SUMMARY

Aldoses and alduronic acids can be simultaneously identified and quantitated by wide-bore capillary gas-liquid chromatography. The aldoses are converted to alditol acetates and the alduronic acids are converted to their corresponding N-propylaldonamide acetates. Standard mixtures containing as little as 100 ng each of arabinose, xylose, mannose, galactose, glucose, inositol, mannanuronic, glucuronic, and galacturonic acids can be readily analyzed. Inositol or phenyl-β-D-glucopyranoside can be used as internal standards.

INTRODUCTION

The analysis of complex hydrolysis mixtures derived from many polysaccharides is often complicated by the presence of alduronic acids in addition to the aldoses normally present. Aldoses can easily be identified and quantitated by gas-liquid chromatography (GLC) as the alditol acetates, aldonitrile acetates, oximes, methyloximes or trimethylsilyl (TMS) or trifluoroacetyl (TFA) derivatives, or by high-performance liquid chromatography (HPLC) on cation- and anion-exchange resins or silica-based columns. Alduronic acids are most often analyzed spectrophotometrically with carbazole, harmine, and m-hydroxydiphenyl, or by decarboxylation and subsequent measurement of carbon dioxide. These commonly used methods are non-specific in that they do not identify which acid is being analyzed.

Enzymatic methods are very specific but of limited scope. HPLC and GLC, which readily identify and quantitate alduronic acids, have had limited use. Aldose and alduronic mixtures can be analyzed by a differential method, but it is tedious and time consuming. It requires three sodium borohydride reductions and a duplicate analysis. A modification reported later in this paper simplifies the method. Other methods produce complex chromatograms that require sophisticated quantitation techniques, require partial separations of acidic and neutral fractions, or combine spectrophotometric and chromatographic techniques, i.e. two different analyses with a degree of uncertainty about the identity of the alduronic
acid. The elegant concept of activating, then reducing the carboxyl group\(^{31}\) in the polyuronide, and then hydrolyzing, still requires the use of radiochromatography or gas chromatography–mass spectrometry (GC–MS) with chemical ionization.

A consolidated method that provides a simple chromatogram using common reagents and instrumentation has heretofore not been available. The present work describes a procedure to simultaneously identify and quantitate mixtures containing the commonly found aldoses and alduronic acids. It combines the widely used alditol acetate method with the new aldonamide acetate method in a manner similar to that recently reported\(^{32}\) for the analysis of mixtures containing aldoses and aldonic acids.

EXPERIMENTAL*

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

**Materials**

Propylamine (Gold label, 99 + %), pyridine (Gold label, 99 + %), L-arabinose, D-xylose, D-mannose, D-galactose, D-glucose, D-galacturonic acid monohydrate, D-gulono-1,4-lactone were obtained from Aldrich. Phenyl-β-D-glucopyranoside, D-glucono-1,5-lactone, D-galactono-1,4-lactone, D-gluconoraphidinose, and myo-inositol were obtained from Pfanziehl Lab. Algicin acid, L-mannono-1,4-lactone, D-ribono-1,4-lactone, D-mannofuranurono-6,3-lactone, and L-rhamnose were obtained from Sigma. Cation-exchange resin (AG 50W-X8, 200–400 mesh, H\(^+\) form), an Aminex HPX-87H organic acid analysis column (30 cm × 7.8 mm) and a micro-guard cation-exchange (H\(^+\) form) column were purchased from Bio-Rad. GLC-coated support (3% SP2340 on 100–120 mesh Supelcoport) and a 30 m × 0.75 mm I.D. capillary column coated with a 1.0 μm film of SP2330 were obtained from Supelco. The capillary column was shortened to 24 m. A 25 cm × 4.6 mm I.D. Spherisorb ODS-5 μm column was obtained from Spectra-Physics.

**GLC analysis**

Analysis by GLC was performed on a Packard Instrument Model 428 gas chromatograph equipped with dual flame ionization detectors and dual electrometers. Per-O-acetylated derivatives (alditol acetates and N-propylaliconamide acetates) were initially separated on a 1 m × 2 mm I.D. glass column packed with 3% SP2340. The temperature was programmed from 190 to 260°C at a rate of 5°C/min. The helium flow-rate was 20 ml/min. A standard mixture was resolved in 18 min (Fig. 1). The 24 m × 0.75 mm I.D. SP2330 wide-bore capillary column gave improved separation in 20 min (Fig. 2) at lower temperatures (200°C for 2 min then 3°C/min to 235°C). The helium flow-rate was 10 ml/min with a make-up helium flow-rate of 10 ml/min. A Spectra-Physics Computing Integrator Model 4270 was used to record the chromatogram and to integrate the peak areas.

**HPLC analysis**

Analysis by HPLC was performed on a Spectra-Physics Model SP8100 liquid chromatograph equipped with a Spectra-Physics autosampler Model SP8110 and outfitted with an Aminex HPX-87H column and a micro-guard cation-exchange (H\(^+\) form).
Fig. 1. Separation of per-P-acetylated N-propylaldonamides and alditols by GLC on a 1 m x 2 mm I.D. 3% SP2340 packed glass column and programmed from 190 to 260°C at a rate of 5°C/min. Peaks: 1 = arabitol, 2 = xylitol, 3 = mannitol, 4 = galactitol, 5 = glucitol, 6 = inositol, 7 = ribonamide, 8 = phenyl-β-D-glucopyranoside, 9 = mannonamide, 10 = gluconamide, and 12 = galactonamide.

form) column. A Spectra-Physics Model SP8440 variable-wavelength UV-VIS detector set at 190 nm or 210 nm and an Altex Model 156 refractive index detector were connected in series. The signal pairs were split. One pair was recorded on a dual pen Houston Omniscribe recorder Series 5000 and the other pair was sent to a central

Fig. 2. Separation of per-O-acetylated N-propylaldonamides and alditols by GLC on a 24 m x 0.75 mm I.D. wide-bore SP2330 column (1.0 μm film) and programmed at 200°C for 2 min, then 3°C/min to 235°C. Peaks: 1 = arabitol, 2 = xylitol, 3 = mannitol, 4 = galactitol, 5 = glucitol, 6 = inositol, 7 = ribonamide, 8 = phenyl-β-D-glucopyranoside, 9 = mannonamide, 10 = gluconamide, 11 = gulonamide, and 12 = galactonamide.
Mod Comp (Modular Computer Systems) computer for integration of peak areas. The eluting solvent (0.6 ml/min) was sulfuric acid (0.01 N) and the temperature was maintained at 30°C. The unacetylated n-propylaldonamides can be chromatographed on a Sperisorb ODS column with water as the mobile phase (Fig. 3).

**Conversion of aldoses and alduronic acids to peracetylated additols and N-propylaldonamides**

To a water solution (1 ml) containing approximately 2 mg each of L-arabinose, d-xylose, d-mannose, d-glucose, d-galactose, myo-inositol, d-mannofuranurono-6,3-lactone, d-glucurono-6,3-lactone, and d-galacturonic acid monohydrate in a 16 x 125 mm culture tube was added 78 μl (13 μl/mg acid) of 0.5 M sodium carbonate. The solution was maintained at 30°C for 45 min and then treated with sodium borohydride (0.5 ml of a 4% solution) for 1.5 h at room temperature (around 22°C). Excess sodium borohydride was decomposed by the dropwise addition of acetic acid (25%) until bubbling stopped (6–8 drops). The solution was poured onto a cation exchange column (2 ml) and eluted with 6 ml water. The collected eluate (16 x 125 mm culture tube) was evaporated to dryness in a vortex evaporator (45°C in vacuo). Borate was removed as trimethyl borate by twice evaporating methanol (3 ml) from the residue. Heating at 85°C in vacuo for 2 h converted the aldonic acids into aldono-lactones. The residue was dissolved in pyridine (1 ml), 1-propylamine (1 ml) was added, and the tube was capped and heated at 55°C for 30 min. The solution was cooled (under 45°C), and nitrogen was bubbled through the reheated solution (55°C) until dry. The residue was dissolved in pyridine (0.5 ml) and acetic anhydride (0.5 ml) and heated at 95°C for 1 h.

The sample is suitable for GLC as is: usual injection size is 0.2 μl. If sample size is small (around 100 ng), tailing from pyridine and acetic anhydride interfere with detection and quantitation. They are removed by bubbling nitrogen through the solution. The dry residue is reconstituted with 25 μl of tetrachloromethane. Suitable injection size is 0.4 μl.

Standards of the N-propylaldonamides for GLC or HPLC can easily be pre-
pared by adding propylamine (0.5 ml) to a culture tube containing the appropriate lactone (1–20 mg), heating at 50°C for 5–10 min and then evaporating the propylamine with a stream of dry nitrogen. The acetates are prepared as above.

Samples for Fig. 1 and 2 were prepared as previously described. For HPLC, the samples were dissolved in water and chromatographed on a C18 column with water as the mobile phase.

Optimization of lactone hydrolysis

Glucuronolactone (4 mg) or mannuronolactone (4 mg) was weighed in a 1.9-ml sample vial and 1 ml water was added. Analysis was by HPLC. To determine the extent of hydrolysis at pH 7 a 10-μl sample was injected onto the HPX-87H column every 15 min for a period of 1 h (five samples). To fresh samples prepared as above, sodium carbonate (0.5 M) was added in different amounts, (0.9–1.4 times the theoretical), and samples were again injected every 15 min.

Quantitation

A series of standard solutions containing 1–5 mg of arabinose, xylose, mannose, galactose, glucose, glucuronolactone and galacturonic acid was prepared and treated as described above. Each tube contained 3 mg of inositol as an internal standard. Samples at five concentration levels were analyzed in triplicate. Calibration curves (relative detector response vs. milligrams of sugar) were calculated for each component and were found to have correlation coefficients in the range 0.997–1.0 and relative response factors of 0.8–1.0.

RESULTS AND DISCUSSION

The component analysis of heteropolysaccharides requires that a simple and precise method for the analysis of aldoses and alduronic acids be available. The method described uses commonly available equipment, reagents, and a well understood reaction sequence with high conversion values. Fig. 4 summarizes the conversion of aldoses and alduronic acids to their respective alditol acetates and N-propylaldonamide acetates, which are then analyzed by GLC. The methodology is anal-

![Diagram of the method for the analysis of aldonic acids and aldoses](attachment://method_diagram.png)

Fig. 4. Summary of the method for the analysis of aldonic acids and aldoses.
ogous to that described for the analysis of aldoses and aldonic acids; however, the chromatography is much improved by the use of the wide-bore capillary column.

The method, for illustrative purposes (Fig. 4) has been compressed into 3 basic steps: A, B and C. In step A, the analyte is treated with a base to ensure the complete hydrolysis of any lactone that might be present. In step B, sodium borohydride reduces all the carbonyl groups. The aldoses are transformed into alditols and the alduronic acids are converted into their corresponding inverted aldonic acids and subsequently into aldonolactones (Fig. 5). In step C, the alditols are converted into alditol acetates and the aldonolactones into N-propylaldonamide acetates.

**Step A alkaline hydrolysis**

The accuracy of the method depends on the stoichiometric conversion of al­
duronic acid to aldonic acid. Almost all of the common alduronic acids (galacturonic acid being an exception) readily equilibrate with their lactones in acidic solutions.

![Fig. 6. (A) GLC analysis of an alginic acid hydrolyzate analyzed without the use of additional base. (B) GLC analysis of an alginic acid hydrolyzate analyzed with the prescribed quantity of additional base. Identification of peracetylated derivatives: 3 = mannitol, 5 = glucitol, 9 = N-propylmannosamide and 10 = N-propylgluconamide.](attachment:fig6.png)
Consequently, alduronic acids formed during the hydrolysis of a glycuronoglycan will, to a great extent, be in the lactone form. If this factor is ignored, subsequent reduction with sodium borohydride will result in partial conversion of the alduronate to alditol. The amount of alditol formed will depend on the experimental conditions and for practical purposes, is not reproducible\(^{33}\). For example, an alginic acid hydrolysate (sulfuric acid) was neutralized with an excess of barium carbonate. No additional base was added. The mixture was reduced and treated as described. The chromatogram (Fig. 6A), shows the presence of mannitol (peak 3) and glucitol (peak 5). These are artifacts absent from the chromatogram of a second aliquot (Fig. 6B) of the same hydrolysate treated with sodium carbonate subsequent to barium carbonate, reduced and then treated as described. Fig. 6 clearly demonstrates that sufficient base must be added before the sodium borohydride reduction.

Epimerization does not occur to any significant degree during the time necessary for complete hydrolysis. Samples containing each of the individual sugars were treated with sodium hydroxide (0.5 \( M \), 13 \( \mu l/mg \)), sodium carbonate (0.5 \( M \), 13 \( \mu l/mg \)), triethylamine (1 \( \mu l/mg \)), or an excess of barium carbonate. Hydrolysis was complete within 30 min at room temperature for sodium hydroxide, sodium carbonate, and triethylamine. The samples were analyzed by HPLC or GLC. After 1 h at room temperature with an excess of barium carbonate, 82\% of the lactone was still present. Although heating for 10 min at 65\(^\circ\)C was reported to hydrolyze the lactones completely\(^{24}\), analysis by HPLC revealed that 17\% of the lactone was still present after 15 min at 65\(^\circ\)C, but none after 40 min. The solutions varied from pH 12.2 for sodium hydroxide to 9.1 for barium carbonate. Because of the relatively short exposure times and moderate temperatures, degrees of epimerization were no greater than 0.3\%. All of the bases listed above work well. Obviously, the amount of base to be added depends on the residual acid left in the sample. The stated amounts assume one starts with an essentially neutral solution. Polysaccharide hydrolysates prepared with trifluoroacetic acid or hydrochloric acid may contain significant amounts of residual acid, even after evaporating on a rotary evaporator or a freeze dryer.

**Step B reduction and lactone formation**

Sodium borohydride readily reduces aldoses to alditols\(^{32,34,35}\) and alduronates to aldonates\(^{24}\). Carboxylic acids or their salts are not reduced by sodium borohydride\(^{36}\). To verify this generalization, galacturonic acid, mannuronolactone, and glucuronolactone were converted to their corresponding sodium salts by treatment with sodium carbonate, then reduced and treated as described. No alditols were found in the GLC. Similar experiments in which sodium carbonate was omitted gave variable amounts of alditols from 40 to 100\%. The variability was found to be a function of the age of the sodium borohydride solution. Solutions of sodium borohydride used within 1–4 min of preparation gave essentially complete reduction of the lactone (Fig. 7A). Approximately 0.3\% acid was formed. A sodium borohydride solution prepared 1 h before the addition of the lactone gave 4\% acid, and a solution stored overnight in the refrigerator before the addition of lactone gave 57\% acid (Fig. 7B), i.e., 57\% of the lactone was hydrolyzed and not reduced. Sodium borohydride was still present in large excess as ascertained by the vigorous bubbling that occurred upon the addition of acetic acid and the complete reduction of aldoses. A
Fig. 7. (A) GLC analysis of glucuronolactone reduced with a freshly prepared sodium borohydride solution. (B) GLC analysis of glucuronolactone reduced with a 16-h old sodium borohydride solution. Identification of peracetylated derivatives: 5 = glucitol and 11 = N-propylgulonamide.

4% sodium borohydride solution becomes more basic with time. A plot of pH vs. time indicates a progressive rise from pH 9.5 to 10.4 after 1 h and after 16 h to 11.3. The high pH of older solutions causes the rate of lactone hydrolysis to increase markedly and to compete more effectively with lactone reduction. This phenomenon probably accounts for the discrepancy in earlier reports on lactone reduction; three sodium borohydride treatments were needed for complete conversion to alditol instead of just one. For complete reduction either a fresh solution or a buffered solution must be used. An earlier procedure for differential determination of carbohydrates can be simplified by taking advantage of this observation. The number of reductions can be reduced from three to two. Because galacturonic acid does not form a lactone, two reductions are still needed. Optimization of lactone formation has been described.

**Step C conversion to N-propylaldonamides**

Optimization of aldonamide acetate formation has been discussed elsewhere. Aldonamide standards for GLC or HPLC do not require pyridine as a cosolvent. Pyridine is required to solubilize alditols to prevent losses from occlusion of lactones in the insoluble alditol matrix. Addition of 0.5 ml of n-propylamine to a tube containing the lactone and heating for 5–10 min at 50°C gives the n-propylamide. Aqueous solutions of the amides can be stored in the refrigerator for over a week or in the frozen state for over a month. Hydrolysis occurs at room temperature. A mixture of the n-propylaldonamides from mannolactone, gluconolactone, gulonolactone, and galactonolactone were not resolved to the baseline. Connecting two
columns in series did not markedly improve the resolution. Nevertheless, it is evident that some mixtures can be analyzed readily by this method. For example, mixtures (Fig. 3) containing only 1, 2, and 4 or 1 and 3, etc., give baseline resolution.

Per-O-acetylated derivatives used for GLC are quite stable. One sample mixture stored in methylene chloride at room temperature has been used for over sixteen months with minimal changes.

The method described permits the accurate determination of some complex carbohydrate mixtures containing aldoses and alduronic acids. The method uses readily available apparatus and reagents. The chromatography is markedly improved by the use of a large-bore capillary column. In addition, the protocol is versatile in that it can be adjusted to handle samples from different sources.

REFERENCES