sealed ampoules, N,N-dioctylmethylamine for neutralization in place of barium hydroxide, a reduced pressure Vortex Evaporator in place of evaporation under a nitrogen stream, disposable extraction columns instead of liquid–liquid extraction and derivatization reaction vials and heating modules in place of test-tubes and water-baths all contribute to decreasing the number of manual operations required and to permitting the simultaneous analysis of multiple samples.

The first step in the determination of the carbohydrate composition of a polysaccharide is the hydrolysis of the polymer to release the monomeric constituents. Sugars are notoriously unstable when heated to high temperatures in strong acid solutions. Any such degradation is undesirable because it results in erroneously low values for the sugars and may introduce additional peaks in the chromatogram. The use of sulfuric acid and the careful exclusion of air has been previously shown to minimize these effects[17]. The neutralization or removal of the acid may also cause degradation of the sugars; for example, if hydrochloric acid is employed and removed by evaporation, extraneous peaks may appear. Sulfuric acid is generally removed by neutralization with barium hydroxide; however, barium hydroxide neutralization is difficult to implement on a micro scale and sample may be lost by coprecipitation with barium sulfate. The use of N,N-dioctylmethylamine to neutralize sulfuric acid eliminates these problems[18]. Following the neutralization step, disposable hydrophobic columns are employed to remove lipids released by the hydrolysis as well as residual N,N-dioctylmethylamine. The internal standards, xylose and methylglucamine, were chosen because they are not normally present in bacterial cell walls; the internal standards were added after the hydrolysis step and carried through the remaining steps.

The next step in the chemical derivatization is a sodium borohydride reduction of the aldoses to alditols and their subsequent acetylation. As stressed by other researchers, multiple evaporations with methanol–acetic acid are required to completely remove borates which will inhibit the acetylation reaction[19,20]. The sample should be thoroughly dried before proceeding to the acetylation step. Pyridine is commonly employed as a catalyst for the acetylation reaction[21]. However, the addition of any catalyst is unnecessary since sufficient sodium acetate catalyst is generated during the methanol–acetic acid evaporations. Furthermore, the addition of pyridine may result in a browning reaction and may produce undesirable extraneous peaks in the chromatogram, even when glass distilled reagents are employed. Following the acetylation reaction, disposable hydrophilic columns are employed to remove the sodium acetate catalyst and other contaminating polar compounds such as amino acids.

Although each of the changes described above contributes only minor improvement individually, the combination of modifications taken together provides considerable advantages over the previously published methods for the preparation of alditol acetate derivatives of carbohydrates.

Improvements in gas chromatography

Cyanosilicone stationary phases have been popular for the GC separation of the alditol acetates of carbohydrates[9,22]. SP-2330 is a polar stationary phase containing 68% cyanopropyl substitution on a methyl silicone polymer and has an upper temperature limit of 275°C. Fig. 1 illustrates the reasonably good separation of a mixture of alditol acetates of neutral and amino sugars that can be achieved on a packed column coated with SP-2330. Two disadvantages, however, are apparent: the first three peaks (deoxyribitol, rhamnitol and fucitol pentaacetates) are not completely resolved, and the time required to elute the last peak (galactosaminitol hexaacetate) is about 36 min. Although a decrease in the initial temperature or the initial program rate might improve the separation of the early eluting peaks, the overall analysis time would suffer. In addition to the problem of low resolution per unit time, this packed column exhibits a decrease in the peak area of amino sugars relative to neutral sugars, perhaps due to undesirable adsorption or decomposition on the column. This decreased sensitivity for amino sugars in comparison to neutral

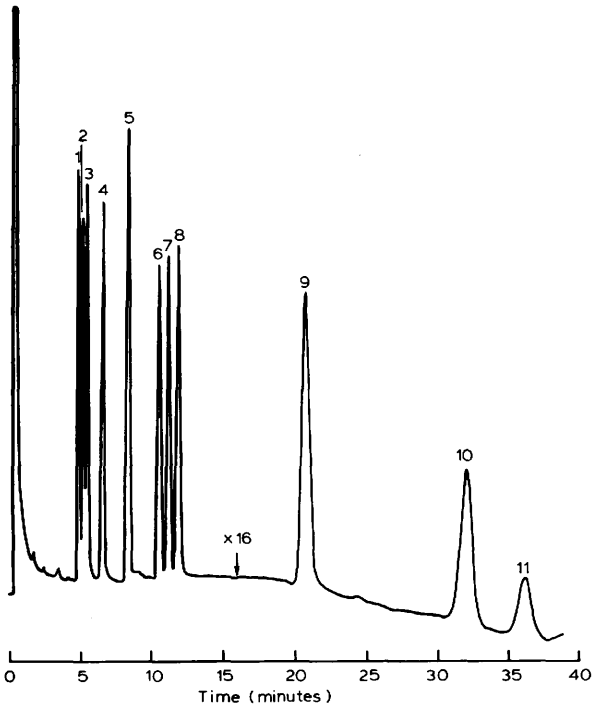


Fig. 1. Packed column chromatogram of the alditol acetate derivatives of a mixture of neutral and amino sugars. Note attenuation change at 16 min. Peaks: 1 = deoxyribose; 2 = rhamnose; 3 = fucose; 4 = ribose; 5 = xylose; 6 = mannose; 7 = galactose; 8 = glucose; 9 = muramic acid; 10 = glucosamine; 11 = galactosamine.

sugars has been noted previously in the alditol acetate method[5–7,22,23] as well as other derivatization procedures[24–29].

We have recently shown[13] that appreciable losses of amino sugars may occur during GC separations on glass capillary columns due to irreversible adsorption or decomposition. Fig. 2 shows a chromatogram obtained on a glass capillary column prepared by leaching with aqueous HCl and then coating with SP-2330. This simple two-step column preparation procedure produces columns that are quite inert to amino sugar alditol acetates and exhibit excellent chromatographic efficiency. The overall analysis time for the capillary separation (Fig. 2) was considerably less than that required for the packed column separation. It might also be noted that rhamnose and fucose are practically baseline separated on the capillary column and that there is no significant column bleed despite temperature programming to 265°C.

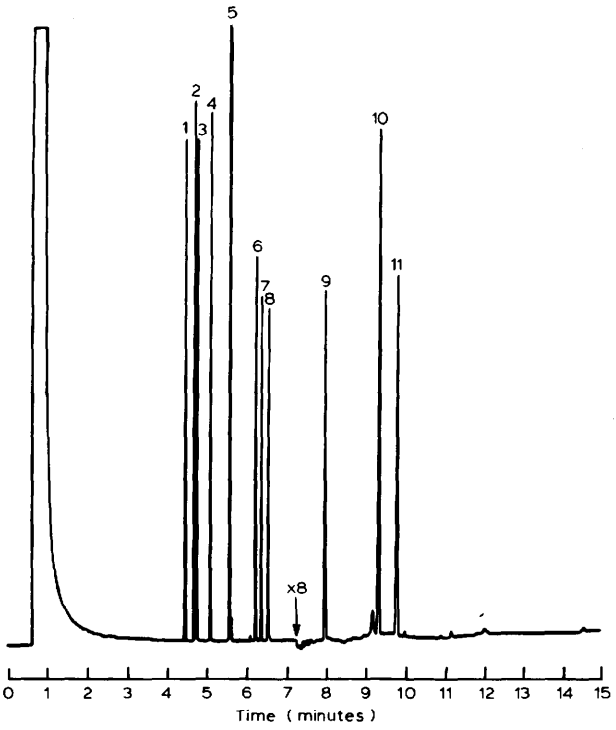


Fig. 2. Capillary column chromatogram of the alditol acetate derivatives of a mixture of neutral and amino sugars. See Fig. 1 for peak identification.

The separation of mixtures containing neutral sugars *or* mixtures containing amino sugars is relatively simple. Oshima *et al.*[12], for example, demonstrate that capillary columns coated with OV-1, SE-54 or Carbowax 20M are suitable for the analysis of amino sugars and that Carbowax 20M is preferred for separating the neutral sugar alditol acetates. However, for the separation of neutral and amino sugar mixtures, long retention times (up to 29 min) are necessary and glucose and galactose are not well resolved. Green *et al.*[13] also separated neutral and amino sugars by capillary GC but required excessive analysis times. The chromatographic columns

used in our work represent an excellent compromise for solving the general elution problem posed by mixtures of both neutral and amino sugars. One final point: although Varma and Varma[30] note that mannosamine is not commonly present in biological samples, it is fundamentally important to be able to resolve mannosamine from its isomers glucosamine and galactosamine; both Green *et al.*[11] and Oshima *et al.*[12] achieved baseline resolution of these three isomers. Under our chromatographic conditions, mannosamine is only partially resolved from galactosamine (chromatogram not shown here); better resolution could be achieved at the cost of a longer analysis time.

Precision of the method

As previously noted by Niedermeier[16] generally most of the error in analyses of alditol acetates is associated with the GC conditions rather than sample preparation. With xylose as an internal standard, five replicate injections of the same sample yielded relative standard deviations of less than 5% for each of the seven neutral sugars; relative standard deviations for the amino sugars were 28.9%, 27.5% and 27.5% for muramic acid, glucosamine and galactosamine, respectively. Following a suggestion by Hicks and Newell[31], we found that when methylglucamine was used as an internal standard for the amino sugars the relative standard deviations were reduced to 4.1%, 2.9% and 4.9% for the same three amino sugars. These results prompted us to employ xylose as an internal standard for neutral sugars and methylglucamine as an internal standard for amino sugars. Calibration curves for the seven neutral sugars and the three amino sugars were obtained by derivatizing solutions of standards over a range of 600 ng to 300 μg of each neutral and amino sugar and with fixed amounts of added xylose and methylglucamine. Calibration curves for rhamnose (Fig. 3) and glucosamine (Fig. 4), typical of neutral and amino sugar calibration curves are shown. Both of these fitted lines gave coefficients of determination of 0.998 and the lack of fits was not significant at the 90% level of confidence.

The method described in this paper is being employed to evaluate different methods of preparing purified bacterial cell walls. Fig. 5 shows a representative chromatogram confirming the presence of rhamnose, muramic acid and glucosamine in a

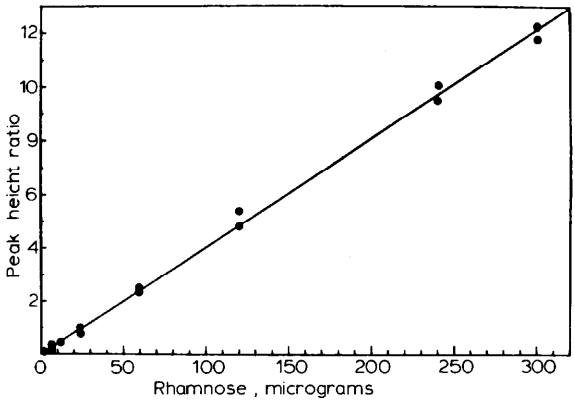


Fig. 3. Standard curve for rhamnose. Peak area ratio of rhamnose to xylose plotted against amount of rhamnose in standard. Calibration data consists of duplicate runs of blank, 600-ng, 3-μg, 6-μg, 12-μg, 60-μg, 120-μg, 240-μg and 300-μg samples and four runs of 24-μg samples, for a total of 22 points.

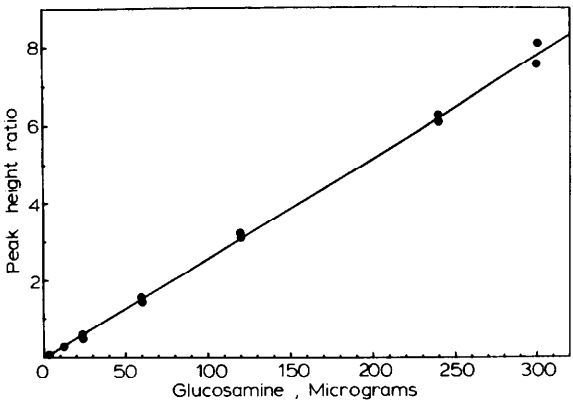
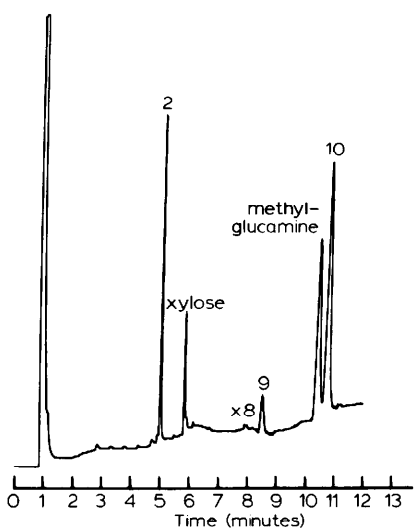


Fig. 4. Standard curve for glucosamine. Peak area ratio of glucosamine to methylglucamine plotted against amount of glucosamine in standard. Calibration data consists of 22 points.

cell wall preparation isolated from *Streptococcus pyogenes*. A chromatogram of a sample blank containing only the internal standards is shown in Fig. 6. In a typical analysis, this cell wall sample was found to contain 17.1% rhamnose, 4.0% muramic acid and 16.0% glucosamine by total weight with relative standard deviations based on four replicates of 2.0%, 5.6% and 4.6%, respectively.



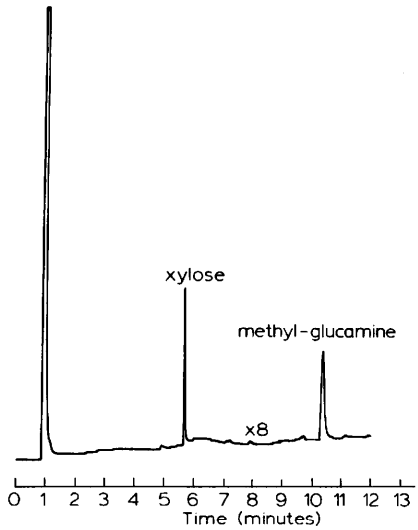


Fig. 5. Capillary column chromatogram of *Streptococcus pyogenes* cell wall hydrolysate. Peak 2 is rhamnose, 9 is muramic acid, 10 is glucosamine and the xylose and methylglucamine internal standard peaks are labelled.

Fig. 6. Capillary column chromatogram of blank run carried through the entire procedure except for hydrolysis.

CONCLUSIONS

The modifications introduced in this paper for the preparation of volatile alditol acetate derivatives of neutral and amino sugars were designed to simplify the manual operations required and to make the method more easily implemented as a routine assay. The method allows a multiple number of samples to be processed simultaneously through the hydrolysis, derivatization and clean-up steps. Additionally, the chosen operating conditions and reagents result in a gas chromatogram that is essentially clear of extraneous peaks that might adversely affect the quality of the separation.

High resolution glass capillary columns can be tailor-made for the efficient separation of alditol acetate derivatives of neutral and amino sugars by the simple two-step procedure of leaching with aqueous hydrochloric acid and then coating with SP-2330. It appears, however, that even with the best deactivated column, there remains some loss of amino sugars relative to neutral sugars. Further improvements in the method, perhaps in the derivatization or clean-up steps, are needed to resolve this problem. Nevertheless, the simplified sample processing and column preparation steps outlined in this article should serve to further popularize the alditol acetate method for the analysis of neutral and amino sugars.

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